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Detection of Genes Regulating β-Lactamase Production in *Enterococcus faecalis* and *Staphylococcus aureus*

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Four β-lactamase-producing clinical isolates (WH245, WH257, CH570, and DEL) of *Enterococcus faecalis* were examined for the presence of the staphylococcal β-lactamase regulatory genes (*blaR1* and *blaI*) by PCR using six primer pairs. All isolates produced small amounts of β-lactamase constitutively. In WH245, CH570, and DEL, the corresponding regions of the regulatory genes have lost sequences of various lengths. However, the regulatory genes in WH257 appeared to be the same as those in staphylococcal plasmid pI258. The β-lactamase genes could be transferred to enterococcal and staphylococcal recipients from WH257 and DEL by conjugation or transformation with selection for gentamicin resistance. After transformation, the expression of β-lactamase from DEL was still constitutive, whereas the gene from WH257 showed inducible expression in *Staphylococcus aureus*. The gene coding for inducible β-lactamase production from pI258 showed constitutive expression in *E. faecalis*. These findings suggest that constitutive β-lactamase production in *E. faecalis* is due not only to the absence of functional regulatory genes but to some other factor(s) as well.

The first β-lactamase-producing (Bla^+) isolate of *Enterococcus faecalis*, HH22, was reported in 1983 (16). Since then, many other Bla^+ enterococci have been isolated in various regions as nosocomial pathogens (5, 11–13, 15, 18, 19, 22, 36). The gene coding for β-lactamase production in *E. faecalis*, as well as in *Staphylococcus aureus*, is located on a plasmid (9, 15, 16, 20) or on the chromosome (1, 24). Recent studies have suggested transposition of the β-lactamase gene in *E. faecalis* (24, 25, 30). In enterococci, β-lactamase is produced constitutively, whereas staphylococci usually produce this enzyme in an inducible fashion. The constitutive production of β-lactamase in enterococci has been extensively studied in strain HH22 (14, 17, 31, 39, 40). Nucleotide sequence analysis of the β-lactamase (*bla*) genes has shown that the structural gene (*blaZ*) and its promoter and operator sequences from HH22 are identical to those of type A *blaZ* genes from *S. aureus* (38, 39) and that HH22 has only 51% of the antirepressor gene (*blaR1*) and has lost the repressor gene (*blaI*) (40), which regulate expression of *blaZ* in *S. aureus* (27, 35). Moreover, in strains CH19 and CH116, an IS*256*-like element inserted within *blaR1* which results in loss of function has been found (23, 26). Therefore, constitutive β-lactamase production in enterococci can be due to the absence of a functional BlaR1 and BlaI (23, 26, 31, 40). In a recent study, Tomayko et al. have shown by PCR that three isolates (WH257, HG9829, and Beirut) of *E. faecalis* have both *blaR1* and *blaI* genes, although β-lactamase production was not inducible (33). However, it was not shown that these genes would be functional in a staphylococcal host.

In this study, we constructed six primer pairs specific for the *blaR1* and *blaI* regions from *S. aureus* and used these in PCR to detect corresponding regulatory genes in four Bla^+ isolates of *E. faecalis*. An isolate which contained these regulatory genes was found, and we then investigated the expression of *blaZ* derived from this isolate in *S. aureus.*

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Four Bla^+ isolates of *E. faecalis* were isolated from clinical specimens in the United States (18, 20) and were kindly provided by Michael A. Cohen, Parke-Davis. *E. faecalis* FA2-2 (2) and *S. aureus* RN4220 (7) are plasmid-free strains which were used in conjugation and transformation experiments as recipients. pI258 (29), which encodes an inducible staphylococcal β-lactamase, was used as a positive control for β-lactamase induction and the PCR assay. pKU101 is a recombinant plasmid containing a 4.1-kb *Hin*cII fragment encoding β-lactamase from pI258 cloned into pND50 (37), which replicates in *S. aureus*, *E. faecalis*, and *Escherichia coli.*

Conjugation and transformation. Tests for transfer of resistance were carried out with *E. faecalis* FA2-2 and *S. aureus* RN4220 as recipients. Mating was carried out by filter-mating techniques, with overnight incubation at 35°C as previously described (14). Transformation was performed by electroporation with a Bio-Rad Gene Pulser and a pulse controller unit (Bio-Rad Laboratories, Hercules, Calif.). An overnight culture grown in brain heart infusion (BHI) broth (Nissui, Tokyo, Japan) at 35°C was diluted 1:20 into 10 ml of fresh BHI broth and grown at 35°C for 3 h. Cells were harvested by centrifugation at 4°C, washed twice with ice-cold 10% (wt/vol) sucrose, washed once with 10% sucrose–10% glycerol, and then resuspended in 0.1 ml of the same solution. After being held on ice until required, 5 μl of DNA solution was thoroughly mixed with 40 μl of cell suspension in a chilled Gene Pulser cuvette (electrode gap of 2 mm). The mixture was exposed to a single electric pulse (peak voltage, 2.5 kV; capacitance, 25 μF; resistance, 200 Ω), which generated a peak field strength of 12.5 kV/cm and a time measured of between 4 and 5 ms. Immediately after delivery of the pulse, the cells were added to 1 ml of BHI broth containing 10% sucrose and incubated at 35°C for 90 min before being spread onto a plate.

β-Lactamase induction and enzyme assay. β-Lactamase expression was determined as follows. Overnight cultures were diluted 1:20 into 10 ml of fresh BHI broth. After incubation for 3 h on a shaker at 35°C, the inducer was added and incubation was continued for 2 h. Cells were harvested, washed once, and suspended in 4 ml of 50 mM phosphate buffer (pH 7.0). The cells were sonicated with an ultrasonic disrupter (model UD-201; Tomy Seiko, Tokyo, Japan), and the cellular debris was removed by centrifugation at 12,000 rpm for 15 min at 4°C. For *S. aureus*, the cells were treated with lysostaphin (25 U/ml; Sigma Chemical Co., St. Louis, Mo.) at 37°C for 20 min before sonication. Methicillin was used as the inducer, at concentrations of 0.8 μg/ml for *S. aureus* and 12.5 μg/ml for *E. faecalis.*

β-Lactamase activity was measured by a colorimetric assay (6, 34) using a spectrophotometer (model UV-2200; Shimadzu Corp., Kyoto, Japan). Enzyme activity was determined at 30°C in 50 mM phosphate buffer (pH 7.0) with piperacillin (λ, 232 nm; ε, 1.64) as a substrate. Protein concentrations were determined by the Bio-Rad protein assay. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of piperacillin in 1 min at 30°C.

Bla^+ colonies were detected by an iodometric method (8) in the conjugation and transformation experiments.

DNA isolation. Plasmid DNA was isolated by the small-scale alkaline method

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype[a]	Source, description, and/or reference(s)
Strains		
E. faecalis		
WH245	Sm^r Em^r Tc^r Cm^r Bla^+	West Haven, Conn. (20, 32).
WH257	Gm^r Sm^r Em^r Tc^r Bla^+	West Haven, Conn. (20, 32)
CH570	Gm^r Sm^r Em^r Tc^r Cm^r Bla^+	Canonsburg, Pa. (18, 32)
DEL	Gm^r Sm^r Em^r Tc^r Cm^r Bla^+	Wilmington, Del. (18, 32)
FA2-2	Rif^r Fus^r	2
S. aureus RN4220		7
Plasmids		
pYN104	Gm^r Sm^r Em^r Bla^+	Isolated from WH257 (18)
pKU201	Gm^r Bla^+	Isolated from DEL (this study)
pKU101	Cm^r Bla^+	Recombinant plasmid containing a 4.1-kb *Hin*cII Bla-encoding fragment from pI258 in pND50 (this study)
pI258	Em^r Bla^+	29
pND50	Cm^r	Shuttle cloning vector (37)

[a] Abbreviations: Bla, β-lactamase; Cm^r, chloramphenicol resistance; Fus^r, fusidic acid resistance; Gm^r, gentamicin resistance; Rif^r, rifampin resistance; Tc^r, tetracycline resistance.

as previously described (28), with mutanolysin (100 U/ml; Sigma Chemical Co.) and lysozyme (5 mg/ml; Sigma Chemical Co.) used to lyse *E. faecalis* and lysostaphin (25 U/ml; Sigma Chemical Co.) used for *S. aureus.*

Total DNA for the PCR was prepared as follows. Cells were suspended in 100 μl of a lytic solution containing 50 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, 25 mM sucrose, RNase (10 μg/ml), and the lytic enzymes described above and incubated at 37°C for 30 min. The resulting protoplasts were lysed by phenol-chloroform extraction, and total DNA was recovered in the supernatant after centrifugation. The DNA extract was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]).

PCR and DNA-DNA hybridization. Primers were obtained from Takara Shuzo Co., Ltd. (Kyoto, Japan). PCRs were carried out according to the instructions of a GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Emeryville, Calif.). All PCRs were performed on a Perkin-Elmer Cetus DNA thermal cycler (model 480).

DNA was transferred to nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using a vacuum blotting system (VacuGene XL; Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (21). DNA-DNA hybridization was carried out with a digoxigenin DNA labeling and detection kit (Boehringer Mannheim Biochemicals) as previously described (24).

RESULTS

Primer selection. PCR primers were chosen on the basis of the published Tn*552* sequence (27) and are listed in Table 2. A series of primer pairs was designed to yield a stepwise increase in product size. Primer P1 was paired with P2 to P7, and each resulting primer pair was evaluated for its ability to amplify the corresponding fragment from plasmid pI258. Each pair allowed specific amplification of a fragment of the predicted size from pI258. Next, DNA-DNA hybridization was performed with a 0.7-kb *Eco*RV fragment of pI258 containing the region flanking the β-lactamase promoter as a probe. All amplification products hybridized with the probe, indicating that the six primer pairs were specific for the regulatory genes for β-lactamase production.

Regulatory genes and β-lactamase production. Four clinical isolates of *E. faecalis* were studied by PCR to determine whether the β-lactamase regulatory genes were present. The results are shown in Table 3. In CH570 and DEL, only the amplification product flanked by P1-P2 was detected, indicating that 57 to 92% and 100%, respectively, of *blaR1* and *blaI* were lost. In WH245, the amplification products flanked by P1-P2 and P1-P3 were detected but not those flanked by other primer pairs, demonstrating that WH245 is missing 22 to 57% and 100%, respectively, of *blaR1* and *blaI*. On the other hand, with WH257, the predicted fragments were amplified with all primer pairs. The *Dra*I restriction patterns of the fragments amplified from WH257 were identical to those from pI258. Moreover, all PCR products obtained from the four isolates hybridized with the probe derived from pI258.

All isolates produced almost the same small amounts of β-lactamase before and after induction with methicillin as well as before and after the use of imipenem, ceftizoxime, or cefixime (Table 4).

Transfer of the β-lactamase gene and its expression in two different hosts. To analyze the expression of the β-lactamase

TABLE 2. PCR primers used in this study[a]

Type and location of primer	Name	Sequence	Positions
Sense primer			
blaZ	P1	5′-ACTCTTTGGCATGTGAACTG-3′	5477–5458
Antisense primers			
blaR1	P2	5′-AATCCTGCAAGAAGAGTTAG-3′	5153–5172
blaR1	P3	5′-GGACAAATCTATCGGCTTCT-3′	4545–4564
blaR1	P4	5′-TGAGTTGAGTCGCAGTATAG-3′	3918–3937
blaI	P5	5′-CATAACATCCCATTCAGCCA-3′	3498–3517
blaI	P6	5′-AACTTTTCATGTCCCCTCCA-3′	3254–3273
Downstream of *blaI*	P7	5′-GAGTCAAGCATAGTTTAC-3′	3117–3134

[a] Sequences and positions were derived from the nucleotide sequence of Tn*552* published by Rowland and Dyke (27).

TABLE 3. Detection of β-lactamase regulatory genes from clinical isolates of *E. faecalis* and plasmid pI258 by PCR assay

Strain or plasmid	Result with the indicated primer pair[a] (predicted size of PCR product)						Gene loss (%)
	P1-P2 (325 bp)	P1-P3 (933 bp)	P1-P4 (1,560 bp)	P1-P5 (1,980 bp)	P1-P6 (2,224 bp)	P1-P7 (2,361 bp)	
WH245	+	+	−	−	−	−	*blaR1*, 22–57; *blaI*, 100
WH257	+	+	+	+	+	+	None
CH570	+	−	−	−	−	−	*blaR1*, 57–92; *blaI*, 100
DEL	+	−	−	−	−	−	*blaR1*, 57–92; *blaI*, 100
pI258	+	+	+	+	+	+	None

[a] +, amplified; −, not amplified.

structural gene in different hosts, transfer of the gene was performed by conjugation and transformation with *E. faecalis* FA2-2 and *S. aureus* RN4220 as the recipients. We selected for transfer of gentamicin or erythromycin resistance and tested these recipients for β-lactamase production by an iodometric method, since β-lactamase-producing transconjugants and transformants could not be selected directly with penicillin because of the inability of single cells to produce sufficient enzyme to overcome the drug in the plates, as previously reported (16, 24). Gentamicin-resistant transconjugants were obtained from WH257 and DEL at frequencies of 5.4×10^{-5} and 1.6×10^{-4}, respectively, and all transconjugants produced β-lactamase when 200 colonies each were tested. However, no transconjugants were obtained from WH245 or CH570 ($<10^{-8}$) when gentamicin or erythromycin was used for the selections. The transconjugants from WH257 and DEL had plasmids of either 68 or 30 kb, respectively designated pYN104 and pKU201. Transfer of the β-lactamase genes was accompanied by resistance to gentamicin, streptomycin, and erythromycin in FA2-2(pYN104) and by resistance to gentamicin in FA2-2(pKU201). Plasmids pYN104 and pKU201 could be transferred to RN4220 at very low frequencies by electroporation. RN4220(pYN104) and RN4220(pKU201) showed the same resistance patterns as FA2-2(pYN104) and FA2-2(pKU201), respectively, and also produced β-lactamase. In each transconjugant and transformant, the expected fragments were amplified by PCR. Although transformation was carried out by electroporation with FA2-2 and RN4220 as the recipients, no transformant producing β-lactamase was obtained from WH245 or CH570.

The expression of β-lactamase genes was analyzed in FA2-2(pYN104), FA2-2(pKU201), RN4220(pYN104), and RN4220(pKU201). FA2-2(pKU101), RN4220(pKU101), and RN4220(pI258) were also used as controls. The results are given in Table 5. FA2-2(pYN104) and FA2-2(pKU201) produced almost the same small amounts of enzyme before and after induction. RN4220(pKU201) also produced the enzyme constitutively. On the other hand, RN4220(pYN104) showed a 46-fold increase of β-lactamase after induction, as did RN4220(pI258) (44-fold). The level of enzyme production in RN4220(pYN104) after induction was higher than that of RN4220(pKU201).

Plasmid pI258 could not be detected after transformation into FA2-2. pKU101 was therefore constructed by cloning a 4.1-kb *Hin*cII fragment from pI258 encoding an inducible β-lactamase gene into pND50, which can replicate in FA2-2. FA2-2(pKU101) produced β-lactamase constitutively, although RN4220(pKU101) produced the enzyme inducibly (28-fold increase after induction). However, RN4220(pKU101) produced about half the amount of enzyme produced by RN4220(pYN104) and RN4220(pI258) after induction. pKU101 was confirmed by PCR assay as having the β-lactamase regulatory genes.

TABLE 4. β-Lactamase activities of clinical isolates of *E. faecalis*

Strain	β-Lactamase activity[a]		Induction ratio
	Uninduced	Induced	
WH245	0.23	0.21	0.91
WH257	0.15	0.15	1.0
CH570	0.36	0.33	0.92
DEL	0.31	0.30	0.97

[a] Methicillin was used as an inducer at a concentration of 12.5 μg/ml. Activity is expressed as micromoles of piperacillin hydrolyzed per minute per milligram of protein.

DISCUSSION

PCR assay provides easy detection of the genes on a plasmid or chromosome after rapid isolation of total DNA. The detection of the regulatory genes for β-lactamase production by PCR using internal primers for *blaR1* and *blaI* of *S. aureus* in enterococci has been reported by Tomayko et al. (33). They found that both *blaR1* and *blaI* fragments were amplified in four clonally distinct isolates analyzed (WH257, HG9829, CH25, and Beirut) but not in six other isolates of a mid-Atlantic clone, including HH22. We designed a series of six primer pairs specific for the regulatory genes to yield a stepwise increase in product size. Deletions of various lengths in the regulatory genes were found in three isolates (WH245, CH570, and DEL). However, WH257 has the full complement of regulatory genes found in plasmid pI258. These findings

TABLE 5. β-Lactamase activities of the clones in enterococcal and staphylococcal hosts

Strain and plasmid	Source of β-lactamase gene	β-Lactamase activity[a]		Induction ratio
		Uninduced	Induced	
E. faecalis FA2-2				
pYN104	Enterococcal	0.14	0.13	0.93
pKU201	Enterococcal	0.30	0.29	0.97
pKU101[b]	Staphylococcal	0.25	0.28	1.1
S. aureus RN4220				
pYN104	Enterococcal	0.19	8.8	46
pKU201	Enterococcal	1.3	1.4	1.1
pI258	Staphylococcal	0.21	9.3	44
pKU101[b]	Staphylococcal	0.16	4.5	28

[a] Activity is expressed as micromoles of piperacillin hydrolyzed per minute per milligram of protein.
[b] See text.

agree with observations previously reported for WH257, CH570, and DEL (33). In addition, it was surprising to find diversity in the *blaR1* and *blaI* sequences between WH245 and WH257 because these isolates belong to the same clonal group as determined by pulsed-field gel electrophoresis and multilocus enzyme electrophoresis (32). A significant sequence divergence could possibly have occurred after acquisition of the genes.

The β-lactamase genes encoded on pYN104 and pKU201, from WH257 and DEL, respectively, could be transferred to *E. faecalis* FA2-2 and *S. aureus* RN4220, so that the expression of these genes was studied in two different hosts. The β-lactamase encoded by pKU201 was produced constitutively in both enterococcal and staphylococcal hosts, probably because of the lack of regulatory genes as previously reported (39, 40). In contrast, the enzyme encoded by pYN104 was produced constitutively in an enterococcal host but was produced inducibly in a staphylococcal host (46-fold increase after induction), as with the enzyme encoded by pI258. Moreover, the β-lactamase gene carried on pKU101, which is derived from pI258, showed constitutive production of the enzyme in an enterococcal host. These findings strongly suggest that constitutive β-lactamase production in *E. faecalis* is attributable to a factor(s) besides the absence of functional regulatory genes that was caused by the deletion of DNA sequences (40), the insertion of an IS*256*-like element within *blaR1* (23, 26), or mutations in *blaR1* and *blaI* (33). There are some possible factors which might be implicated in this constitutive β-lactamase production in *E. faecalis*. First, the staphylococcal regulatory genes could not function in the enterococcal background. Host-dependent gene product function has been described for other systems. When *mecA*, which codes for PBP2′ of methicillin-resistant *S. aureus*, is introduced into *E. coli*, PBP2′ is nonfunctional despite correct localization to the membrane (10). Second, *blaR2*, which is presumed to be the third regulatory gene in *S. aureus* (4) and *B. licheniformis* (3), might be absent in *E. faecalis*. However, the nature of the *blaR2* gene product(s) and the location of *blaR2* on the chromosome are unknown. In addition, it is not known yet whether *blaR2* is on the chromosome of *E. faecalis*. Third, the low level of β-lactamase production in *E. faecalis*, whether induced or noninduced, might indicate that the *E. faecalis* RNA polymerase does not bind very well to this promoter, and perhaps there is not much transcript made. This might also explain the lower level of production of enzyme in FA2-2(pKU201) than in RN4220(pKU201).

RN4220(pKU201) constitutively produced lower levels of enzyme than induced RN4220(pYN104) and RN4220(pI258). This could have been due to differences in plasmid copy number, a mutation in the promoter, or other factors. Further studies designed to sequence the promoter region of the enterococcal β-lactamase gene using products amplified by PCR with the primer pair P1-P2 are in progress. Moreover, RN4220(pKU101) produced about half the amount of enzyme that was produced by RN4220(pI258) after induction, although the *bla* gene on pKU101 is derived from pI258. This is due to instability of pKU101 after induction (personal observation).

In conclusion, we have demonstrated that the β-lactamase encoded by pYN104 and pKU101 shows constitutive expression in *E. faecalis* but inducible expression in *S. aureus*. The constitutive β-lactamase production in *E. faecalis* appears to be due not only to the absence of functional regulatory genes but also to some other, unknown factor(s).

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