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Production of thiol-penicillin-binding protein 3 of *Escherichia coli* using a two primer method of site-directed mutagenesis

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The active site serine residue of penicillin-binding protein 3 of *Escherichia coli* that is acylated by penicillin (Ser-307) has been converted to a cysteine residue using a simple and efficient two primer method of site-directed mutagenesis. The resulting thiol-penicillin-binding protein 3 was expressed under the control of the *lacUV5* promoter in a high copy number plasmid. Constitutive expression of the thiol-enzyme (but not of the wild-type enzyme) was lethal, and the plasmid could only be maintained in *E. coli* strains that carried the *lacI*^{q} mutation. Induction of the expression of the thiol-enzyme resulted in inhibition of cell division and the growth of the bacteria into very long filamentous cells. The inhibition of septation was probably due to interference of the function of the wild-type penicillin-binding protein 3 in cell division by the enzymatically inactive thiol-enzyme, and this implies that penicillin-binding protein 3 acts as part of a complex *in vivo*. We were unable to detect any acylation of the thiol-enzyme by penicillin, but it is not yet clear if this was because the thioester was not formed at an appreciable rate, or if it was formed but was too unstable to be detected by a modified penicillin-binding protein assay.

Key words: penicillin-binding protein/site-directed mutagenesis/active site serine/thiol-enzyme

Introduction

β-Lactam antibiotics kill bacteria by inactivating a set of penicillin-binding proteins (PBPs) that are involved in the final stages of peptidoglycan synthesis (Spratt, 1975, 1983; Waxman and Strominger, 1983). In *E. coli* there are seven well characterised PBPs (Spratt, 1977) and genetic analysis has shown that the high mol. wt. PBPs 1A/1B, 2, and 3 are essential enzymes and are the killing targets for the β-lactam antibiotics (Spratt, 1975, 1983; Suzuki *et al.*, 1978). The combined inactivation of PBP 1A and PBP 1B results in the rapid lysis of *E. coli* cells, inactivation of PBP 2 results in the conversion of the bacteria into spherical cells, and inactivation of PBP 3 results in the inhibition of cell division and the growth of the bacteria into filamentous cells (Spratt, 1983).

The lower mol. wt. PBPs 4, 5, and 6 are non-essential for the growth of *E. coli* under laboratory conditions and these PBPs are therefore not of major importance in the killing action of β-lactam antibiotics (Broome-Smith and Spratt, 1982; Matsuhashi *et al.*, 1977; Spratt, 1980).

The high mol. wt. PBPs 1A/1B, PBP 3, and possibly PBP 2, are bifunctional enzymes catalysing both a penicillin-insensitive peptidoglycan transglycosylase reaction and a penicillin-sensitive peptidoglycan transpeptidase reaction (Matsuhashi *et al.*, 1982). These enzymes appear to be responsible for the synthesis of peptidoglycan from lipid-linked disaccharide pentapeptide precursors and its incorporation into the cell wall by transpeptidation. The lower mol. wt. PBPs catalyse the D-alanine carboxypeptidase reaction *in vitro* but the precise role of these enzymes in peptidoglycan synthesis is not understood (Spratt, 1983; Waxman and Strominger, 1983).

With several D-alanine carboxypeptidases both penicillin and a depsipeptide analogue of the normal peptide substrate acylate a serine residue that is located close to the amino terminus of the proteins, in accordance with the model of Tipper and Strominger (1965) for the interaction of penicillin with PBPs (Frère *et al.*, 1976; Glauner *et al.*, 1984; Waxman and Strominger, 1980; Yocum *et al.*, 1980, 1982). There is also convincing evidence that the hydrolysis of penicillin by both the Class A and Class C β-lactamases occurs by an acyl-enzyme mechanism involving an active site serine residue that is located close to the amino terminus of the proteins (Knott-Hunziker *et al.*, 1979, 1982). Despite the fact that D-alanine carboxypeptidases and β-lactamases both interact with penicillin by an acyl-enzyme mechanism there is very little similarity at the level of their amino acid sequences (Broome-Smith *et al.*, 1983; Spratt, 1983). However, in all D-alanine carboxypeptidases (with one apparent exception, Duez *et al.*, 1981), and all Class A and Class C β-lactamases, the serine residue that is acylated by penicillin occurs within the sequence Ser-Xaa-Xaa-Lys (Ambler, 1980; Broome-Smith *et al.*, 1983; Knott-Hunziker *et al.*, 1979, 1982; Waxman and Strominger, 1980).

It has not yet been established if the transpeptidase domain of the high mol. wt. bifunctional PBPs interacts with penicillin and cell wall peptides by an acyl-enzyme mechanism involving an active site serine residue. We have recently determined the nucleotide sequences of the genes encoding PBP 1A and PBP 1B of *E. coli* (J.K. Broome-Smith, A. Edelman, S. Yousif and B.G. Spratt, in preparation) and the sequence of the gene encoding PBP 3 (*pbpB*) has been reported (Nakamura *et al.*, 1983). PBP 1A and PBP 1B show only ~30% identity in their amino acid sequences and neither of these PBPs shows any extensive sequence similarity to PBP 3. However, despite the low level of similarity between these three high mol. wt. PBPs, the sequence Gly-Ser-Xaa-Xaa-Lys-Pro is found towards the middle of each of the sequences (Figure 1A). The similarity of this sequence to that of the known active site serine regions of D-alanine carboxypeptidases and Class A and Class C β-lactamases, suggests that the high mol. wt. PBPs are also 'serine enzymes', and provides very strong candidates for the active site serine residues of these PBPs.

We describe here the conversion of the putative active site serine of PBP 3 of *E. coli* (Ser-307) to a cysteine using site-directed mutagenesis and the preliminary characterisation of PBP 3$^{Cys-307}$.

A.

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                                        *
PBP 1A  (residues 454-476)    N R A T Q A L R Q V G S N I K P F L Y T A A M
PBP 1B  (residues 499-521)    N R A M Q A R R S I G S L A K P A T Y L T A L
PBP 3   (residues 296-318)    N R T I T D V F E P G S T V K P M V V M T A L
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B.

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                          3'-A CTT GGC CCG ACG TGC CAA TTT GG-5'
                            : ::: ::: ::: :** ::: ::: ::: ::
CTGCAG--174--ACC GAC GTG TTT GAA CCG GGC TCA ACG GTT AAA CCG ATG GTG GTA--543--GGTACC
 PstI         Thr Asp Val Phe Glu Pro Gly Ser Thr Val Lys Pro Met Val Val         KpnI
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Fig. 1. (A) The amino acid sequences around the predicted active site serine residues of PBP 1A, PBP 1B and PBP 3 of *E. coli*. The single-letter code for amino acids is used. The asterisks mark the position of the predicted active site serine residues. The sequences of PBP 1A and PBP 1B are from J.K.Broome-Smith, A.Edelman, S.Yousif and B.G.Spratt (in preparation) and that of PBP 3 from Nakamura *et al.* (1983). **(B)** The annealing of the mutagenesis primer to the non-coding strand of the *pbpB* gene in single-stranded M13mp8/12 DNA. The asterisks mark the positions of the double mismatch. The amino acid sequence shown is that of residues 300–314 of PBP 3. The numbers, 174 and 543, are the number of intervening nucleotides.

Results

A 1448-bp *Pst*I-*Hind*III fragment carrying the coding region for residues 240–588 of PBP 3 was obtained from pPH105 (Hedge and Spratt, 1984; Figure 2) and was cloned into the replicative form of phage M13mp8 DNA to produce M13mp8/12 (Figure 3). The alteration of Ser-307 to Cys-307 in PBP 3 requires the conversion of the serine codon UCA to the cysteine codon UGC. This change was introduced by site-directed mutagenesis using a 24-mer oligodeoxynucleotide primer that was complementary to the appropriate region of the PBP 3 gene (*pbpB*) except for a centrally positioned double mismatch (Figure 1B).

The mutagenesis protocol, a modification of the two primer method of Norris *et al.* (1983), is outlined in Figure 3 and experimental details are given in Materials and methods. The 17-mer universal sequencing primer and the 24-mer double mismatch mutagenesis primer were annealed to single-stranded M13mp8/12 DNA, and both primers were extended by the addition of the Klenow fragment of DNA polymerase and the four deoxynucleoside triphosphates. The resulting partially double-stranded M13mp8/12 DNA was digested with *Pst*I and *Kpn*I and ligated to M13mp18 DNA that had been digested with the same enzymes. The ligation mixture was used to transfect the *sup*^{o} host *E. coli* JM105 and the cells were plated with IPTG and Xgal. The use of a *sup*^{o} strain prevents the growth of phage derived from the M13mp8/12 DNA in the ligation mixture since this phage carries two amber mutations, and the white plaques that arise from the transfection should be recombinants derived from M13mp18 since the latter phage lacks amber mutations.

The ligation yielded ~200 white plaques and single-stranded template DNA was prepared from several of these and sequenced using the universal primer. Of the first four templates sequenced one contained the desired two nucleotide substitution and the others had the wild-type sequence. Phage corresponding to the mutant template were plaque purified and a single plaque was used to prepare both replicative form DNA and single-stranded template DNA. The entire 772-bp *Pst*I-*Kpn*I insert of the mutant template (M13mp18/307/Cys), and of an analogous M13mp18 recombinant carrying the wild-type insert, was then sequenced using the universal primer and three primers from a series that hybridise to regions along the *pbpB* gene. The sequence of the mutant and the wild-type *Pst*I-*Kpn*I fragments was identical except for the two nucleotide substitutions introduced by the site-directed mutagenesis.

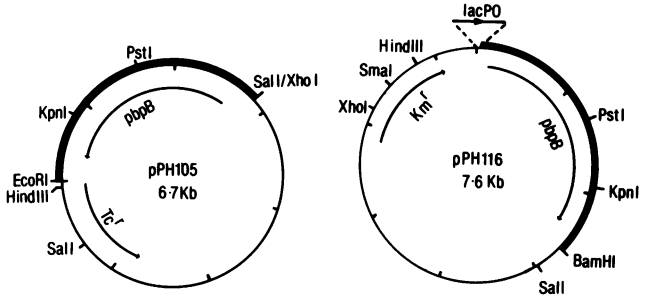

Fig. 2. The structures of pPH105 and pPH116.

The mutant sequence was introduced into the *pbpB* gene by replacing the *Pst*I-*Kpn*I region of the wild-type gene with the *Pst*I-*Kpn*I fragment derived from the replicative form of M13mp18/307/Cys. Initial experiments indicated that the constitutive expression of PBP 3$^{Cys-307}$ was highly toxic to *E. coli* and therefore the mutant sequence was inserted into the *pbpB* gene of the high copy number plasmid pPH116 (Figure 2), where expression of PBP 3 is under the control of the *lacUV5* promoter. The large *Pst*I-*Kpn*I fragment from pPH116 was purified, and was ligated to the small *Pst*I-*Kpn*I fragment derived from M13mp18/307/Cys; the ligation mixture was then used to transform *E. coli* JM101 (*lacI*^{q}) to kanamycin-resistance. The resulting plasmid carrying the mutant allele of *pbpB* was called pJBS615.

pPH116 and pJBS615 were introduced into *E. coli* JM109 (*lacI*^{q} *recA gyrA*) and the plasmid-encoded proteins were analysed by the maxicell technique. Figure 4 shows that a protein which co-migrated with PBP 3 on SDS-polyacrylamide gels was produced, under the control of the *lac* promoter, by both pPH116 and pJBS615. In the absence of IPTG the level of expression of PBP 3 and PBP 3$^{Cys-307}$ was very low but in the presence of IPTG substantial levels of the proteins were produced by both pPH116 and pJBS615.

The effect of the overproduction of wild-type PBP 3 and PBP 3$^{Cys-307}$ on cell morphology was examined. *E. coli* JM101 containing pPH116 or pJBS615 were examined by phase-contrast microscopy before, and 2 h after, the addition of IPTG. In the absence of IPTG, where there was only a low basal level of expression from the *lac* promoter in the *lacI*^{q}

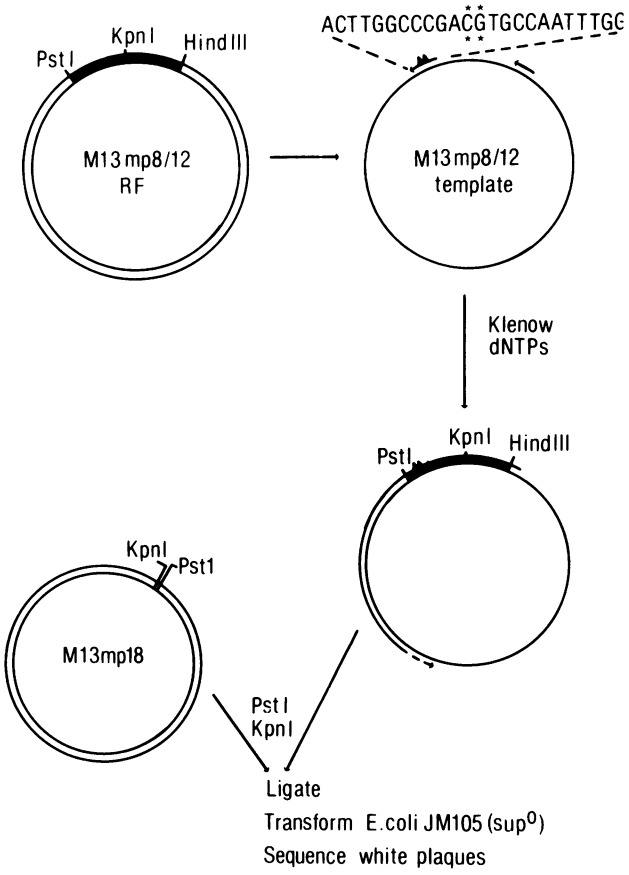

Fig. 3. The protocol for site-directed mutagenesis. See Materials and methods for further details.

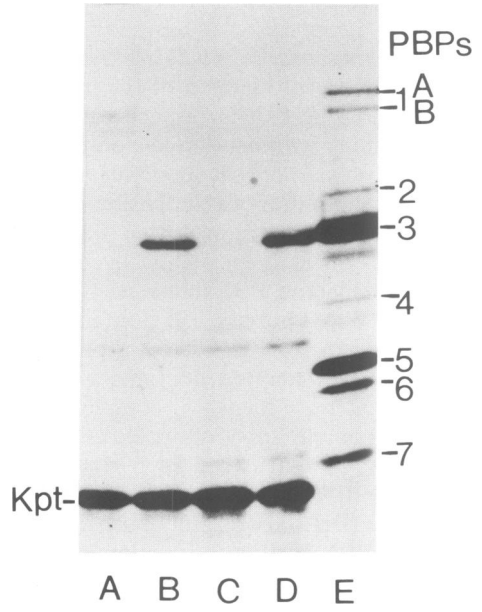

Fig. 4. Expression of PBP 3 and PBP $3^{Cys-307}$ in maxicells. Maxicells were prepared from *E. coli* JM109 carrying pPH116 (**A** and **B**) or pJBS615 (**C** and **D**), and plasmid-encoded proteins were labelled with [^{35}S]methionine, separated on a 12% SDS-polyacrylamide gel, and detected by fluorography. Tracks **B** and **D** were incubated with IPTG before the addition of radioisotope. (**E**) *E. coli* JM101(pPH116) was grown for 2 h in the presence of IPTG (1 mM) and the PBPs were labelled with [^{3}H]benzylpenicillin as described by Broome-Smith and Spratt (1982). Besides PBP 3, the other major plasmid-encoded product was kanamycin phosphotransferase (Kpt).

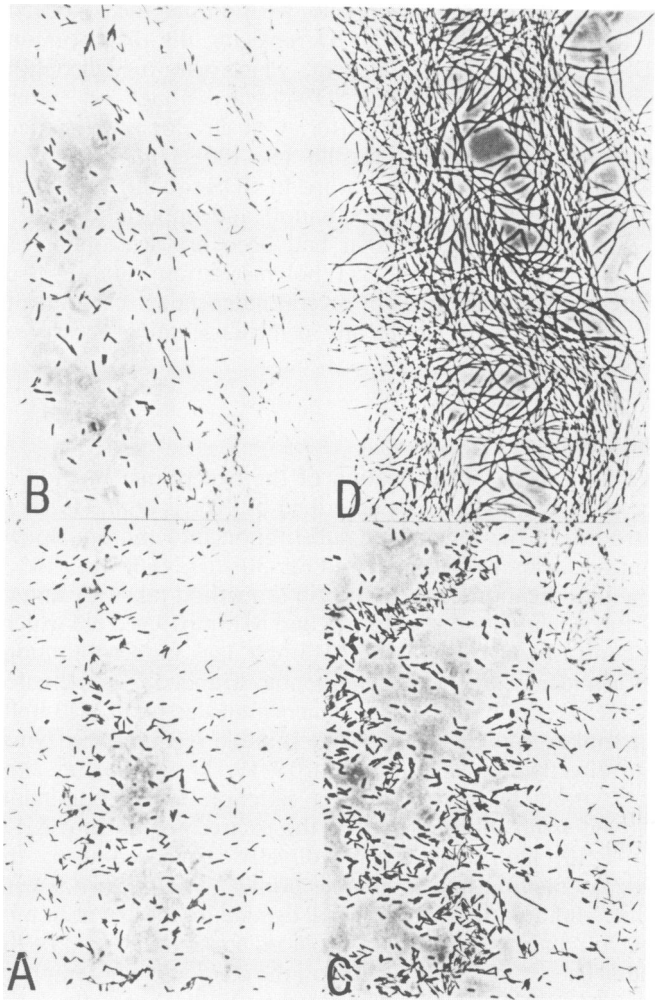

Fig. 5. The morphology of *E. coli* expressing PBP 3 and PBP $3^{Cys-307}$. *E. coli* JM101 carrying pPH116 (**A** and **B**) or pJBS615 (**C** and **D**) were grown in Luria broth at 37°C and were photographed in the exponential phase of growth. **B** and **D** have been grown in the presence of IPTG (1 mM) for 2 h.

background, both strains grew as rod-shaped cells. Overproduction of PBP 3, induced by the addition of IPTG, had no significant effect on cell morphology, but overproduction of PBP $3^{Cys-307}$ had a dramatic effect, resulting in the growth of the bacteria into extremely long filamentous cells (Figure 5). Overproduction of wild-type PBP 3 had no effect on cell viability but overproduction of PBP $3^{Cys-307}$ was lethal since cells carrying pJBS615 plated on Luria agar containing IPTG with an efficiency of 3×10^{-6}. The lethal effect of the expression of the mutant PBP 3 meant that pJBS615 could only be maintained in *E. coli* strains that carried a *lacI*^{q} mutation. The basal level of PBP 3 produced by pPH116 in a *lacI*^{q} background was sufficient to complement the temperature-sensitive mutant SP1000 (*pbpB*(*ts*)*lacI*^{q}). However, the basal level of PBP $3^{Cys-307}$ failed to complement the temperature-sensitivity of SP1000.

Cell envelopes were prepared from JM109(pPH116) and JM109(pJBS615) that had been induced with IPTG for 2 h and these were used in attempts to determine whether PBP $3^{Cys-307}$ retained any penicillin-binding activity. If the mutant PBP still bound penicillin, and if Ser-307 was the acylated residue, penicillin would be attached to PBP $3^{Cys-307}$ by a thioester linkage. As a thioester would be very unstable in the

presence of nitrogen nucleophiles we modified the conditions of the standard PBP assay by replacing the discontinuous SDS-polyacrylamide gel system, which uses a nucleophilic Tris-Glycine buffer, by an SDS-polyacrylamide gel system using sodium phosphate buffer. Cell envelopes were incubated with [^{3}H]benzylpenicillin (5 – 100 μg/ml) and were solubilised at room temperature in SDS-sodium phosphate buffer (without 2-mercaptoethanol) and applied to a 10% SDS-polyacrylamide gel that had been pre-electrophoresed for 24 h to remove the gel polymerisation catalysts (see Materials and methods). In several independent experiments we were unable to detect any penicillin-binding activity of PBP 3$^{Cys-307}$.

Discussion

We have used a modification of the two primer method of site-directed mutagenesis described by Norris *et al.* (1983) to introduce a two nucleotide substitution into the *pbpB* gene that results in the conversion of Ser-307 to Cys-307 in PBP 3. The modification of the two primer method takes advantage of the availability of M13mp18 and M13mp19 vectors which, in contrast to M13mp8 and M13mp9, lack amber mutations (Norrander *et al.*, 1983). The double-stranded region created by the extension of the two primers annealed to an M13mp8/9 template can be excised with suitable restriction enzymes and directly cloned into M13mp18/19. The use of the *sup^o* host strain JM105 provides a selection for recombinants derived from the insertion of the excised region into M13mp18/19, and these can be directly sequenced using the dideoxy-method and a suitable primer. The frequency with which mutants are recovered will depend on the extent of mismatch repair and of strand displacement at the 5′ end of the mutagenesis primer, as well as on the proportion of templates where DNA synthesis was primed by both oligonucleotides. In the mutagenesis experiment described here the desired mutant was found within the first four recombinants that were examined even though no attempt was made to minimise the effects of strand displacement by ligation of the 3′ end of the extended universal primer to the 5′ end of the mutagenesis primer.

The region of the *pbpB* gene that was subjected to site-directed mutagenesis was completely re-sequenced to ensure that no additional sequence alterations had occurred. The plasmids pPH116 and pJBS615 therefore differ only at two nucleotides, and produce normal PBP 3 and PBP 3$^{Cys-307}$ respectively, under the control of the *lacUV5* promoter. The maxicell experiment indicated that the levels of expression of PBP 3 and PBP 3$^{Cys-307}$ from these plasmids were similar, and that the alteration of Ser-307 to Cys-307 did not result in any significant increase in the susceptibility of PBP 3 to proteolysis. The mature form of wild-type PBP 3 contains no cysteine residues (Nakamura *et al.*, 1983). There are, however, two cysteine residues in the signal peptide of pre-PBP 3 but, since this peptide is removed during the insertion of the protein into the cytoplasmic membrane, it is most unlikely that the introduction of cysteine at residue 307 will result in the formation of a disulphide bond.

The conversion of Ser-307 to Cys-307 was designed to test the assignment of this amino acid as the active site residue of PBP 3 that is acylated by penicillin, since the analogous alterations have been carried out with two other classes of 'serine enzymes', and the properties of a thiol-PBP can therefore be predicted. The active site serine of TEM β-lactamase has been converted to a cysteine by site-directed mutagenesis, and the corresponding change has been made by chemical modification of the serine protease, subtilisin (Neet *et al.*, 1968; Sigal *et al.*, 1982, 1984). Thiol-subtilisin was totally inactive with peptide and ethyl ester substrates but retained slight activity with activated ester substrates (e.g., *p*-nitrophenyl acetate). The lack of activity of the thiol-enzyme was largely due to the decreased rate of formation of the acyl-enzyme with only slight effects on the K_m and the rate of hydrolysis of the acyl-enzyme. Apparently the -SH group of the thiol-enzyme is not sufficiently reactive, or is not correctly aligned, to be acylated readily by peptide or ethyl ester substrates. The -SH group can however replace the -OH group in the hydrolysis of activated esters since the increased reactivity of the ester allows the facile formation of an acyl-enzyme (Neet *et al.*, 1968). The thiol-β-lactamase retained some ability to hydrolyse benzylpenicillin and ampicillin (k_{cat} values that are 1 – 2% of that of the wild-type enzyme) and hydrolysed the cephalosporin substrate, nitrocefin, with a k_{cat} that was close to that of the normal enzyme. The ability of the -SH group to replace the -OH group in the hydrolysis of β-lactams has been explained by the reactivity of the β-lactam amide bond (Sigal *et al.*, 1982, 1984).

PBP 3 reacts with both peptide substrates and with β-lactam antibiotics. By analogy with the properties of thiol-subtilisin, and thiol-β-lactamase, the alteration of the active site serine of PBP 3 to a cysteine would be predicted to result in the complete loss of the peptidoglycan transpeptidase activity, which utilises peptide substrates, but the retention of some of the penicillin-binding activity, which involves the more reactive β-lactam molecule.

PBP 3$^{Cys-307}$ was unable to carry out its normal function in the synthesis of peptidoglycan at cell division since pJBS615, in contrast to pPH116, failed to complement the temperature-sensitivity of the *pbpB(ts)* mutant, SP1000. A second indication that the mutant PBP 3 was inactive arises from the lethal effects of the overproduction of the protein. Whereas the amount of wild-type PBP 3 per cell can be elevated at least 100-fold without significant effects on cell morphology or viability, the expression of only moderate amounts of PBP 3$^{Cys-307}$ resulted in inhibition of cell division. The most likely explanation of this phenomenon is that the PBP 3$^{Cys-307}$, expressed from pJBS615, is enzymatically inactive and the molar excess of the inactive PBP interferes with the function of the normal PBP 3 that is expressed from the chromosomal gene. This would occur if PBP 3 acted as part of a complex (e.g., an oligomer) such that one monomer of inactive PBP 3 was sufficient to inactivate the whole complex. The insertion of PBP 3 into a complex *in vivo* would also explain why resistance to β-lactams that is caused by the production of mutant forms of PBP 3 that have decreased affinity for β-lactams is recessive (P.J. Hedge, unpublished experiments).

The inability of PBP 3$^{Cys-307}$ to function *in vivo* is consistent with the predicted lack of activity of a thiol-PBP 3 with its cell wall peptide substrate. Proof of the assignment of Ser-307 as the active site serine could be obtained if the thiol-enzyme retained detectable penicillin-binding activity, since the product of acylation by penicillin would be a thioester, with chemical properties that are very different from those of the ester formed with the serine enzyme. However, we have been unable to detect any penicillin-binding activity of PBP 3$^{Cys-307}$ even when benzylpenicillin concentrations that are 100 times those required to obtain 50% saturation of the wild-type PBP 3 were used.

Recently, however, the assignment of Ser-307 as the residue acylated by penicillin has been established by the isolation and analysis of the peptide of PBP 3 that is labelled with radioactive penicillin (W. Keck, B. Glauner, U. Schwarz, J.K. Broome-Smith and B.G. Spratt, unpublished experiments). PBP $3^{Cys\text{-}307}$ is therefore a thiol-PBP analogous to thiol-β-lactamase and thiol-subtilisin. In common with the other thiol-enzymes, the thiol-PBP 3 was severely impaired in its activity with peptide substrates, as judged by its inability to function in its *in vivo* role as a peptidoglycan transpeptidase. The thiol-PBP 3 would be expected to be poorly acylated by benzylpenicillin but no acylation was detected. At present it is unclear whether penicillin failed to acylate the thiol-PBP, or whether acylation occurred, but the resulting thioester was too labile to remain intact throughout the modified PBP assay.

Materials and methods

Bacterial strains and growth media

E. coli JM101, JM105, JM109, and phage M13mp8 and M13mp18 have been described (Messing and Vieira, 1982; Norrander *et al.*, 1983). *E. coli* SP1000 has the genotype *thr leu thi pbpB(ts)26 recA56/F' lacI^q*.

Luria broth was used for all experiments except that 2YT broth was used for the growth of M13 phage-infected cultures (Miller, 1972). Induction of the *lac* promoter was achieved with 1 mM IPTG.

DNA sequencing

M13 derivatives were sequenced by the dideoxy method using [^{35}S-α-thio]-dATP and gradient gels as described by Biggin *et al.* (1983).

Maxicells

Maxicells were prepared from strain JM109 essentially as described by Sancar *et al.* (1979). IPTG (1 mM) was added 1 h before the addition of [^{35}S]methionine.

Site-directed mutagenesis

A 10 μl hybridisation mixture containing 0.4 pmol of single-stranded M13-mp8/12 DNA, 10 pmol of mutagenesis primer, and 5 pmol of universal sequencing primer, in 15 mM Tris-HCl pH 8.5, 15 mM $MgCl_2$ was incubated at 60°C for 90 min (dideoxy-sequencing reactions on this double primed template showed that both primers annealed specifically and efficiently under these conditions). The annealed mixture was allowed to cool to 21°C, and 10 μl of a solution containing each of the dNTPs (0.2 mM), 15 mM Tris-HCl pH 8.5, 15 mM $MgCl_2$ and five units of DNA polymerase I (Klenow fragment) was added. The primer extension reaction was incubated at 21°C for 60 min and was terminated by heating at 65°C for 10 min. The mixture was diluted 5-fold into 6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10 units of *Pst*I and *Kpn*I were added, and the mixture was incubated at 37°C for 40 min. The digestions were terminated by heating at 65°C for 10 min, the DNA was ethanol precipitated, resuspended in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and ligated with 1 μg of *Pst*I- and *Kpn*I-digested M13mp18 DNA at a final DNA concentration of 20 μg/ml. The ligation mixture was used to transfect JM105 and the cells were plated with IPTG and Xgal (5-bromo-6-chloro-3-indoyl β-D-galactoside; 200 μg/ml). White plaques were dideoxy-sequenced using universal primer to identify those with the mutant sequence.

Assay of PBPs

E. coli cell envelopes were prepared as described (Spratt, 1977) and were finally resuspended at 1 mg protein/ml in 50 mM sodium phosphate buffer pH 7.0. Cell envelopes (20 μl) were incubated for 10 min at 30°C with 5 μl of [^{3}H]benzylpenicillin (5 – 100 μg/ml final concentration) and the reaction was terminated by the addition of 25 μl of solubiliser (4% SDS, 20% glycerol, 0.004% bromophenol blue). After the addition of 2 μl of non-radioactive benzylpenicillin (100 mg/ml) the samples were immediately loaded on a continuous SDS-phosphate polyacrylamide gel (12% acrylamide, 0.16% bisacrylamide, 0.1% SDS, 0.1 M sodium phosphate buffer pH 7.0, polymerised with 0.1% ammonium persulphate and 0.1% tetramethylethylenediamine, using 0.1 M sodium phosphate buffer pH 7.0, 0.1% SDS as the electrophoresis buffer) that had been pre-run for 24 h at 100 V. The gel was fixed for 1 h in several changes of 10% acetic acid, 5% methanol, and was fluorographed using sodium salicylate (Chamberlain, 1979), and exposed to Kodak RP X-ray film at −70°C.

Enzymes and chemicals

Restriction enzymes, T4 DNA ligase, and *E. coli* DNA polymerase I (Klenow fragment) were from Boehringer Mannheim. [^{35}S-α-thio]dATP (410 Ci/mmol) and [^{35}S]methionine (1500 Ci/mmol) were from Amersham International. [^{3}H]benzylpenicillin (27 Ci/mmol) was a gift from Dr P.J.Cassidy of Merck Sharp and Dohme. The mutagenesis primer d(GGTTTAACCG-TGCAGCCCGGTTCA) and the M13 universal sequencing primer d(GTA-AAACGACGGCCAGT) were obtained from Celltech.

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References

Ambler,R.P. (1980) *Phil. Trans. R. Soc. Lond. Ser. B*, **289**, 321-331.
Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963-3965.
Broome-Smith,J.K. and Spratt,B.G. (1982) *J. Bacteriol.*, **152**, 904-906.
Broome-Smith,J.K., Edelman,A. and Spratt,B.G. (1983) in Hakenbeck,R., Höltje,J.-V. and Labischinski,H. (eds.), *The Target of Penicillin*, De Gruyter, Berlin, pp. 403-408.
Chamberlain,J.P. (1979) *Anal. Biochem.*, **98**, 132-135.
Duez,C., Joris,B., Frère,J.-M., Ghuysen,J.-M. and Van Beeumen,J. (1981) *Biochem. J.*, **193**, 83-86.
Hedge,P.J. and Spratt,B.G. (1984) *FEBS Lett.*, **176**, 179-184.
Frère,J.-M., Duez,C., Ghuysen,J.-M. and Vanderkerckhove,J. (1976) *FEBS Lett.*, **70**, 257-260.
Glauner,B., Keck,W. and Schwarz,U. (1984) *FEMS Microbiol. Lett.*, **23**, 151-155.
Knott-Hunziker,V., Waley,S.G., Orlek,B.S. and Sammes,P.G. (1979) *FEBS Lett.*, **99**, 59-61.
Knott-Hunziker,V., Petursson,S., Jayatilake,G.S., Waley,S.G., Jaurin,B. and Grundström,T. (1982) *Biochem. J.*, **201**, 621-627.
Matsuhashi,M., Takagaki,Y., Maruyama,I.N., Tamaki,S., Nishimura,Y., Suzuki,H., Ogino,U. and Hirota,Y. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 2976-2979.
Matsuhashi,M., Nakagawa,J., Tomioka,S., Ishino,F. and Tamaki,S. (1982) in Mitsuhashi,S. (ed.), *Drug Resistance in Bacteria – Genetics, Biochemistry and Molecular Biology*, Japan Scientific Societies Press, Tokyo, Japan, pp. 297-301.
Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269-276.
Miller,J.H. (1972) *Experiments in Molecular Genetics*, published by Cold Spring Harbor Laboratory Press, NY.
Nakamura,M., Maruyama,I.N., Soma,M., Kato,J.-I., Suzuki,H. and Hirota,Y. (1983) *Mol. Gen. Genet.*, **191**, 1-9.
Neet,K.E., Nanci,A. and Koshland,D.E. (1968) *J. Biol. Chem.*, **243**, 6392-6401.
Norrander,J., Kempe,T. and Messing,J. (1983) *Gene*, **26**, 101-106.
Norris,K., Norris,F., Christiansen,L. and Fiil,N. (1983) *Nucleic Acids Res.*, **11**, 5103-5112.
Sancar,A., Hack,A.M. and Rupp,W.D. (1979) *J. Bacteriol.*, **137**, 692-693.
Sigal,I.S., Harwood,B.G. and Arentzen,R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7157-7160.
Sigal,I.S., DeGrado,W.F., Thomas,B.J. and Petteway,S.R. (1984) *J. Biol. Chem.*, **259**, 5327-5332.
Spratt,B.G. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 2999-3003.
Spratt,B.G. (1977) *Eur. J. Biochem.*, **72**, 341-352.
Spratt,B.G. (1980) *J. Bacteriol.*, **144**, 1190-1192.
Spratt,B.G. (1983) *J. Gen. Microbiol.*, **129**, 1247-1260.
Suzuki,H., Nishimura,Y. and Hirota,Y. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 664-668.
Tipper,D.J. and Strominger,J.L. (1965) *Proc. Natl. Acad. Sci. USA*, **54**, 1133-1141.
Waxman,D.J. and Strominger,J.L. (1980) *J. Biol. Chem.*, **255**, 2964-2976.
Waxman,D.J. and Strominger,J.L. (1983) *Annu. Rev. Biochem.*, **52**, 825-870.
Yocum,R.R., Rasmussen,J.R. and Strominger,J.L. (1980) *J. Biol. Chem.*, **255**, 3977-3986.
Yocum,R.R., Amanuma,H., O'Brien,T.A., Waxman,D.J. and Strominger, J.L. (1982) *J. Bacteriol.*, **149**, 1150-1153.

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