STUDIES ON THE PENICILLIN-BINDING COMPONENTS OF *BACILLUS MEGATERIUM*

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1. Introduction

The existence of multiple penicillin-binding proteins has been shown in a wide variety of bacteria [1,2]. Penicillin is thought to kill growing cells either by inhibiting a transpeptidation reaction by which newly synthesized peptidoglycan is incorporated into the cell-wall with the continued action of autolytic enzymes causing cell-lysis, or by directly triggering the autolytic enzymes [3]. It is thus supposed that the binding of penicillins to one or more of the penicillin-binding components (PBCs) is responsible for the death of the cell.

In recent years, much research has been carried out on enzymes possessing D,D-alanine carboxypeptidase activity [1] although a variety of evidence now suggests that inhibition of this enzyme by penicillin is not responsible for cell-death. In order to understand the molecular mechanism of penicillin action, the functions of the other PBCs in the cell must be determined and the PBC whose interaction with penicillin causes cell-death must be identified.

With the exception of the component of lowest molecular weight, which has D,D-alanine carboxypeptidase activity [4,5] none of the PBCs present in Bacilli has been shown to possess enzymic activity although they have been postulated to be transpeptidases, D,D-alanine carboxypeptidases or endopeptidases [1]. Although the PBCs from *Bacillus subtilis* have been separated from other membrane proteins [4], they were not separated from each other and attempts were not made to study possible enzymic activities of the individual PBCs.

This report describes studies on the separation of the PBCs of *Bacillus megaterium* by selective elution from ampicillin-affinose by hydroxylamine and investigations of the enzymic activities of the isolated PBCs.

Abbreviations: Dap 2,6-diaminopimelic acid, Genapol X-100 isotridecanol polyglycol ether (n = 10), PBC penicillin-binding component

2. Materials and methods

Bacillus megaterium KM was grown as described for *Bacillus stearothermophilus* but at 37°C instead of 55°C [6]. Protoplast membranes from exponentially growing cells and UDP–*N*-acetylmuramyl-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala were prepared as described by Barnett [6]. L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala was prepared as described by Nguyen-Distèche et al. [7] and L-Ala-D-Glu-meso-2,6-diamino [1,7-^{14}C]pimelyl-D-Ala-D-Ala as described by Martin, Schilf and Maskos [8]. Ampicillin-affinose was prepared from Affi-Gel 202 (Bio-Rad) by the method of Pollock et al. [9].

In assays of D,D-alanine carboxypeptidase activity, the release of D-alanine was estimated by the method of Johnson et al. [10]. [^{14}C]Benzylpenicillin potassium (54 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. The non-ionic detergent Genapol X-100 was generously donated by Farbwerke Hoechst AG, Frankfurt.

Unless otherwise stated, samples for gel-electrophoresis were treated with [^{14}C]benzylpenicillin (10 μg/ml) for 10 min at 37°C followed by the addition of a 1000-fold excess of [^{12}C]benzylpenicillin. Samples containing Genapol X-100 or samples with a volume greater than 50 μl were precipitated with

acetone (final concentration 80%) and the precipitate collected by centrifugation. Polyacrylamide slab-gel electrophoresis in the presence of sodium dodecyl sulphate was performed essentially as described by Laemmli and Favre [11] and gels were stained with Coomassie Brilliant Blue by the method of Fairbanks, Steck and Wallach [12]. Gels were prepared for fluorography by the method of Bonner and Lasky [13].

3. Results and discussion

Protoplast membranes from *Bacillus megaterium* treated with [^{14}C]benzylpenicillin followed by separation of the proteins by SDS polyacrylamide gel electrophoresis showed five PBCs after fluorography (fig.1a). The molecular weights and relative abundance of the PBCs (numbered in order of decreasing molecular weight) are shown in table 1. The sensitivity of the five PBCs to benzylpenicillin differs substantially with PBC 1 being the most and PBC 5 the least sensitive while PBCs 2, 3 and 4 have similar intermediate sensitivities (table 1, fig.1). PBC 1 was also found to be the most sensitive component in relation to the binding of cephaloridine and cephalothin as measured by competition of the cephalosporin for the [^{14}C]benzylpenicillin binding sites (fig.1b, table 1).

PBCs 1, 3, 4 and 5 could be completely solubilized from protoplast membranes by treatment (20 min, 37°C) with 1% Genapol X-100 in 0.05 M Tris–HCl, pH 8.0, in the presence of 0.5 M NaCl. The solubilization procedure appeared to inhibit the penicillin-binding activity of PBC 2 as this component could not be detected in the solubilized material (i.e., the material not sedimented by centrifugation at 165 000 × *g* for 1 h) or in the residual pellet.

The solubilized PBCs were bound to a column of ampicillin-affinose (5 ml settled volume) which was washed extensively with 1 M NaCl in 0.05 M Tris–HCl, pH 8.0 containing 0.1% Genapol X-100 to remove any proteins bound non-covalently. The PBCs were eluted by batchwise washing at 37°C with neutral 1 M hydroxylamine in 0.5 M Tris–HCl, pH 8.8, containing 0.1% Genapol X-100 (fig.2). Hydroxylamine was removed from samples by dialysis at 4°C against 0.05 M Tris–HCl, pH 8.0, containing 0.1% Genapol X-100. The order of release of the PBCs was PBC 5, PBC 1, PBC 3 and PBC 4.

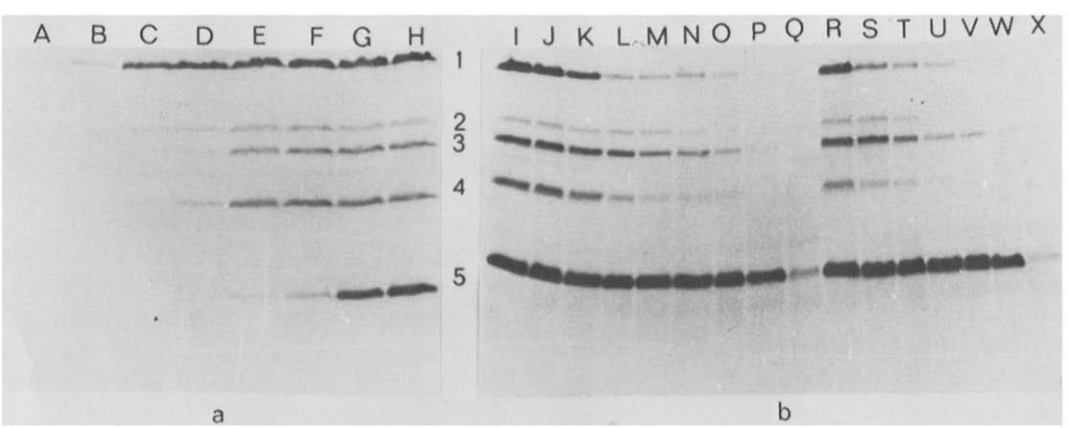

Fig.1. Sensitivity of membrane-bound PBCs to β-lactam antibiotics. Fluorograms showing the PBCs detected by fluorography after fractionation of membranes by SDS–polyacrylamide gel electrophoresis. (a) Membranes were incubated with different concentrations of [^{14}C]benzylpenicillin for 10 min at 37°C. The concentrations of benzylpenicillin used were (μg/ml): (A) 0.005, (B) 0.01, (C) 0.05, (D) 0.1, (E) 0.5, (F) 1.0, (G) 5.0, (H) 10.0. (b) Membranes were preincubated with increasing concentrations of the relevant cephalosporin for 10 min at 37°C before addition of [^{14}C]benzylpenicillin (10 μg/ml) to detect the PBCs that were still accessible. The concentrations of cephalosporins used were (μg/ml), (I) 0 (Control); Cephaloridine: (J) 0.01, (K) 0.03, (L) 0.1, (M) 0.3, (N) 1.0, (O) 10, (P) 100, (Q) 1000. Cephalothin: (R) 0.03, (S) 0.1, (T) 0.3, (U) 1.0, (V) 3.0, (W) 10, (X) 100.

Table 1
Properties of the PBCs from *Bacillus megaterium*

Property	Component				
	1	2	3	4	5
Apparent molecular weight[a]	123000	94000	83000	70000	45000
Binding of [^{14}C]benzylpenicillin[b]	32	7	16	14	31 % of total
Sensitivity to benzylpenicillin[c]	0.04	0.1	0.3	0.3	2 μg/ml
Sensitivity to cephaloridine[d]	0.03	0.3	1.0	0.1	300 μg/ml
Sensitivity to cephalothin[d]	0.05	0.5	2.0	0.2	30 μg/ml

[a] Determined by comparison of the mobilities of the PBCs on SDS polyacrylamide slab-gels with those of proteins of known molecular weight.
[b] Determined by densitometry of fluorograms
[c] Determines as the concentration of [^{14}C]benzylpenicillin required to 50% saturate the relevant PBC present in membranes after incubation for 10 min at 37°C
[d] Determined as the concentration of the cephalosporin which reduced the binding of [^{14}C]benzylpenicillin to the PBC by 50%. Incubation with the cephalosporin was for 10 min at 37°C followed by incubation with [^{14}C]benzylpenicillin (10 μg/ml) for a further 10 min.

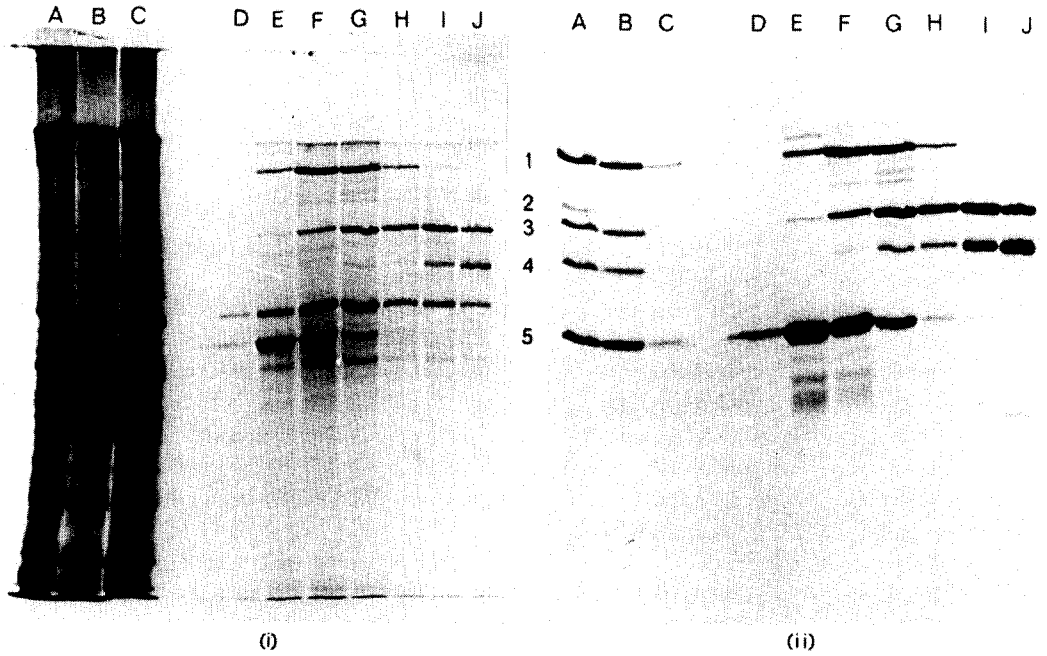

Fig.2. Isolation of the PBCs by covalent affinity chromatography. Analysis of the isolation procedure by SDS–polyacrylamide gel electrophoresis. (i) Proteins as revealed by staining with Coomassie Brilliant Blue. (ii) PBCs as detected by fluorography. Protoplast membranes (A) were treated with Genapol X-100 (as described in the text) to yield solubilized material (B). This material was applied to a column of ampicillin-affinose to which the majority of proteins were unadsorbed (C). The PBCs were eluted from the affinity gel by successive incubations at 37°C with aliquots of hydroxylamine for the following periods: 0 min (D), 0–1 min (E), 1–10 min (F), 10–30 min (G), 30–100 min (H), 100–300 min (I), 300–500 min (J). The faint bands in tracks E, F and G as revealed by fluorography may have arisen by proteolysis of PBC 5 (tracks E and F) and PBC 1 (tracks F and G) during affinity chromatography or the subsequent dialysis as they are not present in the original membranes.

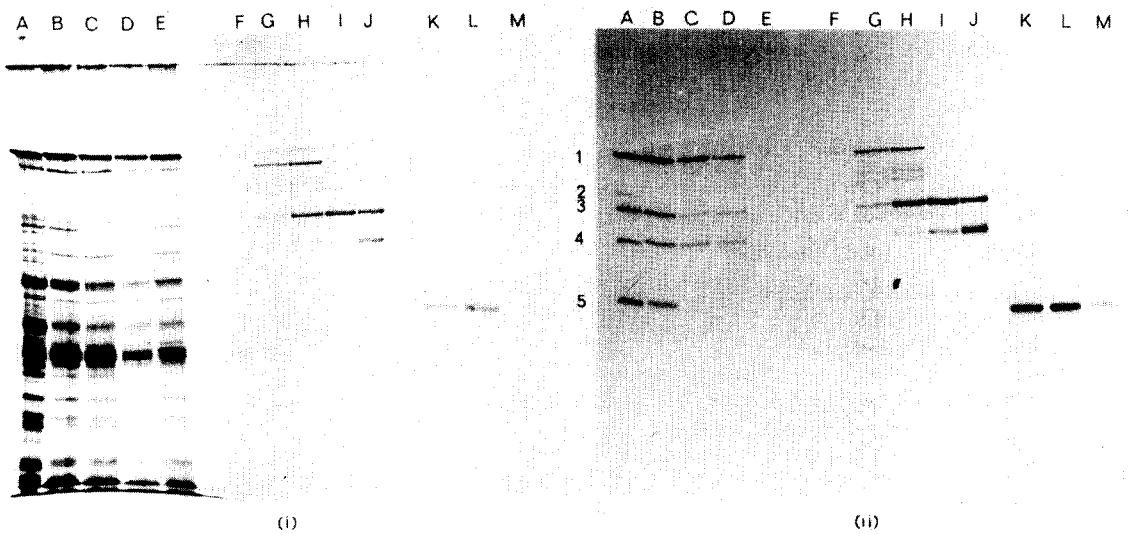

Fig.3. Use of cephalothin pretreatment for the separation of the PBCs. Analysis of the separation procedure by SDS polyacrylamide gel electrophoresis. (i) Proteins as revealed by staining with Coomassie Brilliant Blue. (ii) PBCs as detected by fluorography. Material (B) solubilized from protoplast membranes (A) was treated with cephalothin (4 μg/ml for 10 min at 37°C) and applied to a column of ampicillin-affinose. PBC 5 was adsorbed to the affinity gel and was eluted by successive incubations with hydroxylamine for 0–1 min (K), 1–10 min (L), and 10–30 min (M). The unadsorbed material (C) was treated with hydroxylamine to remove any cephalothin bound to the PBCs (D) (see text for details) and applied to another ampicilline–affinose column to which the majority of proteins were unadsorbed (E). The PBCs were eluted from the affinity gel by successive incubations at 37°C with aliquots of hydroxylamine for the following periods: 0–1 min (F), 1–10 min (G), 10–30 min (H), 30–100 min (I), 100–400 min (J).

The PBCs were not completely separated by this procedure and in order to obtain samples of PBCs 1, 3 and 4 free from PBC 5, the following procedure was adopted. Examination of the sensitivities of the PBCs to cephalothin (fig.1b) indicated that a concentration could be chosen that would bind to all PBCs except PBC 5. Material solubilized from protoplast membranes was treated with cephalothin (4 μg/ml for 10 min at 37°C) before application to an ampicillin–affinose column. PBC 5 (which did not have cephalothin bound to it) was adsorbed to the column and could subsequently be eluted with hydroxylamine (fig.3). PBCs 1, 3 and 4 (which did not have cephalothin bound to them) did not bind to the column. Cephalothin that was bound to PBCs 1, 3 and 4 was removed by treatment with 0.5 M neutral hydroxylamine in 0.5 M Tris–HCl, pH 8.8, for 30 min at 37°C. When these PBCs were applied to another ampicillin–affinose column, they were adsorbed and could be selectively eluted with hydroxylamine in order that the enzymic activities of fractions enriched in particular PBCs (fig.3) could be determined.

Protoplast membranes of *B. megaterium* have D,D-alanine carboxypeptidase activity [14], but it has not been demonstrated previously whether this was due to one or more of the PBCs as occurs with *Salmonella typhimurium* (Shepherd, Chase and Reynolds, submitted for publication) which has at least two PBCs possessing D,D-alanine carboxypeptidase activity. L-Ala–D-[^{14}C]Glu–*meso*-Dap–D-Ala–D-Ala has been used as a substrate for natural model transpeptidation [7] in studies with other organisms in which the dimer produced by transpeptidation is separated from the other radioactive components of the reaction mixture by high-voltage paper electrophoresis. Protoplast membranes of *B. megaterium* incubated at 37°C with this substrate, released D-alanine from it (D,D-alanine carboxypeptidase activity) but no formation of dimer occurred. Possible reasons for this lack of transpeptidase activity are:

(i) The specificity of the natural transpeptidase

is such that it does not catalyse this model reaction.

(ii) Concomitant peptidoglycan synthesis is necessary as for the model transpeptidation reaction in which Dap is incorporated into peptidoglycan [15]

(iii) It is not possible to show transpeptidase activity in the presence of endopeptidase or D,D-alanine carboxypeptidase activities.

Attempts were made to investigate the natural model transpeptidase and D,D-alanine carboxypeptidase activities of the fractions obtained by affinity chromatography. These fractions permitted the study of the activities of some of the PBCs in the absence of the others. Samples containing only PBC 5 (fig.3, tracks K, L. and M) showed D,D-alanine carboxypeptidase activity towards the substrates UDP–*N*-acetylmuramyl–L-Ala–D-Glu–*meso*-Dap–D-Ala–D-Ala and L-Ala–D-Glu–*meso*-Dap–D-Ala–D-Ala. Samples containing PBCs 1, 3 or 4 but not PBC 5 (fig.3) did not show any release of D-alanine from either of the above substrates even with prolonged incubation, suggesting that none of the PBCs 1, 3 or 4 is a D,D-alanine carboxypeptidase. Failure of PBCs 1, 3 and 4 to release D-alanine from the pentapeptide substrate indicates that these three PBCs are not transpeptidases capable of catalysing this model transpeptidation reaction. Incubation of samples containing PBC 5 with L-Ala–D-[^{14}C]Glu–*meso*-Dap–D-Ala–D-Ala showed that although the release of D-alanine was being catalysed by this enzyme, no dimerisation occurred. Thus the only enzymic activity of the PBCs that has been demonstrated is D,D-alanine carboxypeptidase activity by PBC 5.

It is possible that PBCs 1, 3 and 4 lack enzymic activity as a result of denaturation during affinity chromatography although they are still able to bind [^{14}C]benzylpenicillin. Another possibility is that the specificity of the natural transpeptidase is such that it requires a long side-chain as in nascent peptidoglycan. A third possibility, if one accepts the hypothesis proposed by Tomasz and Waks [3], is that the PBC involved in the lethal target might act as a trigger for autolytic activity but lacking enzymic activity itself.

Buchanan and Strominger [16] have attemped to identify the PBC involved in the lethal event in *B. subtilis* by obtaining mutants that were resistant to cloxacillin. The PBCs of these mutants were examined and it was concluded that of the five PBCs found, PBC 2 was the most likely target for killing by penicillins. Treatment of whole cells of *B. megaterium* with [^{14}C]benzylpenicillin followed by fractionation of the membranes by SDS–polyacrylamide slab gel electrophoresis and subsequent fluorography suggests that PBC 1 is the only PBC which binds benzylpenicillin at the minimum growth inhibitory concentration as determined by growth tests on penicillin–agar plates or in liquid media (Reynolds, Shepherd and Chase, submitted for publication). Hence future work will be devoted to identifying the cellular function of PBC 1.

Acknowledgements

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