An amino acid substitution that blocks the deacylation step in the enzyme mechanism of penicillin-binding protein 5 of *Escherichia coli*

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A mutant of *Escherichia coli* has been described that produces an altered form of penicillin-binding protein 5 which still binds penicillin but is unable to catalyse the release of the bound penicilloyl moiety. We show that the mutation is caused by a single nucleotide transition that results in a change from glycine at residue 105 of the wild-type sequence of penicillin-binding protein 5 to aspartate in the mutant.

Penicillin-binding protein *DNA sequencing* *Mutant* *Acyl-enzyme* *Sulfhydryl reagent* Escherichia coli

1. INTRODUCTION

Penicillin-binding proteins (PBPs) catalyse the final stages of peptidoglycan synthesis [1–3]. In *Escherichia coli* PBPs 1A/1B, 2 and 3 have been shown to be the killing targets of the β-lactam antibiotics and to catalyse the polymerisation of peptidoglycan precursors and their incorporation into the cell wall by transpeptidation [2–4]. The PBPs of lower molecular mass (PBPs 4, 5 and 6) are non-essential for bacterial growth and catalyse the D-alanine carboxypeptidase reaction [2,3].

Studies with the D-alanine carboxypeptidases of several bacteria have supported the hypothesis in [5] that penicillin acts an an analogue of the acyl-D-alanyl-D-alanine moiety of the pentapeptide sidechain of the substrates of penicillin-sensitive enzymes [3,6–9]. With these enzymes (e.g., PBP 5 of *Bacillus subtilis* and PBPs 5 and 6 of *E. coli*) penicillin has been shown to acylate an active site residue to form a relatively stable penicilloyl–enzyme complex that is analogous to the transient acyl-enzyme intermediate formed with substrate [7,8]. With some PBPs (e.g., PBPs 5 and 6 of *E. coli* and PBP 4 of *Staphylococcus aureus*) there is a slow enzymically catalysed release of the bound penicilloyl moiety as penicilloic acid with the regeneration of the active enzyme [10–12]. These PBPs therefore catalyse a very weak β-lactamase reaction.

In the case of PBP 5 of *E. coli* (D-alanine carboxypeptidase 1A) the penicilloyl–enzyme complex has a half life of 5 min at 30°C [10]. Authors in [13] have described a mutation (*dacA*11191) that results in the production of an altered PBP 5 which still binds penicillin but is blocked in the release of the bound penicilloyl moiety. The mutant PBP 5 fails to catalyse the D-alanine carboxypeptidase reaction as the hydrolysis of the acyl-enzyme formed with substrate is also blocked and the acyl-enzyme accumulates [3,13]. We have recently obtained the amino acid sequence of PBP 5 from the nucleotide sequence of the *dacA* gene ([14] and in preparation). We here show that the block in the deacylation step of the enzyme mechanism of PBp 5, caused by the *dacA*11191 mutation, is due to a change of a glycine at residue 105 of the wild-type PBP 5 to aspartate in the mutant.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

The following *E. coli* strains were used. TMRL1222, *proA purB his thi galK lacY rpsL dacA*11191 *dacB* [13]; SP5003, *his supF srl*::Tn10 *recA*56 Δ(*lip-dacA-rodA*)/λ*drodA1* [15]. pBS25 contains the *E. coli dacA* gene cloned as a 2.7-kb chromosomal DNA fragment from a partial *Sau*IIIA digest in the low copy number plasmid pSC105 [16]. pBS59 contains the *dacA* gene on a 1597-base pair (bp) *Bam*HI–*Eco*RI fragment inserted in the low copy number vector pLG338 [17]. Restriction maps of these plasmids are shown in fig.1.

2.2. *Assay of PBPs*

PBPs were assayed in whole cells or purified cell envelopes using [^{3}H]benzylpenicillin (10.5 Ci/mmol; kindly provided by Dr Louis Nisbet of Smith, Kline, Beckman Corporation) as in [10,18]. The mutant and wild-type forms of PBP 5 were distinguished by their rate of release of bound [^{14}C]benzylpenicillin. PBPs were labelled in whole cells for 10 min at 30°C with [^{3}H]benzylpenicillin (50 μg/ml final concentration), a 100-fold excess of unlabelled benzylpenicillin was added, and incubation was continued for a further 30 min at 30°C. SDS solubiliser buffer [10] was added and the PBPs were fractionated on a 12% SDS polyacrylamide gel and the amount of [^{3}H]benzylpenicillin remaining bound to PBP 5 was detected by fluorography. Under these conditions less than 5% of the [^{3}H]benzylpenicillin remained bound to the wild-type PBP 5 whereas there was no substantial release from the mutant PBP 5. The affinity of the mutant and wild-type PBP 5 for benzylpenicillin was measured by incubating cell envelopes with 2-fold increasing concentrations of [^{3}H]benzylpenicillin for 10 min at 30°C and quantitating the amount of antibiotic bound as in [10].

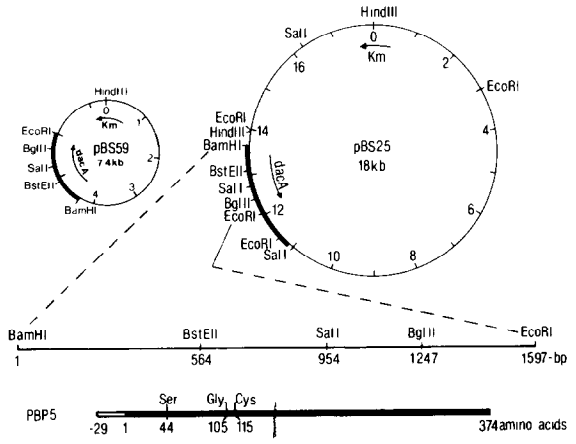

Fig. 1. Structure of pBS25 and pBS59. The location and direction of transcription of the kanamycin phosphotransferase gene (Km) and the *dacA* gene are indicated on the plasmid maps by the arrows. The solid blocks indicate the chromosomal DNA inserts carried by the plasmids. pJBS608 has an identical structure to pBS25 except that it carries the *dacA*11191 mutation (see text). The 1597-bp *Bam*HI-EcoRI fragment that encodes PBP 5, the location of the coding region, and the features of the structure of PBP 5 are shown at the bottom of the figure. The unhatched part of the line diagram of PBP 5 represents the 29 amino acid signal peptide [2,14].

2.3. *Manipulation of DNA and DNA sequencing*

The preparation of plasmid DNA and techniques for the manipulation of DNA and transformation have been described [15,17]. DNA fragments to be sequenced were cloned into the replicative form of bacteriophage M13mp9 [19] and single-stranded template DNA was prepared and sequenced using the 'dideoxy' chain-termination method [20].

3. RESULTS

3.1. *Transfer of the* dacA*11191 mutation from the chromosome onto pBS25*

Strain TMRL1222 carries the *dacA*11191 mutation and produces an altered PBP 5 that still binds benzylpenicillin but is defective in the enzyme catalysed release of the bound penicilloyl moiety [13]. The wild-type *dacA* gene has been cloned as a 2.7-kb DNA fragment in pSC105 to produce pBS25 [20]. *E. coli* TMRL1222 (pBS25) grows as spherical cells which are killed by concentrations of sodium lauroylsarcosinate that have no effect on the viability of normal rod-shaped cells. This effect is due to the elevated levels of PBP 5 [16].

Transfer of the *dacA*111191 mutation from the chromosome onto pBS25 by reciprocal recombination, followed by plasmid segregation, should result in cells that overproduce the mutant enzymatically inactive form of PBP 5. Overproduc-

tion of enzymatically inactive PBP 5 is not expected to result in the growth of *E. coli* as spherical cells and this provides a selection for the in vivo transfer of the *dacA*11191 mutation to pBS25 by plating strain TMRL1222 (pBS25) on penassay agar containing 1% sodium lauroylsarcosinate where only rod-shaped cells will survive. In practice strain TMRL1222 (pBS25) plated on the above agar with a frequency of about 10^{-4}. The majority of the survivors were rod-shaped and produced elevated levels of the mutant form of PBP 5. Plasmid carrying the *dacA*11191 mutation was prepared from one of these strains and was purified genetically by transformation into strain SP5003, which is deleted of the entire chromosomal *dacA* gene [15]. SP5003 carrying the plasmid (pJBS608) produced elevated levels of the mutant form of PBP 5 (fig.2).

The half time for the release of the penicilloyl moiety from the wild-type and mutant forms of PBP 5 at 30°C were 4 min and about 60 min, respectively (fig.2). The affinities of the mutant and wild-type forms of PBP 5 for benzylpenicillin were estimated by measuring the concentration required to obtain 50% saturation of the PBPs using cell envelopes prepared from the overproducing strains SP5003(pBS25) and SP5003(pJBS608). The affinity of the mutant form of PBP 5 for benzylpenicillin was 2 times less than that of the wild-type enzyme.

3.2. *Location of the* dacA*11191 mutation to a* Bst*EII*-Bgl*II fragment and its sequencing*

The location of the mutation that prevents the release of bound penicillin was determined by reconstructing the *dac*A gene from fragments of the wild-type and mutant genes. The 683-bp *Bst*EII-*Bgl*II fragment from pJBS608 was purified and ligated to the purified large *Bst*EII-BglIII fragment of pBS69 to regenerate the *dacA* gene (see fig.1). The ligation mixture was used to transform *E. coli* SP5003 to kanamycin resistance. Transformants were examined for the production of the mutant or wild-type forms of PBP 5 by measuring the rate of release of bound [^{14}C]benzylpenicillin. Thirteen out of 26 transformants produced by the mutant form of PBP 5 and all of these contained plasmids that were the same size as pBS59 and were indistinguishable from pBS59 by restriction mapping. They must therefore have arisen by the replacement of the 683-bp *Bst*EII-*Bgl*II fragment of wild-type *dacA* in pBS59 with the corresponding fragment from the mutant *dacA* gene of pJBS608. The *dacA*11191 mutation is therefore located in the 683-bp *Bst*EII-*Bgl*II fragment.

To sequence this region, the 1247-bp *Bam*HI-*Bgl*II fragment was isolated from pJB608 (fig.1) and was digested with *Sau*IIIA. The *Sau*IIIA fragments were cloned into the *Bam*HI site of the replicative form of M13mp9

Fig. 2. Release of bound [^{3}H]benzylpenicilloyl moiety by wild-type and mutant forms of PBP 5. Cell envelopes of SP5003(pBS25) (tracks a–g) and SP5003 (pJBS608) (tracks h–n) were incubated with [^{3}H]benzylpenicillin (30 μg/ml final concentration) for 10 min at 30°C, a 100-fold excess of unlabelled benzylpenicillin was added, and samples were taken after further incubation at 30°C for 0 (a,h); 5 (b,i); 10 (c,j); 20 (d,k); 30 (e,l); 45 (f,m) and 60 min (g,n). The PBPs were fractionated on a 12% SDS polyacrylamide gel and the level of radioactivity bound to each protein was detected by fluorography as in [10].

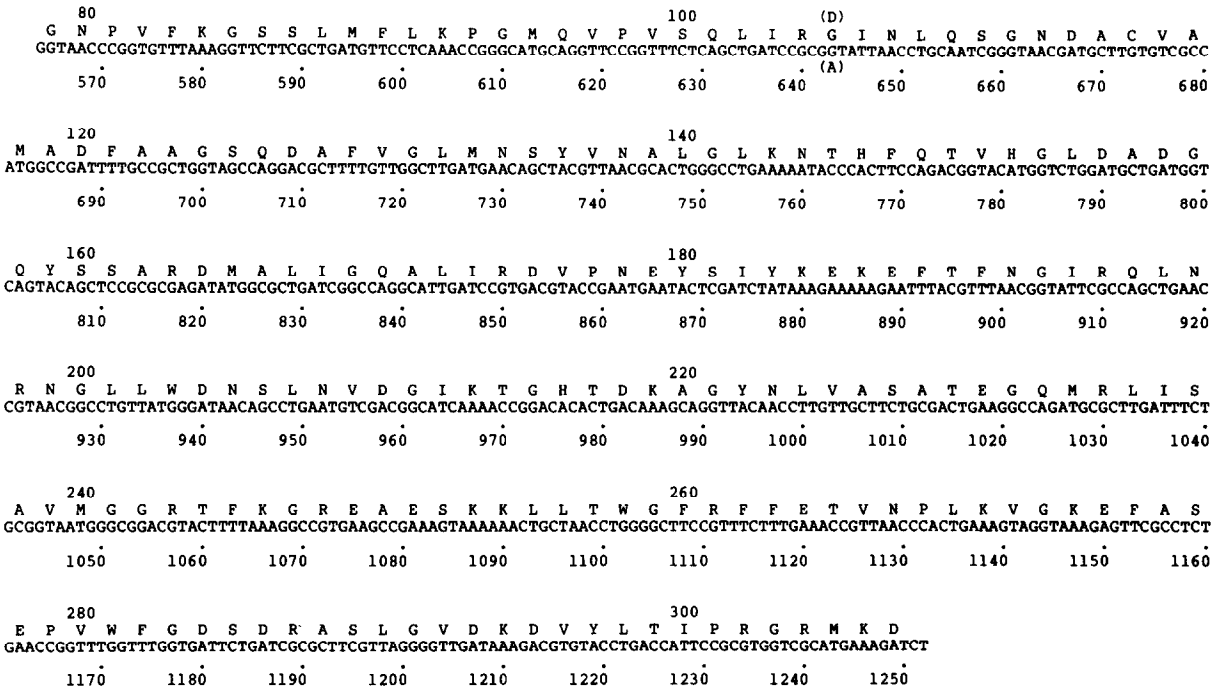

Fig. 3. The sequence of the *Bst*EII–*Bgl*II fragment of the mutant and wild-type *dacA* gene. The sequence shown is that of the wild-type gene. The alteration caused by the *dacA*11191 mutation is shown in brackets at nucleotide 643 and at amino acid 105. The single-letter notation for amino acids is used.

bacteriophage and were sequenced. All of the sequence of the 683-bp *Bst*EII–*Bgl*II fragment of pJBS608 was determined and the sequence was found to be identical to the wild-type sequence except at position 643 (fig. 1) where a G–A transition had occurred resulting in a change of glycine-105 to aspartate-105 in PBP 5. The sequence of this region in the wild-type and mutant is shown in fig. 3.

4. DISCUSSION

The block in the deacylation step of the enzyme mechanism of PBP 5 in the *dacA*11191 mutant is caused by a single nucleotide substitution that results in the change of glycine-105 to aspartate-105. The rate of release of the bound benzylpenicilloyl moiety in the mutant was about 6% of that from wild-type PBP 5. The affinity of the mutant PBP 5 for benzylpenicillin was also altered, being about 2 times lower than that of the normal enzyme. This may be an underestimate of the real difference in the affinity between the mutant and wild-type enzymes since the concentration of benzylpenicillin required to obtain 50% saturation is likely to be influenced by the differences in the rates of deacylation. The *dacA*11191 mutation therefore results in a PBP 5 that is altered in both the acylation and deacylation steps of the enzyme mechanism. The effect of the deacylation step appears, however, to be substantially greater than that on the acylation step. This is supported by the accumulation of acyl-enzyme when the mutant PBP 5 (but not the wild-type PBP 5) is incubated with peptide substrates [3].

In [21] it was reported that pCMB has the same effect on the activity of D-alanine carboxypeptidase 1A as that produced by the *dacA*11191 mutation and it was suggested that a cysteine residue is involved in the deacylation step of the enzyme mechanism but not in the acylation event [3] We have reinvestigated the effect of pCMB on PBP 5 (in preparation) and have shown that both the binding and release of penicillin are prevented by this reagent at relatively low concentrations (<0.2 mM) and the effect of pCMB on PBP 5 is

therefore different from the effect of the *dacA*11191 mutation which preferentially affects the deacylation step of the enzyme mechanism. PBP 5 contains only a single cysteine residue (cysteine-115) [14] and the inhibition of the binding and release of penicillin by pCMB can be attributed to the modification of this residue. It is interesting that modification of two closely positioned residues (glycine-105 and cysteine-115) drastically affects the enzyme mechanism. This region may in the three-dimensional structure be a part of, or close to, the active site of PBP 5. The active site residue that is acylated by penicillin (and by substrate) has been assigned as serine-44 on the basis of homologies with those D-alanine carboxypeptidases where the acive site serine has been determined by chemical methods [2,14].

Cysteine-115 could have a catalytic role in the mechanism of PBP 5 although modification of this residue by pCMB may inactivate the enzyme indirectly. Glycine-105 is unlikely to have a catalytic role and the effect of the change from glycine-105 to aspartate-105 caused by the *dacA*11191 mutation may result from an alteration of the secondary structure of this region. An understanding of the importance of these residues for the activity of PBP 5 must await the solution of the three-dimensional structure of PBPs.

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