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A water-soluble form of penicillin-binding protein 2 of *Escherichia coli* constructed by site-directed mutagenesis

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Penicillin-binding protein (PBP) 2 of *Escherichia coli* is located in the cytoplasmic membrane. The N-terminal hydrophobic segment (31 amino acids, residues 15–45) of PBP2 was removed by a deletion in the PBP2 gene by site-directed mutagenesis, resulting in the production of a water-soluble form of PBP2 (called PBP2*). PBP2* retained the penicillin-binding activity, was localized in the cytoplasm and was overproduced under the control of the *lpp-lac* promoter. This indicates that the removed hydrophobic segment is an uncleaved signal sequence required for translocation of PBP2 across the cytoplasmic membrane, and also suggests that the segment anchors the protein to the membrane.

Peptidoglycan; Penicillin-binding protein; Ectoprotein; Amino-terminal anchor; Water-soluble enzyme; Site-directed mutagenesis

1. INTRODUCTION

Seven penicillin-binding proteins have been found in the cytoplasmic membrane of *Escherichia coli* [1]. Among them, high-molecular-mass PBPs (PBP1A [2], PBP1B [3–5], PBP2 [6] and PBP3 [7]) are bifunctional enzymes showing both penicillin-insensitive peptidoglycan transglycosylase and penicillin-sensitive peptidoglycan transpeptidase activities. In the case of PBP2, the two activities are detected only in the presence of the RodA protein in membrane preparations [6]. These PBPs are essential for normal cell growth and are the killing targets for penicillin and other β-lactam antibiotics.

The *pbpA* gene that encodes PBP2 has been cloned [8]. Recently, its nucleotide sequence and the deduced amino acid sequence were reported [9]. The hydropathy profile of PBP2 suggested that a hydrophobic segment close to the N-terminus of the protein is the only sequence that can span the membrane [9]. Therefore, the N-terminal region of PBP2 seems not only an uncleaved signal sequence [9] but also an anchor of the protein to the cytoplasmic membrane. Thus, we attempted to make the protein soluble by removing the N-terminal hydrophobic segment through a deletion. In this paper, the production of a water-soluble form of PBP2 (called PBP2*) and its properties are described.

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Abbreviations: PBP, penicillin-binding protein; IPTG, isopropyl β-D-thiogalactoside

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E. coli MV1184 [*ara Δ(lac-pro) rpsL thi* φ80d*lacZ*ΔM15 *Δ(srl-recA)306*::Tn10, F′:*traD36 proAB lacI*^{q}ZΔM15] was used as a host for plasmids. The following plasmids were used:

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pMA102 [8], containing the *pbpA* and *rodA* genes; expression vector pIN-II-A1 [10], containing a strong *lpp-lac* fused promoter (shown by a thick arrow in fig.1) and an *lpp* ribosome-binding site, followed by the initiation codon and cloning sites; pUC13; and pUC4K [11], containing the kanamycin-resistance gene. The methods used for gene engineering were essentially the same as those described in [12]. Restriction endonucleases and other modifying enzymes were standard commercial products.

2.2. *Oligonucleotide-directed site-specific mutagenesis*

A deletion was made by oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA, according to Morinaga et al. [13]. The oligodeoxyribonucleotide, CGGCAGAGTCCCGCTTTACCGA, used as a mutagenic primer was synthesized with a DNA synthesizer system 1 plus (Beckman) and purified by reverse-phase high-performance liquid chromatography. [γ-^{32}P]ATP (~5000 μCi/mmol, Amersham) was used for labeling the hybridization probe. DNA sequencing was carried out by the dideoxy method using an M13 sequencing kit (Takara Shuzo Co.) and [α-^{32}P]dCTP (~400 μCi/mmol; Amersham).

2.3. *Growth and fractionation of cells*

E. coli MV1184 harboring a plasmid was grown in RH medium (15 g of Bactotryptone and 8 g of NaCl per liter, pH 7.0) with thiamine-HCl (1 μg/ml) at 37°C to the early logarithmic phase, and then expression was induced by the addition of IPTG to a final concentration of 1 mM. After induction for 3 h at 37°C, the cells were harvested. The periplasmic fraction was obtained by the cold osmotic shock method [14]. Cells, after removal of the periplasmic fraction, were resuspended in ice cold 10 mM sodium phosphate buffer (pH 7.0) and then sonicated on ice. After removal of debris, the supernatant was centrifuged at 100000 × *g* for 40 min at 4°C. The supernatant and precipitate thus obtained were termed the cytoplasmic and membrane fractions, respectively. The cyclic phosphodiesterase activity of each fraction was measured using bis(*p*-nitrophenyl)phosphate as a substrate [14]. The PBP assay was carried out using [^{3}H]benzylpenicillin (*N*-ethylpiperidinium salt) (16.4 μCi/μl, 56.9 μCi/μg; a gift from Dr P.J. Cassidy, Merck Sharp and Dohme, NJ, USA), as described [15].

3. RESULTS

Expression vectors for PBP2, pHA102 and pHA103, were constructed as shown in fig.1. The *pbpA* gene that encodes PBP2 can be transcribed under the control of the *lpp-lac* fused promoter in the presence of the *lac* inducer, IPTG. These plasmids carry the kanamycin-resistance gene instead of the ampicillin-resistance gene. The N-terminal sequence of PBP2 encoded by the *pbpA* gene in pHA102 and pHA103 was a little different from that of the original PBP2 owing to a change in the N-terminal coding region; Met-Lys-Leu-Gln- changed to Met-Lys-Gly-Asn-Ser-. Both pHA102 and pHA103 complemented a *pbpA* mutant.

The N-terminal hydrophobic region of PBP2 contains a long stretch of 25 non-ionic amino acids (residues 21–45; average hydropathy, +2.1) [9] (fig.2). A deletion of 93 nucleotides encoding 31 amino acids including the long stretch (residues 15–45) (fig.2) was introduced into the *pbpA* gene of pHA102 by oligonucleotide-directed site-specific mutagenesis, and plasmid pHA102Δ93 was obtained. Then, pHA103Δ93 was derived from pHA102Δ93 by removing the 1.1-kb *Apa*I-*Bam*HI fragment containing the *rodA* gene. The deletion was verified by DNA sequencing. The resulting N-terminal sequence of PBP2 is shown in fig.2, and we called the resulting protein, PBP2*.

To determine the localization of PBP2*, IPTG-induced *E. coli* cells harboring pHA103Δ93 were separated into periplasmic, membrane and cytoplasmic fractions. The ratio of the relative activity of cyclic phosphodiesterase, a periplasmic enzyme, in each fraction obtained from the same amount of cells was 78:21:1 (periplasmic/cytoplasmic/membrane fractions, respectively), showing that the fractionation was good but not complete. The PBP assay results for each fraction, obtained from the same amount of cells, are shown in fig.3. PBP2* was overproduced and located only in the cytoplasmic fraction (fig.3g), i.e., not in the membrane (fig.3h,l) and periplasmic (fig.3i) fractions. These results indicate that PBP2* remained in the cytoplasm in a water-soluble form and retained the penicillin-binding activity. The

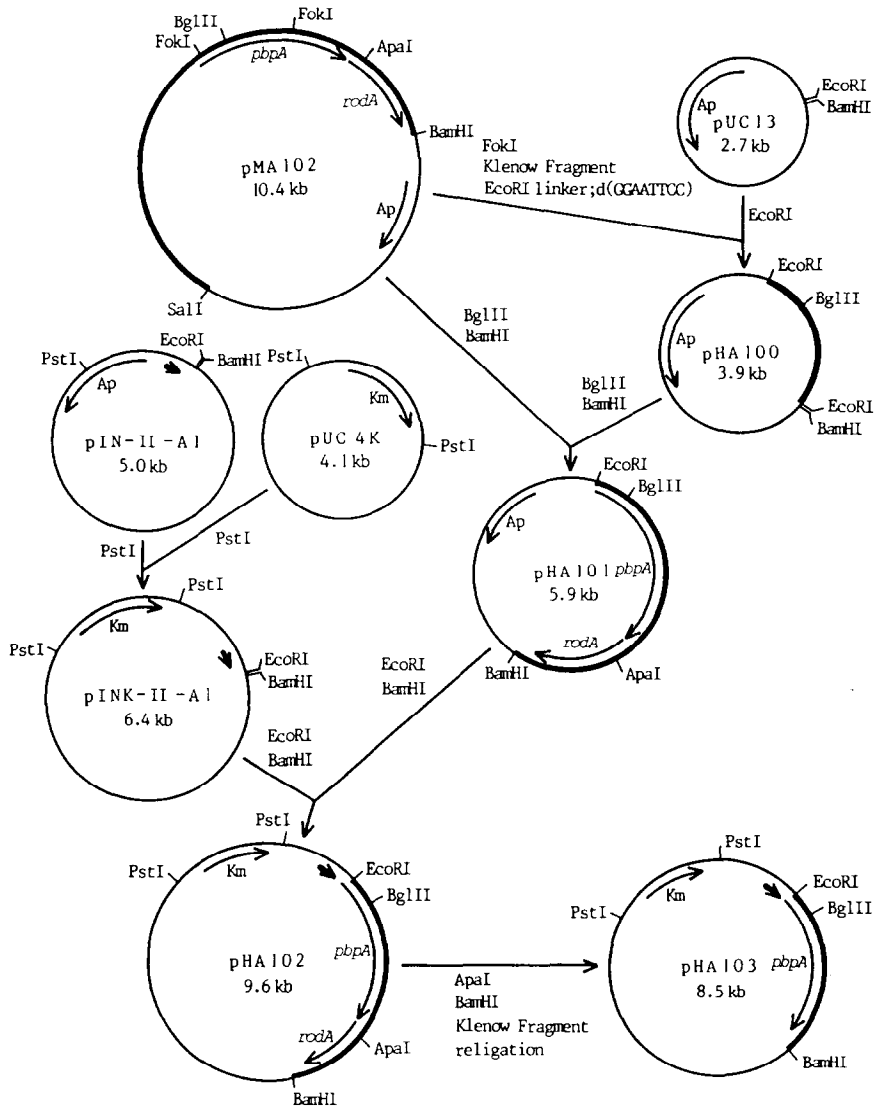

Fig.1. Construction of PBP2 expression vectors, pHA102 and pHA103. The *pbpA* gene was obtained from pMA102 [8]. The *Fok*I site, located immediately after the initiation codon of the *pbpA* gene, was converted to an *Eco*RI site. The 3.2-kb *Eco*RI-*Bam*HI fragment of pHA101 was inserted in frame between the *Eco*RI and *Bam*HI sites, preceded by the initiation codon, of pINK-II-A1, which is a kanamycin-resistant derivative of an expression vector, pIN-II-A1 [10]. From the resulting plasmid, pHA102, the 1.1-kb *Apa*I-*Bam*HI fragment containing the *rodA* region was removed, and pHA103 was obtained.

production of PBP2* (fig.3g) was greater than that of PBP2 expressed by the same promoter (fig.3e). The extents of overproduction of PBP2* (fig.3g) and PBP2 (fig.3e,k) relative to production of PBP2 in control cells were calculated to be about 500- and 300-fold, respectively. The calculation was carried out on the basis of the amounts of

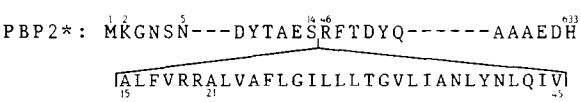

Fig.2. Amino acid sequence of PBP2* (upper line) and the removed N-terminal hydrophobic segment of PBP2 (residues 15–45) (lower line). Residues Leu-3–Gln-4 of PBP2 changed to Gly-Asn-Ser in PBP2*.

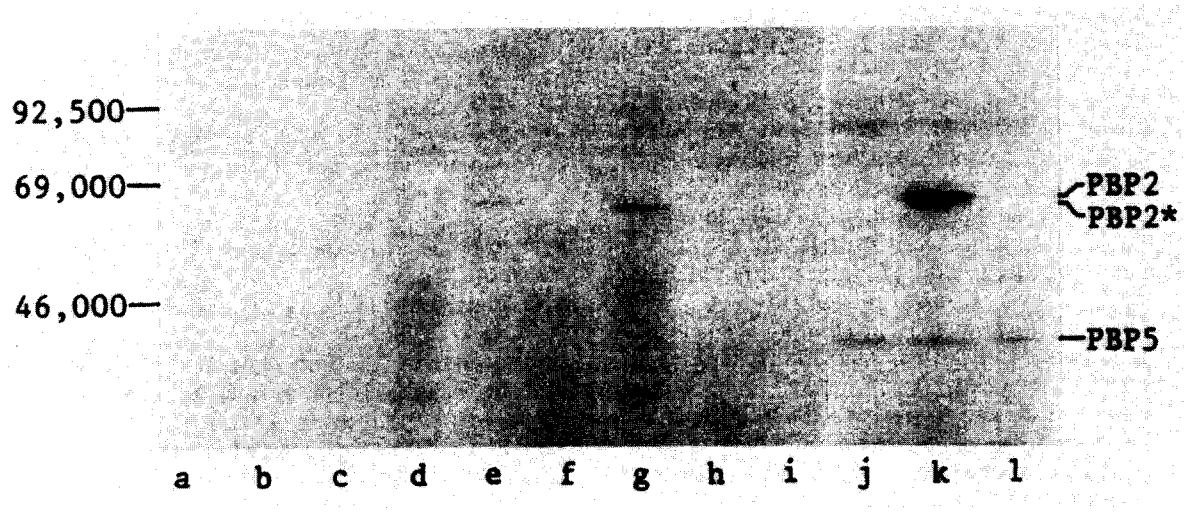

Fig.3. Detection of PBP2* in cytoplasmic (a,d,g), membrane (b,e,h,j–l) and periplasmic (c,f,i) fractions prepared from IPTG-induced *E. coli* cells of MV1184 (a–c,j), MV1184 (pHA103) (d–f,k) and MV1184 (pHA103Δ93) (g–i,l). Each fraction (29 μl) derived from cells corresponding to 0.53 (a–i) or 5.3 (j–l) *A* units at 660 nm was incubated at 30°C with 1 μl of [^{3}H]benzylpenicillin (16.4 μCi/μl), followed by separation by 10% SDS-polyacrylamide slab gel electrophoresis, and then PBPs were localized by fluorography.

PBP2 and PBP5 in control cells, 20 and 1800 molecules/cell, respectively [16].

4. DISCUSSION

PBPs are considered to be ectoproteins [17], which are anchored to the cytoplasmic membrane by membrane-spanning segment(s), with a substantial portion of each protein protruding into the periplasmic space. PBP5, PBP6 (both D-alanine carboxypeptidases) [18] and PBP3 [19] have N-terminal cleavable signal sequences that are essential for protein export, whereas the N-terminal sequence of PBP2 is not cleaved [9]. Our finding that PBP2* is localized in the cytoplasm indicates that the N-terminal hydrophobic region is an uncleaved signal peptide, and also suggests that the region anchors the protein to the cytoplasmic membrane. In contrast, the C-terminus of PBP5 is essential for its localization to the cytoplasmic membrane [20].

Both pHA102Δ93 and pHA103Δ93 were unable to complement a *pbpA* mutant (not shown), because of the localization of PBP2* in the cytoplasm. However, it is essential to determine whether or not PBP2* is able to catalyze both the transglycosylase and transpeptidase reactions by means of in vitro reconstitution experiments with the RodA protein.

PBP2* could be purified from the soluble fraction of cells by affinity chromatography much more easily than membrane-bound PBP2, and was used to determine the penicillin-binding active site (Takasuga, A., Adachi, H., Ishino, F., Matsuhashi, M., Ohta, T. and Matsuzawa, H., in preparation). The purified PBP2* is expected to be suitable for crystallization for X-ray studies on one of the penicillin-target enzymes.

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