The Gene Encoding the Low-Affinity Penicillin-Binding Protein 3r in Enterococcus hirae S185R Is Borne on a Plasmid Carrying Other Antibiotic Resistance Determinants

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ABSTRACT

Two plasmid-derived NcoI DNA fragments of 14 and 4.5 kb, respectively, have been isolated from the multidrug-resistant strain Enterococcus hirae S185R and analyzed. The 14-kb fragment contains two inverted (L and R) IS1216 insertion modules of the ISS1 family. These modules define a Tn5466 transposon-like structure that contains one copy of the methylase-encoding ermAM conferring erythromycin resistance and one copy of the adenylyl-transferase-encoding aadE conferring streptomycin resistance. Immediately on the left side of IS1216L there occurs a copy of pbp3r encoding the low-affinity penicillin-binding protein (PBP) PBP3r, itself preceded by a psr-like gene (psr3r) that controls the synthesis of PBP3r, ermAM, aadE, and the transposase gene (tnp) of IS1216R have the same polarities, and these are opposite those of psr3r, pbp3r, and the tnp gene of IS1216L. The 4.5-kb fragment is a copy of the 4.5-kb sequence at the 5′ end of the 14-kb fragment, although it is not a restriction product of the 14-kb fragment. It contains three genes with the same polarity: psr3r, pbp3r, and tnp in an IS1216 element. Because of the very high degree of identity (99%) with the chromosomal psrfm and pbp5fm genes of Enterococcus faecium D63R, it is proposed that both the psr3r and pbp3r genes were transferred from an E. faecium strain and inserted in a plasmid of E. hirae. E. hirae is the first known bacterial species in which a low-affinity PBP-encoding gene has been found to be plasmid borne.

The penicillin-binding proteins (PBPs) are membrane-bound serine transferases involved in wall peptidoglycan synthesis. Penicillin inactivates the PBPs in the form of stable serine-linked penicilloyl enzymes (14).

Resistance to penicillin in the absence of β-lactamase production can be mediated by PBPs. Enterococci gain resistance either by the overproduction of a constitutive low-affinity PBP (11, 12) or by a further reduction of the affinity of that PBP (19, 41). In some resistant enterococcal strains, two low-affinity PBPs may be present at the same time (27, 38). Resistance among staphylococci occurs by acquisition of a single PBP which has a low affinity for all the usual β-lactam antibiotics (5, 16) and has probably originated from another species within the genus Staphylococcus (39). The low-affinity PBPs confer penicillin resistance because they are able to perform the functions needed for wall peptidoglycan synthesis under conditions in which all the other PBPs are inactivated (5, 11, 16, 38).

Enterococcus hirae ATCC 9790 is moderately resistant to benzylpenicillin and produces a chromosome-encoded low-affinity PBP, PBP5 (9, 11). Overproduction of PBP5 in the highly penicillin-resistant laboratory mutant E. hirae R40 has been related to the inactivation of a negative regulatory gene psr that is located immediately upstream from pbp5 and that encodes a 33-kDa protein (20, 22).

E. hirae S185, a clinical isolate from pig intestine, produces two low-affinity PBPs, PBP5 and PBP3r (27, 28). Chemical mutagenesis of E. hirae S185 has led to the isolation of a penicillin hypersusceptible mutant, E. hirae SS22, which still produces very low levels of PBP5 but which has lost the capacity to produce PBP3r (28). Conversely, six successive passages of E. hirae S185 in broth containing benzylpenicillin (32 µg/ml) has led to the isolation of a penicillin-resistant mutant, E. hirae S185R, which selectively overproduces PBP3r (28). PBP5 and PBP3r of E. hirae S185 and S185R are structurally related to each other (78.5% amino acid sequence identity) and to the low-affinity PBP, PBP2′, of methicillin-resistant Staphylococcus aureus (33% amino acid identity).

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sequence identity). In addition, PBP3r is almost identical (99.8% amino acid sequence identity) to PBP5fm found in Enterococcus faecium strains.

Comparison of the E. hirae wild-type strains and mutants with respect to their susceptibilities to benzylpenicillin and erythromycin suggested that pbp3r, but not pbp5, might be linked to an erythromycin resistance determinant, erm (28). The aim of the study described in this paper is to show that E. hirae S185R possesses a plasmid-borne pbp3r gene linked to two resistance determinants, those for resistance to erythromycin and streptomycin, respectively.

(The work described in this paper is part of a dissertation presented by D.R. in partial fulfilment of a Ph.D. at the University of Liège.)

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and enzymes. E. hirae S185, SS22, and S185R were grown as described previously (27, 28). Strain S185 was shown to belong to the E. hirae species by using the API 20 Strep and API Staph test kits (BioMérieux, Marcy l’Etoile, France) and by analyzing the PBP patterns in comparison with those of the type strains of different species. In all cases, strain S185 behaved exactly like the E. hirae type strain, ATCC 9790. By a similar approach, strains D63 and D63R were shown to belong to the species E. faecium.

The plasmids and E. coli hosts used in this study are listed in Table 1. Three different plasmids (pDML501, pDML508, and pDML510) which each contained an insert from E. hirae S185R were analyzed in the course of this study. pDML501 and pDML508 had an NcoI fragment, and pDML510 had an EcoRI fragment (28). The 4.5-kb inserts of pDML501 and pDML510 and the 14-kb insert of pDML508, obtained by restriction digestion and agarose gel electro-phoresis, each hybridized with oligonucleotide O1 (see below) derived from pbp3r. In contrast, the 14-kb insert of pDML508, but not the 4.5-kb inserts of pDML501 and pDML510, hybridized with oligonucleotide O4 (erm) (see below), showing that pbp3r and erm are somehow linked to each other in the larger 14-kb DNA fragment.

The restriction endonucleases (Boehringer, Mannheim, Germany) and the AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) were used as recommended by the manufacturers.

DNA preparations. Plasmids of Escherichia coli were prepared as described previously (28) or by using the Nucleobond kit (Macherey and Nagel, Düren, Germany). Plasmids of E. hirae S185, SS22, and S185R each were prepared by using the method of Anderson and McKay (1), which was modified as follows. Cells were grown in 100 ml of brain heart medium for 4 to 5 h (optical density at 550 nm = 2), harvested by centrifugation, and resuspended in 5 ml of 10 mM Tris-HCl (pH 8.0)-1 mM EDTA-25% (wt/vol) sucrose-7 mg of lysozyme per ml-1 µg of mutanolysin per ml, and the suspension was incubated at 37°C for 15 min. The lysate was centrifuged for 10 min at 9,000 × g, and the pellet was resuspended in 1.5 ml of Tris-EDTA buffer. The suspension was supplemented with 1 ml of a 5% (wt/vol) sodium dodecyl sulfate (SDS) solution in Tris-EDTA buffer. After gentle mixing, the solution was incubated for 20 min at 37°C. The alkaline denaturation was performed as described by Currier and Nester (8), followed by phenol-chloroform extractions and ethanol precipitation.

DNA fragments were eluted from the agarose gels by using Sephaglas Band-Prep kits (Pharmacia Biotech, Brussels, Belgium) according to the manufacturer’s recommendations.

Nucleotide sequencing. Nucleotide sequencing was carried out as described by Sanger et al. (33) with M13 universal and reverse oligonucleotides or oligonucleotides complementary to inserts as primers. Denaturation of double-stranded DNA was performed as described by Zhang et al. (40). Sequencing reactions were carried out by using the Sequenase kit (United States Biochemicals, Cleveland, Ohio), the T7 sequencing kit (Pharmacia Biotech, Brussels, Belgium) with [35]SdATP labeling, or the Autoread sequencing kit (Pharmacia Biotech) with fluorescent primers or by the incorporation of fluorescent dUTP. For the sequencing reactions with the Autoread kit, electrophoresis was performed with an ALFexpress DNA sequencer (Pharmacia Biotech) (2).

Oligonucleotides and hybridization. The oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). Their positions are indicated in Fig. 1A and B. Oligonucleotides GCAGGAATGGCATCGAAAAAGGCAG (O1) and GCGG AAATCAGAAGAACACG (O2) started at 193 and 1,861 bp from the ATG of pbp3r, respectively. Oligonucleotide CCGTACGGTTTCTGTGCAAAGTT (O3), designated an IS1216 repeat, started at 80 bp upstream from the ATG of the transposase-encoding gene, tmp. The boxed sequence followed by T is the
sequence of the 18-bp IS1216 repeats. Oligonucleotides GGGCATTTAACGA CGAAACTGGCTA (O4) and ACCTCTGTTTGTAGGGAATTGAAA (O5) started 120 and 283 bp from the ATG of the *ermAM* gene of *Enterococcus faecalis* pAMβ1, respectively. Oligonucleotide O5 was based on the sequence of the complementary strand.

The oligonucleotide probes used for hybridization were labeled at the 3'-OH end with digoxigenin-ddUTP by using the terminal transferase from Boehringer. The hybridizations were performed under stringent conditions at temperatures 4°C below the melting temperature of the probe. Nylon filters were washed twice for 5 min each time at 64°C in 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) containing 0.1% (wt/vol) SDS and twice for 5 min at 50°C in 0.1× SSC containing 0.1% (wt/vol) SDS. Hybridizations were detected by chemiluminescence with Lumigen-PPD provided by Boehringer following the instructions of the manufacturer.

**Slot blot hybridization.** Samples (62.5, 125, 250, and 500 ng) of DNA preparations (denatured at 95°C for 10 min) were deposited on a nylon membrane in a Bio-Dot SF blotting microfiltration unit (Bio-Rad, Richmond, Calif.). The DNA samples were bound to the membrane by exposure to UV light (312 nm) for 3 min. Hybridization with oligonucleotide O1 (pbp3r) was performed as described above. Quantification of the hybridization bands was done by two-dimensional densitometry of pictures taken with a video camera and processed with CAM software (Cybertech-Dalton, Berlin, Germany).

**Homology searches.** Searches of the protein and DNA sequence databases were performed as described by Pearson and Lipman (26) by using the FASTA and TFASTA software packages. Alignments of nucleotide and amino acid sequences were made with BESTFIT according to the algorithm of Smith and Waterman (36) (GCG package).

**Nucleotide sequence accession number.** The EMBL accession number for the sequence of the 4.5-kb *NcoI* insert of pDML501 is X69092, that for the sequence of IS1216 inserted in pDML501 is X81654, and that for the sequence of *ermAMEH*, the *ermAM* found in *E. hirae* S185, is X81655.

### TABLE 1. Plasmids and *E. coli* strains used in this study

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<td>pBR325 with a 4.5-kb <em>NcoI</em> insert from <em>E. hirae</em> S185R&lt;br&gt;Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; pbp3r</td>
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<td>pDML508</td>
<td>pBR325 with a 14-kb <em>NcoI</em> insert from <em>E. hirae</em> S185R&lt;br&gt;Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; <em>erm</em> pbp3r</td>
<td>28</td>
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<td>pDML510</td>
<td>pBR322 with a 4.5-kb <em>EcoRI</em> insert from <em>E. hirae</em> S185R&lt;br&gt;Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; pbp3r</td>
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<td>pDML540</td>
<td>pBR322 with a 7-kb <em>EcoRI</em> insert from <em>E. hirae</em> R40&lt;br&gt;Tc&lt;sup&gt;r&lt;/sup&gt; Ap&lt;sup&gt;r&lt;/sup&gt; pbp5</td>
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<td>DH5α</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; Δ80lacZ M15 (lacZYA-argF) U169 dcoR recA1 endA1 phoA hsdR17 (r&lt;sub&gt;K&lt;/sub&gt;, m&lt;sub&gt;K&lt;/sub&gt;) supE44 χ&lt;sup&gt;-&lt;/sup&gt; thi-1 gyrA96 relA1</td>
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RESULTS

The \(\text{pbp3r}\)-containing 4.5-kb DNA insert of pDML501 possesses a \(\text{psr3r}\) gene and an insertion module IS1216. It was previously shown that \(\text{pbp3r}\) is located in the middle of the 4.5-kb \(\text{NcoI}\) insert of pDML501 (28). Sequencing of the complete insert confirmed the restriction map shown in Fig. 1A. It also led to the identification, 417 bp upstream from \(\text{pbp3r}\), of a 517-bp open reading frame (ORF) that was identical to the 597-bp \(\text{psr}\) gene of \(E.\ faecium\) D63R (\(\text{psrfm}\)) except that it lacked the first 80 bp (41). In all likelihood, the missing 80 bp at the 5’ end of \(\text{psr3r}\) of the 4.5-kb \(\text{NcoI}\) fragment of pDML501 was lost by restriction during cloning since a complete 597-bp gene, called \(\text{psr3r}\), was subsequently identified at one end of a 4.5-kb \(\text{pbp3r}\)-containing EcoRI fragment cloned in pDML510 from a genomic library of \(E.\ hirae\) S185R (28). The sequence of \(\text{psr3r}\) was identical to that of \(\text{psrfm}\). In both cases, the ATG codons each occurred 10 bp downstream from the first nucleotide of the EcoRI site and 80 bp upstream from the first nucleotide of the \(\text{NcoI}\) site (41).

The identity also extended over the 417-bp intergenic region between \(\text{psr3r}\) and \(\text{pbp3r}\) and the 219-bp segment downstream from the \(\text{pbp}\) genes (Fig. 2). The intervening 417-bp sequence found in the \(\text{NcoI}\) insert of pDML501 consisted of two uneven parts. The sequence of a 331-bp segment was identical to that of the 331-bp segment identified immediately downstream from \(\text{psrfm}\). The sequence of the remaining 86-bp segment was identical to that of an 86-bp segment immediately upstream from the \(\text{pbp5fm}\). Hence, with the exception of a 1.2-kb fragment of unknown origin inserted between these two sequences in \(E.\ faecium\) D63R (8a, 41), the sequences of the \(E.\ hirae\) S185R and the \(E.\ faecium\) D63R DNA fragments were identical over a length of 3,267 bp (Fig. 2).

An insertion module designated IS1216 was also identified in the 4.5-kb \(\text{NcoI}\) insert of pDML501. IS1216 started 637 bp downstream from the 3’ end of \(\text{pbp3r}\) and shared 65% nucleotide sequence identities with the methicillin-resistant strain \(S.\ aureus\) IS257 sequence (also called \(\text{IS431mec}\) [4]) and 75 to 76% sequence identities with the \(Lactococcus\ lactis\) IS946 (31) and ISS1 sequences (29), and its transposase-encoding gene \(\text{tnp}\) had the same orientation as \(\text{pbp3r}\).

Finally, one may mention that the EcoRI site at one end of the EcoRI insert of pDML510 was only 82 bp away from the inverted repeat (IR) sequence of one IS1216 element. This latter element was partially sequenced. A 411-bp sequence was found to be identical to the sequence occurring immediately upstream from the EcoRI site at the 3’ end of the 4.5-kb \(\text{NcoI}\) insert in pDML501 (as depicted in Fig. 1A). Hence, in all likelihood, the \(\text{NcoI}\) and EcoRI inserts of pDML501 and pDML510, respectively, were identical.

The 14-kb DNA insert of pDML508 has a \(\text{pbp3r}\) gene adjacent to a transposon-like structure that carries erythromycin and streptomycin resistance markers. The 14-kb insert of pDML508, the restriction map of which is also shown in Fig. 1B, comprises three regions, as described below.

(i) The 4.5-kb terminal portion of the 6.5-kb \(\text{NcoI-EcoRI}\) fragment at the 5’ end of the insert (as defined in Fig. 1B) had the same restriction map as the 4.5-kb \(\text{NcoI}\) insert of pDML501 except that one \(\text{ClaI}\) site was not detected by restriction analysis. Upon PCR amplification with oligonucleotides O2 (\(\text{pbp3r}\)) and O3 (IS1216 repeats), both the \(\text{NcoI-EcoRI}\) fragment of pDML508 and the \(\text{NcoI}\) insert of pDML501 generated several 0.8-kb \(\text{IS1216}\) copies (because O3 annealed on both inverted repeats) and one 1.6-kb DNA fragment that encompasses the 637 bp of intervening \(\text{pbp3r-IS1216}\) sequence. The restriction fragments of the PCR products of IS1216 and the 1.6-kb fragment were those expected from the restriction map (Fig. 1B). As a consequence, the 4.5-kb terminal portion of the 5’ region of the pDML508 14-kb insert was a duplicate of the 4.5-kb \(\text{NcoI}\) fragment of pDML501 except that it lacked one \(\text{ClaI}\) site and the EcoRI and \(\text{NcoI}\) sites which, in the 4.5-kb insert, were immediately downstream from IS1216. However (as also shown in Fig. 1B), the 14-kb insert of pDML508 possessed an EcoRI site 6.5 kb downstream from the 5’ end. Partial sequencing of that 6.5-kb fragment showed that the \(\text{NcoI-EcoRI}\) fragments of the pDML508 and pDML501 inserts had identical 809-bp IS1216 elements and \(\text{psr3r}\) sequences (300 bp) but different 3’ end sequences.

(ii) The 2.6-kb EcoRI-HindIII central fragment of the 14-kb insert of pDML508 possessed the unique \(\text{ClaI}, \text{SphI}, \text{PvuII},\) and \(\text{AccI}\) sites. It did not hybridize with any of the \(\text{pbp3r}\), IS1216, and \(\text{erm}\) probes.

(iii) The 4.6-kb HindIII-\(\text{NcoI}\) fragment of the 3’ region of the 14-kb insert of pDML508, shown in Fig. 1B (cloned in pUCBM20; plasmid pDML528), did not hybridize with oligonucleotide O1 (\(\text{pbp3r}\)) but it hybridized with oligonucleotides O3 (IS1216 repeats) and O4 (\(\text{erm}\)). The HindIII-\(\text{NcoI}\) fragment was restricted with Seal and with \(\text{ScaI-PvuII}\), respectively, and the products were subcloned into pUCBM20 or pGEM3-Zf(+). Double-stranded DNA sequencing of the subclones led to the identification of the insertion module IS1216R, the erythromycin resistance determinant, \(\text{erm}\) (consisting of the methylase-encoding gene and the flanking ORFs,
ORFs 1 and 3), and the streptomycin resistance determinant, *aad*. These ORFs each had the same orientation opposite that of *mp* in IS1216 of the 6.5-kb *NcoI-EcoRI* fragment.

**FIG. 1.** Restriction maps of the 4.5-kb insert of pDML501 (A) and the 14-kb *NcoI* insert of pDML508 (B). The restriction and probe hybridization sites shown are only those necessary to understand the products generated by restriction and PCR experiments. The brackets above the maps represent the PCR products. The restriction sites are indicated by the following letters: A, *AccI*; C, *Clal*; H, *HindIII*; and S, *Seal*. Broken arrows identify the DNA regions whose sequences are known.

**FIG. 2.** Comparison of the *pbp3r* and *pbp5fm* loci in *E. hirae* S185R and *E. faecium* D63R, respectively. The scheme highlights the genes and DNA regions that are identical in both strains. The 1.2-kb insert of *E. faecium* D63R is hatched.

IS1216R was identical to IS1216 (of the pDML501 insert) and to IS1216L (of the *NcoI-EcoRI* fragment of the pDML508 insert). The result of the analysis of the 80-bp flanking sequences was that the regions upstream from both IS1216 and IS1216L (oriented as shown in Fig. 1) were identical. In contrast, the downstream sequences diverged immediately after the right IR sequence, with IS1216R being inserted in a totally different DNA sequence (as shown in Fig. 3). More importantly, short direct repeats (3 to 14 bp) were not identified in the sequences on both sides of IS1216, IS1216L, and IS1216R.
The entire sequence of the erm determinant was almost identical (98.5% identities) to those of the ermAM determinants of pAM77 (21), pAM77 (18), and Tn917 (35). Accordingly, it was designated ermAAMEH because it was found in E. hirae S185. The ATG codon of ermAAMEH ORF1 and that of the methylase-encoding gene started 73 and 276 bp downstream from the left inverted repeat of IS1216R, respectively.

The 73-bp sequence was identical to those of pAM77 and Tn917. It contained a -10 motif that might be involved in the formation of a hybrid promoter with a putative -35 hexamer found in the left IR of IS1216R (13). Note that the sequences of the ermAM in Tn917 and pAM77 diverged from that of ermAAMEH 2 and 71 bp downstream from the stop codon of ORF3, respectively.

The streptomycin resistance gene started 169 bp downstream from the ermAAMEH ORF3 stop codon. Its established 864-bp sequence had 89.6% identity with the 906-nucleotide ant(6)Ia (or aadE) of E. faecalis that encodes the 302-amino-acid residue adenyllyl-transferase (25). It was called aadEEH.

The sequence of the 8.8-kb structure comprising ermAAMEH, aadEEH, IS1216L and IS1216R described above resembled those of composite transposons (13). It was designated Tn5466.

**FIG. 3.** Comparison of the 30-bp sequences identified immediately upstream and downstream from each of the IS1216 elements.

 Amplification of the sequence pbp3r-IS1216 of E. hirae S185R. As described below, restriction digests of a plasmid-enriched preparation of E. hirae S185R led to the identification of two EcoRI fragments of 4.5 and 6.6 kb, respectively, and two NcoI fragments of 4.5 and 14 kb, respectively. The four fragments each hybridized with oligonucleotide O1 (pbp3r), indicating that E. hirae S185R contained at least two copies of pbp3r. However, the intensities of the 4.5-kb bands were higher than those of the 6.6- and 14-kb bands, respectively, suggesting that the 4.5-kb fragments occurred at a higher copy number (data not shown).

At variance with the plasmid-enriched preparations of E. hirae S185R, only a 6.6-kb EcoRI fragment and a 14-kb NcoI fragment were identified by hybridization in restricted total DNA and plasmid-enriched preparations of E. hirae S185 (from which mutant S185R was isolated). These two fragments each hybridized with oligonucleotides O1 (pbp3r) and O3 (IS1216 repeats), and the 14-kb NcoI fragment also hybridized with oligonucleotide O4 (erm). In all likelihood, these fragments were similar to those present in the E. hirae S185R digestion products, and they almost completely overlapped (Fig. 1B). Arising from this view, the 4.5-kb EcoRI and NcoI fragments derived from the E. hirae S185R plasmid preparations might be the result of rearrangements at the 5′ end of the 14-kb NcoI fragment. The 20-fold increase in the pbp3r content of resistant strain S185R in comparison with that of the wild-type strain S185 (shown by the slot blot technique) supported this hypothesis. One may note that in these experiments, E. hirae SS22 DNA and pDML501 were used as negative and positive controls, respectively (see below).

The antibiotic resistance determinants are plasmid borne. E. hirae S185, SS22, and S185R (see the Introduction) were examined with respect to their susceptibilities to antibiotics and their plasmid patterns. In comparison with the wild-type strain S185, which was moderately susceptible to gentamicin, resistant to benzylpenicillin, and highly resistant to erythromycin and streptomycin, strain SS22, which was derived from strain S185 after chemical mutagenesis (28), had greatly increased susceptibilities to benzylpenicillin, erythromycin, and streptomycin (MICs, 0.1, 1, and 50 µg/ml, respectively), while strain S185R was sixfold more resistant to benzylpenicillin (MIC, 100 µg/ml) and had the same high levels of resistance to erythromycin (MIC, >360 µg/ml) and streptomycin (MIC, >2,000 µg/ml). Expression of erythromycin resistance in E. hirae S185 was inducible by a 1-h pretreatment with a low concentration of the antibiotic (0.5 µg/ml). Pretreated and control cells were grown in the presence of a high concentration of erythromycin (500 µg/ml). The antibiotic did not inhibit the pretreated cells, which grew as well as untreated control cells, but it retarded the growth of control cells.
E. hirae S185, SS22, and S185R were analyzed by procedures which allowed small and large plasmids (up to 130 kb) to be prepared. Agarose gel electrophoresis and detection with ethidium bromide (Fig. 4A) showed that (i) each of the preparations obtained from the three strains each contained an 80-kb plasmid; (ii) the preparations obtained from strains S185 and S185R, but not that from strain SS22, contained a 40-kb plasmid; and (iii) the preparations obtained from strain S185R contained additional plasmid bands, the origins of which remain unknown. The smallest plasmid was 14 kb. The bands between 40 and 80 kb were not visible in 0.7 or 0.8% agarose gels, but they were detected in small quantities just below the thick 80-kb band in more concentrated agarose gels (Fig. 4C). A supercoiled DNA ladder (not shown in Fig. 4) and plasmids whose sizes were known (e.g., pIP964) were used to determine the sizes of the different bands. Oligonucleotide O1 (pbp3r) hybridized with the 40-kb plasmid of strains S185 and S185R and with one of the upper bands present in strain S185R. It did not hybridize with the NcoI- or EcoRI-digested total DNA of SS22 (data not shown) or with the 80-kb plasmid-enriched preparation of the same strain (Fig. 4B), showing that pbp3r is exclusively plasmid borne in E. hirae S185 and S185R and is borne mainly on the 40-kb plasmid. One may note that the 164-bp erm PCR product (Fig. 1B) corresponding to the segment of ermAM from nucleotides 120 to 283 (oligonucleotides O4 and O5) also hybridized with the 40-kb plasmids of strains S185 and S185R and the 14-kb plasmid and probably with one of the upper bands of strain S185R. Finally, oligonucleotide O3 (IS1216 repeats) hybridized with the genomic DNA and all the plasmids of the three strains (data not shown).

**Fig. 4.** Plasmid patterns. Agarose gel electrophoresis of plasmid preparations of E. hirae S185R, S185, and SS22. (A and C) Detection with ethidium bromide on 0.8 and 1% (wt/vol) agarose gels, respectively. (B) Southern blot of the gel described in panel A with oligonucleotide O1 (pbp3r). Controls were pDML501 with a 4.5-kb Ncol insert containing pbp3r and pDML540 with a 7-kb EcoRI insert containing pbp5. The upper bands observed in the pDML501 preparation are probably another form and dimers of pDML501, because restrictions yielded only the 4.5-kb insert and the pBR325 vector. Standards were linear bacteriophage - HindIII fragments and supercoiled pIP964 (a generous gift from T. Horaud, Pasteur Institute, Paris, France). Ch, linearized chromosomal DNA contaminant.

**DISCUSSION**

E. hirae S185R carries several copies of the insertion module IS1216 and penicillin resistance marker pbp3r, as well as the streptomycin resistance marker aadeEH and the erythromycin resistance marker ermAM EH. These elements are plasmid borne, although some IS1216 modules also seem to be present on the chromosome. Most likely, aadeEH, ermAMEH, and the two IS1216 modules form a transposon-like structure: IS1216L-aadeEH-ermAMEH-IS1216R. Since the unknown portion of the plasmid replicon may contain additional insertion sequence modules and markers, this putative transposon only has been numbered Tn5466.

The enterococcal IS1216 and the *S. aureus* IS257 are highly similar. They belong to the ISSI family (32). Members of this family are known to be involved in cointegration and recombination processes in *L. lactis* (29).

Recently, IS1216-like modules were found to be associated with antibiotic resistance determinants in other enterococcal strains. IS1216V was proposed to mediate the horizontal spread of the vancomycin resistance
transposon Tn5506 in *E. faecium* (17). IS1216V modules were also described in another vancomycin resistance transposon, Tn5482 (15). A similar module was found on the chromosome of *E. faecalis* CX19 downstream from the β-lactamase gene (30).

IS257 is probably involved in the integration of the low-affinity PBP2'-encoding *mecA* in the chromosomes of methicillin-resistant *S. aureus* strains, in the amplification phenomena observed in highly resistant mutants, and in the evolution of the *mec* locus (23). In the multidrug-resistant methicillin-resistant *S. aureus* strains, *mecA* is surrounded by Tn554 or Tn544, by the heavy metal resistance determinant *mer*, and/or by an integrated plasmid, pUB110 or pT181. All of these elements except the transposons are flanked by IS257 copies, and the transposons and plasmids each carry additional antibiotic resistance genes (3, 37), suggesting that IS257 is involved in the clustering of the resistance genes. IS257 also facilitates the cointegration of plasmids in staphylococci (24).

The IS1216 modules of *E. hirae* lack adjacent direct target sequences, a situation which is reminiscent of IS257 in the *mec* region of the chromosome of *S. aureus* (37). Yet, by analogy with IS257 and ISS1, the *E. hirae* IS1216 modules might be responsible for the transposition of *pbp3r* in *E. hirae* S185R and for the emergence of other plasmids. Further studies are needed to understand how IS1216 elements mediate DNA rearrangements and exchanges leading to an increase in the *pbp3r* copy number. However, because of the almost complete overlap of the 6.6-kb *EcoRI* fragment and the 14-kb *Ncol* fragment in strain S185 and probably in strain S185R, it is highly probable that both the *EcoRI* and the *Ncol* sites at the 5’ ends of these fragments are separated only by a 90-bp sequence identical to that found in pDML510. Assuming that transposition occurred during selection and led to the formation of a repetitive DNA structure on one plasmid, restriction with *Ncol* or *EcoRI* could produce only the 4.5-kb fragments, in addition to either the 14-kb fragment or the 6.6-kb fragment. Thus, one of the plasmids, at least in strain S185R, might have a repetitive DNA organization.

In addition to DNA rearrangements, an increase in the copy number of the *pbp3r* bearing plasmids might also have occurred due to a mutation that modified the regulatory mechanism of the copy number. Copy numbers were not determined during this work mainly because the yields of the plasmid preparations of *E. hirae* S185 and S185R were not highly reproducible.

By analogy with *mecA* in methicillin-resistant *S. aureus* strains, *pbp3r* in *E. hirae* S185R (and probably S185) occurs next to Tn5466. An answer to the question of whether *pbp3r* could be transferred with the help of Tn5466 or a larger Tn5466-containing composite transposon requires further investigation. At variance with the location of integration of *mecA* in the naturally occurring MRSA strains, *pbp3r* is integrated into a large plasmid, not in the chromosome. *E. hirae* is the first known bacterial species in which a low-affinity PBP-encoding gene that confers penicillin resistance is plasmid borne.

The origins of *pbp3r* and *psr3r* are still unknown. Because both genes are inserted in a 3.2-kb DNA portion whose sequence is 99% homologous to the sequence of a fragment cloned from the *E. faecium* D63R chromosome (41), one may hypothesize that a DNA fragment of at least 3.2 kb was derived from an *E. faecium* strain and inserted directly in a plasmid of *E. hirae* or in a plasmid of *E. faecium* which was then transferred to *E. hirae*.

The elements so far identified highlight the strong potential for the spread of resistance to penicillin (mediated by PBP3r), erythromycin, and streptomycin among enterococci and other bacteria by transposition and/or cointegration.

Finally, one may note that as observed in most of the enterococcal species except *E. faecalis*, *E. hirae* has an L-Lys-D-Asp type of peptidoglycan (6, 7, 10, 34). Given that PBP3r must function as a transpeptidase, it is highly probable that the spread of PBP3r-mediated penicillin resistance remains limited to bacterial species whose peptidoglycan is structurally related to that of *E. hirae*.

**ACKNOWLEDGMENTS**

This work was supported in part by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian Prime Minister’s Office, Services Fédéraux des Affaires Scientifiques, Techniques et Culturelles (PAI no. 19 and P4/03).
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