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Genes Involved in the Regulation of β-Lactamase Production in Enterococci and Staphylococci

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In enterococci, the structural gene for β-lactamase (*blaZ*) is identical to *blaZ* from *Staphylococcus aureus*. However, in the enterococci studied to date, β-lactamase is produced constitutively, whereas in staphylococci it is often inducible. Recent reports have revealed the presence of two adjacent genes upstream of the staphylococcal *blaZ* thought to be the antirepressor (*blaR1*) and repressor (*blaI*) genes. In the present study, β-lactamase expression mutants of the staphylococcal β-lactamase plasmid pI524 were generated by transposon mutagenesis with the transposon Tn*917*. Tn*917* insertions upstream of *blaZ* in either *blaR1* or *blaI* resulted in constitutive β-lactamase production, indicating that the repressor function is lost with insertion of Tn*917* into either gene. This finding supports the concept that the staphylococcal β-lactamase regulatory genes are encoded on a polycistronic mRNA. The corresponding region upstream of the enterococcal *blaZ* from *Enterococcus faecalis* HH22 was sequenced and compared with the staphylococcal *blaR1* sequence. The two sequences were identical for 893 nucleotides, and then the sequences diverged completely. Therefore, in strain HH22, only 51% of the putative antirepressor gene is present and the repressor gene is also absent. In conclusion, constitutive β-lactamase production in HH22 appears to be due to a lack of the regulatory genes *blaR1* and *blaI* which regulate expression of *blaZ* in staphylococci.

Penicillinase production among gram-positive bacteria is usually inducible; that is, enzyme levels increase manyfold over baseline levels upon exposure to beta-lactam antibiotics. Of the major β-lactamase producers among gram-positive organisms, namely, *Bacillus*, *Staphylococcus*, and *Enterococcus* spp., the *Bacillus* induction mechanism has been studied in the greatest detail. It has been shown through DNA nucleotide sequence determination and deletion analysis that β-lactamase synthesis in *Bacillus licheniformis* is under the control of a repressor (*penI*) and an antirepressor (*penJ*) (2–4). *penI* is located 5′ to the promoter for the β-lactamase gene (*penZ*) and is transcribed in the opposite direction. The start of *penJ* is located 2 bases downstream of the termination codon of *penI* and is translated along with *penI* from a polycistronic mRNA (11). On the basis of the predicted amino acid sequence of *penJ*, the protein is thought to be a transmembrane protein, with the amino-terminal portion in the cytoplasm and the carboxy-terminal portion facing outside the cell (3, 4); further studies have shown that the carboxy-terminal domain of the antirepressor protein can bind beta-lactam antibiotics and has high sequence homology with the OXA-2 β-lactamase of *Salmonella typhimurium* (15). On the basis of these findings, it has been speculated that the antirepressor is a sensory transducer protein which binds the beta-lactam antibiotic and then delivers the signal from outside of the cell to the inside to begin β-lactamase production (4). Less is known about β-lactamase induction in *Staphylococcus* spp. Recently, Rowland and Dyke (10) and Wang et al. (18) sequenced the putative regulatory region of the staphylococcal β-lactamase gene. They found two open reading frames upstream of *blaZ*. These open reading frames are thought to be the staphylococcal repressor (*blaI*) and antirepressor (*blaR1*) on the basis of the predicted amino acid sequence homology with the *B. licheniformis* repressor and antirepressor. The staphylococcal repressor and antirepressor proteins have 39 and 27% identities, respectively, with the corresponding *Bacillus* proteins but are arranged in the reverse order on the DNA strand. The hydrophobicity plots for the two antirepressors are basically the same, and the carboxy-terminal domain of the staphylococcal protein, like that of the *Bacillus* protein, shows significant homology with the OXA-2 β-lactamase (10). However, there are as yet no reports of functional studies with the staphylococcal regulatory genes.

β-Lactamase production in enterococci is a fairly recent discovery (7). The nucleotide sequence of *blaZ* from *Enterococcus faecalis* HH22 has been determined, and the predicted amino acid sequence is identical to the published sequences of three of four staphylococcal type A β-lactamases (23). Although the β-lactamases of enterococci and some staphylococci are identical, regulation of β-lactamase production is different. In enterococci, the enzyme is produced constitutively, as opposed to inducible expression in staphylococci. An explanation for the difference in expression was not evident by DNA sequence analysis since the two genes had identical promoter and operator regions and expression of the enterococcal enzyme could be repressed by the staphylococcal repressor in complementation studies (23). In the study described here, we sequenced the region farther upstream of the HH22 β-lactamase gene to determine whether the antirepressor and repressor genes are present. We also used the transposon Tn*917* to construct β-lactamase regulatory mutants of the staphylococcal β-lactamase plasmid pI524 to investigate the functions of the regions thought to be the antirepressor and repressor genes on the basis of DNA sequence determination.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. *Staphylococcus aureus* RN4 (5) is a naturally occurring staphylococ-

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cal isolate containing the wild-type β-lactamase, heavy metal resistance plasmid pI524. *S. aureus* RN1 (9) is a plasmid-free strain used in the β-lactamase assays as a negative control. *S. aureus* RN2677 (8) is a plasmid-free strain which was used in the transposon mutagenesis studies. *S. aureus* RN4220 (8) containing the temperature-sensitive plasmid pTV1-ts (22) and the staphylococcal serogroup B bacteriophage φ11 (8) were kindly provided by Gordon Archer.

DNA sequencing. The 1.3-kb region of DNA directly upstream of the enterococcal β-lactamase gene was subcloned into plasmid pUC18 (21). Nested deletions were generated with exonuclease III (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by a previously described method (12). Exonuclease digestions were performed at 30°C, with aliquots taken every minute. The nucleotide sequences of the deletion clones were determined by the Sanger dideoxynucleotide chain termination method by using the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio). The sequence was submitted to GenBank under accession number L07892.

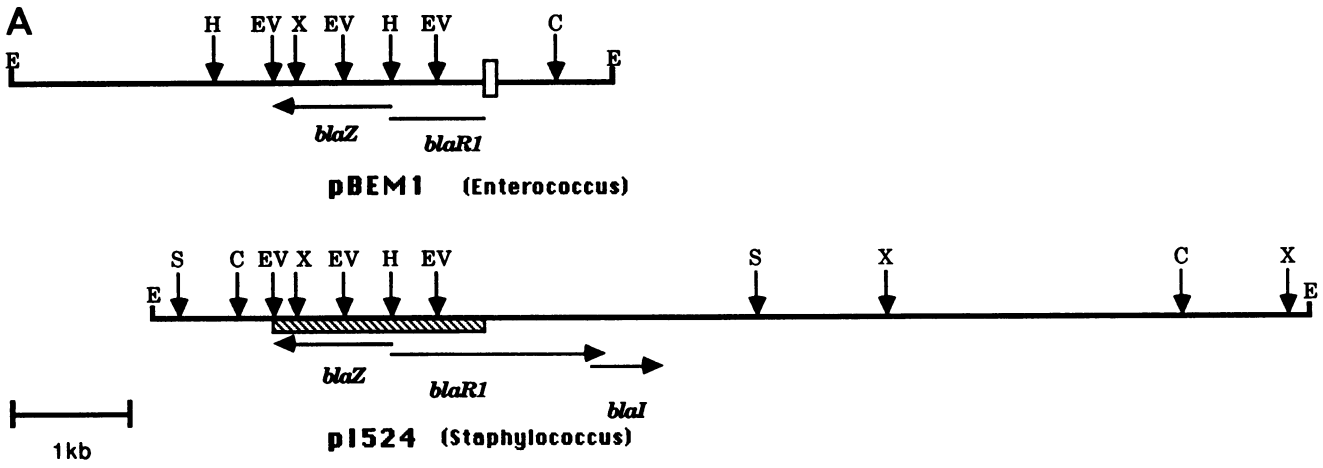
DNA isolation. pI524 was isolated from strain RN4 by a dye-buoyant density gradient centrifugation procedure which has been described previously (19). Minipreparation plasmid isolation from staphylococci was done by a method modified from that of Goering and Ruff (1). Staphylococcal cells grown overnight in 5 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) were pelleted for 10 min at 5,000 rpm. The pellet was resuspended in 0.4 ml of STET buffer (0.8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris [pH 8.0]), and the mixture was transferred to microcentrifuge tubes. After the addition of 7 μl of lysostaphin (5,000 U/ml in 0.05 M Tris [pH 7.5]–0.15 M NaCl; Sigma Chemical Co., St. Louis, Mo.), the cells were incubated at 37°C for 12 min. The tubes were placed in a boiling water bath for 1 min and were then immediately centrifuged for 8 min at room temperature. The pellet was carefully removed with a toothpick, and 100 μl of 10 M ammonium acetate and 0.5 ml of isopropyl alcohol were added to the supernatant to precipitate the DNA. After incubation at room temperature for 15 min, the DNA was pelleted for 30 min. The pellet was resuspended in 250 μl of TE (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]), 5 μl of RNase (10 mg/ml in water; Sigma Chemical Co.) was added, and then the mixture was incubated at 37°C for 30 min. The solution was extracted once with an equal volume of chloroform-phenol-isoamyl alcohol (25:24:1), and the DNA was precipitated again with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of ethanol. The DNA pellet was resuspended in 35 μl of water.

Generation of pI524::Tn*917* β-lactamase regulatory mutants. pTV1-ts (22) is a temperature-sensitive plasmid which was used in the present study as the delivery vehicle for transposon Tn*917* (13). The temperature-sensitive replication defect allows the plasmid to replicate at 30°C but not at 42°C. pTV1-ts has a chloramphenicol (Cm) resistance determinant, in addition to the erythromycin (Em) resistance determinant carried by Tn*917*. To deliver Tn*917* in a background with pI524, pI524 was first used to transform RN2677 protoplasts by the procedure of Wirth et al. (20). RN2677 was selected as the host because RN4 (the native host of pI524) could not be infected with φ11. RN4220 harboring pTV1-ts was infected with bacteriophage φ11 by previously described methods (17), and the phage lysate was then used to infect RN2677 carrying plasmid pI524. The infected cells were plated onto Mueller-Hinton agar (MHA; Difco Laboratories) containing Cm at 20 μg/ml (for selection of pTV1-ts) and cadmium at 8 μg/ml (for selection of pI524) at the permissive temperature (30°C). Colonies growing on the Mueller-Hinton plates were screened by plasmid analysis to confirm the presence of both plasmids.

RN2677(pI524, pTV1-ts) was grown overnight at 30°C on MHA with Cm and cadmium. Cells were resuspended in saline to an optical density at 550 nm of 0.3, and 20 μl of adjusted culture was added to 20 ml of Penassay broth (Difco Laboratories) with 1 μg of Em per ml (for transposition induction). The culture was incubated for 16 h at the nonpermissive temperature (42°C). The overnight culture was diluted and plated onto MHA containing Em (20 μg/ml; selection for Tn*917*). Colonies growing on the Em plates were also tested for the loss of Cm resistance to be sure that pTV1-ts was lost. Of 400 Em^r colonies tested, 91% were Cm^s.

β-Lactamase assays. Em^r Cm^s colonies were screened for β-lactamase regulatory mutations with a polyvinyl alcohol (PVA)-iodine β-lactamase plate assay (14). In the assay, PVA (0.75%; Sigma Chemical Co.) was incorporated into the agar. Colonies were placed with a toothpick onto a PVA plate and a corresponding PVA-methicillin (0.5 μg/ml for induction of β-lactamase production) plate, and the plates were incubated overnight at 37°C. Both plates were flooded with a KI-I_2 solution so that a blue I_2-PVA complex was formed. After draining the plate, a 1% solution of penicillin was added and penicilloic acid was produced by the action of the β-lactamase on penicillin. The penicilloic acid reacted with the iodine, resulting in a clearing around the β-lactamase-producing colonies. The phenotype (constitutive or inducible) of the colony could be determined by comparing the size of the clearing on the plate containing methicillin with that of the clearing on the plate without methicillin. Constitutive and inducible controls previously described (23) were included on each plate for comparison. In addition to the plate assay, a more sensitive spectrophotometric assay with nitrocefin was used in order to determine the quantity of β-lactamase produced by the regulatory mutants.

Mapping the Tn*917* transpositions in plasmid pI524. Staphylococcal plasmid DNA from Em^r Cm^s colonies which showed β-lactamase regulatory differences in the PVA plate assay was isolated by the minipreparation procedure described above. Tn*917* insertions into plasmid pI524 were mapped by using restriction endonuclease *Eco*RI (six sites in pI524 and none in Tn*917*), *Sal*I (six sites in pI524 and one site in Tn*917*), and *Xba*I (six sites in pI524 and two sites in Tn*917*). More detailed mapping with a few selected pI524::Tn*917* mutants was carried out with the enzymes *Sfu*I and *Xho*I. Restriction enzyme digestions were carried out according to the instructions of the manufacturer (Boehringer Mannheim Biochemicals). Digested plasmid DNA was electrophoresed through 0.7% agarose gels in 1× Tris-borate running buffer (10× TBE is 0.89M Tris base, 0.025 M EDTA, 0.89 M boric acid [pH 8.0]). DNA fragments were transferred from agarose gels to Hybond-N membranes (Amersham Corp., Arlington Heights, Ill.) by the method of Southern (12). To locate the position of Tn*917* on pI524, a DNA probe for Tn*917* was used. It consisted of a 4.2-kb *Xba*I-*Hpa*I fragment from pTV1-ts which contained only internal transposon sequences. The probe was made by cloning the 4.2-kb fragment from pTV1-ts into pUC18, and this plasmid is referred to as pBEM100 (21). To isolate the probe fragment, digested DNA was separated on a 0.7% agarose gel in 1× TBE buffer, and the probe fragment was excised and then extracted from the agarose with Prep-A-Gene (Bio-Rad Laboratories, Richmond, Calif.). The DNA



B

E. faecalis 5'...CATTCATTTAATGGTAAAAAGTCATTACTCAAAAGAAGATTAATT
S. aureus 5'...CATTCATTTAATGGTAAAAAGTCATTTCTCAAAAGAAGATTAATT

E. faecalis AATATGGTTCTGTTGCAAAGTTTTAAATAAAGAATAAAATCCCTT
S. aureus AATATAAAAGAAGCCAATCTGAAAAAACAGTCCAAATTAATTCTA

E. faecalis ACGGTATCTATGATTTAAGCTGGGATTCCCAATAATACCTTGATT
S. aureus ATTTTTATTTGTATTTTTACTTTTTTGCTCATGGTAATTCAAAGC

E. faecalis TCAGTACAGACCGAAAACC...3'
S. aureus CAGTTTTTGATGGGGCAAT...3'

FIG. 1. (A) Restriction enzyme maps of enterococcal and staphylococcal β-lactamase regions (dark lines). pBEM1 contains the 5.1-kb *Eco*RI fragment encoding *blaZ* from *E. faecalis* HH22. pI524, a naturally occurring β-lactamase plasmid from *S. aureus*, contains a 9.7-kb *Eco*RI fragment encoding the β-lactamase structural and regulatory genes. The β-lactamase gene *blaZ*, antirepressor gene *blaR1*, and repressor gene *blaI* are indicated by arrows below the enzyme maps. The direction of the arrows represents the direction of transcription. The hatched box on the pI524 map indicates the region of identity shared between the enterococcal and staphylococcal (10) β-lactamase regions. The point of sequence divergence between the two *blaR1* genes is shown with an open box on the map of pBEM1. Although the region downstream of the two β-lactamase genes has not been sequenced, the enzyme maps are clearly different in this area. Restriction enzyme sites are indicated by C (*Cla*I), E (*Eco*RI), EV (*Eco*RV), H (*Hin*dIII), S (*Sal*I), and X (*Xba*I). (B) A partial DNA sequence of the enterococcal and staphylococcal (10, 18) *blaR1* genes. The area of sequence divergence is indicated by an arrow. The sequence shown here corresponds to the region contained within the open box on pBEM1 in panel A.

was radioactively labeled by using the Random Primed DNA Labeling kit (Boehringer Mannheim Biochemicals) and [α-^{32}P]dCTP. Hybridization was performed under stringent conditions (42°C and 50% formamide) with the prehybridization and hybridization solutions suggested by Amersham Corp.

RESULTS

Nucleotide sequence of enterococcal putative regulatory region. We have previously reported the nucleotide sequence of the *E. faecalis* HH22 β-lactamase structural gene and the sequence 70 nucleotides upstream of the gene (proximal to the β-lactamase promoter) (23). In the current study, the 1-kb region directly upstream of the enterococcal β-lactamase promoter was sequenced. This upstream sequence was compared with the corresponding region of the previously published staphylococcal β-lactamase-encoding transposon Tn*552* (10). The restriction enzyme maps of the two β-lactamase-encoding fragments and the regions of sequence homology are shown in Fig. 1A. In the region directly upstream of the β-lactamase gene, the enterococcal sequence was identical to the staphylococcal sequence for 893 nucleotides, after which the two sequences diverged completely. The sequences of the two genes before and after the point of divergence are shown in Fig. 1B. The point of divergence occurred in the region which corresponded to the staphylococcal antirepressor gene (*blaR1*). According to the nucleotide sequence, the enterococcus has only 51% of *blaR1*. In HH22, *blaI* appears to be missing, and this is in agreement with results of a previous study which showed that genomic DNA from HH22 did not hybridize with a DNA probe to *blaI* (16).

Tn*917*-generated constitutively producing β-lactamase mutants of pI524. Four hundred Em^r and Cm^s colonies were selected for testing by the PVA plate assay for differences in β-lactamase regulation. Thirty of the 400 colonies showed a difference in zone size between the uninduced and induced (containing methicillin) plates. Twenty of the 30 (66%) demonstrated a constitutive phenotype; that is, they had large zones of the same size on both plates; 5 of the 30 colonies (17%) had no zones on either plate and were considered to be β-lactamase negative and not inducible. The remaining five colonies (17%) were inducible (larger zone on methicillin plate) but, on the basis of their zone sizes, showed differences in the amount of β-lactamase they produced. Restriction enzymes were used to map the Tn*917*

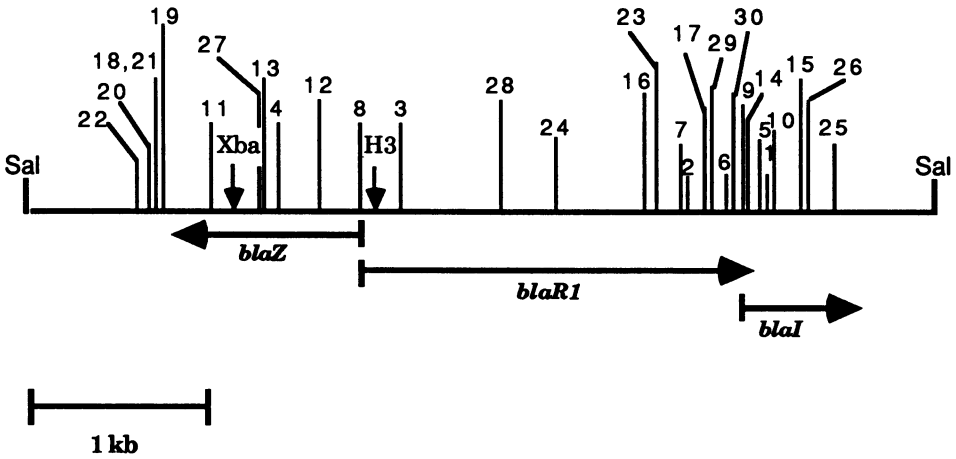


FIG. 2. Tn*917* insertions into the β-lactamase structural and regulatory genes of pI524. The Tn*917* insertions are shown on the 5-kb *Sal*I fragment from pI524 encoding *blaZ*, *blaR1*, and *blaI* and are numbered 1 to 30. Insertions 1 to 3, 5 to 10, 14 to 17, 23 to 26, and 28 to 30 resulted in constitutive expression of β-lactamase, whereas insertions 4, 11 to 13, and 27 were β-lactamase negative. Insertions 18 to 22 did not map in the β-lactamase genes, and these mutants were not studied further.

insertions in the regulatory mutants of pI524, which are shown in Fig. 2. In colonies with the constitutive phenotype, the transposon was localized to *blaR1* or *blaI*. The β-lactamase-negative phenotype mapped in the structural β-lactamase gene. In the five colonies that were inducible but that produced different amounts of enzyme, the transposon mapped outside the β-lactamase region. These colonies were not studied further.

***blaR1* and *blaI* mutants in β-lactamase assay.** Enzyme levels in three transposon-generated constitutive β-lactamase mutants, two with an insertion in *blaR1* (pI524::Tn*917*-24 and pI524::Tn*917*-28) and the other with an insertion in *blaI* (pI524::Tn*917*-5), were determined in a spectrophotometric β-lactamase assay. The results are shown in Table 1. pI524::Tn*917*-24 and pI524::Tn*917*-28 (transpositions in *blaR1*) showed only a 2-fold increase after induction, whereas the wild-type plasmid pI524 showed a 21-fold increase. The results for pI524::Tn*917*-5 (*blaI*) were similar to those for transpositions in *blaR1*. There was very little increase in the amount of enzyme after induction (1.5-fold), although preinduction enzyme levels were higher than those in the *blaR1* mutants. In pI524::Tn*917*-4, the transposon was localized to *blaZ*, and this strain did not produce any detectable β-lactamase.

TABLE 1. β-Lactamase activities of pI524::Tn*917* *bla* regulatory mutants

Plasmid	Tn*917* location	β-Lactamase activity[a]		Induction ratio[b]
		Uninduced	Induced	
pI524	None	6.3	134	21
pI524::Tn*917*-24	*blaR1*	95	183	2
pI524::Tn*917*-28	*blaR1*	70	144	2
pI524::Tn*917*-4	*blaZ*	0	0	0
pI524::Tn*917*-5	*blaI*	123	184	1.5

[a] Expressed as nanomoles of nitrocefin hydrolyzed per minute per milliliter of standardized culture (all cultures were adjusted to an optical density at 600 nm of 0.8). Each value represents the average of two determinations.

[b] Induction ratio = induced activity/uninduced activity.

DISCUSSION

The first β-lactamase-producing enterococcal strain was reported in 1983 (7), and since then many other β-lactamase-producing enterococcal strains have been found in various geographical regions (6). Recent studies (23) have shown that the DNA sequence of *blaZ* from *E. faecalis* HH22 is identical to the sequences of the staphylococcal type A β-lactamases from pPC1 and pS1 and differed by only 2 base changes with the type A gene from pI258, which resulted in silent amino acid substitutions. However, though the staphylococcal and enterococcal enzymes are the same, the β-lactamases are regulated differently in the two bacteria. We could not attribute the difference in regulation between the two genes to nucleotide changes in the promoter, operator, or structural gene or to host differences (23). Another possible explanation was mutated or absent regulatory genes in the enterococcus. We previously noticed that a short segment of nucleotides directly upstream of the enterococcal β-lactamase promoter was identical to sequences of the staphylococcal putative antirepressor gene published by Rowland and Dyke (10) and Wang et al. (18). Thus, HH22 did contain sequences identical to those of the putative staphylococcal antirepressor gene, but we had not sequenced far enough upstream of the enterococcal *blaZ* to determine how much of the antirepressor was present. In the present study, we sequenced farther upstream of *blaZ* to find out how much of the gene was present. The sequence upstream of the enterococcal *blaZ* gene was identical to the corresponding region in the staphylococcal sequence for 893 nucleotides. After this region, the two sequences diverged completely. The 893 nucleotides code for only 51% of the antirepressor gene. The repressor gene was not contiguous to this truncated *blaR1*. Moreover, a probe for the staphylococcal repressor gene did not hybridize with HH22 DNA (16). In this case, the constitutive nature of the enterococcal β-lactamase gene from *E. faecalis* HH22 is apparently due to the absence of a functional repressor and antirepressor. All previous findings indicate that the enterococcal enzyme originated from a staphylococcal source. However, studies have shown that staphylococcal and enterococcal β-lactamase plasmids are very different (19), which means that the

gene is not present in enterococci on one of the previously well described staphylococcal β-lactamase plasmids. Transfer to enterococci may have occurred through either a recombination or a transposition event, and at some point, loss of the regulatory genes occurred. Most of the other reported β-lactamase-producing enterococci are also constitutive producers of penicillinase (personal observation), although it is not known whether the regulatory genes are present in these other strains.

In the present study, we also investigated the regulation of β-lactamase expression in staphylococci. The nucleotide sequences of the staphylococcal regulatory genes *blaR1* and *blaI* have been reported by two different laboratories (10, 18), but functional studies which relate these genes to regulatory function have not been performed. Transposon insertions generating β-lactamase-negative mutants mapped in *blaZ*, as would be expected. In the inducible strains with different enzyme levels, the transposon mapped outside the β-lactamase region, which could suggest they were normal inducible isolates but were picked as mutants by accident because of the crude method chosen for mutant screening. In the constitutive β-lactamase-producing mutants, the transposon insertions mapped to either *blaR1* or *blaI*, indicating that these regions are involved in regulating enzyme expression. Rowland and Dyke (10) and Wang et al. (18) suggested that *blaR1* and *blaI* are on a polycistronic mRNA because a separate promoter was not found for *blaI*, and our new data support their findings. Although insertions in *blaR1* and *blaI* showed some differences in the amount of enzyme produced before induction, the difference was small and could be due to some repressor being produced from another promoter or to variability in the assay.

In conclusion, the β-lactamase gene in the staphylococcus is regulated by upstream sequences previously suggested to code for the antirepressor and repressor. The constitutive β-lactamase production in *E. faecalis* HH22 appears to be due to missing regulatory genes.

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