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Identification of penicillin-binding protein 5a of *Bacillus megaterium* KM as a DD-carboxypeptidase

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Measurement of the stabilities of DD-carboxypeptidase activity and the penicillin-binding activity of proteins 5 and 5a in membranes isolated from vegetative cells and stage-V forespores suggests that the unique sporulation-specific protein 5a may be a penicillin-sensitive DD-carboxypeptidase.

During endospore development in *Bacillus megaterium* KM (Todd & Ellar, 1982) and *Bacillus subtilis* (Sowell & Buchanan, 1983; Todd *et al.*, 1983) unique sporulation-specific penicillin-binding proteins are synthesized and inserted into the forespore membranes (Todd & Ellar, 1982; Todd *et al.*, 1983). This differentiation of the penicillin-binding profile begins at the time of engulfment of the forespore compartment (stages II–III) concomitant with the initiation of spore-specific peptidoglycan synthesis. The new peptidoglycan (cortex), deposited between the double membranes of the foresore, has been cited as the major structure responsible for the production (Gould & Dring, 1975) or maintenance (Ellar, 1978) of the dehydrated spore cytoplasm that is essential for the acquisition of spore heat-resistance and dormancy (Imae *et al.*, 1978; Keynan, 1978; Ellar, 1978). This role of cortex in determining spore properties has been attributed to its unique chemical structure (Tipper & Gauthier, 1972; Gould & Dring, 1975; Rogers, 1977), which contains spore-specific muramic lactam residues and a greatly diminished content of cross-linked peptides compared with the vegetative polymer. The cortex may also be responsible for the spherical ellipsoidal shape of the spore (Imae *et al.*, 1978; Todd & Ellar, 1982).

We have previously proposed (Todd & Ellar, 1982) that the sporulation-specific penicillin-binding proteins 3e and 5a of *B. megaterium* KM may be involved in the modifications necessary for cortex maturation, such as the cleavage of terminal D-alanine residues from peptide side chains and the hydrolysis of peptide cross-links by DD-carboxypeptidase and endopeptidase activities.

In order to test these proposals we have investigated the properties of penicillin-binding protein 5a and present evidence indicating that it may possess DD-carboxypeptidase activity *in vitro*.

Abbreviation used: UDP-MurNAc-pentapeptide, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine.

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Experimental

Bacterial strain and assay of penicillin-binding proteins

The conditions for growth and sporulation were as described previously (Ellar & Posgate, 1974; Stewart *et al.*, 1981). Intact forespores from sporangia at stage V of sporulation were isolated by the method of Ellar & Posgate (1974), and membranes, prepared from forespores and vegetative cells, were treated with [^{3}H]benzylpenicillin (20 μg/ml; 31 Ci/mmol) as previously described (Todd & Ellar, 1982). After reaction with [^{3}H]-benzylpenicillin, the penicillin-binding proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and detected by fluorography (Todd & Ellar, 1982). Polyacrylamide gels were prepared for fluorography by the method of Chamberlain (1979).

Assay of DD-*carboxypeptidase activity*

DD-Carboxypeptidase activity was assayed by measuring the release of D-alanine from UDP-MurNAc-pentapeptide substrate by using the colorimetric method of Johnson *et al.* (1975) as modified by Shepherd *et al.* (1977). All incubations were performed at 37°C for 10 min in a final volume of 30 μl containing 1.67 mM-UDP-MurNAc-pentapeptide in 50 mM-potassium phosphate buffer, pH 5.4, containing 10 mM-$MgCl_2$. The pH and Mg^{2+} concentration used were approximately those re-

ported by Diaz-Maurino *et al.* (1974) to be optimal for DD-carboxypeptidase activity in *B. megaterium* KM vegetative membrane.

Stability of penicillin-binding and DD-*carboxypeptidase activities in vitro*

Membranes were incubated in 50mM-Tris/HCl buffer, pH 7.5, containing 5mM-$MgCl_2$ and in 33mM-potassium phosphate buffer, pH 5.4, containing 7mM-$MgCl_2$ at 37°C and then assayed for binding of penicillin to penicillin-binding proteins 5 and 5a and for DD-carboxypeptidase activity respectively. The amount of penicillin bound was determined by microdensitometry of the fluorogram with a double-beam recording microdensitometer (Joyce, Loebl and Co., Gateshead, Tyne and Wear, U.K.).

Materials

UDP-MurNAc-pentapeptide, made by the method of Barnett (1973), was generously given by Dr. C. Harrington (Department of Biochemistry, University of Cambridge, Cambridge, U.K.), and the [^{3}H]benzylpenicillin was kindly given by Dr. Stapley (Merck, Sharp and Dohme, Rahway, NJ, U.S.A.). Non-radioactive benzylpenicillin was from Sigma Chemical Co., and *p*-chloromercuribenzoate was from Hopkin and Williams.

Results and discussion

Peptide 'mapping' of penicillin-binding proteins 5 and 5a from *B. megaterium* KM (Todd & Ellar, 1982) and *B. subtilis* Sueoka (Todd *et al.*, 1983) showed that for each organism these proteins are structurally distinct, and that protein 5a is probably unique. A comparison of the properties of proteins 5 and 5a of *B. megaterium* KM revealed that they have the same affinity for benzylpenicillin, cefmetazole, methicillin and nocardicin A (results not shown), and both these proteins and penicillin-binding protein 3e are sensitive to *p*-chloromercuribenzoate (Fig. 1). The stability of penicillin-binding of proteins 5 and 5a did, however, differ markedly, as shown in Fig. 2. The half-life of the binding activity of protein 5a was approx. 18min, compared with 885 and 1100min for proteins 5 and 3e respectively.

Attempts to purify penicillin-binding protein 5a by using the methods described by Chase (1980) were therefore unsuccessful, as the protein could not be detected after 10min at 37°C in the presence of 1% Genapol X-100 and 1.0M-NaCl (results not shown).

Indirect evidence for the enzyme activity of penicillin-binding protein 5a was obtained by measuring the stability of the DD-carboxypeptidase activity of membranes isolated from exponentially growing *B. megaterium* KM and forespores from sporulating cells at stage V. Fig. 3 shows that the

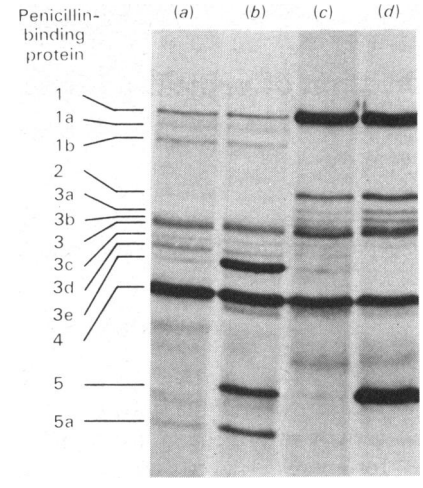

Fig. 1. *Sensitivity of penicillin-binding proteins to p-chloromercuribenzoate*

The Figure is a fluorogram showing the penicillin-binding protein of state-V forespore membranes after incubation at 37°C for 10min with an equal volume of *p*-chloromercuribenzoate (2.0mM, in water at pH 9.0) (track *a*) and water (track *b*) and the penicillin-binding proteins of vegetative membranes incubated with *p*-chloromercuribenzoate (track *c*) and water (track *d*). Membranes were prepared and assayed for penicillin-binding activity as previously described (Todd & Ellar, 1982).

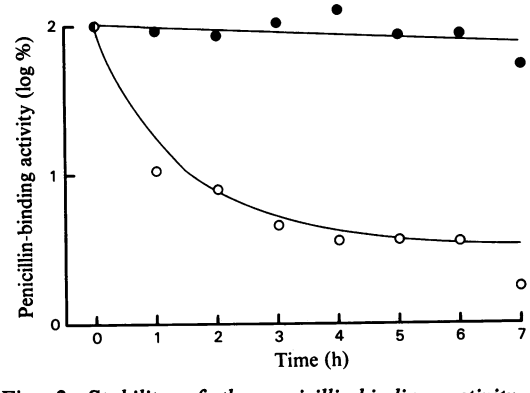

Fig. 2. *Stability of the penicillin-binding activity of penicillin-binding proteins 5 and 5a in vitro*

Membranes from isolated stage-V forespores in 50mM-Tris/HCl buffer, pH 7.5, containing 5mM-$MgCl_2$ were incubated at 37°C, and the penicillin-binding activities of penicillin-binding proteins 5 (●) and 5a (○) were determined as described in the Experimental section.

DD-carboxypeptidase activity of the forespore membranes has a half-life of approx. 47min compared with 180min for vegetative membranes. The

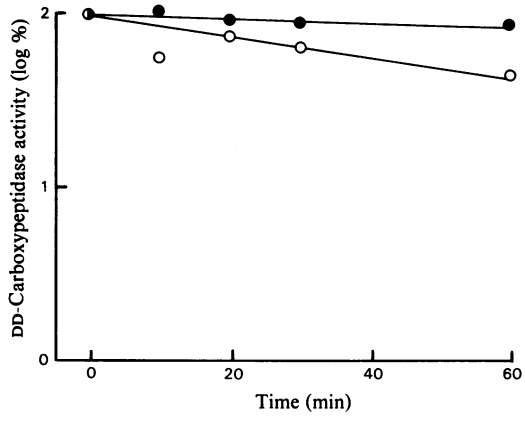

Fig. 3. *Stability of* DD-*carboxypeptidase activity in vitro*
The DD-carboxypeptidase activities of vegetative (●) and stage-V forespore (○) membranes in 33 mM-potassium phosphate buffer, pH 5.4, containing 7 mM-$MgCl_2$ were measured after incubation at 37°C as described in the Experimental section.

measured stability of the stage-V DD-carboxypeptidase activity is therefore similar to that of the binding activity of penicillin-binding protein 5a *in vitro*, indicating that protein 5a may have DD-carboxypeptidase activity *in vitro*.

The pH optimum for both vegetative and stage-V forespore membrane DD-carboxypeptidase activities was approx. pH 5.4 in 50 mM-potassium phosphate buffer containing 10 mM-$MgCl_2$. However, since the total DD-carboxypeptidase activity of the forespore membrane was 34% that of the vegetative membrane, it is likely that these assay conditions are not optimal for the DD-carboxypeptidase activity of penicillin-binding protein 5a. Both enzyme activities were sensitive to *p*-chloromercuribenzoate (100% inhibited by 1 mM-*p*-chloromercuribenzoate at 37°C for 10 min in DD-carboxypeptidase assay buffer) and benzylpenicillin (80% inhibited by 100 μg of benzylpenicillin/ml).

These results indicate that *in vivo* penicillin-binding protein 5a may function as a penicillin-sensitive DD-carboxypeptidase (or endopeptidase) in the regulation of the cross-linkage of spore cortex.

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