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The sporulation-specific penicillin-binding protein 5a from *Bacillus subtilis* is a DD-carboxypeptidase *in vitro*

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The sporulation-specific penicillin-binding protein 5a was purified from *Bacillus subtilis* and shown to possess DD-carboxypeptidase activity *in vitro*.

The synthesis of the sporulation-specific penicillin-binding protein (PBP) 5a during sporulation of bacilli is temporally and spatially regulated: it is only detected in large amounts during spore peptidoglycan (cortex) synthesis from stage II to stage V (Todd & Ellar, 1982; Sowell & Buchanan, 1983; Todd *et al*, 1983*a*) and is found principally in the membranes of the developing forespore compartment (Todd & Ellar, 1982; Todd *et al.*, 1983*a*).

Peptide 'mapping' of PBPs 5 and 5a from *Bacillus megaterium* KM (Todd & Ellar, 1982) and *Bacillus subtilis* 168 Sueoka (Todd *et al.*, 1983*a*) showed that for each organism these proteins are structurally distinct, and that PBP 5a is probably unique. We have obtained indirect evidence that *B. megaterium* KM PBP 5a possesses DD-carboxypeptidase activity *in vitro* and proposed (Todd & Ellar, 1983) that *in vivo* PBP 5a is partly responsible for the smaller degree of peptide cross-linkage observed in spore peptidoglycan compared with the vegetative polymer (Tipper & Gauthier, 1972). The spore cortex is probably a major structure responsible for spore heat-resistance and dormancy (Tipper & Gauthier, 1972; Gould & Dring, 1975; Rogers, 1977; Ellar, 1978; Imae *et al.*, 1978; Keynan, 1978).

In the present investigation we have partially purified PBP 5a from a mutant *B. subtilis* that lacks PBP 5 and demonstrated that it has DD-carboxypeptidase activity *in vitro* with a pH optimum distinct from the vegetative DD-carboxypeptidase activity of PBP 5.

Abbreviations used: ^{14}C-labelled UDP-MurAc-pentapeptide, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-2,6-diaminopimelyl-D-alanyl-D-[^{14}C]alanine; PBP, penicillin-binding protein.

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Materials and methods

Bacterial strains

B. subtilis JT175 (*trpC2 cam1*) was derived from *B. subtilis* 168 Sueoka *trpC2* (Leighton & Doi, 1971) by plasmid-mediated insertional mutagenesis (Ferrari *et al.*, 1983) of the structural gene of PBP 5 (J. A. Todd, unpublished work). This mutant lacked detectable PBP 5, as shown in Fig. 1.

Preparation of membranes

The conditions for growth and sporulation of *B. subtilis* JT175 in modified Schaeffer's medium were as described previously (Leighton & Doi, 1971) except that the medium was supplemented with chloramphenicol (5 μg/ml) and L-tryptophan (50 μg/ml). Sporulation cells of *B. subtilis* JT175 (about 200 g wet wt.) were harvested at stage IV (as described by Todd *et al.*, 1983*a*) and resuspended in 400 ml of 50 mM-potassium phosphate buffer, pH 7, containing 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 0.5 mM-dithiothreitol (PMSF buffer). Cells were disrupted and membranes recovered as previously described (Todd *et al.*, 1983*b*).

Partial purification of PBP 5a

PBPs were solubilized by stirring the membranes with an equal volume of PMSF buffer containing 2% (v/v) Triton X-100 and 1 M-NaCl for 20 min at 37°C. Insoluble material was removed by centrifugation (240000*g* for 60 min).

PBP 5a was partially purified by 6-aminopenicillanic acid affinity chromatography (Blumberg & Strominger, 1972) essentially as described previously (Waxman & Strominger, 1979; Todd *et al.*, 1983*b*). Solubilized membranes were preheated to 35°C, treated with cephalothin (10 μg/ml) for 10 min and then allowed to react for 20 min with CM-Sepharose CL-6B coupled to 6-aminopenicil-

lanic acid. After extensive washing in 50mM-potassium phosphate buffer, pH7, containing 1M-NaCl and 0.1% (v/v) Triton X-100 the column was poured (approx. 75ml packed volume). PBP 5a was released from the column by batch elution with neutralized hydroxylamine buffer (Todd *et al.*, 1983*b*) at 0, 30 and 90min at 35°C. PBP 5a was further purified by applying the column fraction that contained the bulk of PBP 5a (the 30min elution fraction) to a second 6-aminopenicillanic acid affinity column (7ml packed volume) as described above. PBPs were detected as described by Todd *et al.* (1983*a*) except that 10μg of [^{3}H]benzylpenicillin/ml was used.

Assay of DD-*carboxypeptidase activity*

The DD-carboxypeptidase activity present in the 30min elution fraction from the second affinity column was measured by release of D-[^{14}C]alanine from ^{14}C-labelled UDP-MurAc-pentapeptide under the following assay conditions. Three 100μl portions of the partially purified PBP 5a preparation (about 400μg of PBP 5a) in 10mM-potassium phosphate buffer, pH7, containing 0.1% (v/v) Triton X-100 were adjusted to pH5.5, 7 and 8.5 by the addition of 25μl of 250mM-potassium phosphate buffer, pH5.5, 250mM-Tris/HCl buffer, pH7, and 250mM-Tris/HCl buffer, pH8.5, respectively. Two samples (4 and 40μg) of membrane protein from vegetative cells of *B. subtilis* 168 Sueoka, suspended in 125μl of 50mM-potassium phosphate buffer, pH5.5, were assayed as controls. Each assay mixture contained 10mM-$MgCl_2$. Radioactive UDP-MurAc-pentapeptide (5μl of 0.3mM at 40Ci/mmol) was added and the reaction carried out at 37°C for 2h. The reaction was terminated by boiling for 3min, denatured protein removed by centrifugation (10000*g* for 10min) and the supernatant subjected to descending chromatography (Todd *et al.*, 1983*b*). The percentage of D-[^{14}C]alanine released was determined by cutting out the radioactive areas of the chromatogram, followed by H_2O_2 treatment and determination of the radioactivity as described previously (Todd & Ellar, 1982; Stewart *et al.*, 1981).

Results and discussion

The relatively low stability *in vitro* of the penicillin-binding activity of PBP 5a from *B. megaterium* KM precluded its purification by affinity chromatography (Todd & Ellar, 1983). In

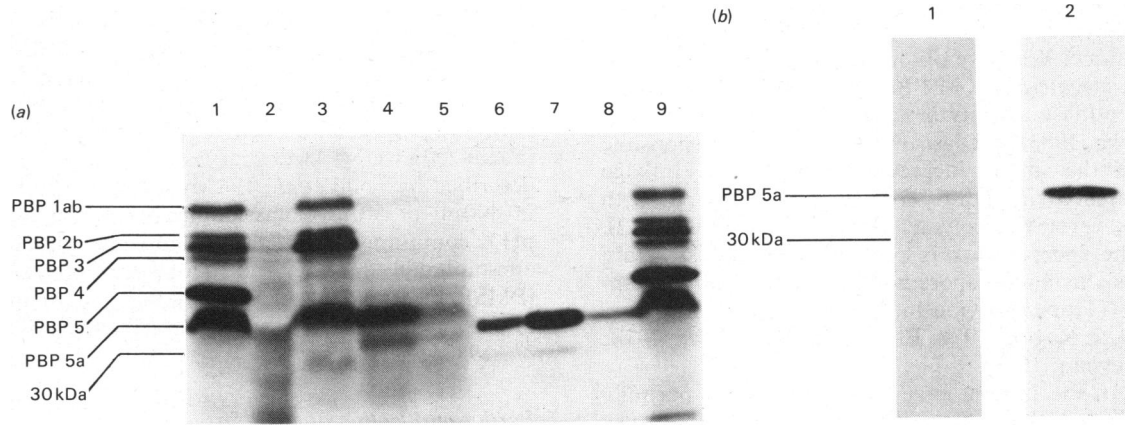

Fig. 1. *Purification of PBP 5a*

(*a*) is a fluorogram showing the PBPs present in sporangial membranes isolated from *B. subtilis* SL 557 (Todd *et al.*, 1983*a*) (tracks 1 and 9), and fractions recovered during the purification: insoluble pellet (track 2), solubilized membrane material after the removal of insoluble material (track 3), solubilized membrane material after treatment with cephalothin (track 4), the solubilized membrane material that did not bind to the 6-aminopenicillanic acid affinity column (track 5), and 240μl of the hydroxylamine batch elutions of the affinity column taken at 0, 30 and 90min (tracks 6–8 respectively). About 100μg of protein was loaded on each track except for tracks 6–8. PBPs were assayed as previously described (Todd *et al.*, 1983*b*). PBP 5a was partially purified by 6-aminopenicillanic acid affinity chromatography (Waxman & Strominger, 1979; Todd *et al.*, 1983*b*) and as detailed in the Materials and methods section. In (*b*) track 1 is a Coomassie Brilliant Blue R stain of a sodium dodecyl sulphate/10%-polyacrylamide slab gel showing the proteins (about 2μg of PBP 5a) present in 500μl of the 30min elution fraction of the second 6-aminopenicillanic acid affinity column, and track 2 is a fluorogram of this slab gel. The procedures are outlined in the Materials and methods section. Fluorography was carried out for 2 days at −80°C with Kodak X-Omat S X-ray film.

contrast, the binding between *B. subtilis* PBP 5a and benzylpenicillin *in vitro* is as stable as that between the antibiotic and PBP 5 from *B. subtilis*. No change in the binding of [^{3}H]benzylpenicillin to PBPs 5 and 5a was observed after preincubation of the stage IV sporangial membranes in PMSF buffer for 60min at 37°C (results not shown).

Previously purification of PBP 5 from *B. subtilis* by affinity chromatography was facilitated by preincubation of solubilized membranes with cephalothin (Blumberg & Strominger, 1972). Similarly, we found that PBP 5a was insensitive to concentrations of cephalothin of up to 100μg/ml (results not shown) and therefore it could be used to purify selectively PBPs 5 and 5a from the high-M_r proteins.

Preliminary purification studies revealed that PBPs 5 and 5a are released from a 6-aminopenicillanic acid affinity column by elution with hydroxylamine at exactly the same time (results not shown), resulting in co-purification of the proteins. To obtain PBP 5a free of PBP 5 we constructed a mutant (*B. subtilis* JT175) that lacked PBP 5 (J. A. Todd, unpublished work). Fig. 1 shows the partial purification of PBP 5a. The 30min elution fraction, containing the bulk of PBP 5a (Fig. 1*a*, lane 7), was preincubated with cephalothin (10μg/ml) and subjected to a second 6-aminopenicillanic acid affinity chromatography step. After dialysis, 500μl of the 30min elution fraction from the second column was treated with [^{3}H]benzylpenicillin and subjected to sodium dodecyl sulphate/10%-polyacrylamide-gel electrophoresis and fluorography. Coomassie Brilliant Blue R staining (Fig. 1*b*, lane 1) revealed that PBP 5a was approx. 80–90% pure. Fluorography of this slab gel confirmed that PBP 5a was the only PBP present (Fig. 1*b*, lane 2). The protein marked 30kDa in Fig. 1 was the major contaminant. This protein has a very low affinity for benzylpenicillin and accounted for less than 5% of the total protein detected in Fig. 1*b*, lane 1.

The DD-carboxypeptidase activity of the partially purified preparation of PBP 5a was analysed by measuring the release of D-[^{14}C]alanine from the substrate, ^{14}C-labelled UDP-MurAc-pentapeptide. The results (Fig. 2 and Table 1) indicate that PBP 5a has DD-carboxypeptidase activity *in vitro*

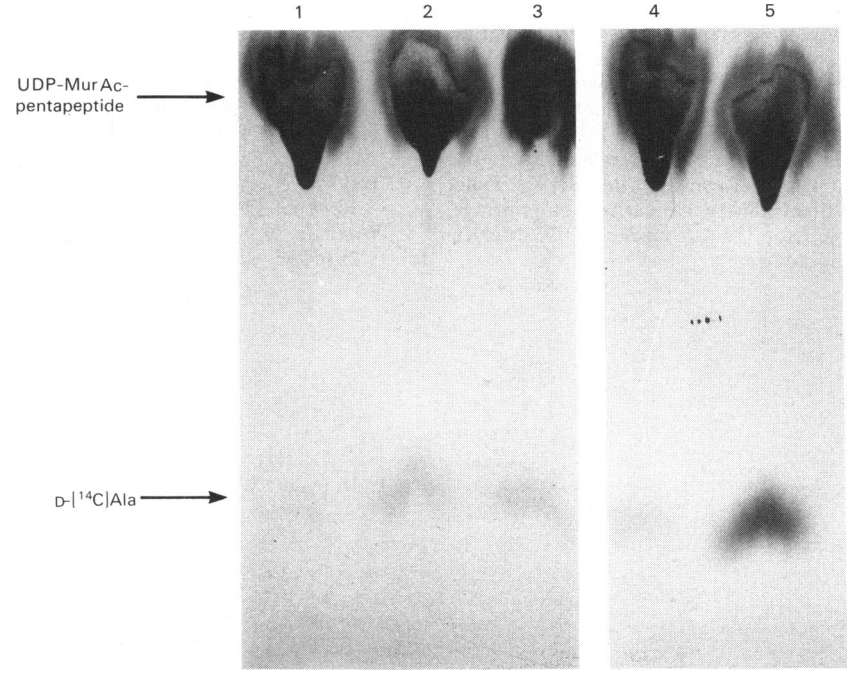

Fig. 2. *Assay of* DD-*carboxypeptidase activity of PBP 5a*

The Figure is an autoradiogram of a chromatogram showing the release of D-[^{14}C]alanine from the substrate ^{14}C-labelled UDP-MurAc-pentapeptide incubated at three different pH values, pH 5.5, 7 and 8.5 (lanes 1–3 respectively), with approx. 400μg of the partially purified preparation of PBP 5a analysed (Fig. 1*b*). Lanes 4 and 5 show the release of D-[^{14}C]alanine by 4μg and 40μg respectively of membrane protein isolated from exponentially growing cells of *B. subtilis* 168 Sueoka. The assay conditions are described in the Materials and methods section and the products were analysed by descending chromatography (Todd *et al.*, 1983*b*). Autoradiography was for 10 days with Kodak X-Omat S X-ray film.

Table 1. *Assay of* DD-*carboxypeptidase activity of PBP 5a*
The radioactive areas of the chromatogram shown in Fig. 2 were cut out and the radioactivities determined as described previously (Stewart *et al.*, 1981; Todd & Ellar, 1982). For details see the legend to Fig. 2 and the Materials and methods section.

Assay	Release (%)
PBP 5a preparation at pH 5.5	0.8
PBP 5a preparation at pH 7	3.1
PBP 5a preparation at pH 8.5	3.0
4 μg of membrane protein at pH 5.5	2.0
40 μg of membrane protein at pH 5.5	15

with a pH optimum between pH 7 and 8.5. This activity is distinct from those of *B. subtilis* PBP 5 (Lawrence & Strominger, 1970) and *B. megaterium* PBP 5 (Chase *et al.*, 1977), both of which show optimal DD-carboxypeptidase activity *in vitro* at about pH 5.5.

PBP 5a is likely therefore to function *in vivo* as a DD-carboxypeptidase or 'endopeptidase' specifically during cortex synthesis. This suggests that the degree of peptide cross-linkage in cortex is important.

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