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

DOMINIQUE RAZE,† OLIVIER DARDENNE, SÉVERINE HALLUT, MANUEL MARTINEZ-BUENO,‡ JACQUES COYETTE,* AND JEAN-MARIE GHUYSEN

Centre d’Ingénierie des Protéines, Institut de Chimie, B6, Université de Liège, B-4000 Sart Tilman, Belgium

Received 31 March 1997/Returned for modification 30 May 1997/Accepted 20 November 1997

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 \textit{ermAM} conferring erythromycin resistance and one copy of the adenylyl-transferase-encoding \textit{aadE} conferring streptomycin resistance. Immediately on the left side of IS1216L, there occurs a copy of \textit{psr3} coding for the low-affinity penicillin-binding protein (PBP) PBP3r, itself preceded by a \textit{psr}-like gene (\textit{psr3r}) that controls the synthesis of \textit{psr3} and PBP3r, \textit{ermAM}, \textit{aadE}, and the transposase gene (\textit{tnp}) of \textit{IS1216R} have the same polarities, and these are opposite those of \textit{psr3}, \textit{psr3r}, and the \textit{tnp} gene of \textit{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: \textit{psr3}, \textit{pbp3r}, and \textit{tnp} in an \textit{IS1216} element. Because of the very high degree of identity (99%) with the chromosomal \textit{psr3} and \textit{pbp3r} genes of \textit{Enterococcus faecium} D63R, it is proposed that both the \textit{psr3} and \textit{pbp3r} genes were transferred from an \textit{E. faecium} strain and inserted in a plasmid of \textit{E. hirae}. \textit{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 ester-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 staphyloccocci 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 \textit{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).

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

\textit{E. hirae} S185, a clinical isolate from pig intestine, produces two low-affinity PBPs, \textit{PBP5} and \textit{PBP3r} (27, 28). Chemical mutagenesis of \textit{E. hirae} S185 has led to the isolation of a penicillin hypersusceptible mutant, \textit{E. hirae} SS22, which still produces very low levels of \textit{PBP5} but which has lost the capacity to produce \textit{PBP3r} (28). Conversely, six successive passages of \textit{E. hirae} S185 in broth containing benzylpenicillin (32 μg/ml) has led to the isolation of a penicillin-resistant mutant, \textit{E. hirae} S185R, which selectively overproduces \textit{PBP3r} (28). \textit{PBP5} and \textit{PBP3r} of \textit{E. hirae} S185 and S185R are structurally related to each other (78.5% amino acid sequence identity) and to the low-affinity PBP, \textit{PBPO}, of methicillin-resistant \textit{Staphylococcus aureus} (33% amino acid sequence identity). In addition, \textit{PBP3r} is almost identical (99.8% amino acid sequence identity) to \textit{PBP5} found in \textit{Enterococcus faecium} strains (41).

Comparison of the \textit{E. hirae} wild-type strains and mutants with respect to their susceptibilities to benzylpenicillin and erythromycin suggested that \textit{pbp3r}, but not \textit{pbp3}, might be linked to an erythromycin resistance determinant, \textit{erm} (28). The aim of the study described in this paper is to show that \textit{E. hirae} S185R possesses a plasmid-borne \textit{pbp3} 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. \textit{E. hirae} S185, SS22, and S185R were grown as described previously (27, 28). Strain S185 was shown to belong to the \textit{E. hirae} species by using the API 20 Strap 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 \textit{E. hirae} type strain, ATCC 9790. By a similar approach, strains D65 and D63R were shown to belong to the species \textit{E. faecium}.

The plasmids and \textit{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 \textit{E. hirae} S185R were analyzed in the course of this study. pDML501 and pDML508 had an \textit{NcoI} fragment, and pDML510 had an \textit{EcoRI} fragment (28). The 4.5-kb inserts of pDML501 and pDML510 and the 14-kb

* Corresponding author. Mailing address: Centre d’Ingénierie des Protéines, Institut de Chimie, B6, Université de Liège, B-4000 Sart Tilman, Belgium. Phone: 32-4-366.33.99. Fax: 32-4-366.33.64. E-mail: jcoyette@ulg.ac.be.
† Present address: Laboratoire de Microbiologie Génétique et Moléculaire, Institut Pasteur, BP 245, F-59039 Lille, France.
‡ Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, E-18071 Granada, Spain.
insert of pDML508, obtained by restriction digestion and agarose gel electro-
phoresis, was 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 (Machery and Nagel, Düren, Germany). Plasmids of *E. hirae* S185, S22, 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, 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 solution 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 [³²P]dATP 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 GCGGCGGGCGGGTTTCTA (O2) started from 193 and 1,861 bp from the ATG of pbp3r, respectively. Oligonucleotide CCTCGCTGGTCTTGGCAGAACTGT (O3), designated an IS1216 repeat, started 80 bp upstream from the ATG of the transposase-encoding gene, tnp. The box sequence followed by a T is the sequence of the 18-bp IS1216 repeats. Oligonucleotides GCAGAAATGGCATCGAAAAAGGCAG (O1) and GCGGCGGGCGGGTTTCTA (O2) started from 193 and 1,861 bp from the ATG of pbp3r, respectively. Oligonucleotide O5 was based on the sequence of the complementary strand.

The oligonucleotide probes used for hybridization were labeled at the 5′-OH end with digoxigenin-dUTP 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 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-PDD 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) (GGC package).

**Nucleotide sequence accession number.** The EMBL accession number for the sequence of the 4.5-kb NcoI insert of pDML501 is X90992, that for the sequence of IS1216 inserted in pDML501 is X81654, and that for the sequence of erm41-MEH, the ermAM found in *E. hirae* S185, is X81655.

## RESULTS

The pbp3r-containing 4.5-kb DNA insert of pDML501 possesses a psr3r gene and an insertion module IS1216. It was previously shown that pbp3r is located in the middle of the 4.5-kb NcoI insert of pDML501 (28). Sequencing of the complete insert confirmed the restriction map shown in Fig. 1A. It also led to the identification of 417 bp upstream from pbp3r, of a 517-bp open reading frame (ORF) that was identical to the 597-bp psr gene of *E. faecium* D63R (psrfm) except that it lacked the first 80 bp (41). In all likelihood, the missing 80 bp at the 5′ end of psr3r of the 4.5-kb NcoI fragment of pDML501 was lost by restriction during cloning since a complete 597-bp gene, called psr3r, was subsequently identified at one end of a 4.5-kb pbp3r-containing EcoRI fragment cloned in pDM510 from a genomic library of *E. hirae* S185R (28). The sequence of psr3r was identical to that of 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 NcoI site (41).

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

An insertion module designated IS\textsubscript{1216} was also identified in the 4.5-kb \textit{Nco}I insert of pDML501. IS\textsubscript{1216} started 637 bp downstream from the 3' end of \textit{pbp3r} and shared 65\% nucleotide sequence identities with the methicillin-resistant strain \textit{S. aureus} IS257 sequence (also called IS\textsubscript{431mec} [4]) and 75 to 76\% sequence identities with the \textit{Lactococcus lactis} IS\textsubscript{946} (31) and ISS\textsubscript{1} sequences (29), and its transposase-encoding gene \textit{tnp} had the same orientation as \textit{pbp3r}.

Finally, one may mention that the \textit{EcoRI} site at one end of the \textit{EcoRI} insert of pDML510 was only 82 bp away from the inverted repeat (IR) sequence of one IS\textsubscript{1216} element. This latter element was partially sequenced. A 411-bp sequence was found to be identical to the sequence occurring immediately upstream from the \textit{EcoRI} site at the 3' end of the 4.5-kb \textit{Nco}I insert in pDML501 (as depicted in Fig. 1A). Hence, in all likelihood, the \textit{NcoI} and \textit{EcoRI} inserts of pDML501 and pDML510, respectively, were identical.

The 14-kb DNA insert of pDML508 has a \textit{pbp3r} gene adjacent to a transposon-like structure that carries erythromycin and streptomycin resistance markers. The 14-kb insert of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Restriction maps of the 4.5-kb insert of pDML501 (A) and the 14-kb \textit{Nco}I 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, \textit{Acc}I; C, \textit{Cla}I; H, \textit{Hind}III; and S, \textit{Sca}I. Broken arrows identify the DNA regions whose sequences are known.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Comparison of the \textit{pbp3r} and \textit{pbp5fm} loci in \textit{E. hirae} S185R and \textit{E. faecium} D63R, respectively. The scheme highlights the genes and DNA regions that are identical in both strains. The 1.2-kb insert of \textit{E. faecium} D63R is hatched.}
\end{figure}
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 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 NcoI insert of pDML501 except that one CiaI site was not detected by restriction analysis. Upon PCR amplification with oligonucleotides O2 (pbp3r) and O3 (IS1216 repeats), both the NcoI-EcoRI fragment of pDML508 and the NcoI insert of pDML501 generated several 0.8-kb IS1216 copies (because O3 annealed on both inverted repeats) and one 1.6-kb DNA fragment that encompasses the 637 bp of intervening 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 NcoI fragment of pDML501 except that it lacked one CiaI site and the EcoRI and 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 NcoI-EcoRI fragments of the pDML508 and pDML501 inserts had identical 809-bp IS1216 elements and par3r 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 CiaI, SphI, PvuII, and AccI sites. It did not hybridize with any of the pbp3r, IS1216, and erm probes.

(iii) The 4.6-kb HindIII-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 oligonucleotides O1 (pbp3r) but it hybridized with oligonucleotides O3 (IS1216 repeats) and O4 (erm). The HindIII-NcoI fragment was restricted with ScaI and with 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, 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 tcp in IS1216L of the 6.5-kb NcoI-EcoRI fragment.

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 pAM61 (21), pAM77 (18), and Tn917 (35). Accordingly, it was designated ermAMEH because it was found in E. hirae S185. The ATG codon of ermAMEH ORF1 and that of the methylese-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 ermAMEH 2 and 71 bp downstream from the stop codon of ORF3, respectively.

The streptomycin resistance gene started 169 bp downstream from the ermAMEH 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 adenyltransferase (25). It was called aadEEH.

The sequence of the 8.8-kb structure comprising ermAMEH, aadEEH, IS1216L and IS1216R described above resembled those of composite transposons (13). It was designated Tns5466.

Amplification of the sequence pbp3r-IS1216 of E. hirae S185R. As described below, restriction digestions 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 80-fold 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, S522, 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 contained an 80-kb plasmid; (ii) the preparations obtained from strains S185 and S185R, but not that from strain S522, 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 S522 (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 S522. (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 NcoI insert containing pbp3r and pDML540 with a 7-kb EcoRI insert containing pbp3.

**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 *ermAMEH*. 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 ISS1 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, Tn5852 (15). A similar module was found on the chromosome of *E. faecalis* C19 downstream from the β-lactamase gene (30).

IS257 is probably involved in the integration of the low-affinity PB2-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 *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 NcoI fragment in strain S185 and probably in strain S185R, it is highly probable that both the EcoRI and the NcoI 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 repetitive DNA structure on one plasmid, restriction with NcoI 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 \textit{pbb3r} and \textit{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 \textit{E. faecium} \textit{D63R} chromosome (41), one may hypothesize that a DNA fragment of at least 3.2 kb was derived from an \textit{E. faecium} strain and inserted directly in a plasmid of \textit{E. hirae} or in a plasmid of \textit{E. faecium} which was then transferred to \textit{E. hirae}.

The elements so far identified highlight the strong potential for the spread of resistance to penicillin (mediated by \textit{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 \textit{E. faecalis}, \textit{E. hirae} has an \textit{L-Lys-to-Asp} type of teipidoglycan (6, 7, 10, 34). Given that \textit{PBP3r} must function as a transpeptidase, it is highly probable that the spread of \textit{PBP3r}-mediated penicillin resistance remains limited to bacterial species whose teipidoglycan is structurally related to that of \textit{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).

REFERENCES