The *Enterococcus hirae* R40 penicillin-binding protein 5 and the methicillin-resistant *Staphylococcus aureus* penicillin-binding protein 2' are similar

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The penicillin-resistant *Enterococcus hirae* R40 has a typical profile of membrane-bound penicillin-binding proteins (PBPs) except that the 71 kDa PBP5 of low penicillin affinity represents about 50 % of all the PBPs present. Water-soluble tryptic-digest peptides were selectively produced from PBP5, their N-terminal regions were sequenced and synthetic oligonucleotides were used as primers to generate a 476 bp DNA fragment by polymerase chain reaction. On the basis of these data, the PBP5-encoding gene was cloned in *Escherichia coli* by using pBR322 as vector. The gene, included in a 7.1 kb insert, had the information for a 678-amino acid-residue protein. PBP5 shows similarity, in the primary structure, with the high-molecular-mass PBPs of class B. In particular, amino acid alignment of the enterococcal PBP5 and the methicillin-resistant staphylococcal PBP2' generates scores that are 30, for the N-terminal domains, and 53, for the C-terminal domains, standard deviations above that expected for a run of 20 randomized pairs of proteins having the same amino acid compositions as the two proteins under consideration.

INTRODUCTION

Bacteria possess multiple membrane-bound proteins that bind penicillin in the form of stable serine-ester-linked penicilloyl derivatives [1]. The penicillin-binding proteins (PBPs) fall in two major groups. The monofunctional low-molecular-mass PBPs are active-site-serine DD-peptidases; they probably control the extent of wall peptidoglycan cross-linking. The high-molecular-mass PBPs are two-domain proteins in which the active-site-serine-containing penicillin-binding C-terminal domain is preceded by a several-hundred-amino acid-residue N-terminal extension [1,2]. They fulfil multiple functions. In *Bacillus licheniformis* the high-molecular-mass PBP BLAR is the penicillin sensory-transducer required for the specific inducibility of β-lactamase synthesis [3,4]. PBPs 1A and 1B of *Escherichia coli* catalyse peptidoglycan transglycosylation (presumably at a site in the N-terminal domain) and peptidoglycan transpeptidation (presumably at the serine-containing site of the C-terminal domain) [2]. PBPs 2 and 3 of *Esch. coli*, in conjunction with other life-cycle proteins, play important roles in cell division and determination of cell shape [1,2,5–9].

Resistance to β-lactam antibiotics may arise by the acquisition of a novel PBP of low penicillin affinity, for example the PBP2' of *Staphylococcus aureus* [10–12], or by the introduction of multiple amino acid substitutions resulting in the remodelling of the penicillin-binding domain of some targeted high-molecular-mass PBPs, for example the PBPs 3 of *Escherichia coli* [13], 2B and 2X of *Streptococcus pneumoniae* [14–16] and 2 of *Neisseria gonorrhoeae* and *Neisseria meningitidis* [17–20]. Sequence-similarity searches suggest that these high-molecular-mass PBPs are related to each other and probably form a distinct class B of PBPs of great physiological importance [1,16].

The relatively low susceptibility to penicillin of *Enterococcus hirae* A.T.C.C. 9790 [21] and *Ent. hirae* S185, a clinical isolate

from pig intestine [22], has been attributed to the presence of a high-molecular-mass PBP of low affinity for the drug, the 71 kDa PBP5 and the 77 kDa PBP3^r respectively. Laboratory mutants have been obtained that overproduce PBP5 (*Ent. hirae* R40) and PBP3^r (*Ent. hirae* S185^r) and, in parallel with this, have a much increased penicillin-resistance [21,22]. Experiments were undertaken with the aim of unravelling the primary structure of the *Ent. hirae* R40 PBP5.

MATERIALS AND METHODS

Bacterial strains

Ent. hirae strains R40 and Rev14 [21,23] were gifts from Dr. R. Fontana and Dr. P. Canepari (University of Verona, Verona, Italy). MAX Efficiency *Esch. coli* DH5αF'IQ competent strain was from Bethesda Research Laboratories (Gaithersburg MD, U.S.A.). *Esch. coli* HB101 and JM105 were also used.

Membranes

Ent. hirae cells were grown unshaken at 37 °C in 500 ml of SB medium [24] and collected at the late exponential phase ($A_{550} = 6.0$). The membranes were prepared as described previously [22,24,25]. The proteins were measured by the Lowry method as modified by Coyette et al. [26].

Labelling with benzyl[^{14}C]penicillin, SDS/PAGE and fluorography

Samples were labelled with benzyl[^{14}C]penicillin (54 Ci/mol; Amersham International, Amersham, Bucks., U.K.) and submitted to SDS/PAGE. Fluorography of the gels and estimation of the PBPs by densitometry of the fluorograms were performed as described previously [22,25]. Mean values (ten assays) of the molecular masses of the PBPs were determined by using BSA (66.3 kDa), ovalbumin (42.7 kDa), *Streptomyces* R61 DD-

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carboxypeptidase (38 kDa), carbonic anhydrase (29 kDa), myo-globin CNBr-cleavage products (17.2, 14.6, 8.24, 6.4 and 2.6 kDa) and hen egg-white lysozyme (14 kDa) as standards. The values of the second-order rate constant of protein acylation and first-order rate of protein deacylation were measured, and the antibiotic concentrations and incubation times necessary to achieve a given extent of saturation of the PBPs were calculated as described previously [27,28].

Purification of the PBP5 tryptic-digest derivatives and amino acid microsequencing

The PBP5 tryptic-digest derivatives, referred to as t-PBP5s, were purified essentially as described by Piras et al. [22] but with the following modifications. (1) The Q-Sepharose column was equilibrated and eluted with 25 mM-Tris/borate buffer, pH 8.0. (2) Fractions from the first chromatography were concentrated to 15 ml, dialysed, divided into 5 ml samples and filtered on a 1 ml MonoQ HR 5/5 column equilibrated with 20 mM-Bistris/ HCl buffer, pH 6.3, and eluted with the same buffer and an NaCl gradient (0–1 M). (3) Fractions from step (2) were concentrated to 2 ml by ultrafiltration, adjusted to 0.85 M-$(NH_4)_2SO_4$ in 50 mM-sodium phosphate buffer, pH 7.0, and filtered on the phenyl-Superose column used previously [22]. Each step of the purification procedure was monitored by SDS/PAGE of samples previously labelled with benzyl[^{14}C]penicillin. Electroblotted peptides were microsequenced as described by Piras et al. [22].

Antibodies and immunoassays

Adult rabbits were injected subcutaneously with a preparation emulsified in complete Freund's adjuvant and containing 80 μg (total amount) of several peptide fragments of PBP5 purified from Ent. hirae R40 [29]. The same injection was repeated four times at 15-day intervals. A final injection was made with a preparation containing 140 μg of 67 kDa and 63 kDa peptide fragments ([29] and the Results section) in a ratio of 4:1. Elimination of non-specific antibodies was made by immuno-adsorption of the antiserum by using a mixture of Esch. coli HB101 and Ent. hirae Rev14 cell lysates as described below. (Note that strain Rev14 lacks PBP5.)

Late-exponential-phase cells of Esch. coli and Ent. hirae Rev14 were collected separately from 1-litre cultures and re-suspended separately in 50 ml of lysozyme solution (1 mg/ml). The Esch. coli cell suspension was supplemented with 5 mM-EDTA and kept on ice for 30 min. The Rev14 cell suspension was incubated at 37 °C for 30 min. The two cell lysates were mixed and the final 100 ml preparation was (i) boiled for 15 min in a microwave oven, (ii) cooled and treated for 30 min at 37 °C with 2 mg of DNAase and 2 mg of RNAase, (iii) boiled for 5 min as above, (iv) cooled and shaken for 90 min at 22 °C in the presence of a 30 cm × 30 cm nitrocellulose sheet cut into small pieces, (v) supplemented with 1.5 g of BSA, 1.5 ml of 1 % (w/v) NaN_3, 30 ml of water and 15 ml of 10 × TBS (5 M-Nacl/200 mM-Tris/HCl buffer, pH 7.5), and (vi) incubated at 22 °C for 30 min. The antiserum was diluted 100-fold in the mixture and shaken for 16 h at 22 °C. After centrifugation (40000 g for 20 min), the purified antiserum was diluted 50-fold with TTBS [TBS containing 0.05 % (v/v) Tween 20 and 1 % (w/v) BSA] before use. Immunodetection was performed on proteins or peptides transferred from polyacrylamide slab gels on to 0.45 μm-pore-size Millipore HA type nitrocellulose sheets as described by Piras et al. [22].

DNA recombinant techniques and nucleotide sequencing

Restriction endonucleases, T7 DNA polymerase, calf intestinal alkaline phosphatase and T4 DNA ligase were from Boehringer (Mannheim, Germany) and Bethesda Research Laboratories

(Gaithersburg, MD, U.S.A.). [γ-^{32}P]dATP and [α-^{35}S]thio]ATP were from Amersham International and ampicillin was from Beecham (Brussels, Belgium). Oligonucleotides were synthesized by Eurogentec (Liège, Belgium). The Ent. hirae R40 chromosomal DNA was prepared as described previously [30]. This procedure includes treatments with lysozyme in the presence of sucrose, SDS and proteinase K. Preparation of plasmid DNA, transformation of Esch. coli, digestion of DNA with restriction enzymes, treatment with calf intestinal alkaline phosphatase, ligation and agarose-gel electrophoresis of plasmids and digested DNAs were performed essentially as described by Sambrook et al. [31]. DNA fragments were purified by using the Geneclean procedure (Bio-101, La Jolla, CA, U.S.A.), subcloned into bacteriophages M13mp18 and M13mp19 and sequenced with the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH, U.S.A.) by using the dideoxynucleotide chain-termination method [32].

Amplification by PCR

PCR amplification was performed as described by Piras et al. [22] except that before the first cycle the DNA template was treated at 94 °C for 5 min and after the 30th cycle the time at 72 °C was 7 min. The PCR reaction product (i.e. a 476 bp DNA segment) was (i) submitted to PAGE (6 % acrylamide) in non-denaturing TBE buffer (1 mM-EDTA/40 mM-Tris/borate buffer, pH 8 in a Bio-Rad Mini Protean apparatus, (ii) electroeluted from the gel in 10 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-NaCl and 1 mM-EDTA, (iii) extracted twice with phenol/ chloroform (1:1, v/v) and precipitated with ethanol, (iv) digested with BamHI and EcoRI restriction enzymes (Bethesda Research Laboratories), and (v) cloned in bacteriophages M13mp18 and M13mp19 [31]. MAX Efficiency Esch. coli
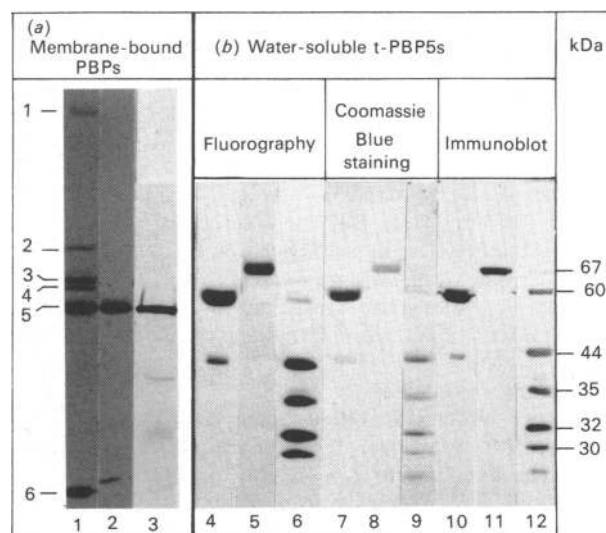
Fig. 1. SDS/PAGE of the Ent. hirae R40 membrane-bound PBPs (a) and the water-soluble PBP5 tryptic-digest peptides (b)

Conditions of electrophoresis: (a) 7.2 % (w/v) acrylamide; 16 h; gel length 32 cm; (b) 8.5 % (w/v) acrylamide; 5 h; gel length 16 cm. (a) Membranes (200 μg of protein) were labelled with 100 μM-benzyl-[^{14}C]penicillin for 60 min either directly (lane 1) or after previous treatment with 15 μM non-radioactive benzylpenicillin for 10 min (lane 2), and the PBPs were detected by fluorography of the gels. Immunodetection (lane 3) of PBP5 in isolated membranes (100 μg of total protein) with the anti-PBP5 serum was also performed. (b) Isolation of the 60 kDa t-PBP5 (lanes 4, 7 and 10), the 67 kDa t-PBP5 (lanes 5, 8 and 11) and tryptic digestion of the purified 67 kDa t-PBP5 (lanes 6, 9 and 12). The peptides seen by fluorography in lanes 4–6 (5 μg, 2 μg and 10 μg respectively) were labelled with 100 μM-benzyl[^{14}C]penicillin for 60 min after proteolysis.

DH5αF′IQ competent cells were used for the transformation experiments.

RESULTS

Specific labelling of PBP5 in *Ent. hirae* R40 membranes with benzyl[^{14}C]penicillin

Ent. hirae R40 contains the six species-specific membrane-bound PBPs [33]: PBP1 (119±8 kDa), PBP2 (84±1.7 kDa), PBP3 (77±0.6 kDa), PBP4 (75±0.7 kDa), PBP5 (71±0.7 kDa) and PBP6 (43±1 kDa) (Fig. 1, lane 1). When compared with *Ent. hirae* A.T.C.C. 9790, the peculiarity of *Ent. hirae* R40 is that

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(1) 67 kDa t-PBP5: TISEKEALEKYQNIYGAVAIKGLEISN...

(2) 60 kDa t-PBP5: INWQPNLIFPQMEDTDKVSLTSEEAKXGDILDRNGKKLA...

(3) 44 kDa t-PBP5: NAELSXNGKIGRSGLEMTYDKELRGTNGGKLSITDADG...

(4) 32 kDa t-PBP5: MTNGISQEDYQAYTENKDQPFI...

(5) 30 kDa t-PBP5: DQPFISRFATGYAXGSTFKMITAAIGLDNQTLNP...

Fig. 2. *N*-Terminal amino acid sequences of the PBP5 tryptic-digest fragments

The underlined sequences were those on the basis of which the two oligonucleotides shown in Fig. 3 were synthesized. Note the overlap DQPFI between the 32 kDa t-PBP5 and the 30 kDa t-PBP5.

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the 71 kDa PBP5 of low penicillin affinity represents about one-half of all the PBPs present. In order to map out fragments specifically produced by tryptic digestion of PBP5, membranes of *Ent. hirae* R40 were prepared in which PBP5 was virtually the only PBP to be labelled with benzyl[^{14}C]penicillin (Fig. 1, lane 2). For this purpose membranes were treated first with 15 μM non-radioactive benzylpenicillin for 10 min at 37 °C (under which conditions PBPs 1, 2, 3 and 4 were fully saturated, PBP6 was saturated by 90 % and PBP5 remained in a free form), and then with 100 μM-benzyl[^{14}C]penicillin for 60 min at 37 °C, thus achieving complete labelling of PBP5.

Tryptic digestion of the *Ent. hirae* R40 membranes: the 67 kDa t-PBP5 and 60 kDa t-PBP5 tryptic-digest fragments
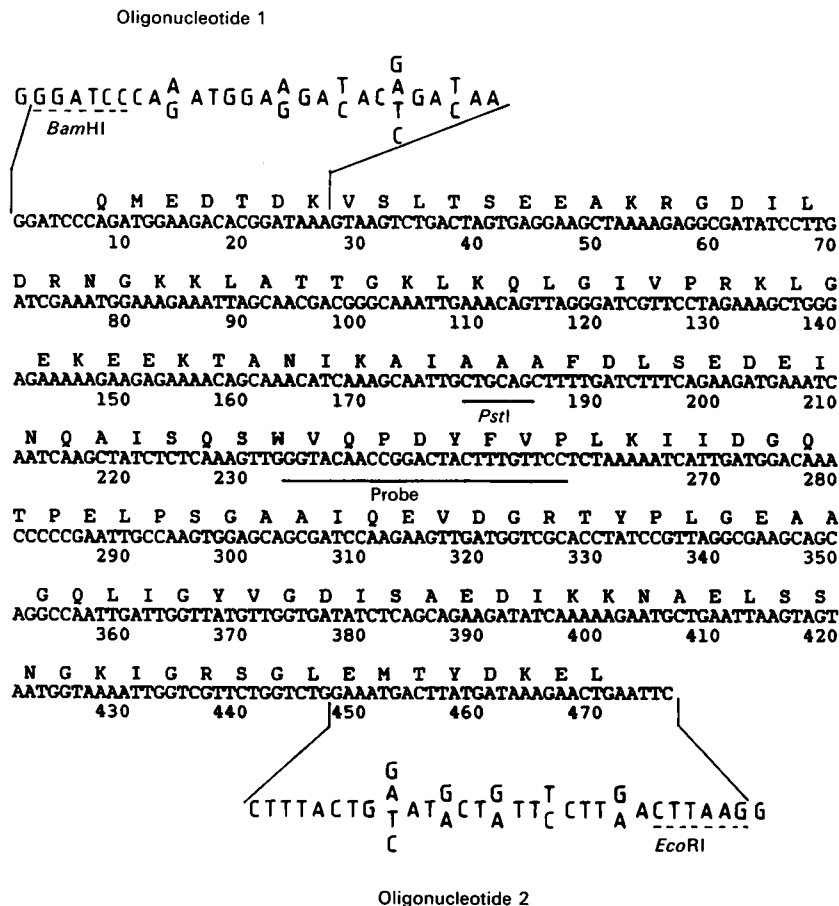
It was known that PBP5 could be quantitatively converted into water-soluble penicillin-binding derivatives by trypsin treatment of isolated membranes of *Ent. hirae* R40 [29,34]. On the basis of these data, a membrane suspension [1.5 g of total protein in 120 ml of 40 mM-sodium phosphate buffer, pH 6.5, containing 1 mM-MgCl$_2$ and 3 % (v/v) glycerol] was incubated with 0.5 % (w/w) trypsin (type XI; Sigma Chemical Co.) for 15 min at 37 °C. The reaction was stopped by addition of 10 mg of soyabean trypsin inhibitor, and the preparation was submitted to ultracentrifugation at 105 000 *g* for 1 h.

Analysis of the supernatant showed that, as a result of this relatively mild proteolytic treatment, 85 % of the membrane-bound PBP5 (initial amount 120 nmol or 8.3 mg) was converted into four water-soluble fragments, of 67±0.6 kDa (55 %),

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Oligonucleotide 1

Fig. 3. Oligonucleotides used as primers for PCR amplification, PCR product and amino acid sequence of PBP5-PCR

The probe used to screen the clones and the *Pst*I cleavage site are underlined.

63 ± 0.7 kDa (14 %), 60 ± 1.4 kDa (7 %) and 44 ± 1.2 kDa (9 %). The fragments bound benzyl[^{14}C]penicillin with a low affinity comparable with that of the membrane-bound PBP5 (see below). They had apparent molecular masses comparable with those of the radioactive fragments obtained from membranes whose PBP5 had been selectively labelled with benzyl[^{14}C]penicillin before trypsin treatment.

The 67 kDa t-PBP5 tryptic-digest fragment was isolated and the 60 kDa t-PBP5 fragment was partially purified (see the Materials and methods section). (1) The supernatant fraction (120 ml; 860 mg of protein) was filtered on the Q-Sepharose FF column and the four aforementioned tryptic-digest fragments were eluted at 0.19–0.24 M-NaCl, with a 20-fold specific enrichment. (2) The pooled fractions (treated as described in the Materials and methods section) were filtered on the 1 ml MonoQ HR5/5 column. Elution with the NaCl gradient yielded two fractions. Fraction A, which was eluted at 0.21–0.24 M-NaCl, was considerably enriched in the 67 kDa t-PBP5. Fraction B, which was eluted at 0.19–0.21 M-NaCl, contained almost equivalent amounts of 67 kDa t-PBP5 and 60 kDa t-PBP5 plus much smaller amounts of 44 kDa t-PBP5. (3) Fraction A and fraction B (treated as described in the Materials and methods section) were loaded on to the phenyl-Superose HR5/5 column. The 60 kDa t-PBP5 from fraction B was eluted at 0.3–0.15 M-$(NH_4)_2SO_4$. It was about 85 % pure (Fig. 1, lanes 4 and 7). The 67 kDa t-PBP5 from fraction A was eluted at an $(NH_4)_2SO_4$ concentration of less than 0.15 M. It was at least 95 % pure (Fig. 1, lanes 5 and 8).

Tryptic digestion of the purified 67 kDa t-PBP5: the 44 kDa t-PBP5, 35 kDa t-PBP5, 32 kDa PBP5 and 30 kDa t-PBP5 tryptic-digest fragments

The 67 kDa t-PBP5 (0.4 mg in 0.4 ml of 40 mM-sodium phosphate buffer, pH 6.5, containing 1 mM-$MgCl_2$) was incubated with 10 % (w/w) trypsin for 60 min at 37 °C. As a result of this drastic proteolytic treatment, the 67 kDa t-PBP5 was degraded into six fragments (Fig. 1, lane 9). Fragments of molecular mass smaller than 30 kDa lacked penicillin-binding capacity (Fig. 1, lanes 6 and 9). With the procedure described previously [27,28], the membrane-bound PBP5 and the 67 kDa, 60 kDa and 44 kDa fragments reacted with benzylpenicillin with the same low second-order rate constant of protein acylation (5–9 $M^{-1} \cdot s^{-1}$) and the same low first-order rate constant of protein deacylation (6×10^{-6}–13×10^{-6} s^{-1}). Note that, at those concentrations needed to saturate PBP5, penicillin partially acylated BSA.

Specificity profile of the anti-(67 kDa t-PBP5) antibodies

Antibodies raised against the tryptic derivatives of PBP5 (see the Materials and methods section) reacted only with PBP5 (plus two weaker bands) but not with the other PBPs present in the membranes of Ent. hirae R40 (Fig. 1, lane 3). They also reacted with all the tryptic-digest PBP5 fragments described above (Fig. 1, lanes 10–12).

Amino acid sequencing data and PCR amplification

The peptide fragments (usually 600 pmol) separated by SDS/PAGE as shown in Fig. 1 (lanes 4–to 12) were electroblotted and the amino acid sequence of the N-terminal region of each individual peptide was determined by automated microsequence analysis (Fig. 2). On the basis of these data, the two oligonucleotides shown in Fig. 3 were synthesized. Oligonucleotide 1 had a BamHI site at the 5'-end and coded for the sequence Q11–K17 of the 60 kDa t-PBP5. Oligonucleotide 2 had an EcoRI site at the 5'-end and was complementary to the nucleotide sequence coding for the sequence E16–L23 of the 44 kDa t-PBP5. Amplification by the PCR technique with the oligo-
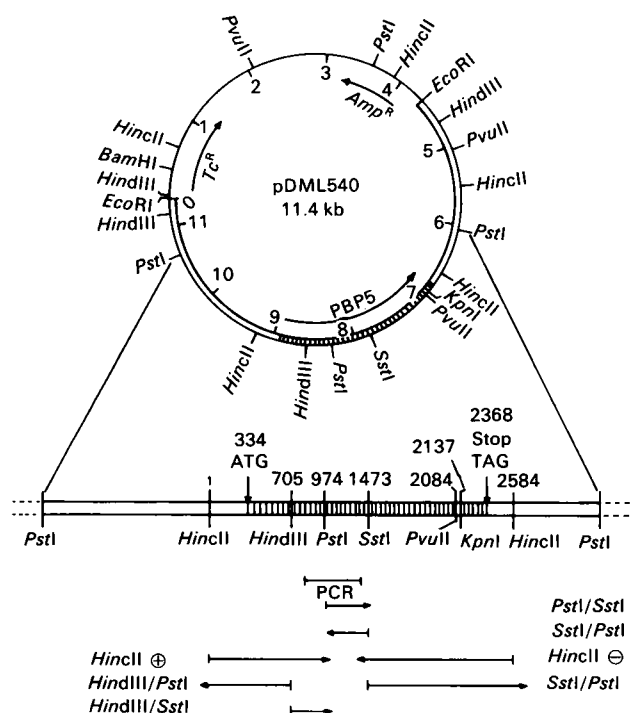
Fig. 4. Restriction map of pDML540 and strategy used for nucleotide sequencing of the Ent. hirae R40 PBP5-encoding gene

nucleotides as primers and the Ent. hirae R40 DNA as template generated a 476 bp DNA segment the sequence of which translated into a 155-amino acid-residue segment. This peptide, called PBP5-PCR, is also shown in Fig. 3.

Gene cloning and sequencing

The Ent. hirae R40 DNA (2 μg) was partially digested with EcoRI, the DNA fragments were inserted into pBR322 (1 μg) previously cut by EcoRI and dephosphorylated, and the ligation mixture (5 μl out of 20 μl) served to transform Esh. coli HB101 cells. Transformants were selected on solid LB medium containing 50 μg of ampicillin/ml, transferred on to nylon filters (Amersham International) and screened with the [γ-^{32}P]ATP-labelled 24-mer probe (Fig. 3). Hybridization was carried out at 50 °C and post-hybridization washing at 65 °C (T_m 72 °C). Of the 2150 ampicillin-resistant Esch. coli transformants tested, one clone gave a strong positive reaction with the probe. The harboured plasmid pDML540 (11.4 kb) had acquired a 7.1 kb insert. pDML540, whose restriction map is shown in Fig. 4, was digested separately by HincII and by PstI and SstI. The HincII 2.6 kb DNA fragment and the PstI–SstI 0.5 kb DNA fragment hybridized with the probe (results not shown). Nucleotide sequencing of the PstI–SstI fragment allowed, on the basis of the position of the PstI site in the PBP5-PCR product, establishment of the orientation of the PBP5-encoding gene, from PstI to SstI. The HincII 2.6 kb DNA fragment was sequenced on both strands, by use of the strategy also shown in Fig. 4. A 2034-nucleotide-residue open reading frame (Fig. 5) started at position 334 with an ATG codon and terminated at position 2368 with a TAG stop codon.

Amino acid sequence of PBP5 and sequence-similarity searches

The open reading frame translated into a 678-amino acid-residue protein. As derived from the amino acid sequence (Fig. 5), the 67 kDa, 60 kDa, 44 kDa, 32 kDa and 30 kDa peptide fragments were produced by tryptic cleavage of the bonds K76–T,

*Hinc*II
GTCAACTCTTGGGAAGTATTAAAACCAATTTACCTGAAAGTGTATTAATTGATTGTGGCATTGATTTCTTAAAAGATGACAAACCAATTGATACGTTATCTGTACCAGTTGAAGGTAGTT
1   120

GGAATTTTAATGATTACACACCTTCTGGTAGTGTACTAGAACTTGATTTAGAAAAAAATCAATTAGCAATCAATCAGTTTTTAGACCAATAGATTTACATTTTATTTAAAGTGTAAGAAG
121   240

                    (-35)   (-10)   RBS   M K R S D N H R R 9
TTAGAAACAAGCGATTGTCGTTGTGTTTTAGCTTCTTTTCTTATGGTAAAATGGATAGAAGTTAAGATAAGAAAATGTGAGCGGAGGGACAAAATGAAACGAAGTGACAATCATCGTAGG
241   360

   N K T G V Y L T S A L V L V L A A G G G Y F Y Y Q K T Q E E Q V V S A G E K K I 49
AACAAGACTGGTGTTTATCTAACAAGCGCCCTTGTTTTGGTTCTTGCGGCTGGTGGCGGGTATTTTTACTATCAAAAAACGCAAGAAGAGCAAGTGGTTTCAGCAGGAGAAAAAAGATC
361   480

   Q Q F A E R L S T G D Y K K A M S Y T Q I H S Q T K K T I S E K E A L E K Y Q N 89
CAACAGTTTGCGGAACGTTTGAGTACTGGTGATTATAAAAAGGCGATGTCATACACGCAAATTCATTCACAGACTAAGAAGACAATTTCTGAAAAAGAAGCTCTTGAAAAATACCAAAAT
481   600

   I Y G A V A I K G L E I S N L K V T K K D S E T Y S F S Y K A K M N T S L G E L 129
ATTTACGGAGCTGTGGCCATAAAAGGCTTGGAAATCTCTAATTTAAAAGTGACGAAAAAAGATAGTGAAACCTATTCTTTTTCTTATAAAGCTAAAATGAATACAAGCTTAGGCGAATTA
601   *Hind*III   720

   K D L S Y K G T L T N K N D Q I K I N W Q P N L I F P Q M E D T D K V S L T S E 169
AAAGACCTATCATATAAAGGGACTTTGACAAATAAAAATGATCAGATCAAAATCAACTGGCAGCCGAACTTGATTTTTCCTCAAATGGAAGATACAGACAAAGTAAGTCTGACTAGTGAG
721   840

   E A K R G D I L D R N G K K L A T T G K L K Q L G I V P R K L G E K E E K T A N 209
GAAGCTAAAAGAGGCGATATCCTTGATCGAAATGGAAAGAAATTAGCAACGACGGGCAAATTGAAACAGTTAGGGATCGTTCCTAGAAAGCTGGGAGAAAAAGAAGAGAAAACAGCAAAC
841   960

   I K A I A A A F D L S E D E I N Q A I S Q S W V Q P D Y F V P L K I I D G Q T P 249
ATCAAAGCAATTGCTGCAGCTTTTGATCTTTCAGAAGATGAAATCAATCAAGCTATCTCTCAAAGTTGGGTACAACCGGACTACTTTGTTCCTCTAAAAATCATTGATGGACAAACCCCC
961   *Pst*I   Probe   1080

   E L P S G R A I Q E V D G R T Y P L G E R A A Q L I G Y V G D I S A E D I K K N 289
GAATTGCCAAGCGGACGAGCGATCCAAGAAGTTGATGGTCGCACCTATCCGTTAGGCGAACGAGCAGCGCAATTGATTGGTTATGTTGGTGATATCTCAGCAGAAGATATCAAAAAGAAT
1081   1200

   A E L S S N G K I G R S G L E M T Y D K E L R G T N G G K L S I T D A D G T E K 329
GCTGAATTAAGTAGTAATGGTAAAATTGGTCGTTCTGGTCTGGAAATGACTTATGACAAAGAACTACGTGGCACCAATGGAGGGAAATTGAGTATTACGGATGCTGATGGGACAGAAAAA
1201   1320

   E V L I D Q E V K N G Q D I K L T L D A D A Q K I A F D S L G G K A G S T V A T 369
GAAGTATTGATTGATCAAGAAGTGAAAAATGGTCAGGATATCAAATTAACTCTTGATGCCGATGCTCAAAAAATCGCATTTGATAGTTTAGGTGGAAAAGCGGGTTCAACAGTAGCGACT
1321   1440

   S P K T G E L L V L A S S P S Y D P N K M T N G I S Q E D Y Q A Y T E N K D Q P 409
TCGCCAAAAACGGGTGAGTTGTTAGTATTAGCGAGCTCTCCAAGTTATGATCCAAACAAAATGACAAATGGAATCTCTCAAGAAGATTATCAAGCCTATACGGAAAATAAAGATCAACCA
1441   *Sst*I   1560

   F I S R F A T G Y A P G S T F K M I T A A I G L D N Q T L N P D E V L T I N G L 449
TTTATTAGTCGTTTTGCTACCGGTTACGCTCCCGGATCTACATTTAAAATGATTACTGCCGCAATCGGATTAGATAATCAAACGCTGAATCCAGACGAAGTATTGACGATCAATGGATTG
1561   1680

   K W Q K D Q S W G S Y Q V T R V S D V P Q V N L R N A M I Y S D N I Y M A Q E T 489
AAATGGCAAAAAGACCAATCATGGGGATCGTATCAAGTTACTCGTGTCAGCGATGTGCCTCAAGTCAATCTGAGAAACGCAATGATTTATTCAGATAATATCTATATGGCTCAAGAAACC
1681   1800

   L K M G E K K F R E G L N K F I F G E D L D L P I S M N P A Q I S N K D S F K S 529
TTGAAAATGGGTGAGAAGAAATTCCGCGAAGGATTAAATAAATTCATTTTTGGGGAAGACTTAGATTTGCCGATCAGCATGAATCCCGCACAAATCTCAAATAAGGATAGTTTCAAGTCA
1801   1920

   E I L L A D T G Y G Q G E L L I N P I Q Q A T M Y S V F A N N G N L V Y P K L E 569
GAAATCTTATTAGCAGATACTGGTTATGGGCAGGGAGAACTATTGATCAATCCGATCCAACAAGCAACGATGTATTCTGTTTTTGCTAATAATGGAAATCTCGTTTATCCAAAATTAGAG
1921   2040

   M D K E T K V K Q N V I A S T A V Q T I L P D L K D V V Q D T N G T A H S L A T 609
ATGGATAAAGAAACGAAAGTTAAACAAAATGTCATTGCTTCAACAGCTGTACAGACGATTCTGCCAGATTTGAAAGATGTTGTTCAAGATACGAATGGTACCGCTCATTCTTTAGCTACT
2041   *Pvu*II   *Kpn*I   2160

   L G I P I A A K T G T A E I K E K Q D E K G Q E N S F L F A V N P D T N G Y L M 649
TTAGGCATACCAATAGCAGCAAAAACTGGTACAGCGGAAATCAAAGAAAAACAAGATGAAAAAGGACAAGAAAATAGCTTCCTATTTGCTGTGAATCCTGATACAAATGGTTATCTCATG
2160   2280

   I S M L E D K A E G D S A T K R A P E L L Q Y L N Q N Y Q *   678
ATCAGCATGTTAGAAGATAAAGCAGAAGGTGATTCTGCAACGAAACGTGCACCTGAGTTGCTCCAATACTTGAATCAAAACTATCAATAGACGAATGAATAAAACTAATCAATGCATGTA
2281   2400

GTCACTACTTACATTTGGGCTAGGAAAGGTAAATTATTTAGAATACTACAGTCACTCGATTAGTTTTTTTTATATAGGGATGCTTTAGAATGATCTTTTTACCTTTGGTTTAACGATAAAA
2401   2520

GTAGCTAAACCTAATAAAATGAATCCAATAATGAATGGCATCGTATGATGGAAATTAGCATATGTTGAC
2521   2580   *Hinc*II

Fig. 5. Nucleotide sequence of the *Ent. hirae* R40 PBP5-encoding gene and deduced amino acid sequence of the protein

The potential promoter −35, −10 hexamers, the Shine–Dalgarno sequence (RBS), the restriction sites and the probe used to screen the clones are underlined. The inverted repeats of the putative transcription termination signal are indicated by horizontal arrows.
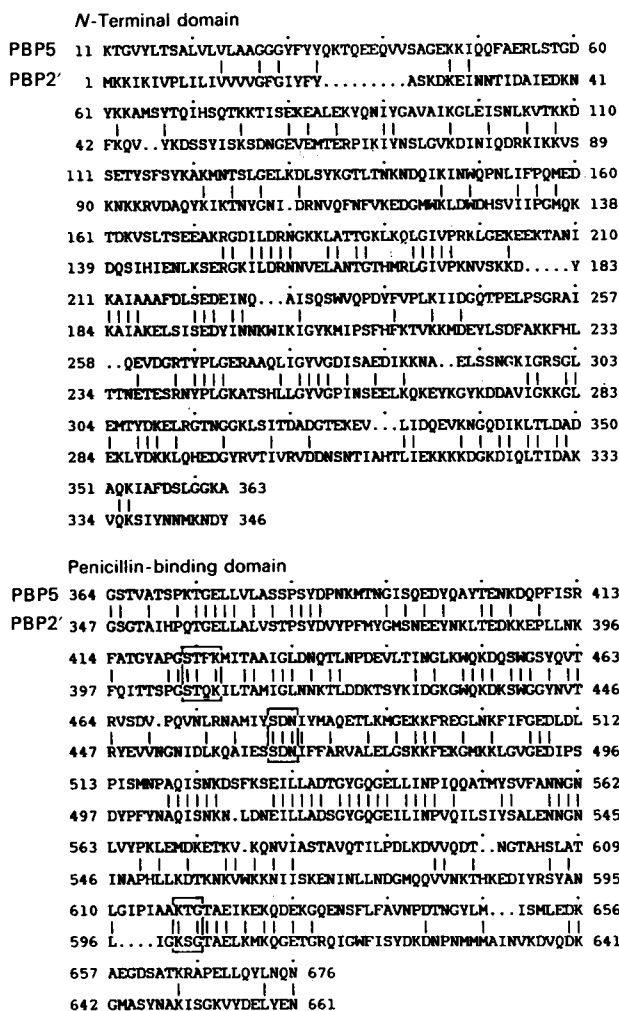
```
                 N-Terminal domain

PBP5   11  KTGVYLTSALVLVLAAGGGVFYYQKTQEEQVVSAGEKKIQQFAERLSTGD  60
PBP2'   1  MKKIKIVPLILIVVVVGFGIYFY.........ASKDKEIWNTIDAIEDKN  41

       61  YKKAMSYTQIHSQTKKTISEKEALEKYQNIYGAVAIKGLEISNLKVTKKD  110
       42  FKQV..YKDSSYISKSDNGEVEMTERPIKIVNSLGVKDINIQDRKIKKVS  89

      111  SETYSFSYKAKMNTSLGELKDLSYKGTLTNKNDQIKINWQPNLIFPQMED  160
       90  KNKKRVDAQYKIKTNYGNI.DRNVQFNFVKEDGMWKLDWDHSVIIPCMQK  138

      161  TDKVSLTSEEAKRGDILDRNGKKLATTGKLKQLGIVPRKLGEKEEKTANI  210
      139  DQSIHIENLKSERGKILDRNNVELANTGTHMRLGIVPKNVSKKD.....Y  183

      211  KAIAAAFDLSEDEINQ...AISQSWVQPDYFVPLKIIDGQTPELPSGRAI  257
      184  KAIAKELSISEDYINNKWIKIGYKMIPSFHFKTVKKMDEYLSDFAKKFHL  233

      258  ..QEVDGRTYPLGERAAQLIGYVGDISAEDIKKNA..ELSSNGKIGRSGL  303
      234  TTNETESRNYPLGKATSHLLGYVGPINSEELKQKEYKGYKDDAVIGKKGL  283

      304  EMTYDKELRGTNGGKLSITDADGTEKEV...LIDQEVKNGQDIKLTLDAD  350
      284  EKLYDKKLQHEDGYRVTIVRVDDNSNTIAHTLIEKKKKDGKDIQLTIDAK  333

      351  AQKIAFDSLGGKA  363
      334  VQKSIYNNMKNDY  346


                 Penicillin-binding domain

PBP5  364  GSTVATSPKTGELLVLASSPSYDPNKMTNGISQEDYQAYTENKDQPFISR  413
PBP2' 347  GSGTAIHPQTGELLALVSTPSYDVYPFMYGMSNEEYNKLTEDKKEPLLNK  396

      414  FATGYAPGSTFKMITAAIGLDNQTLNPDEVLTIMGLKWQKDQSWGSYQVT  463
      397  FQITTSPGSTQKILTAMIGLNNKTLDDKTSYKIDGKGWQKDKSWGGYNVT  446

      464  RVSDV.PQVNLRNAMIVSDNIYMAQETLKMGEKKFREGLNKFIFGEDLDL  512
      447  RYEVVNGNIDLKQAIESSDNIFFARVALELGSKKFEKGMKKLGVGEDIPS  496

      513  PISMNPAQISNKDSFKSEILLADTGYGQGELLINPIQQATMYSVFANNGN  562
      497  DYPFYNAQISNKN.LDNEILLADSGYGQGEILINPVQILSIYSALENNGN  545

      563  LVYPKLEMDKETKV.KQNVIASTAVQTILPDLKDVVQDT..NGTAHSLAT  609
      546  INAPHLLKDTKNKVWKKNIISKENINLLNDGMQQVVNKTHKEDIYRSYAN  595

      610  LGIPIAAKTGTAEIKEKQDEKGQENSFLFAVNPDTNGYLM...ISMLEDK  656
      596  L....IGKSGTAELKMKQGETGRQIGWFISYDKDNPNMMMAINVKDVQDK  641

      657  AEGDSATKRAPELLQYLNQN  676
      642  GMASYNAKISGKVYDELYEN  661
```

Fig. 6. Amino acid sequence alignment of the Ent. hirae R40 PBP5 and Staph. aureus PBP2'

Vertical lines indicate strict identities. The S*XXK, SDN and KT(S)G motifs the penicillin-binding domains are boxed.

K146–I, K288–N, K389–M and K406–D respectively. The *Ent. hirae* R40 PBP5 had significant similarity in the primary structure to the *Esch. coli* PBPs 2 [35] and 3 [36] (27% and 22% strict identities respectively), the *Strep. pneumoniae* PBPs 2X [16] and 2B [37] (24% and 25% respectively), the *N. gonorrhoeae* PBP2 [18] (25%) and the methicillin-resistant *Staph. aureus* PBP2' [12] (33%). Alignment of the *Ent. hirae* PBP5 and the *Staph. aureus* PBP2', by using the Bestfit program in the GCG package (gap weight: 5.0; length weight: 0.3), generated 223 strict identities (Fig. 6). The significance of the degree of similarity found between the two aligned amino acid sequences was estimated with the seqdp program of Goad & Kanehisa [38]. The alignment had scores of −400 for the *N*-terminal domains (27% strict identities) and −490 for the *C*-terminal domain (40% strict identities). These scores were 30 and 53 standard deviations respectively above that expected from a run of 20 randomized pairs of proteins having the same amino acid compositions as the two domains under consideration.

DISCUSSION

Upstream from the ATG initiation codon of the *Ent. hirae* R40 PBP5-encoding gene there occur nucleotide sequences that

are analogous to the canonical −35 and −10 promoter sequences found in *Esch. coli* [39], lactococci [40] and pneumococci [41]. Of the two potential −35 hexamers, that starting at position T261 fits best the known rules for an active promoter sequence. The GGAGG pentamer located 11 nucleotide residues upstream from the ATG codon has the characteristics of the Shine–Dalgarno sequences found in *Staph. aureus* and *Bacillus* species [42]. The TAG stop codon is followed by an inverted repeat, which might act as a terminator.

The *β*-lactamases of class A and the *Streptomyces* R61 low-molecular-mass PBP of known three-dimensional structure have a unique signature in the form of four conserved amino acid groupings, which, as a result of the folding of the polypeptide chain, are brought close to each other and define the enzyme active site. The S*XXK motif, about 60 residues downstream from the *N*-terminus of the protein, is central to the cavity with the active-site serine S* located at the *N*-terminus of one *a*-helix. The S(Y)XN motif on a loop that connects two *α*-helices forms one side of the cavity. The triad K(H)T(S)G, about 60 residues upstream from the *C*-terminus of the protein, occurs on the innermost strand of a five-stranded *β*-sheet and forms the other side of the cavity. Finally, a peptide segment, the D/E motif, containing two dicarboxylic amino acid residues occurs, in the primary structure, downstream of the S(Y)XN motif and, in the tertiary structure, on a loop or small *α*-helix at the entry of the cavity [1,43].

Assuming that this model applies to the high-molecular-mass PBPs, the methicillin-resistant *Staph. aureus* 670-amino acid-residue PBP2' [12] consists of a ~ 345-amino acid-residue *N*-terminal domain connected to a ~ 325-amino acid-residue *C*-terminal penicillin-binding domain and has the active-site serine residue at position 405 (Fig. 6). Similarly, the penicillin-resistant *Ent. hirae* R40 678-amino acid-residue PBP5 consists of a ~ 360-amino acid-residue *N*-terminal domain connected to a ~ 320-amino acid-residue penicillin-binding domain and has the active-site serine residue at position 422. Amino acid alignment and sequence-similarity searches reveal that the *N*-terminal domains and the *C*-terminal domains of PBP2' and PBP5 are very similar. Alignment also highlights the occurrence of the conserved S*XXK, SDN and KT(S)G motifs. E508 or E542 (in the numbering of PBP5) are possible candidates for the E/D motif.

It has been proposed that the methicillin-resistant *Staph. aureus* strains have imported an inducible high-molecular-mass PBP2' and that the encoding gene has probably evolved by recombination of a PBP gene of some bacterium and an inducible *β*-lactamase gene [12]. In contrast, the enterococcal PBP5 gene seems to be constitutive. Whatever their origin, the *Staph. aureus* PBP2' and *Ent. hirae* PBP5 are only about 25 times more susceptible to penicillin than BSA. Each of these PBPs can take over the functions needed for wall peptidoglycan assembly under conditions where all the other PBPs are inactivated by reaction with *β*-lactam antibiotics. These high-molecular-mass PBPs probably represent the ultimate achievement in intrinsic resistance to these antibiotics.

Tryptic cleavage of the K76–T77 bond causes release of PBP5 from the *Ent. hirae* R40 membranes in the form of a pencillin-binding water-soluble derivative, showing that the membrane anchor of the PBP is located upstream from K76. Comparison of the molecular mass of the membrane-bound PBP (71 000 Da, as derived from migration on SDS/PAGE) with that of the precursor (74 862 Da), suggests that the precursor may undergo processing by elimination of a 30–40-amino acid-residue peptide at the *N*-terminus of the protein. This region possesses six diamino acids between M1 and T12, a long G13–G29 hydrophobic segment and several putative leader-peptidase-cleavage sites (S18–A19, A25–A26, A26–G27, S43–A44 or A44–A45) [44].

The exact mode of membrane anchoring of PBP5 will remain unknown as long as the membrane-bound form has not been isolated and its *N*-terminal region has not been sequenced. Note that immunological and penicillin-binding analyses (results not shown) indicate that PBP5 is expressed in *Esch. coli* and co-migrates with the natural form, thus suggesting that in *Ent. hirae* R40 PBP5 is probably not processed.

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