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Cloning and Sequencing of the Low-Affinity Penicillin-Binding Protein 3^r-Encoding Gene of *Enterococcus hirae* S185: Modular Design and Structural Organization of the Protein

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The clinical isolate *Enterococcus hirae* S185 has a peculiar mode of resistance to penicillin in that it possesses two low-affinity penicillin-binding proteins (PBPs): the 71-kDa PBP5, also found in other enterococci, and the 77-kDa PBP3^r. The two PBPs have the same low affinity for the drug and are immunochemically related to each other. The PBP3^r-encoding gene has been cloned and sequenced, and the derived amino acid sequence has been compared by computer-assisted hydrophobic cluster analysis with that of the low-affinity PBP5 of *E. hirae* R40, the low-affinity PBP2′ of *Staphylococcus aureus*, and the PBP2 of *Escherichia coli* used as the standard of reference of the high-M_r PBPs of class B. On the basis of the shapes, sizes, and distributions of the hydrophobic and nonhydrophobic clusters along the sequences and the linear amino acid alignments derived from this analysis, the dyad PBP3^r-PBP5 has an identity index of 78.5%, the triad PBP3^r-PBP5-PBP2′ has an identity index of 29%, and the tetrad PBP3^r-PBP5-PBP2′-PBP2 (of *E. coli*) has an identity index of 13%. In spite of this divergence, the low-affinity PBPs are of identical modular design and possess the nine amino acid groupings (boxes) typical of the N-terminal and C-terminal domains of the high-M_r PBPs of class B. At variance with the latter PBPs, however, the low-affinity PBPs have an additional ≈110-amino-acid polypeptide stretch that is inserted between the amino end of the N-terminal domain and the carboxy end of the membrane anchor. While the enterococcal PBP5 gene is chromosome borne, the PBP3^r gene appears to be physically linked to the *erm* gene, which confers resistance to erythromycin and is known to be plasmid borne in almost all the *Streptococcus* spp. examined.

The relatively low susceptibility to β-lactam antibiotics of enterococci compared with that of other streptococci is attributed to the presence of membrane-bound penicillin-binding proteins (PBPs) with low affinity for the drug (10, 22). Presumably, these PBPs are able to take over the functions of the other PBPs when the cells are grown in the presence of β-lactam antibiotics (10, 23). *Enterococcus hirae* ATCC 9790 and *E. hirae* S185, a clinical isolate from swine intestine, have been studied in some detail (6, 7, 11, 23). Benzylpenicillin has a MIC for *E. hirae* ATCC 9790 of 1 $\mu g \cdot ml^{-1}$. *E. hirae* ATCC 9790 possesses a low-affinity PBP which, on the basis of its migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is referred to as the 71-kDa PBP5. Serial cultures in the presence of increasing concentrations of benzylpenicillin have led to the isolation of a mutant, strain R40, for which the MIC is 80 $\mu g \cdot ml^{-1}$ and which, in parallel to this, overproduces PBP5 (11). The MIC for *E. hirae* S185 is 16 $\mu g \cdot ml^{-1}$. Unexpectedly, that strain possesses two low-affinity PBPs, the 71-kDa PBP5 and 77-kDa PBP3^r. Exposure to increasing concentrations of penicillin has led to the isolation of a mutant, strain S185^r, for which the MIC is considerably increased (175 $\mu g \cdot ml^{-1}$) and which, in parallel to this, selectively overproduces the 77-kDa PBP3^r. Irrespective of the strains from which they are isolated, PBP3^r and PBP5 have the same low affinity for penicillin as expressed by the same low value (20 $M^{-1}\ s^{-1}$) of the second-order rate constant of protein acylation. The two proteins have distinct tryptic digestion patterns but are nevertheless immunochemically related (7, 23).

With this information provided, questions arose regarding the molecular organization of PBP3^r compared with that of PBP5 and the expression of the PBP3^r and PBP5 genes. As a prerequisite to an answer to these questions, the PBP3^r gene has been cloned and sequenced and the modular design of PBP3^r has been investigated by hydrophobic cluster analysis of the amino acid sequence. The possible existence of a specific linkage between the low-affinity PBP-encoding genes and other non-β-lactam antibiotic resistance determinants has also been examined. The results of these investigations are described below.

(Most of this work was conducted by G. Piras in partial fulfillment of the requirements for a Ph.D. degree from the University of Liège, Liège, Belgium.)

MATERIALS AND METHODS

Bacterial strains, MICs, and DNA recombination techniques. *E. hirae* S185 and S185^r were grown as described elsewhere (23). MICs were determined in liquid SB medium (6). *Escherichia coli* HB101 and JM105 (grown in Luria broth or 2XYT broth) and plasmids pBR322 and pBR325 were used for gene cloning experiments (25). The DNA recombination techniques, the enzymes, and the *E. hirae* S185^r total DNA were used as described in references 7 and 23. DNA

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fragments were purified by the GeneClean procedure (Bio 101, La Jolla, Calif.) or by electroelution in 10 mM Tris-HCl (pH 8.0) containing 5 mM NaCl and 1 mM EDTA. The 29-mer probe (T_m = 80°C) and the other oligonucleotides described in Results were from Eurogentec, Liège, Belgium. Hybridization and posthybridization washings were carried out at 55 and 70°C, respectively. Double-strand sequencing was carried out as described previously (29).

PBP analysis and immunoassays. Membranes were prepared, protein contents were estimated, PBPs were labeled with benzyl[^{14}C]penicillin, and SDS-PAGE and fluorography were performed as described elsewhere (5, 23). Rabbit anti-PBP3^r and anti-PBP5 antibodies prepared and partially purified by immunoadsorption as described previously (7, 23) were used for immunoblotting experiments and detection of both PBP3^r and PBP5 (see the introduction).

Isolation from *E. hirae* S185 of penicillin-sensitive mutants (strain SS22) and penicillin-resistant revertants (strain SS22^r). Samples of a stationary-phase culture of *E. hirae* S185 grown in SB medium were treated with 50 mM sodium nitrite in sodium acetate buffer, pH 4.6, for 30 or 60 min at 37°C (4). Alternatively, samples of an exponential-phase culture of strain S185 grown in brain heart (BH) medium were diluted to 10^4 cells · ml^{-1} (in fivefold-diluted BH medium) and treated with acridine half-mustard ICR-191 (Serva, Feinbiochemica, Heidelberg, Germany) at final concentrations of 5 and 12.5 μg · ml^{-1}, at 37°C for 4 to 5 h (4). After nitrous acid or acridine half-mustard ICR-191 treatment, samples (0.25 ml) of diluted cell suspensions were spread on BH agar plates and maintained at 37°C for 24 h. Randomly chosen, surviving colonies were streaked on penicillin-free and penicillin-containing (0.25 μg · ml^{-1}) BH agar plates. After incubation at 37°C for 24 h, penicillin-sensitive clones (selected on penicillin-free agar plates) were grown in liquid SB medium and the benzylpenicillin MICs for them were determined. The two mutagens yielded 70 mutants for which the MIC was very low (0.1 μg · ml^{-1}). These mutants represented about 9% of the surviving colonies. Strain SS22, obtained by nitrous acid treatment, was one of them.

Penicillin-resistant revertants were obtained by serial subcultures of strain SS22 on agar plates containing increasing amounts of penicillin, from 0.1 to 30 μg · ml^{-1}. The MIC for these revertants was considerably increased (35 μg · ml^{-1}). Strain SS22^r was one of these revertants.

Hydrophobic cluster analysis. Hydrophobic cluster analysis is a powerful method for comparing proteins that are weakly related in the primary structure (12). It rests upon a duplicated representation of the amino acid sequences on an α-helical two-dimensional pattern (in which the hydrophobic residues tend to form clusters) and compares the distribution of the hydrophobic clusters along the sequences. The shapes of the clusters are usually associated with definite secondary structures, and therefore, clusters of similar shapes, sizes, and relative positions express similarity in the polypeptide folding of the proteins. In this method, the six residues adjacent to the amino acid i are $i - 4$, $i - 3$, $i - 1$, $i + 1$, $i + 3$, and $i + 4$. Hence, compared with methods based only on a single amino acid property or identity, hydrophobic cluster analysis allows distant information to become visible more readily and allows deletions or insertions to be introduced more easily between the secondary structures.

Nucleotide sequence accession number. The EMBL accession number for the nucleotide sequence shown in Fig. 3 (see below) is X69092.

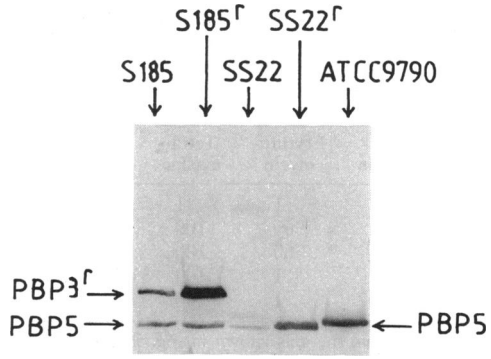

FIG. 1. Membrane-bound PBP3^r and PBP5 of *E. hirae* ATCC 9790, S185, S185^r, SS22, and SS22^r. SDS-PAGE and immunodetection of the PBPs with the anti-PBP3^r and anti-PBP5 antibodies.

RESULTS

Linkage between the PBP3^r and *erm* genes. It was known (see the introduction) that *E. hirae* S185 has two low-affinity PBPs, the 71-kDa PBP5 and the 77-kDa PBP3^r, and that, by exposure to increasing concentrations of penicillin, *E. hirae* S185 gave rise to penicillin-resistant mutants, in particular, strain S185^r, which selectively overproduced PBP3^r (see Fig. 1 and 5 in reference 23). The first run of exposure to penicillin caused a 6-fold-increased MIC, from 16 to 100 μg · ml^{-1}, and a concomitant 3- to 4-fold-increased amount of PBP3^r. In contrast, the subsequent runs resulted, each, in a small increment in the MIC, which reached 175 μg · ml^{-1} after the fourth run. In parallel to this, the amount of PBP3^r seemed to remain constant but there was a detectable, progressive decrease in the amounts of the other PBPs present in the membranes.

Chemical mutagenesis of strain S185 under the conditions described in Materials and Methods led to the isolation of hypersensitive mutants, in particular, strain SS22. SDS-PAGE of the isolated membranes and Western blot (immunoblot) analysis with the anti-PBP3^r and anti-PBP5 antibodies showed that, to all appearances, strain SS22 had lost the capacity of producing PBP3^r but still contained low levels of PBP5 (about 25 and 8% of the amounts of PBP5 present in strain S185 and strain ATCC 9790, respectively) (Fig. 1). Consistent with this observation, serial cultures of strain SS22 in the presence of increasing concentrations of penicillin led to the isolation of resistant revertants, in particular, strain SS22^r, which overproduced PBP5 but still lacked PBP3^r (Fig. 1). In contrast with penicillin-resistant S185^r mutants, the increase in penicillin resistance of SS22 revertants was progressive and apparently associated with regular increases in PBP5 amounts in the cell membranes. Note that the PBP pattern of the mutants of each class derived from strain S185 was similar to that of the respective prototypic strains S185^r, SS22, and SS22^r. Sometimes, but not always, PBP5 of strain ATCC 9790 and PBP5 of strain S185 (and its derivatives) migrated somewhat differently, suggesting that minor modifications in the proteins might occur.

Given that the parental strain S185 was also resistant to erythromycin and tetracycline, thereby probably possessing the *erm* and *tet* genes, the susceptibility of the mutants to these non-β-lactam antibiotics was determined. The MICs (Table 1) revealed that strains SS22 and SS22^r, which had lost the capacity of producing PBP3^r but not PBP5, had also lost the ability to resist erythromycin, suggesting that the

TABLE 1. Susceptibility to three antimicrobial agents and content of membrane-bound low-affinity PBPs in five *E. hirae* strains

Strain	MIC ($\mu g \cdot ml^{-1}$) of:			Level[a] of:	
	Benzyl-penicillin	Erythro-mycin	Tetra-cycline	PBP5	PBP3^r
ATCC 9790	1	1	1	+	0
S185	16	360	100	+	++
S185^r	175	360	100	+	++++
SS22	0.1	1	100	Very low	0
SS22^r	35	1	100	++	0

[a] Visual estimation from the gels shown in Fig. 1 and in Fig. 1 and 5 of reference 23, on a scale from very low to ++++ (0, not present).

PBP3^r and *erm* genes were linked physically and deleted concomitantly by chemical mutagenesis of strain S185.

Cloning of the PBP3^r gene. In a previous study (23), two degenerated oligodeoxynucleotides were synthesized on the basis of the known N-terminal sequences of peptides isolated from a tryptic digest of the *E. hirae* PBP3^r and used to amplify a 233-bp DNA segment by the polymerase chain reaction procedure. This segment was cloned, and on the basis of its established nucleotide sequence, the nondegenerated 5′-CATTTTGTTTGGATCATAGCTTGGAGAGC-3′ 29-mer probe (complementary to the SSPSYDPNKK decapeptide-encoding DNA strand) was synthesized and γ-^{32}P labeled at the 5′ end. Libraries of *E. hirae* S185^r total DNA were prepared into pBR322 or pBR325 depending on the available restriction sites and used to transform *E. coli* HB101 cells. Of the 7,400 colonies cloned, 16 gave a strong hybridization signal with the radioactive 29-mer probe after extensive washings under stringent conditions. Recombinant plasmids whose inserts ranged from 0.7 to 14 kb in size were identified by Southern blot analysis. To prove that the inserts contained the PBP3^r gene, membranes of transformed *E. coli* cells were isolated and subjected to SDS-PAGE followed by Western blot analysis with anti-PBP3^r (and anti-PBP5) antibodies. *E. coli* actually produced a novel, membrane-bound, low-affinity PBP which migrated with an apparent molecular mass of 77 kDa (and thus distinguished itself from the 71-kDa PBP5). Of the available plasmids, pDML501, i.e., pBR325 harboring a 4.5-kb *Nco*I insert, served to sequence the PBP3^r gene. Comparison of the restriction map of pDML501 with that of the PBP5 gene-containing pDML540 (7) confirmed that pDML501 did contain the PBP3^r gene (Fig. 2).

Nucleotide sequence of the PBP3^r gene and amino acid sequence of PBP3^r. By using the strategy shown in Fig. 2, sequencing of the pDML501 insert on both strands yielded an open reading frame which started at position 261 with an ATG codon and terminated at position 2095 by a TAA stop codon (or from position 6010 to position 8044 by using the numbering of pDML501) (Fig. 3). A perfect ribosome binding site, AGGAGG, which matched exactly the sequence consensus (27) occurred 5 bp upstream from the ATG start codon. Computer analysis of the secondary structure of the corresponding RNA showed that the AGGAGG sequence was on a single-stranded region optimal for the expression of prokaryotic genes (19). The TAA stop codon was followed by palindromic regions able to form a hairpin or stem-loop in the corresponding RNA, a structure typical of many prokaryotic terminators (19). The 39.7% GC content of the sequence was similar to that of other streptococcal genes (9).

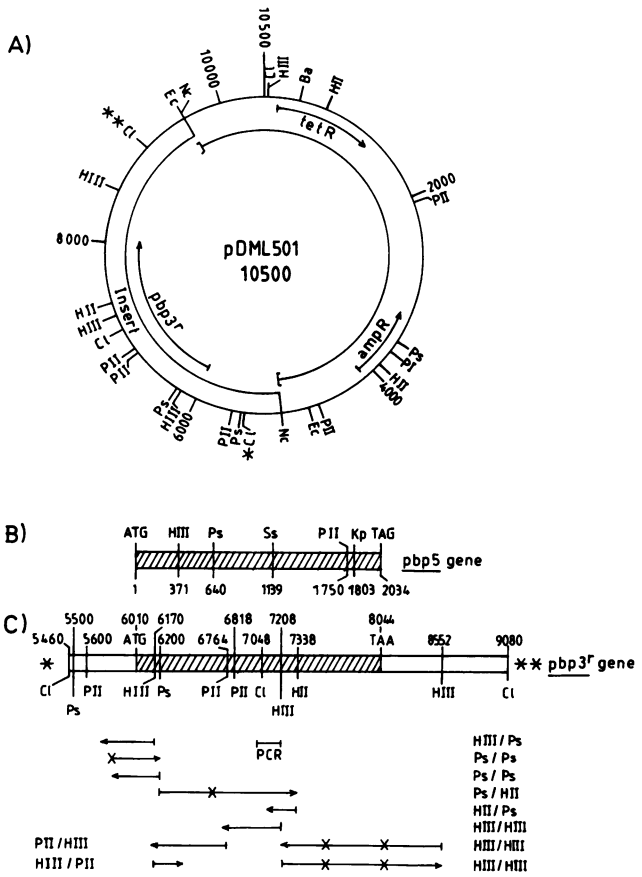

FIG. 2. Cloning and sequencing of the PBP3^r gene of *E. hirae* S185^r. (A) Restriction map of pDML501. The arrow in the insert indicates the position and orientation of the PBP3^r gene. (B) Restriction map of the PBP5 gene (see reference 7). (C) Restriction map of the PBP3^r gene and sequencing strategy. Restriction sites: Ba, *Bam*HI; Cl, *Cla*I; Ec, *Eco*RI; HII, *Hin*dII; HIII, *Hin*dIII; Kp, *Kpn*I; Nc, *Nco*I; Ps, *Pst*I; PI, *Pvu*I; PII, *Pvu*II; Ss, *Sst*I. The 0.6-kb PII-HIII, 0.7-kb Ps-Ps, 1.1-kb Ps-HII, and 1.0- and 1.3-kb HIII-HIII DNA fragments were cloned into M13mp18 or mp19 phages and sequenced on both strands (in most cases) by using M13 universal or synthetic primers. The Ps-Ps segment was sequenced by the double-strand method with a specific synthetic oligonucleotide.

The PBP3^r gene translated into a 678-amino-acid-residue protein (Fig. 3). The theoretical 4.73 pI value (Genetics Computer Group program) was very close to the experimental 4.5 pI value measured for the 63-kDa t-PBP3^r tryptic peptide isolated previously (23).

Hydrophobic cluster analysis of PBP3^r. The amino acid sequence of the *E. hirae* PBP3^r was compared with those of the low-affinity PBP5 of *E. hirae* and the low-affinity PBP2′ of *Staphylococcus aureus*, using the *E. coli* PBP2 as the standard of reference. The staphylococcal PBP2′, which is responsible for acquired resistance to methicillin, is structurally related to the enterococcal PBP5 (7). The 633-amino-acid-residue PBP2 of *E. coli* is involved, together with the intrinsic membrane protein RodA (3, 15, 21), in the formation of the rod shape of the cell. Of the high-M_r PBPs of known primary structure, the *E. coli* PBP2 shows the highest similarity with the low-affinity PBPs.

The high-M_r PBPs are three module proteins (13). A membrane anchor, usually 30 to 60 amino acid residues long,

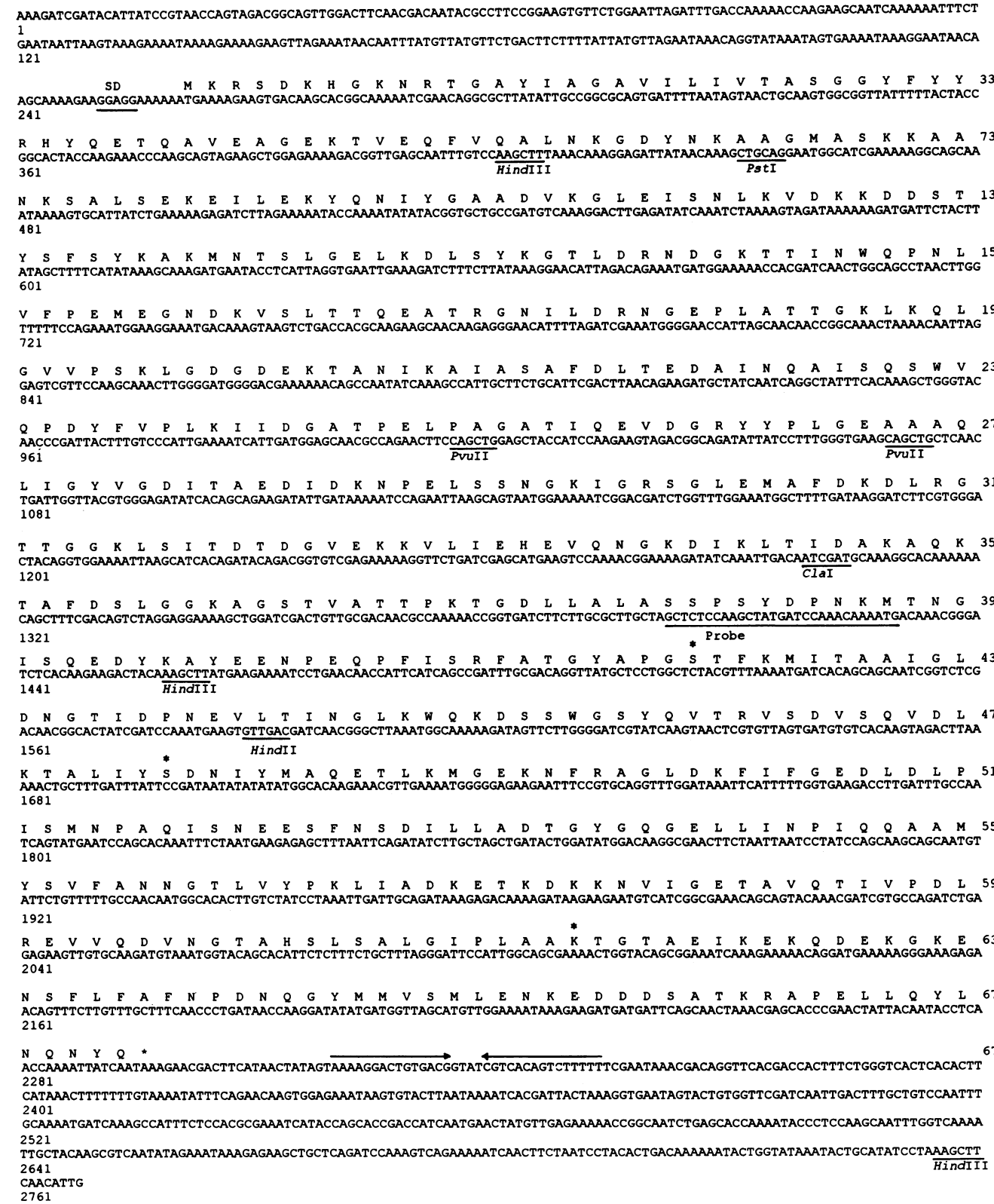

FIG. 3. Nucleotide sequence of the PBP3r gene and amino acid sequence of PBP3r of *E. hirae* S185r. SD, Shine-Dalgarno sequence. Arrows indicate inverted repeats forming the putative terminator. Restriction sites and the position of the cloning probe are underlined. *, active-site-defining motifs of the penicilloyl serine transferase family. PBP3r has a calculated molecular mass of 73,822 kDa.

is linked to a several hundred-amino-acid-residue N-terminal domain which is linked to a several hundred-amino-acid-residue penicillin-binding C-terminal domain. The high-M_r PBPs fall into two classes, A and B, whose members differ in their N-terminal domains (8). Those of class B possess nine conserved motifs or boxes along the amino acid sequences (8, 13). Box 1 and box 4 are at the amino end and the carboxy end of the N-terminal domain, respectively. Box 5 and box 9 are at the amino end and the carboxy end of the C-terminal domain, respectively. Boxes 6, 7, and 8 of the C-terminal domain are the active-site-defining motifs characteristic of the penicilloyl serine transferase family, i.e., in the indicated order, the tetrad S*XXK (where S* is the active-site serine), the triad SDN (or analog), and the triad KT(S)G (or analog).

Consistent with the modular design of the proteins, the patterns of hydrophobic and nonhydrophobic clusters of the membrane anchors and N-terminal domains of the four PBPs under comparison are shown in Fig. 4. The corresponding C-terminal domains are shown in Fig. 5. In this representation, each hydrophobic amino acid residue (F, I, L, M, V, W, and Y) and each hydrophobic cluster are delineated; the hydrophobic residues and clusters occurring at equivalent places along the sequences of the low-affinity PBPs 3^r, 5, and 2′ are in boldface; and the nonhydrophobic residues occurring as strict identities are marked by scattered points. The residues and clusters marked in boldface or by scattered points along the amino acid sequence of the *E. coli* PBP2 occur at places equivalent to those found in the low-affinity PBPs. Figure 6 is the linear amino acid alignment derived from the data of Fig. 4 and 5.

DISCUSSION

The low-affinity PBPs 3^r and 5 of *E. hirae* S185 and PBP2′ of *S. aureus* are proteins of very similar size, containing 678, 678, and 667 amino acid residues, respectively (7, 26). The pair PBP3^r-PBP5 has 532 identities (identity index: 78.5%), the pair PBP3^r-PBP2′ has 216 identities (33%), and the triad PBP3^r-PBP5-PBP2′ has 195 identities (29%) (Fig. 6). In spite of this divergence, the hydrophobic and nonhydrophobic cluster patterns of the three low-affinity PBPs are almost superimposable (Fig. 4 and 5). Few deletions or insertions have to be made between the conserved clusters to obtain an optimal match.

The cluster pattern of the low-affinity PBPs is similar to that of the *E. coli* PBP2 except that the low-affinity PBPs have an additional ≈110-amino-acid-residue stretch that extends from the carboxy end of the membrane anchor to the amino end of the N-terminal domain. When this N-terminal extension is excluded from the analysis, the four PBPs under comparison have 82 identities (identity index: 13%) (Fig. 6). This low index is not due to the random distribution of a limited number of conserved amino acid residues but results from the occurrence of 10 definite amino acid groupings of high homology or identity along the amino acid sequences (Fig. 4 and 5). When the comparison is restricted to the nine boxes conserved in the high-M_r PBPs of class B, the identity scores are 85% for the pair *E. hirae* PBP3^r-*E. hirae* PBP5, 70% for the pair *E. hirae* PBP3^r-*S. aureus* PBP2′, and 64% for the pair *E. hirae* PBP3^r-*E. coli*PBP2. The 10th conserved grouping, which is located immediately downstream from D-535 or D-518 in the low-affinity PBPs, aligns with the grouping located immediately downstream from D-447 in the *E. coli* PBP2. Site-directed mutagenesis experiments (1) suggest that D-447 is an important component of the catalytic machinery of the *E. coli* PBP2. It may be equivalent to the active-site E-166 of the β-lactamase of class A (17). Note also that the SDN motif (box 7) of the low-affinity PBPs aligns with the SAD motif of the *E. coli* PBP2.

From the above analysis, one can safely conclude that the enterococcal and staphylococcal low-affinity PBPs (i) have very similar polypeptide scaffoldings and thereby perform similar functions and (ii) have a basic multimodule design and structural organization comparable to those of the *E. coli* PBP2 but (iii) differ from the *E. coli* PBP2 by the presence of an ≈110-amino-acid residue polypeptide inserted immediately downstream from the membrane anchor. This polypeptide is large enough to provide the low-affinity PBPs with an additional domain having a particular folding and performing a separate function. Its possible role in the low-affinity PBP-mediated penicillin resistance remains to be established.

PBP3^r and PBP5 of *E. hirae* S185 are extremely similar with respect to structure and low susceptibility to acylation by penicillin. Yet expression of PBP3^r is selectively enhanced when strain S185 is submitted to penicillin pressure (strain S185^r), expression of PBP3^r is selectively annihilated when strain S185 is submitted to chemical mutagenesis (strain SS22), and exposure of this latter strain to penicillin pressure causes overexpression of PBP5 (strain SS22^r). These differences in regulation and chemical susceptibility as well as the linkage of the erythromycin resistance-encoding *erm* gene to the PBP3^r gene, but not to the PBP5 gene, indicate that the PBP3^r and PBP5 genes are borne by different DNA segments. Recent results from this lab show that both the PBP3^r and *erm* genes are present on a large plasmid (24). Note that *erm* is plasmid borne in almost all streptococci examined except *S. pneumoniae* (14, 18). Similarly, the *S. aureus mec* region, which contains the low-affinity PBP2′-encoding *mecA* gene, is present in a transposon that carries other antibiotic resistance determinants (i.e., *aadD*, *tet*, and *erm*) (16, 20, 28). The enterococcal PBP3^r and staphylococcal *mec* genes code for similar PBPs; they may also be integrated into similar DNA structures.

The accumulation of antibiotic resistance determinants in autotransmissible plasmids facilitates the lateral or horizontal mobility of the corresponding genes between bacterial species (2). It is a serious threat for antibacterial chemotherapy. By acting as collectors of transposons in which genes move as integron cassettes, these plasmids increase the gene pool and thereby increase the gene flux between bacterial species. Intraspecies transfer of low-affinity PBP-encoding genes may be accompanied by the immediate acquisition of penicillin resistance. Given the structural variations of the wall peptidoglycans and the variations in the specificity profiles of the PBP-catalyzed reactions, acquired resistance to penicillin by interspecies transfer of low-affinity PBP-encoding genes may require remodeling of the PBP active sites. The relatively low index of identity (28%) between the enterococcal and staphylococcal low-affinity PBPs may be the expression of a species-specific adjustment of the enzyme active-site configuration.

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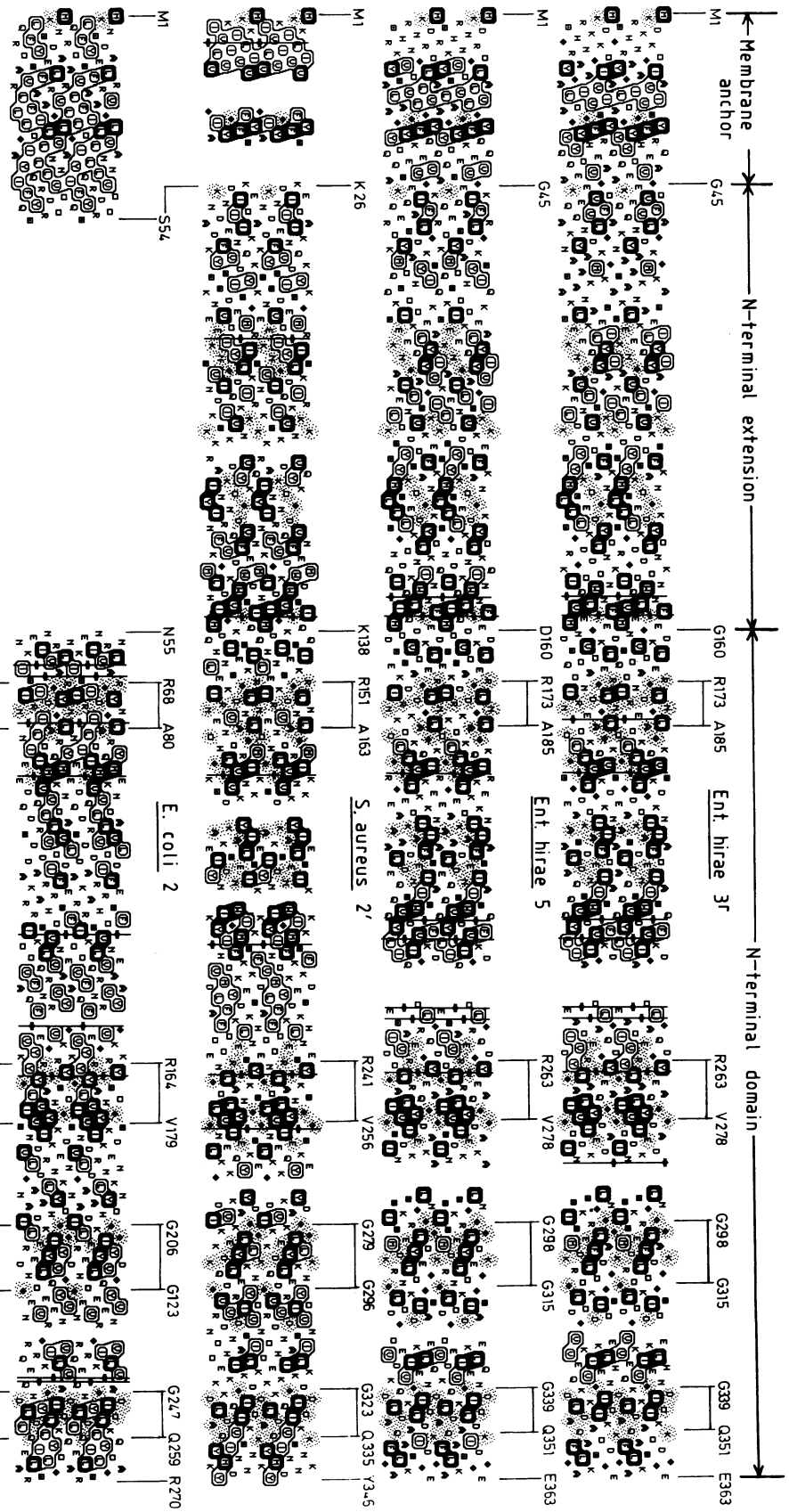

FIG. 4. Hydrophobic cluster analysis of the membrane anchors and N-terminal domains of *E. hirae* PBP3r, *E. hirae* PBP5, *S. aureus* PBP2', and *E. coli* PBP2. Symbols: □, serine; ○, threonine; ◆, glycine; ★, proline. For details, see the text.

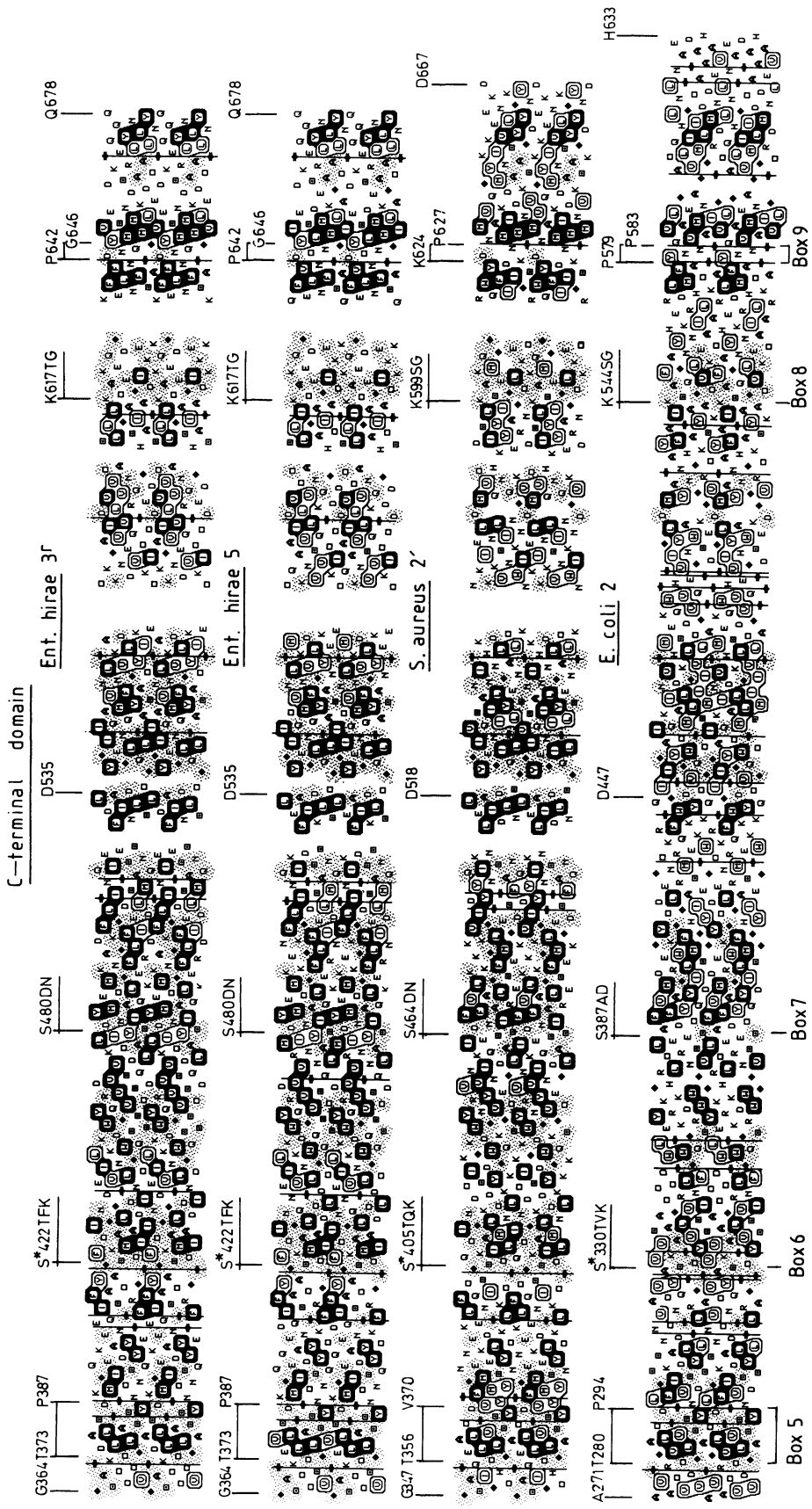

FIG. 5. Hydrophobic cluster analysis of the penicillin-binding, C-terminal domains of *E. hirae* PBP3^r, *E. hirae* PBP5, *S. aureus* PBP2', and *E. coli* PBP2. For symbols, see the legend to Fig. 4.

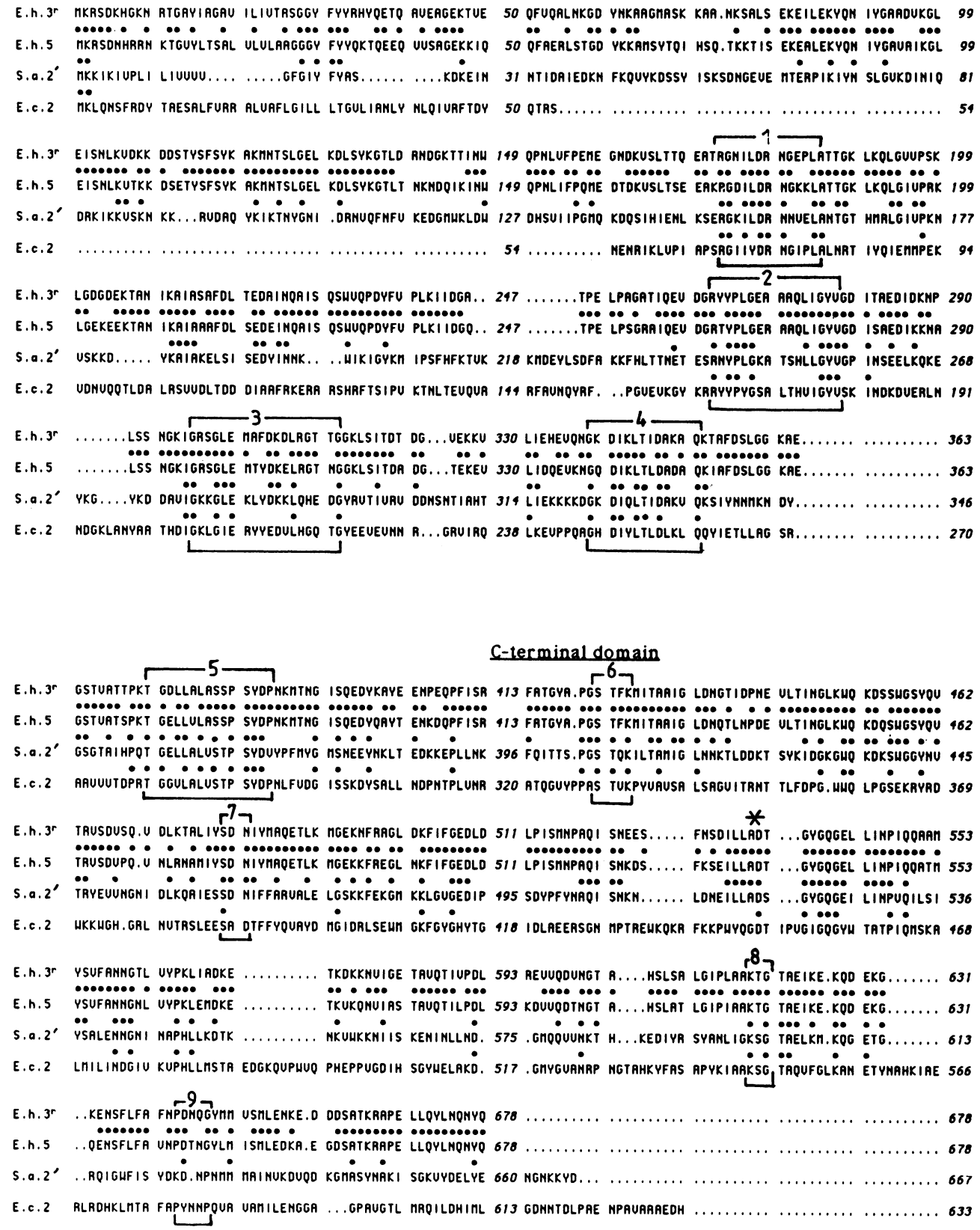

FIG. 6. Amino acid alignment of *E. hirae* PBP3^r (E.h.3^r), *E. hirae* PBP5 (E.h.5) *S. aureus* PBP2′ (S.a.2′), and *E. coli* PBP2 (E.c.2). Black dots, strict identities in the pair PBP3^r and PBP5; the triad PBPs 3^r, 5, and 2′; and the tetrad PBPs 3^r, 5, 2′, and 2. *, conserved aspartic residue. The alignments derive from the data of Fig. 4 and 5. The nine conserved boxes are numbered.

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