A gene fusion that localises the penicillin-binding domain of penicillin-binding protein 3 of *Escherichia coli*

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A gene fusion that links the COOH-terminal 349 amino acids of penicillin-binding protein 3 (60 kDa) of *E.coli* to the NH_2-terminus of β-galactosidase has been constructed. The fusion protein (38.5 kDa) retains the ability to bind benzylpenicillin with high affinity, establishing that the penicillin-binding domain (and presumably the penicillin-sensitive transpeptidase activity) of this high molecular mass penicillin-binding protein is located on a COOH-terminal functional domain.

Escherichia coli *Penicillin-binding protein* *Gene fusion* *β-Lactam antibiotic* *Peptidoglycan* *Transglycosylase-transpeptidase*

1. INTRODUCTION

Penicillin-binding proteins (PBPs) and β-lactamases are two classes of enzymes that interact with penicillin [1,2]. The PBPs are involved in peptidoglycan synthesis and are inhibited by penicillin, whereas the β-lactamases de-toxify penicillin by hydrolysing it to penicilloic acid. In *Escherichia coli* the higher molecular mass penicillin-binding proteins are the killing targets for β-lactam antibiotics [1], and are believed to be bifunctional enzymes catalyzing both the penicillin-insensitive peptidoglycan transglycosylase reaction and the penicillin-sensitive transpeptidase reaction [3–6]. The lower molecular mass PBPs catalyse the D-alanine carboxypeptidase reaction and are non-essential for bacterial growth [1,2]. The class A and class C β-lactamases, and the low molecular mass PBPs, are serine enzymes in which penicillin (and peptidoglycan substrates in the case of PBPs) acylates an active site serine residue that is located close to the NH_2-terminus of the proteins [7–9]. The higher molecular mass PBPs are also presumed to be 'serine enzymes' but this has yet to be demonstrated.

Sequence comparisons of high molecular mass PBPs, low molecular mass PBPs, and class A β-lactamases have identified one small region of similarity between all these proteins ([1,10], unpublished). In the low molecular mass PBPs, and in the β-lactamases, this region occurs just to the NH_2-terminal side of the active site serine, i.e., very close to the NH_2-terminus of these enzymes. In the high molecular mass bifunctional PBPs, this region occurs towards the middle of the primary sequence (e.g., residues 261–277 in PBP 3) and we have suggested that the penicillin-sensitive transpeptidase domain of these PBPs extends from the middle of the proteins towards the COOH-terminus and that the transglycosylase domain is NH_2-terminal [1,10]. We show here, using gene fusions, that a protein which contains only the COOH-terminal 349 residues of PBP 3 of *E. coli* retains the ability to bind penicillin with high affinity. Constructions of this type may allow the production of soluble penicillin-binding domains that are suitable for X-ray analysis of the penicillin-binding site of a membrane-bound killing target for penicillin.

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2. MATERIALS AND METHODS

E. coli JM101 was used in all experiments [11]. The PBP 3 gene was obtained from pLC26-6 of the collection of Clark and Carbon [12,13]. The plasmid vector pLG339 [14] and the phage vector M13mp8 [11] have been described, and pteH8 and pteH9 are analogues of the vectors pUC8 and pUC9 [15] in which the Ap^r gene has been replaced by Km^r (unpublished).

Techniques for the purification and manipulation of DNA have been described. Restriction endonucleases were obtained from Boehringer or from Amersham International and were used according to the recommendation of the suppliers.

E. coli JM101 (pPH153) was grown in Luria broth and expression from the *lac* promoter was induced by the addition of isopropylthiogalactoside (IPTG, Sigma) to a final concentration of 1 mM. The assay of PBPs was carried out using freeze-thawed bacteria and [^{3}H]benzylpenicillin (27 Ci/mmol, kindly provided by Dr P.J. Cassidy of Merck Sharp and Dohme, Rahway, NJ, USA) as in [16]. Fractionation of bacterial cells into soluble (cytoplasm and periplasm) and cell envelope fractions was carried out as in [17].

An estimate of the affinity of the β-galactosidase-PBP 3 fusion protein for benzylpenicillin was carried out as in [17] except that crude extracts of bacteria were used. *E. coli* JM101 (pPH153) that had been induced with IPTG were resuspended in ice-cold 50 mM sodium phosphate buffer (pH 7.0) at 2×10^{10} bacteria/ml and were disrupted by sonication (0°C). Unbroken bacteria and debris were removed by centrifugation at $10\,000\times g$ for 20 min at 2°C and the supernatant was used as a crude extract containing cell envelope, periplasmic, and cytoplasmic fractions.

3. RESULTS

The PBP3 gene (*pbp*B) has been sequenced in [18]. A *Pst*I site is located at a position corresponding to amino acids 240/241 and divides the coding region at the predicted junction between the transglycosylase and transpeptidase domains. To confirm the location of the penicillin-sensitive transpeptidase domain, and in an attempt to produce a soluble fragment of PBP3 that retains penicillin-binding activity and which might be suitable for X-ray studies, we have utilized the *Pst*I site to create an in-phase fusion to β-galactosidase. The coding region of the *pbp*B gene was obtained as a 2608-base pair (bp) *Pvu*II fragment from pLC26-6 of the collection of Clark and Carbon [12,18] and was cloned into the *Sma*I site of the replicative form of M13mp8 to produce M13mp8/PBP3A (fig.1). The gene was subcloned from the M13 recombinant into pLG339 to produce pPH105 (fig.1), and a 1448-bp *Pst*I–*Hind*III fragment, encoding the COOH-terminal region of PBP 3, was obtained from pPH105 and was inserted between the *Pst*I and *Hind*III sites of pteH8 to produce pPH153 (fig.1).

E. coli JM101 (pPH153) should express a fusion protein (38.5 kDa), under the control of the *lac* promoter, which is comprised of the first 6 amino acids of β-galactosidase, followed by 5 amino acids encoded by the multiple cloning region of the vector, and residues 240–588 of PBP 3 (fig.2). In the absence of IPTG the pattern of PBPs in *E. coli* JM101 (pPH153) was identical to that in the parent strain but in the presence of IPTG a new PBP appeared that had the predicted molecular mass of the PBP 3 fusion protein (fig.3). This protein was identified as the fusion product since its expression was under the control of the *lac* promoter and it was not made by cells carrying the vector plasmid or by constructs (e.g., pPH154) that contained the same fragment of the PBP3 gene cloned in the opposite (inappropriate) orientation in pteH9 (fig.3).

The amount of [^{3}H]benzylpenicillin bound to the fusion protein was 150–200% of that bound to PBP 5 (fig.3). *E. coli* contains about 1800 molecules of PBP 5 per bacterium [17], and therefore about 3000 molecules of the fusion protein per bacterium were detected by the PBP assay. This level is far below that expected for a protein expressed from the *lac* promoter and the *lacZ* ribosome-binding site on a high copy number vector, suggesting that only a portion of the fusion protein that was synthesized was being detected by the PBP assay. The sensitivity of the fusion protein to proteolysis was examined by pre-labelling cells with [^{3}H]benzylpenicillin, adding a 200-fold excess of unlabelled benzylpenicillin, and following the disappearance of the labelled fusion protein during incubation at 37°C. After 30 min incubation there was no significant loss of the labelled fusion protein. The addition of the protease inhibitor, *p*-

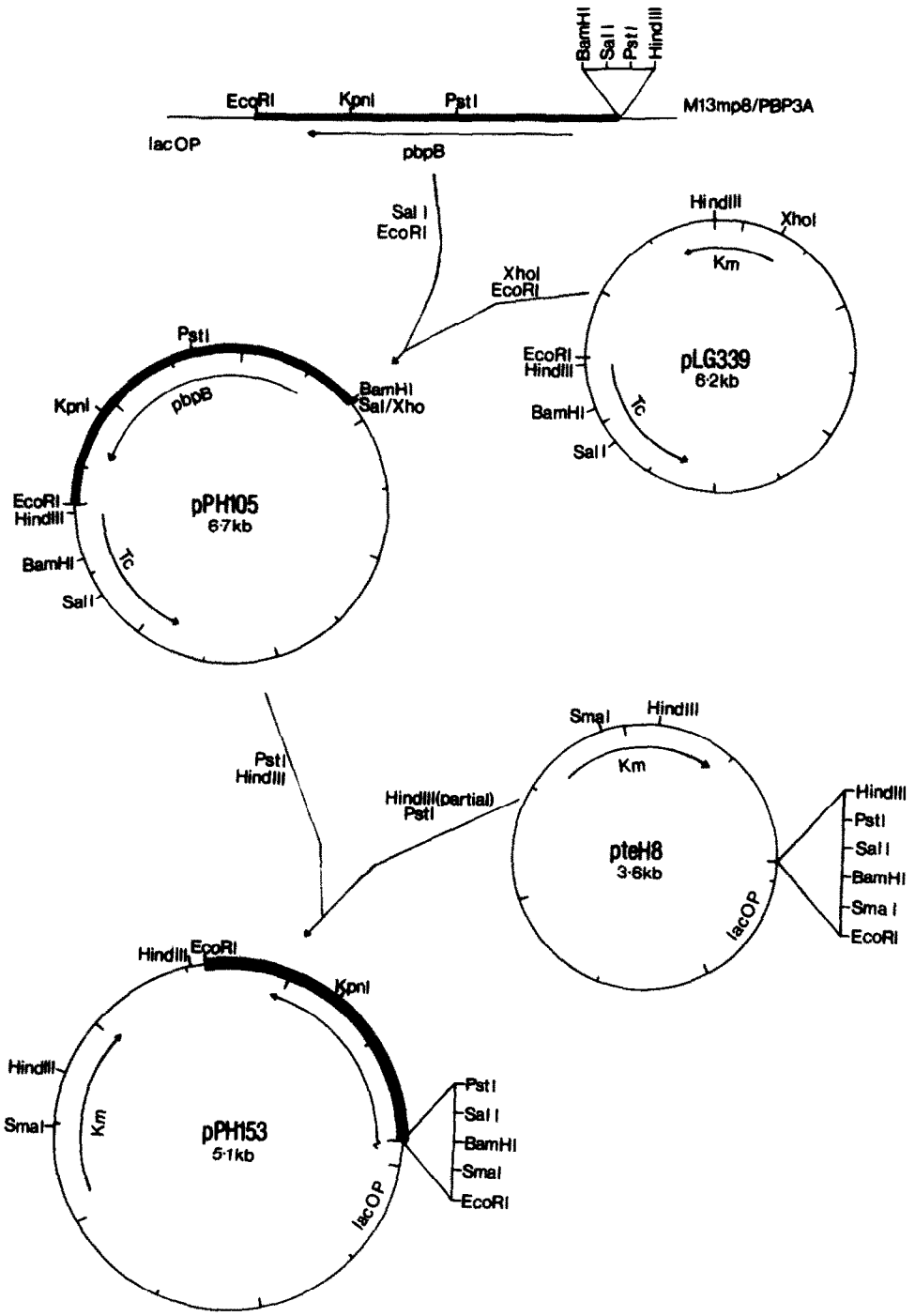

Fig.1. Construction of a *lacZ-pbp*B fusion. The PBP 3 gene (*pbp*B) was obtained on a 2.6-kb *Pvu*II fragment from the Clarke and Carbon plasmid pLC26-6 [18] and was inserted into M13mp8 to produce M13mp8/PBP3A. The gene was subcloned into pLG339 [14] and a 1404-bp *Pst*I–*Hin*dIII fragment was obtained from the resulting plasmid and was inserted into pteH8. A second construction (pPH154) was made that contained the same 1404-bp fragment inserted in the opposite orientation by cloning into pteH9.

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      1   2   3   4   5   6   7   8   9  10  11 | 240 241 242 243 244        587 588
(MET) THR MET ILE THR ASN SER ARG GLY SER VAL ASP | LEU GLN ALA LEU VAL ..... ARG SER  *
 ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC | CTG CAG GCG CTG GTT ..... AGA TCG TAA
                                                 |  PstI
```

Fig.2. The sequence across the *lacZ–pbp*B fusion junction in pPH153. Amino acids 1–6 are derived from *lacZ*, residues 7–11 are encoded by the multiple cloning region of the vector, and residues 240–588 are from the *pbp*B gene.

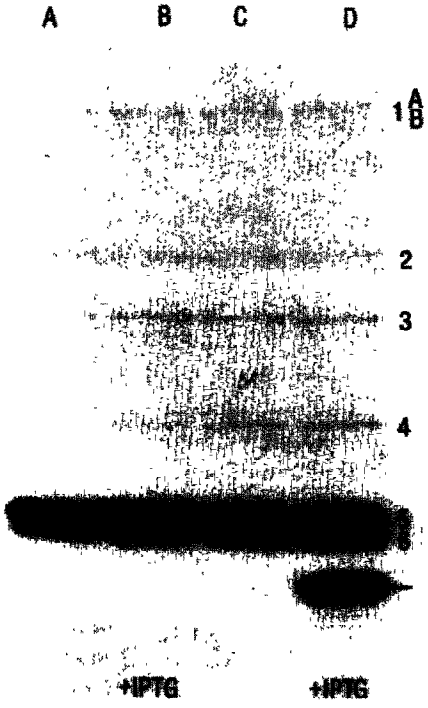

Fig.3. Detection of a penicillin-binding β-galactosidase-PBP 3 fusion protein. *E. coli* JM101 (pPH154) (A,B) and JM101 (pPH153) (C,D) were grown in Luria broth to a cell density of 5×10^7/ml. Part of each culture was removed and IPTG was added to a final concentration of 1 mM and the cultures were shaken for a further hour at 37°C. The bacteria from 0.5 ml of each culture were pelleted in an Eppendorf centrifuge for 2 min, the supernatant was removed, and the cells were resuspended in 20 μl of 50 mM sodium phosphate buffer (pH 7.0). After two cycles of freeze-thawing in a dry ice–ethanol bath, 5 μl of [^{3}H]benzylpenicillin (27 Ci/mmol) were added to give a final concentration of 50 μg/ml. After 10 min at 30°C the binding was terminated by the addition of 25 μl of SDS gel solubiliser buffer [17] and the PBPs were fractionated on a 12% SDS polyacrylamide gel and were detected by fluorography for 3 days at −70°C [17]. The arrow shows the position of the β-galactosidase–PBP 3 fusion protein.

phenylmethylsulfonyl fluoride (1.2 mM), had no effect on the amount of the fusion protein detected by the PBP assay. Proteolysis is therefore not the major cause for the unexpectedly low levels of the fusion protein.

The possibility that the activity of the protein was unstable was examined by taking an aliquot of *E. coli* JM101 (pPH153) that had been induced with IPTG, resuspending the cells in 50 mM sodium phosphate buffer (pH 7.0) and pre-incubating the bacteria at 37°C prior to the addition of [^{3}H]benzylpenicillin. The half-life of the penicillin-binding activity of the fusion protein was less than 1 min at 37°C. The relatively low level of the fusion protein that we observed is therefore due to the very rapid inactivation of the protein so that only a fraction of that synthesized is detected by the PBP assay. The stability of the protein was higher at 30°C and increased levels could be obtained in cells grown and assayed at this temperature.

Measurement of the affinity of the fusion protein for [^{3}H]benzylpenicillin was complicated by the loss of activity of the protein during the assay. The concentrations of radioactive benzylpenicillin required to obtain 50% saturation of the fusion protein and the normal PBP 3 were, however, indistinguishable (not shown).

PBP 3 is located in the cytoplasmic membrane and is synthesized as a preprotein with an NH_2-terminal signal peptide [18]. The fusion of the COOH-terminal part of PBP3 to the NH_2-terminus of β-galactosidase would therefore be expected to result in the appearance of the fusion protein in the cytoplasm. The instability of the fusion protein made it difficult to convincingly demonstrate its cellular location. However, experiments in which the fusion protein was labelled with [^{3}H]benzylpenicillin before cell fractionation indicated that the protein was equally distributed between

the cytoplasmic and cell envelope fractions whereas the normal PBP 3 was exclusively found in the cell envelope fraction. Further experiments with more stable fusion proteins are needed to unambiguously demonstrate the cellular location and solubility properties of the protein.

4. DISCUSSION

The properties of the PBP 3 fusion protein establish that the whole polypeptide chain that constitutes the penicillin-binding site is contained within amino acids 240–588. The removal of the NH_2-terminal 240 amino acids does not appear to substantially alter the affinity of PBP 3 for benzylpenicillin although it has a drastic effect on the stability of the protein. The reason for the decreased stability of the protein is unknown.

The active site residue of PBP 3 that is acylated by penicillin must also be located between residues 240 and 588. This contrasts strikingly with the findings with all of the class A and class C β-lactamases, and the D-alanine carboxypeptidases, where the acylated amino acid is a serine residue located close to the NH_2-terminus [7–9]. Strong candidates for the active site serine residues of PBP 1A, PBP 1B and PBP 3 have been obtained by comparing the amino acid sequences of these PBPs with the sequences around the active site serine residues of D-alanine carboxypeptidases and β-lactamases (in preparation). In each of these high molecular mass PBPs the assigned active site serine occurs towards the middle of the primary sequence and in PBP 3 the assignment of serine-307 has been supported by site-directed mutagenesis (in preparation).

The peptidoglycan transpeptidase activity may also be contained on the PBP 3 fusion protein but direct assay of transpeptidase activity in this unstable protein is not possible. Our results are therefore consistent with the suggestion that the penicillin-sensitive transpeptidase activity is located on a COOH-terminal functional domain. One obvious possibility is that the transglycosylase domain of PBP 3 is located within the NH_2-terminal 240 residues but there is at present no information on this matter.

The organisation of high molecular mass PBPs in the cytoplasmic membrane has not been studied. A logical organisation for a bifunctional peptidoglycan transglycosylase-transpeptidase would be to have the transglycosylase domain within, or on the outer surface of, the membrane where it acts on its membrane-inserted disaccharide pentapeptide substrate, and the transpeptidase extending into the periplasm where it can catalyse the crosslinking of glycan strands to the preexisting peptidoglycan. The transpeptidase domain may therefore normally exist within an aqueous environment and may be readily crystallised if removed from the transglycosylase domain. Removal of the NH_2-terminus of PBP 3 resulted in at least some of the protein being found in the cytoplasm but substantial amounts were also found in the cell envelope fraction. The instability of the protein made further analysis of its solubility properties difficult, and further fusions are being constructed to see if alteration of the position in PBP 3 where the protein is fused to β-galactosidase can result in more stable proteins. The production of a smaller, soluble, penicillin-binding domain from a high molecular mass PBP would be an attractive prospect for X-ray studies of a killing target for β-lactam antibiotics.

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