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Importance of the E-46–D-160 Polypeptide Segment of the Non-Penicillin-Binding Module for the Folding of the Low-Affinity, Multimodular Class B Penicillin-Binding Protein 5 of *Enterococcus hirae*

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Compared with the other class B multimodular penicillin-binding proteins (PBPs), the low-affinity PBP5 responsible for penicillin resistance in *Enterococcus hirae* R40, has an extended non-penicillin-binding module because of the presence of an ≈110-amino-acid E-46–D-160 insert downstream from the membrane anchor. Expression of *pbp5* genes lacking various parts of the insert-encoding region gives rise to proteins that are inert in terms of penicillin binding, showing that during folding of the PBP, the insert plays a role in the acquisition of a correct penicillin-binding configuration by the G-364–Q-678 carboxy-terminal module.

Enterococci are the agents of many nosocomial infections in humans (2, 11) and the second leading cause of hospital-acquired infections in the United States (19). Penicillin resistance is most often mediated by penicillin-binding proteins (PBPs) and rarely by β-lactamase (1, 10, 18). *Enterococcus hirae* ATCC 9790, having moderate resistance to penicillin, produces small amounts of the low-affinity PBP5. *E. hirae* R40, a laboratory mutant derived from ATCC 9790, overproduces PBP5 and has a considerably increased resistance (6). *E. hirae* S185, a clinical isolate from the pig intestine, is of intermediate penicillin resistance. It produces both PBP5 and another low-affinity PBP, PBP3r (13). Similarly, penicillin resistance among *Staphylococcus aureus* and *Staphylococcus epidermidis* strains is due to the production of the *mecA*-encoded low-affinity PBP2′ (also called PBP2a) (9). *pbp5* and *mecA* are chromosomal (4, 9). In contrast, *pbp3r* is plasmid borne (15).

As derived from gene sequencing and hydrophobic cluster analysis, PBP5, PBP3r, and PBP2′ have an identity index of 29% (4, 14, 17). They belong to the multimodular class B PBPs (5). At variance with the other members of the same class, however, each of the low-affinity PBPs has an extended non-penicillin-binding (non-PB) module because of the presence of an ≈110-amino-acid segment, E-46–D-160, that is inserted immediately downstream from the transmembrane anchor (5, 8, 14). The inserts of the three PBPs under consideration are related in amino acid sequence and, presumably, have the same polypeptide fold. The modular design of PBP5 is shown in Fig. 1A. In order to establish whether the E-46–D-160 insert plays a role during biosynthesis of PBP5, *pbp5* genes lacking large portions of the insert-encoding sequence were expressed in *Escherichia coli* and the abilities of the produced, membrane-bound truncated proteins to bind penicillin were estimated by using standard penicillin-binding assay procedures.

pDML540, a pBR322 derivative bearing a 7.1-kb *Eco*RI *pbp5*-containing fragment (4), was digested with *Hin*cII. The released 2.6-kb *pbp5*-containing fragment was subcloned into the *Sma*I site of the M13mp18 replicative form. From this, the 2.7-kb *Eco*RI-*Pvu*I fragment was reinserted into pBR322, yielding pDML541. Finally, the 2.6-kb *Eco*RI-*Sal*I fragment was inserted in the high-copy-number pUC18, generating pDML546.

Deletions in *pbp5* (using the nested deletion kit of Pharmacia Biotech) were made as follows. pDML546 was cleaved with *Bal*I (in the middle part of the insert-encoding sequence) and treated with exonuclease III from 8 to 18 min at 20°C in ExoIII buffer (Pharmacia Biotech kit)–100 mM NaCl (to slow down the rate of the reaction). Samples were removed at 1-min intervals and incubated with S1 nuclease for 30 min at 24°C, and the reaction was stopped by adding the S1 stop solution (303 mM Tris base–50 mM EDTA) and heating the samples for 10 min at 65°C. The DNA was recircularized in 5% polyethylene glycol with the ligase mix provided with the kit and used to transform *E. coli* DH5α (16). Transformants were selected on Luria-Bertani plates (1.5% agar–75 μg of ampicillin ml^{-1}).

Plasmids of randomly chosen clones (two for each incubation time with exonuclease III) were digested with *Eco*RI and *Sal*I and subjected to agarose gel electrophoresis. Clones harboring plasmids the insert of which was smaller than 2.6 kb were grown in liquid medium, and the presence of PBP5 derivatives in the isolated membranes was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using anti-PBP5 antibodies (4, 14) (Fig. 1B).

Plasmids encoding PBP5 derivatives were isolated from the corresponding clones by the alkaline lysis method (16) and purified on Macherey-Nagel Nucleobond columns. By using the dideoxyribonucleotide chain termination reaction with the AutoRead sequencing kit and the ALF DNA sequencer of Pharmacia Biotech, the regions which encoded the 110-amino-acid insert in the wild-type *pbp5* were sequenced, allowing the deletions to be identified. Membranes (total protein, 200 μg) of the same clones were treated with 100 μM benzyl[^{14}C] penicillin for 60 min either immediately or after a 30-min preincubation with 1 μM nonradioactive penicillin and submitted to SDS-PAGE, and the gels were analyzed by fluorography (Fig. 1B) (3, 4, 14). Membranes of *E. coli*/pDML546 and *E. hirae* R40 served as positive controls. Membranes of *E. coli* DH5α served as a negative control.

The outcome of these experiments was that (i) eight trans-

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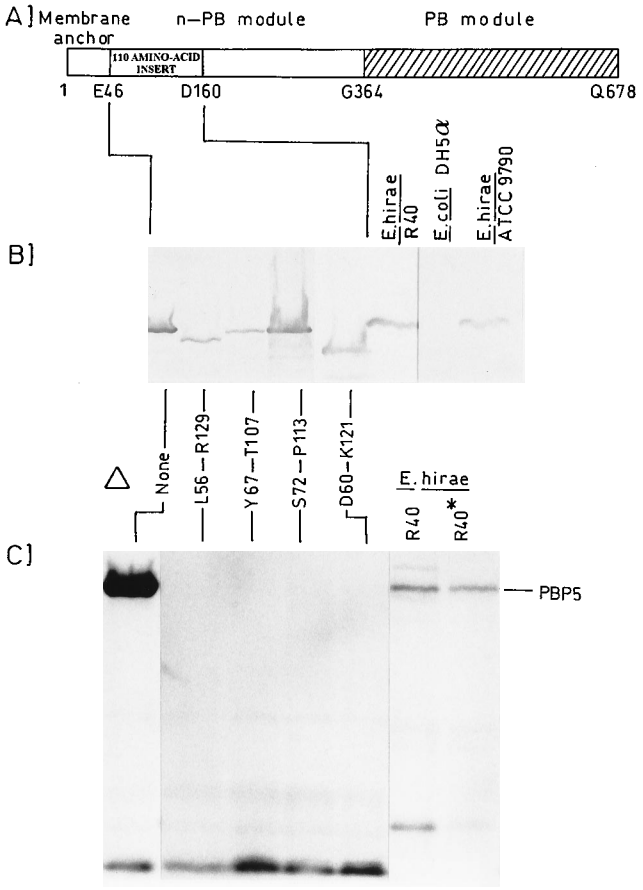

FIG. 1. (A) Modular design of the *E. hirae* PBP5; (B) Western blot (immunoblot) of the wild-type and truncated PBP5 forms with the positions of the deleted segments indicated; (C) SDS-PAGE and fluorography of isolated membranes labeled with benzyl[^{14}C]penicillin. The negative control in the Western blot is a sample of *E. coli* DH5α membranes. Positive controls in the Western blot and fluorography are *E. hirae* ATCC 9790 and R40 membranes. R40*, labeled R40 membranes which were pretreated with 1 μM nonradioactive penicillin. The lower radioactive bands are the DD-carboxypeptidases of the *E. coli* and *E. hirae* membranes, respectively.

formants each of which contained a plasmid with a truncated *pbp5* gene were obtained; (ii) of these, only four (corresponding to the 14- to 17-min exonuclease III treatments) gave positive immunoblot results; (iii) the deletions introduced in the E-46–D-160 insert extended from Y-67 to T-107 (39 amino acid residues), from S-72 to P-113, substituting T-113 (40 amino acid residues), from D-60 to K-121 (60 amino acid residues), and from L-56 to R-129, substituting L-129 (72 amino acid residues); and (iv) these truncated forms of PBP5, when produced in *E. coli*, were inert in terms of penicillin binding (Fig. 1C).

The monofunctional penicilloyl serine transferases, i.e., the β-lactamases and low-molecular-mass PBPs, seem to fold rapidly presumably via the transitory formation of a molten globule (as shown with the TEM β-lactamase [20]). As a polypeptide chain increases in length, finding the right fold becomes more difficult. The unsuccessful attempts to produce the PB module of PBP5 independently from the rest of the protein (12) support the results of the present investigation. Moreover, upon treatment of isolated membranes of *E. hirae* R40 with trypsin, the preformed PBP5 gives rise to penicillin-binding fragments the size of which, 30 to 40 kDa, is equivalent to that of the PB module (4). All these results lead to the same conclusion. Acquisition of stable penicillin-binding folding by the PB module of PBP5 requires concomitant biosynthesis of the complete, extended non-PB module. Studies of derivatives of PBP2′ of *Staphylococcus aureus* and PBP1b and PBP3 of *E. coli* overproduced from appropriate expression/secretion vectors suggest that this property is common to the multimodular, class A and class B PBPs (7, 21).

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