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Overexpression, solubilization and refolding of a genetically engineered derivative of the penicillin-binding protein 3 of *Escherichia coli* K12

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Summary

Replacement of the amino-terminal 40-amino-acid region of the 588-amino-acid precursor of the membrane-bound penicillin-binding protein 3 (PBP3) by the decapeptide MKGKEFQAWI was carried out by altering the amino-coding end of the *ftsI* gene. Insertion of the modified gene into a runaway-replication plasmid under the control of a fused *lpp* promoter and *lac* promoter/operator, resulted in the overexpression by *Escherichia coli* of the modified PBP3 (designated PBP3) in the cytoplasm. About 80% of the accumulated PBP3** underwent sequestration in the form of insoluble protein granules that were isolated by cell breakage or cell lysis. After selective removal of contaminants by an EDTA-lysozyme/DNase (deoxyribonuclease)/Nonidet extraction, treatment of the granules with guanidinium chloride followed by dialysis against buffer containing 0.5 M NaCl yielded a refolded, water-soluble PBP3**, which, upon chromatography on Superose 12, exhibited the expected 60000 molecular mass. The refolded PBP3** bound benzylpenicillin in a 1 to 1 molar ratio, was highly sensitive to aztreonam and showed the same degree of thermostability, in terms of penicillin-binding capacity, as the parent, membrane-bound PBP3, suggesting that protein refolding occurred with formation of the correct intramolecular interactions. Two to three mg of refolded PBP3** can be obtained from 1 litre of culture of the overproducing strain.**

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Introduction

The term 'active-site serine, penicillin-interactive protein or protein domain' applies to a whole set of bacterial enzymes: the β-lactamases of class A, C and D, the low-M_r DD-peptidases/penicillin-binding proteins (PBPs), and the DD-peptidase/penicillin-binding domains of the high-M_r PBPs (Joris *et al.*, 1988). A common feature of these enzymes is reaction with penicillin and other β-lactam antibiotics in a 1 to 1 molar ratio via formation of a serine ester-linked acyl (penicilloyl, cephalosporoyl) enzyme. Gene sequencing has yielded the primary structure of about 20 of these enzymes (see Joris *et al.*, 1988) and X-ray crystallography has revealed details of the three-dimensional structure and active-site environment of several β-lactamases of class A (Kelly *et al.*, 1986; Samraoui *et al.*, 1986; Herzberg and Moult, 1987; Dideberg *et al.*, 1987) and the naturally occurring, water-soluble, low-M_r DD-peptidase/PBP of *Streptomyces* R61 (Kelly *et al.*, 1986). On the basis of these structural data, it has been proposed that, in all likelihood, all the penicillin-interactive proteins or domains form a 'superfamily' of evolutionarily related enzymes (Joris *et al.*, 1988). Although they have diverged greatly in terms of primary structure, and acquired varying specificities and functionalities, they would have conserved substantial similarities in their polypeptide 'scaffolding'.

In spite of these advances, determination of the three-dimensional structure of the physiologically-important, high-M_r PBPs 1A, 1B, 2 and 3 of *E. coli* remains an important aim of the research in this field. As the results of biochemical and recombinant DNA experiments suggest, these high-M_r PBPs contain both an amino-terminal domain that catalyses a penicillin-insensitive peptidoglycan-transglycosylase reaction and a carboxy-terminal penicillin-binding domain that catalyses a penicillin-sensitive peptidoglycan transpeptidase reaction. With the exception of the aforementioned *Streptomyces* R61 DD-peptidase/PBP, the PBPs are membrane-bound. As a corollary, their crystallization must be achieved in the presence of detergent, an approach which so far has not yielded crystals suitable for X-ray diffraction analysis

(Ishino *et al.*, 1988; Ferreira *et al.*, 1988). Alternatively, catalytically active, water-soluble derivatives can be prepared. This strategy makes use of the fact that the PBPs are essentially periplasmic proteins anchored within the membrane via their carboxy-termini (the low-M_r PBPs) or their amino-termini (the high-M_r PBPs). Genetically engineered, water-soluble derivatives of the *E. coli* PBPs 1B (Spratt *et al.*, 1988), 2 (Adachi *et al.*, 1988), 3 (Spratt *et al.*, 1988) and 5 (Ferreira *et al.*, 1988) have been obtained. The water-soluble PBP5 has yielded crystals suitable for crystallography studies (Ferreira *et al.*, 1988).

The strategy used by Spratt *et al.* (1988) to obtain a water-soluble derivative of the *E. coli* PBP3 was to construct a plasmid that encoded a hybrid protein in which residues 53–588 of the PBP3 precursor were fused to the signal peptide and the first ten residues of the mature PBP5. Translocation and processing resulted in the expression of a modified PBP3 in the periplasm of *E. coli*, but the yield was low. Given that (as observed with PBP1B) the occurrence of large concentrations of PBPs in the periplasm may be lethal for *E. coli* (Spratt *et al.*, 1988), experiments were undertaken with the objective of overproducing in the cytoplasm a modified PBP3 (designated PBP3**) that lacked the signal peptide of the precursor. As observed with many proteins synthesized intracellularly in high levels in *E. coli* (Marston, 1986), the overexpressed PBP3** partitioned into a soluble form but predominantly underwent sequestration into cytoplasmic granules. Guanidinium chloride (GuCl) treatment of these bodies followed by dialysis against buffer containing 0.5 M NaCl yielded a 'refolded', water-soluble PBP3** which was indistinguishable from the cytoplasmic 'native' water-soluble PBP3**.

Results

*Intracellular synthesis of PBP3***

A low-copy-number plasmid (pDML210; derived from pBR322) and a high-copy-number plasmid (pDML211; derived from the kanamycin-resistant, runaway-replication plasmid pIM-IIA$_3$) were constructed as described in *Experimental procedures* and in Figs 1 and 2. These plasmids possessed a *ftsI*** gene that coded for a modified PBP3**, in which the amino-terminal 40-amino-acid region of the PBP3 precursor was replaced by the decapeptide MKGKEFQAWI. In both plasmids, the *ftsI*** gene was under the control of a fused lipoprotein (*lpp*) promoter and lactose (*lac*) promoter/operator system. *E. coli* K12 derivatives were used as recipients. Bacterial growth was carried out with vigorous shaking in Luria-Bertani medium supplemented with thiamine (20 μg ml^{-1}) (Maniatis *et al.*, 1982).

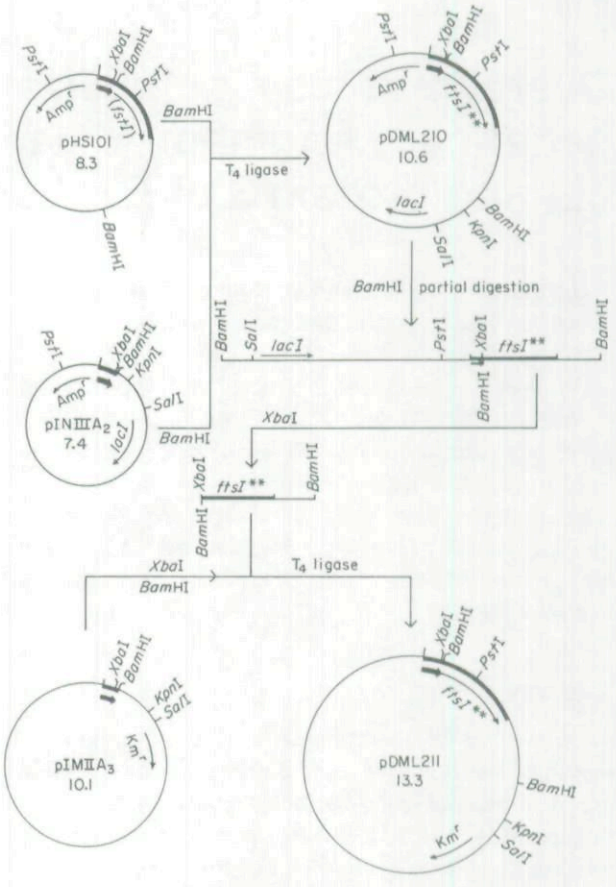

Fig. 1. Construction of plasmids pDML210 and pDML211. Heavy arrow: fused *lpp* promoter, *lac* promoter/operator (see Fig. 2). Partial digestion of pDML210 with *Bam*HI yielded two linearized plasmids: only one of them is represented.

The low-copy-number plasmid pDML210 (20 copies per cell) was introduced into F′lacI^q *E. coli* JM105. The resulting strain, JDB2, was grown at 37°C in the presence of ampicillin (50 μg ml^{-1}) until the culture reached an optimal density of 0.6 at 660 nm. Then 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture incubated for a further 3 h. Strain JDB2 accumulated small amounts of cytoplasmic granules and did not exhibit morphological abnormalities.

The high-copy-number plasmid pDML211 was introduced into F′lacI^q *E. coli* JM105, giving rise to strain JDB4 and into (F′lacI^q)$^-$ and lon$^-$ *E. coli* GC4670, giving rise to strain JDB5. Both strains were grown at 30°C in the presence of kanamycin (25 μg ml^{-1}) until the cultures reached an optical density of 0.1, at which time the temperature was shifted to 37°C. After 2 h, 2 mM IPTG was added and the incubation of the cultures was continued for a further 3 h. With both strains, the temperature shift led to amplification of the plasmid copy number to about 2000 per cell. In parallel to this, the cells became abnormally

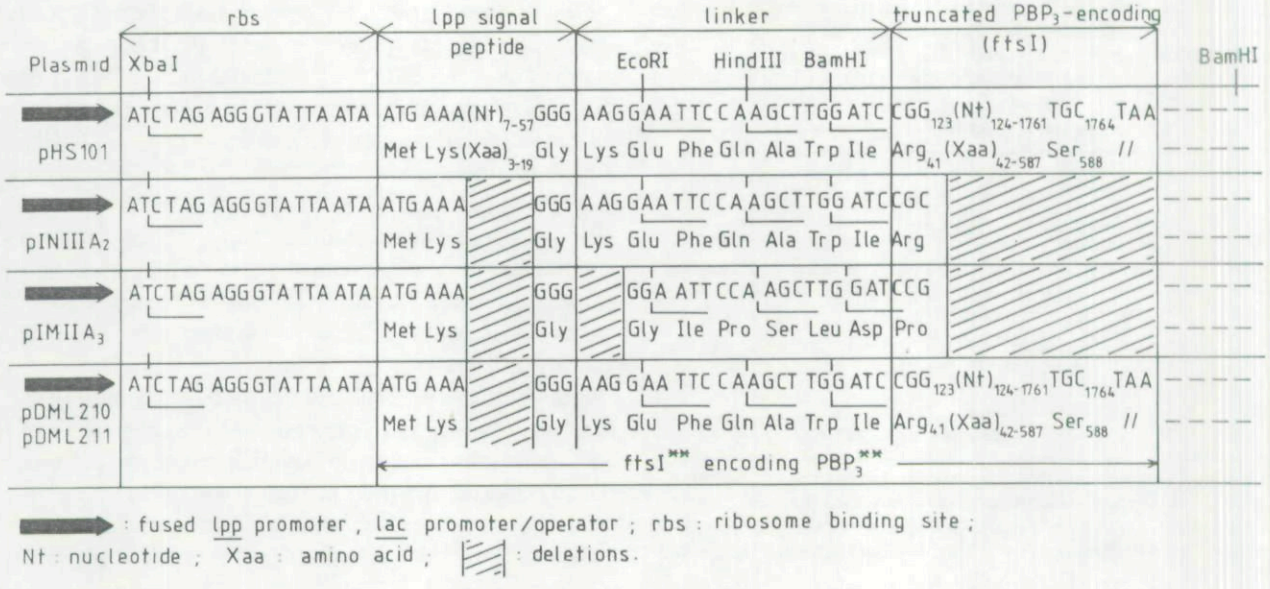

Fig. 2. The *Xba*I-*Bam*HI DNA sequences in plasmids pHS101, pIN-IIIA$_2$, pIM-IIA$_3$, pDML210 and pDML211, with the translated polypeptide segments.

elongated with multiple refractile cytoplasmic bodies (protein granules) that were already visible 2 h after the temperature shift.

*Partitioning of the intracellularily synthesized PBP3***

By definition, PBP3**, like the parent PBP3, is a 60000-M$_r$ protein that has the ability to bind benzylpenicillin in a 1 to 1 molar ratio. Consequently, PBP3** was quantitatively estimated by submitting to gel electrophoresis in the presence of sodium dodecylsulphate (SDS), samples previously derivatized by [^{35}S]-benzylpenicillin under saturating conditions, and by performing microdensity measurements of the Coomassie-stained and radioactive 60000-M$_r$ protein band. In this procedure, the 349-amino-acid DD-peptidase/PBP of *Streptomyces* R61 (Duez *et al.*, 1987) and its [^{35}S]-benzylpenicilloyl derivative were used as references. Another feature of PBP3** which was observed in the course of these studies, was its requirement for a high salt concentration to remain water soluble. For this purpose, a 10 mM Tris-borate pH 8 buffer containing 0.5 M NaCl, 10% (w/v) glycerol and 0.2 mM dithiothreitol was used: it was called buffer D. All the operations described below were carried out at 4°C unless otherwise stated.

Cells collected from 1 litre of cultures of strains JDB2, JDB4 and JDB5, and stored frozen at −20°C, were suspended in 20 ml buffer D supplemented with 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5 mM ethylene diamine tetraacetate (EDTA). They were disrupted by sonication (two successive treatments at 0°C for 1 min each, using an MSE150 watt ultrasonic disintegrator MK2 (Crawley, Sussex, UK). Suspensions of the disrupted cells were submitted to two successive centrifugations at 3000 × g for 5 min and 100000 × g for 4 h, respectively. The supernatants, after a 3-fold concentration by ultrafiltration, contained the native, water-soluble PBP3**. In turn, the pellets obtained by centrifugation at 3000 × g were washed with buffer D (by centrifugation at 3000 × g for 10 min), yielding the protein granules.

Table 1 gives the yield in nmoles of PBP3**, as defined above, that were obtained from 1 litre of cultures in the form of native, water-soluble protein and protein granules. Strain JDB5 was lon$^-$, i.e. it lacked protease L, which has an important role in the degradation of abnormal proteins (Marston, 1986). It was the best PBP3** producer and accumulated protein granules to levels ranging to 9% of total cell protein. Exposure to IPTG had little or no effect as the *lac* repressor was titrated out.

*Properties of the native, water-soluble PBP3** and refolded water-soluble PBP3** from overproducing* E. coli *JDB5: comparison with the membrane-bound PBP3 of the parent strain GC4670*

The native PBP3** fraction contained all the intracellular soluble proteins and thus had a very low specific activity. It was not further studied except that upon chromatography on Superose 12 in buffer D, the PBP3** eluted just after the 68000-M$_r$ serum albumin, i.e. it behaved as a true water-soluble protein (Fig. 3, lanes 2, 3). As shown by gel electrophoresis, the 60000-M$_r$ PBP3** occurred as a

Table 1. Amounts of PBP3** synthesized by the recombinant *E. coli* strains grown in the absence and the presence of 2 mM IPTG, and occurring in the form of native, water-soluble protein and protein granules. Results are given in nmoles of PBP3** produced per litre of culture, i.e. in nmoles of 60000-M_r protein that binds [^{35}S]-benzylpenicillin in a 1 to 1 molar ratio. For growth conditions, see text.

Strain	Harboured plasmid	PBP3** forms	PBP3** produced (nmoles l^{-1} culture) IPTG absent	IPTG present
JDB2 (F'lacI^q)	pDML210	Native	2	4
		Granules	0.5	0.5
JDB4 (F'lacI^q)	pDML211	Native	4	6
		Granules	6	30
JDB5 (F'lacI^q)$^-$	pDML211	Native	9	9
		Granules	40–50	40–50

For the purposes of comparison, the amounts of membrane-bound PBP3 in strain GC4670 grown under the same conditions as its derivative, JDB5, were about 0.1 nmole l^{-1} of culture. This value translates into about 30 copies of PBP3 per cell. A value of 50 copies was estimated by Spratt (1977).

doublet (Fig. 3, lane 3). The two protein bands reacted with the anti-membrane-bound PBP3 antibodies.

The protein granules prepared by cell breakage and centrifugation (Fig. 3, lane 4) had a specific activity of 0.15 nmoles PBP3** per mg protein (estimated purity: 1%). Subsequently, protein granules were prepared, contaminants were selectively removed and the sequestered PBP3 was solubilized as described in *Experimental procedures* and illustrated in Fig. 3, lanes 4–6. Lysis of the JDB5 cells by the EDTA/lysozyme/DNase/Nonidet treatment yielded protein granules with a specific activity of 0.6 nmoles PBP3** per mg protein (estimated purity: 4%). Gel electrophoretic analysis of these granules showed that the 60000-M_r protein band contained only 0.1 equivalent [^{35}S]-benzylpenicilloyl moiety. Hence, 90% of this material consisted of a protein (or proteins) having a molecular mass identical to that of PBP3** but lacking penicillin-binding capacity. Treatment of the protein granules with guanidinium chloride (1.5 M, 3 M and 6 M concentrations gave similar results) and protein refolding by dialysis (yields: 20% of total proteins; 75% of PBP3**) generated a water-soluble preparation with a specific activity of 2.5 nmoles PBP3** per mg protein. Though the degree of purity was still low (15%), the refolded PBP3** was the only PBP present, it bound benzylpenicillin in a 1 to 1 molar ratio, and, upon chromatography on Superose 12 in buffer D, it eluted just after the 68000-M_r serum albumin, as observed with the native, water-soluble PBP3**. The yield in refolded PBP3** was 30–35 nmoles l^{-1} of culture of JDB5.

The native and refolded water-soluble PBPs3** and the membrane-bound PBP3 exhibited exactly the same thermostability in buffer D (Fig. 4). The two water-soluble PBPs3** were half-saturated at a 0.5–0.6 μM benzylpenicillin concentration. Half-saturation of the membrane-bound enzyme occurred at 2.5 μM benzylpenicillin but one should note that, in this case, the experiments were carried out in the presence of vast amounts of other, penicillin-competing PBPs. Finally, the two water-soluble PBPs3** and the membrane-bound PBP3 were half-saturated by aztreonam at a concentration lower than 0.1 μM. PBP3 is known to be hypersensitive to this monocyclic β-lactam antibiotic.

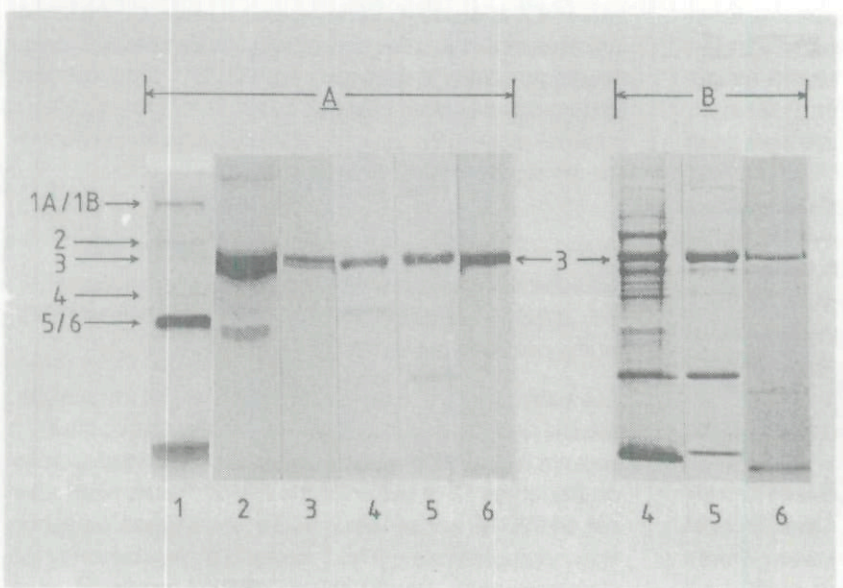

Fig. 3. Fluorograms (A) and Coomassie Brilliant Blue-stained gels (B) obtained after polyacrylamide gel electrophoresis in SDS of [^{35}S]-benzylpenicillin-labelled enzyme samples. 1: membranes of the host *E. coli* GC4670 (110 μg protein); 2 and 3: native, water-soluble PBP3** from *E. coli* JDB5 before (2) (70 μg protein) and after (3) (10 μg protein) chromatography on Superose 12; 4 and 5: protein granules isolated from *E. coli* JDB5 by cell breakage and centrifugation (4) (90 μg protein) and by cell lysis and EDTA/lysozyme/DNase/Nonidet treatment (5) (20 μg protein); 6: refolded PBP3** from *E. coli* JDB5 (A; 1.2 μg protein) and (B; 2 μg protein).

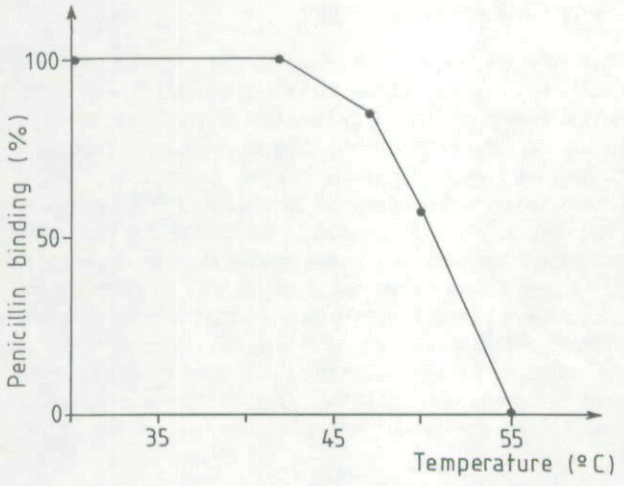

Fig. 4. Thermostability of the membrane-bound PBP3 of the host *E. coli* GC4670, and the native water-soluble PBP3** and refolded water-soluble PBP3** of *E. coli* JDB5. Isolated membranes from *E. coli* GC4670 (144 μg protein) and the soluble PBP3s** (1.5 picomoles), were incubated in 12 μl and 8 μl, respectively, of buffer D for 10 min at the indicated temperatures. The samples were then incubated for 10 min at 30°C with 0.1 mM [^{35}S]-benzylpenicillin and the amounts of [^{35}S]-benzylpenicilloyl enzymes estimated after polyacrylamide gel electrophoresis in SDS. The three enzymes behaved identically.

Discussion

In the literature, there is an increasing number of reports showing that, when expressed intracellularly in high levels in *E. coli* by means of DNA recombinant techniques, 'foreign' proteins such as eukaryotic polypeptides (Marston, 1986) and both 'abnormal' and 'normal' *E. coli* proteins (for example the RTEM β-lactamase and EnvA protein) (Ghrayeb *et al.*, 1984; Masui *et al.*, 1984) accumulate as inclusion bodies, even if the authentic proteins are produced in a soluble form. This particular response of the bacterial cell caused by the accumulation of a high concentration of protein is not just a precipitation phenomenon. Solublization of the accumulated protein from these bodies requires stringent chemical conditions (the use of high concentrations of guanidinium chloride, urea, detergents, alkaline pH, organic solvents): i.e. it can be achieved only by disrupting non-covalent hydrogen bonds, ionic and hydrophobic interactions and/or polypeptide unfolding.

In the present work, the *ftsI* gene that encodes the membrane-bound PBP3 of *E. coli* has been genetically engineered with the objective of producing, in *E. coli*, an intracellular derivative in which the amino-terminal 40 amino acids of the precursors have been replaced by the decapeptide MKGKEFQAWI. Insertion of the modified gene into a high-level expression cloning vector results in the synthesis of the modified PBP3** in the cytoplasm with a 500-fold amplification, when compared with the amounts of naturally occurring membrane-bound PBP3. PBP3** overproduction causes filamentation of the cells but is not a lethal event perhaps because most of the synthesized PBP3** (80%) is sequestered in the form of protein granules. After selective elimination of contaminants from these bodies, conversion of the insoluble PBP3** to an unfolded random coil by guanidinium chloride treatment and refolding by dialysis against buffer containing 0.5 M NaCl yields a PBP3** that is undistinguishable from the small proportion of intracellular PBP3** that partitions in the soluble fraction. On the basis of their thermostability, their affinity to benzylpenicillin and their susceptibility to aztreonam, the penicillin-binding domain of both native and refolded water-soluble PBP3s** appear to have conserved or re-acquired a polypeptide folding which is identical, or very similar, to that of the native state. The refolded PBP3** represents less than 10% of the protein content of the protein granules. It seems that during sequestration by aggregation, a high proportion of the synthesized PBP3** is denatured into a form that escapes recovery. In any case, these protein granules enable us to obtain, using a two-step procedure, a preparation of active PBP3** which is about 15% pure, with a yield of 2–3 mg per litre of culture.

Experimental procedures

Bacterial strains

All strains were *E. coli* K12 derivatives. Strain JE7611 (F$^-$ *recA1 ftsI730 str thi metE proC xyl lacZ tonA tsx*) harboured the originial plasmid pHS101 (Houba-Herin *et al.*, 1985) (see below). Strain HB101 (F$^-$ *hsdS20* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ$^-$) (Boyer and Rouland-Dussoix, 1969), strain JM105 (Δ(*lac-pro*) *thi strA endA sbcB hsdR*/F′ *traD36 proAB lacI*^{q} *lacZΔM15*) (Messing *et al.*, 1981), and strain GC4670 (*lon*::Tn*10 thr leu lacY*) (a gift from Dr B. Holland, University of Leicester, UK) were used as recipients of the recombinant plasmids.

Plasmids (Figs 1 and 2)

Plasmid pHS101 contained the truncated PBP3-encoding (*ftsI*) gene (Houba-Herin *et al.*, 1985). Plasmids pIN-IIIA_2 (a derivative of pBR322) and pIM-IIA_3 (a kanamycin-resistant, runaway-replication plasmid derived from RI) were used as vectors (Masui *et al.*, 1983). The general characteristics of these plasmids and a detailed map of their *Xba*I-*Bam*HI sequences are shown in Fig. 1 and Fig. 2, respectively. Each plasmid possesses an ORF that is under the control of a fused *lpp* promoter and *lac* promoter/operator system. In pHS101, the ORF codes for a chimeric protein in which the signal peptide of the murein lipoprotein is linked via heptapeptide KEFQAWI, to a truncated PBP3 that lacks the amino-terminal 40-amino-acid region of the precursor and thus lacks the cysteine residues at position 28 and 30 (so that the

lipoprotein cleavage site Gly-Cys is modified into Gly-Lys, which is not recognized by signal peptidase 1). Downstream of this ORF, there is a second *Bam*HI site. pIN-IIIA_2 lacks the *ftsI* gene, possesses one single *Bam*HI site and has an *Xba*I-*Bam*HI insert identical to that of pHS101 except that there is a 51 nucleotide deletion in the gene encoding the signal peptide of the murein lipoprotein. The runaway-replication plasmid pIM-IIA_3 also has a unique *Bam*HI site, but it is in a different reading frame from the corresponding site in pIN-IIIA_2.

Construction of the low-level expression vector, pDML210 (Figs 1 and 2)

The *Bam*HI-(*ftsI*)-*Bam*HI segment (3.2 kb) was excised from pHS101, gel-purified and inserted in the right orientation, in the *Bam*HI site of pIN-IIIA_2 which had been previously treated with alkaline phosphatase. The ligated mixture was used to transform *E. coli* HB101 and the transformants were selected on LB agar containing ampicillin (50 μg ml^{-1}). Insertion of the DNA fragment in the right orientation conferred to the recombinant plasmid the distinctive property to yield, upon digestion with *Pst*I, two fragments of 1.8 kb and 8.8 kb, respectively. Ten transformants were tested; five gave the desired response. As a result of this construction, plasmid pDML210 had under the control of the *lpp* promoter and *lac* promoter operator system, the gene *ftsI*** coding for the desired PBP3**.

Construction of the high-level expression vector, pDML211 (Figs 1 and 2)

pDML210 was partially digested with *Bam*HI. The linearized plasmids were isolated and digested with *Xba*I. The desired 3.2 kb fragment was isolated by agarose gel electrophoresis and ligated to pIM-IIA_3, which had been previously digested with *Xba*I and *Bam*HI. The ligated mixture was used to transform *E. coli* HB101 and the transformants were selected on LB agar containing kanamycin (25 μg ml^{-1}). The resulting plasmid was called pDML211.

Enzymes, antibodies, antibiotics and recombinant DNA techniques

Restriction endonucleases, T4 DNA ligase and bacterial alkaline phosphatase were from Amersham International, Amersham, UK. DNaseI was from Boehringer–Mannheim (FRG). Lysozyme was from Sigma Chemical Co., St Louis, MO, USA. The *Streptomyces* R61 DD-peptidase/PBP (> 95% purity) was that described in Duez *et al.* (1987). Rabbit antisera directed against the Triton X-100-purified membrane-bound PBP3 of *E. coli* was a gift from Dr W. Keck (University of Gröningen, The Netherlands). [^{35}S]-Benzylpenicillin (500 mCi mmole^{-1}) was from New England Nuclear (Dupont de Nemours, Belgium). Ampicillin was from Beecham (Brussels, Belgium). Kanamycin was from Boehringer-Mannheim (FRG). Benzylpenicillin was from Rhône-Poulenc (Paris, France). Aztreonam was a gift from Dr Lucania (The Squibb Institute for Medical Research, Princeton, NJ, USA). β-Iodopenicillanate was a gift from Dr J. Kemp (Pfizer Central Research, Sandwich, Kent, UK). All recombinant DNA techniques were based on Maniatis *et al.* (1982).

*PBP3** and protein estimation*

The PBPs were labelled with [^{35}S]-benzylpenicillin in buffer D under conditions which caused complete derivatization of PBP3 (0.1 mM benzylpenicillin; 10 min of incubation at 37°C). Polyacrylamide gel electrophoresis in SDS, Coomassie Brilliant Blue staining and fluorography of the gels were carried out according to standard procedures (Laemmli and Favre, 1973; Spratt, 1977; Chamberlain, 1979). Strain JDB2 was an RTEM β-lactamase producer. In this case, the enzyme samples were treated with 5 × 10^{-5} M β-iodopenicillanate for 20 min at 30°C (to inactivate the β-lactamase) before the PBPs were labelled with [^{35}S]-benzylpenicillin. Proteins were estimated using Lowry's procedure with the addition of SDS (Shepherd *et al.*, 1977). Alternatively, samples were hydrolysed with 6 M HCl at 100°C for 20 h and the amino groups were estimated after dinitrophenylation (Frère *et al.*, 1973).

*Preparation of the refolded, water-soluble PBP3** from strain JDB5*

The procedure was an adaptation (A. Renard, personal communication) of methods used for the isolation of polypeptides from protein granules accumulated in *E. coli* (Marston, 1986). Frozen cells (from 1 l of culture) were suspended in 160 ml of a lysozyme solution (1 mg ml^{-1}) made in 50 mM Tris-HCl pH 8 containing 0.5 mM EDTA. The cell suspension was incubated for 15 min at 4°C, and then 40 ml of 2 M NaCl, 2.5 mM $MgCl_2$, 0.5 mM PMSF 2.5% (v/v) were added. This was Nonidet, 50 mM Tris-HCl pH 8 incubated for 15 min at 4°C. Finally, DNase (10 μg ml^{-1}, final concentration) was added and the mixture was incubated for 30 min at 4°C. The protein granules were collected by centrifugation at 10000 × g for 10 min, washed three times with 20 mM Tris-HCl–5 mM EDTA pH 8, and suspended in buffer D at a final concentration of 10 mg protein ml^{-1}. The suspension was sonicated using the MSE 150 watt disintegrator MK2 (two successive treatments at 0°C for 15 s each), diluted 10 times with 1.65 M guanidine chloride previously dissolved in buffer D without NaCl, incubated for 30 min at 0°C and centrifuged at 30000 × g for 45 min. The supernatant, dialysed against buffer D and centrifuged at 30000 × g for 45 min, contained the refolded PBP3**.

Chromatography on Superose 12

Chromatography of the water-soluble PBPs3** was performed in buffer D using the FPLC Pharmacia system. Detection of the eluted PBP3** was carried out by [^{35}S]-benzylpenicillin binding and by immunoblotting using anti-membrane-bound PBP3 antibodies as reagent (Bio-Rad Immun Blot alkaline phosphatase Assay system).

Plasma membrane

Plasma membranes were prepared from *E. coli* GC4670 and the membrane-bound PBP3 (after labelling with [^{35}S]-benzylpenicillin) was estimated as described above.

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