MUTANTS OF *ESCHERICHIA COLI* WHICH LACK A COMPONENT OF PENICILLIN-BINDING PROTEIN 1 ARE VIABLE

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1. Introduction

The study of penicillin-binding proteins (PBPs) provides a convenient approach to a biochemical understanding of the complex morphological responses produced by β-lactam antibiotics [1,2]. In *Escherichia coli* six PBPs have been consistently detected in the cytoplasmic membrane. PBP 1 has been proposed to be the target with which penicillins and cephalosporins interact to inhibit cell elongation and thereby to lead to cell lysis [1,2]. This assignment has been complicated by recent experiments that have suggested that PBP 1 may not be a single polypeptide. Partial resolution of PBP 1 into two components has been occasionally observed and the binding of some cephalosporins to this PBP were markedly biphasic [3].

In this paper we describe a modification of the sodium dodecylsulphate polyacrylamide gel system that we have used previously. This modification results in a clear and reproducible separation of PBP 1 into two or possibly more components. A mutant which shows no binding of (^{14}C)benzylpenicillin to one of these components has been shown to grow normally. The other component of PBP 1 has the properties expected of the target at which β-lactams interact to inhibit cell elongation.

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2. Materials and methods

E. coli KN126 has been described previously [3]. Strain KN126 was mutagenised with nitrosoguanidine and a derivative (SP6, previously called B6 [1]) was isolated which was resistant to mecillinam (10 μg/ml) at 30°C. SP6 grew as spherical cells at 30°C and failed to form colonies on penassay agar at 42°C. SP61 was a spontaneous revertant of SP6 which grew as normal rods at 42°C. The mutation which results in the growth of SP6 as spherical cells has been located (Iwaya M. and Spratt, B. G. unpublished experiments) at 14 min on the *E. coli* linkage map [4]. Strain CGSC4282 harbours the F-prime, F254, was obtained from Dr B. J. Bachmann [5]. This F-prime carries the genes from 7–15 min and therefore carries a normal copy of the gene which is defective in SP6. The plasmid, F254, was transferred to SP6 by conjugation. SP6 grows slowly and derivatives of this strain harbouring F254 were readily detected as faster growing colonies. Examination of these latter colonies showed that they were invariably rod-shaped and had the same nutritional requirements as SP6. One of these derivatives was chosen for further study and was designated SP62. During growth SP62 segregated rare spherical cells due to the occasional loss of F254.

Cells were grown in penassay broth (Difco antibiotic medium number 3) and washed cell envelopes were prepared as described [3]. PBPs were detected by

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binding [^{14}C]benzylpenicillin (51 mCi/mmol, Radiochemical Centre, Amersham, England) to washed cell envelopes, selectively solubilising the proteins of the cytoplasmic membrane with Sarkosyl NL-97, and fractionating them on a sodium dodecylsulphate (SDS) polyacrylamide slab-gel. Three different SDS polyacrylamide gel systems were used. System 1 was a 10% gel with the gel composition and buffers described by Laemmli [6] as used previously [3]. System 2 was the above system with the single modification that the running gel was composed of 10% wt/vol acrylamide and 0.068% wt/vol methylene bisacrylamide. System 3 was that of Lugtenberg et al. [7] using a running gel of 6.5% wt/vol acrylamide and 0.12% wt/vol methylene bisacrylamide. The dried gels were fluorographed on pre-fogged Kodak RP Royal X-ray film [8]. Binding of non-radioactive β-lactams to the PBPs was measured by their competition for the binding of [^{14}C]benzylpenicillin as described previously [3].

3. Results

Previous experiments using gel system 1 showed PBP 1 to be a single band [3]. This band could not be resolved into sub-components by running longer gels or by reducing the acrylamide concentration from 10% wt/vol to 7.5% wt/vol (without altering the ratio of acrylamide/bisacrylamide). Gel systems 2 and 3, which differed from system 1 in their lower level of crosslinking due to an increased ratio of acrylamide/bisacrylamide, resulted in the resolution of PBP 1 into at least two components. These are referred to as PBPs 1A and 1B. Figure 1 shows the PBPs of *E. coli* KN126 fractionated using gel systems 1 and 2.

PBPs 1A and 1B differed markedly in their affinities for several β-lactams. Since PBP 1 has been suggested to be the target at which β-lactams interact to inhibit cell elongation [1,2], we compared the binding to PBPs 1A and 1B of cephalexin, which has little effect on cell elongation, and cephaloridine which is a potent inhibitor of cell elongation. Both β-lactams bound with relatively high affinity to PBP 1A; cephaloridine also showed high affinity for PBP 1B whereas cephalexin had a very low affinity for this PBP. Mecillinam showed no affinity for either PBP 1A or 1B.

The mecillinam-resistant spherical mutant SP6 was previously shown to fail to bind [^{14}C]benzylpenicillin or [^{14}C]mecillinam to PBP 2 [1]. Re-examination of the PBPs of this mutant using gel systems 2 and 3 showed that it also failed to bind [^{14}C]benzylpenicillin to PBP 1A (fig.2C,D). No trace of any binding to PBPs 1A or 2 has been observed in experiments with envelopes of SP6. Increasing the duration of the incubation with [^{14}C]benzylpenicillin from 10–20 min still failed to result in any detectable binding to these PBPs. In addition the relative proportions of PBP 1B, and in some experiments PBP 3, were increased in comparison to the parent strain KN126.

In order to assess the relevance of the defect in PBP 1A in the phenotype of SP6 we have examined the PBPs in derivatives of the mutant which have regained the normal rod morphology. Introduction

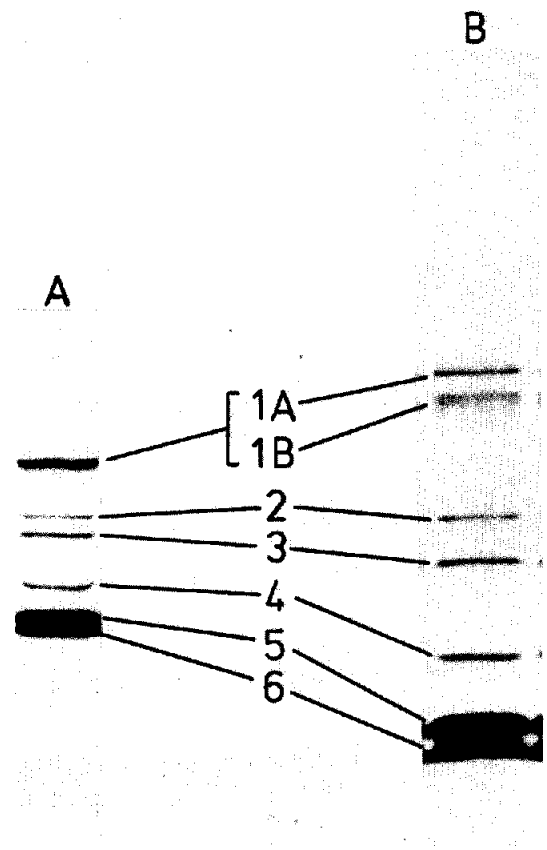

Fig.1. [^{14}C]Benzylpenicillin (34 μg/ml) was bound to envelopes of *E. coli* KN126 for 10 min at 30°C and the PBPs were fractionated using gel system 1 (A) or system 2 (B). PBPs were detected by fluorography for 20 days.

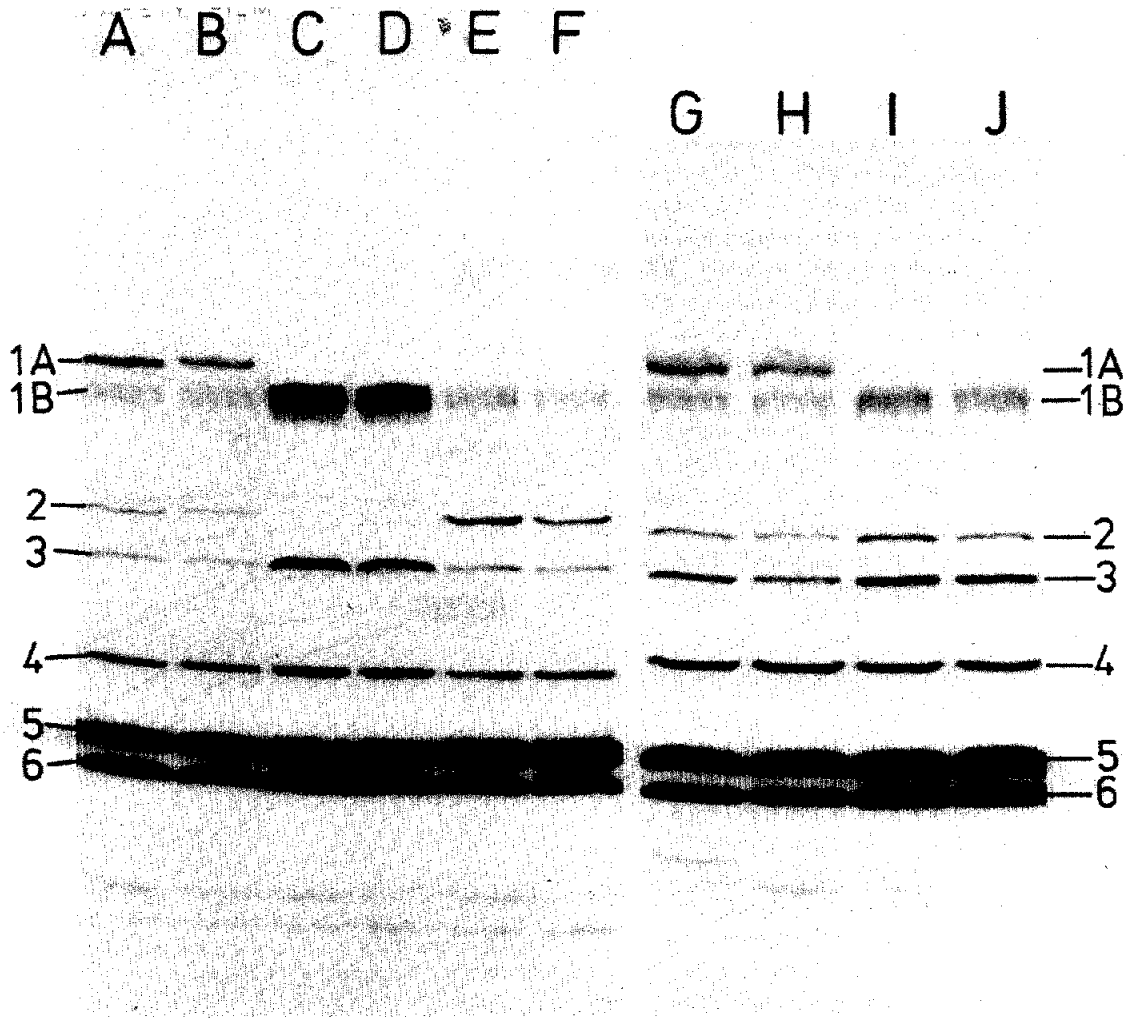

Fig.2. [^{14}C]Benzylpenicillin (34 μg/ml) was bound to envelopes of KN126 (A,B and G,H), SP6 (C,D), SP62 (E,F), or SP61 (I,J). Binding was either for 10 min at 30°C (A,C,E,G,I) or for 5 min at 42°C (B,D,F,H,J). PBPs were fractionated using gel system 2 and detected by fluorography.

of F254 into SP6 to give the F-prime derivative SP62 resulted in the re-appearance of the normal rod shape and of PBP 2 (at a level considerably higher than in KN126) but had no effect on the defect in PBP 1A (fig.2E,F). Similarly the rod-shaped temperature-resistant revertant SP61 regained PBP 2 but still lacked PBP 1A (Fig.2I,J).

The derivatives SP61 and SP62 have the full complement of PBPs with the exception of PBP 1A. We compared the growth of SP61 with that of KN126 to ascertain whether the loss of PBP 1A activity resulted in any marked growth defect. Both strains (and also SP62) grew well in penassay broth at 30°C and 42°C and looked normal under the phase-contrast microscope. The mean generation times of KN126 and SP61 in penassay broth at 37°C were 23.7 min and 25.3 min, respectively (average of three measurements). The morphological responses of SP61 and KN126 to growth in the presence of mecillinam, benzylpenicillin, ampicillin, cephalexin and cephaloridine, and the concentrations

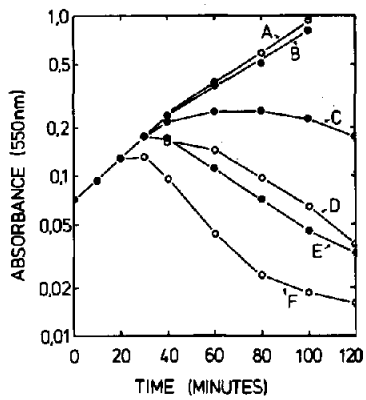

Fig.3. Growth of KN126 in penassay broth at 37°C in the presence of 0 (A), 50 (D) and 100 (F) μg/ml benzylpenicillin. Growth of SP61 in the presence of 0 (B), 50 (C) and 100 (E) μg/ml benzylpenicillin.

at which these responses occurred, were almost identical. The rate of lysis of SP61 in the presence of several β-lactams was however slower than that of KN126. Figure 3 shows the lysis of KN126 and SP61 in the presence of benzyl-penicillin.

4. Discussion

PBP 1 was resolved on loosely crosslinked SDS polyacrylamide gels into at least two components. PBP 1A was probably a single polypeptide but PBP 1B may still consist of more than one polypeptide as partial resolution of this component into two bands was sometimes observed.

Although SP6 lacks both PBP 1A and PBP 2 activity it is clear that the spherical shape of the cells and their mecillinam-resistance were due to the defect in PBP 2 and were not related to the loss of PBP 1A. Inactivation of PBP 2 by mecillinam, or in mutants which produce a highly thermolabile form of PBP 2, is known to result in the formation of spherical cells [1,2].

The isolation of the revertant SP61, which had all PBP activities except that of PBP 1A, allowed the examination of the role of the latter PBP in cell growth. No defects in the growth of SP61 (or the F-prime derivative SP62) were observed which suggests that the activity of PBP 1A is not essential for the growth and normal morphology of *E. coli* under laboratory conditions and also indicates that this protein is not one of the targets at which β-lactams bind to produce their lethal effects. It is of course difficult to eliminate the possibility that some activity of PBP 1A remains under in vivo conditions or the unlikely possibility that penicillin-binding activity has been lost without loss of the other enzymatic properties of the protein. The only significant difference between SP61 and KN126 was the slower rate of lysis of the former strain in the presence of β-lactam antibiotics. This may reflect a subtle role of PBP 1A peptidoglycan metabolism.

The level of the PBPs in different cell envelope preparations is to some extent variable and this makes comparisons between strains difficult. The level of PBP 1B in the mutant SP6 was, however, consistently higher than in the parent KN126 and the level returned to approximately normal in both SP61 and SP62 (fig.2). Apparently the loss of PBP 2 activity results in the overproduction of PBP 1B. The level of PBP 3 in the mutant SP6 was higher than normal in some experiments (e.g., that shown in fig.2) but in other experiments this was not observed and therefore its significance is unclear.

The reason why the mutant SP6 simultaneously lost two PBPs is unclear. Several alternatives can be considered:

(i) The genes for PBPs 1A and 2 map contiguously and both genes are deleted in SP6.
(ii) There are two independent mutations in the genes for PBPs 1A and 2.
(iii) There is a mutation in a control gene which is required for the synthesis of both PBPs.
(iv) There is a mutation in the gene coding for PBP 1A and PBP 2 is a cleavage product of this protein.
(v) The two PBPs exist in a complex in vivo such that inactivation of one can alter the activity of the other.

The recovery of PBP 2 activity without the simultaneous recovery of PBP 1A activity in both the revertant SP61 and the F-prime derivative SP62 excludes all possibilities that are based on some interdependence between PBPs 1A and 2 (c–e). The isolation of a spontaneous revertant which regained PBP 2 activity excludes the possibility of a deletion and therefore we assume that strain SP6 carries two separate mutations in the genes for PBPs 1A and 2. Nitrosoguanidine was used in the isolation of SP6 and this mutagen is known

to produce multiple closely-linked mutations [9]. However, the genes for PBPs 1A and 2 are not known to be closely-linked as F254 carried the gene for PBP 2 but not that for PBP 1A. The gene for PBP 1A could map just to the right of the limits of F254 and still be within the region in which nitrosoguanidine produces multiple lesions. If the PBP 1A gene is found to map far from the PBP 2 gene it seems unlikely that a mutant would be obtained by chance with two independent and unrelated PBP mutations unless the survival of mutants which lack PBP 2 are enhanced by an additional mutation in the gene for PBP 1A. However, one other mecillinam-resistant mutant which lacked PBP 2 activity still had a normal PBP 1A (Spratt, B. G. unpublished experiments).

Finally, the high affinity of cephaloridine for PBP 1B and the low affinity of cephalexin makes this protein the likely target at which β-lactams bind to inhibit cell elongation and we would predict that mutants which produced a defective form of this protein would be found among temperature-sensitive cell lysis mutants.

Acknowledgements

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