The association of penicillin-binding proteins with cell elongation and septum formation in *Bacillus megaterium*

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Analysis of the penicillin-binding proteins in the membranes from germinated spores of *Bacillus megaterium* and a filamentous mutant indicated that (a) no specific synthesis of any penicillin-binding protein occurred before or during two relatively synchronous cell divisions, (b) penicillin-binding proteins 1, 3a and 3b may be involved in cell elongation and (c) filamentation of the mutant may be due to a decrease in the concentration of penicillin-binding protein 1.

Certain PBPs of *Escherichia coli* have been shown to be involved in the determination of cell shape, elongation and septum formation (Spratt, 1983). It has also been demonstrated that the activities of several enzymes of peptidoglycan metabolism fluctuate during the *E. coli* cell cycle (Mirelman, 1979). Therefore periodic changes in the concentrations of PBPs might account for specific peptidoglycan synthesis at a certain time in the cell cycle. The results reported by Buchanan (1980) and Wientjes *et al.* (1983), however, indicated that peptidoglycan synthesis in *E. coli* is probably not regulated by controlling the amounts of these enzymes.

The spore-forming bacilli offer a convenient system for the study of cell-cycle-specific events: germination of dormant spores culminates in a phase of vegetative growth, when the cells elongate and divide with a relatively high degree of synchrony (Dawes & Hanson, 1972). Germination triggering (Keynan, 1978) is followed by a period of germination-specific development that includes the irreversible loss of the unique properties of the spore, the onset of metabolism and the initiation of macromolecular synthesis. These processes are independent of exogenous amino acids, whereas vegetative growth, beginning at approx. 70 min in *Bacillus megaterium*, is dependent on the addition of nutrients to the medium (Setlow & Primus, 1975). Germination therefore provides an opportu-

nity to study changes in the concentrations and types of PBPs that are inserted into the membrane as the cell initiates elongation and proceeds in a relatively synchronous manner through the first and second cell divisions.

Previous work (Todd & Ellar, 1982) has shown that the profile of PBPs in the *B. megaterium* dormant-spore inner membrane differs radically from that of the vegetative plasma membrane and is restored to the vegetative profile during germination. Similar results have been reported for *Bacillus subtilis* (Neyman & Buchanan, 1985). In the present study we have assayed the PBPs in the membranes from germinated spores of *B. megaterium* KM at regular intervals throughout germination and in the two successive vegetative cell divisions. In addition, the penicillin-binding profiles of the filamentous temperature-sensitive mutant TH14 of *B. megaterium* A.T.C.C. 19213 (Hitchins & Sadoff, 1972a,b) have been investigated.

A comparison of the PBPs of mutant cells dividing synchronously at the permissive temperature (30°C) with those of the elongating non-dividing cells at the restrictive temperature indicated that specific PBP synthesis does not occur when the septum is formed during *B. megaterium* cell division. However, correlative evidence is presented that indicates (a) cell elongation catalysed by PBP1 may be a requirement for the initiation of cross-wall formation, and (b) elongation requires PBPs 3a and 3b.

Experimental

The conditions for growth, sporulation and germination of dormant spores of *B. megaterium*

Abbreviation used: PBP, penicillin-binding protein.

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KM were as described previously (Stewart *et al.*, 1981; Todd & Ellar, 1982).

The parent *B. megaterium* A.T.C.C. 19213 and the temperature-sensitive strain, TH14, originally described by Hitchins & Sadoff (1972a) were kindly provided by the Bacillus Stock Center, Ohio State University, Columbus, OH, U.S.A. These strains were cultured under the same conditions as *B. megaterium* KM. Dormant spores were prepared and germinated exactly as described for *B. megaterium* KM (Todd & Ellar, 1982) except that 500 µg of L-leucine/ml was added to the germination medium. Germination was monitored by phase-contrast microscopy and septa were made visible by treatment of the cells with lysozyme (20 mg/ml) (Ellar & Posgate, 1974).

Membranes were prepared and treated with [³H]benzylpenicillin (20 µg/ml; 31 Ci/mmol) as previously described (Todd *et al.*, 1983a). After reaction with [³H]benzylpenicillin, the PBPs were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and detected by fluorography (Chamberlain, 1979; Todd & Ellar, 1982).

Results and discussion

Fig. 1 shows the PBPs present in the membranes isolated from dormant and germinated spores of *B.*

megaterium KM assayed every 15 min during germination. As observed by phase-contrast microscopy, dormant spores rapidly lost refractility during the first 15 min (t_{15}) followed by swelling (t_{15} to t_{60}), outgrowth and elongation (from approximately t_{45}) and the first (t_{125} – t_{165}) and second (t_{185} – t_{230}) cell divisions.

PBP 1, absent from dormant-spore membranes, was first detected in germinating *B. megaterium* KM at t_{30} . At t_{45} , PBPs 2, 3a and 3b re-appeared and increased concentrations of PBP3 were apparent (Fig. 1). The concentrations of these proteins continued to rise steadily throughout germination until at t_{75} – t_{90} the penicillin-binding profile was essentially identical with that of an exponentially growing cell. The spore-specific high concentrations of PBPs 3d and 4 (Todd & Ellar, 1982) decrease markedly from t_{60} (Fig. 1), as do those of PBPs 1a and 1b, which are formed during sporulation by proteolysis of PBP 1 (Todd & Ellar, 1982). For *B. sphaericus*, Tipper *et al.* (1977) have proposed that rapid cell-wall synthesis must occur early in germination as the cell swells and begins elongation, to ensure survival of the germinated spore. The high concentrations of PBPs 3d and 4 detected in the dormant-spore inner membranes (Fig. 1, t_0) that persist until t_{60} may indicate a

Fig. 1. Penicillin-binding profiles of dormant and germinated spores of *B. megaterium* KM

The Figure is a fluorogram showing the PBPs of membranes isolated from dormant (0 min) and germinated spores of *B. megaterium* KM assayed at 15 min intervals throughout germination and two cell divisions. Membranes were prepared and treated with [³H]benzylpenicillin (20 µg/ml), and the PBPs were identified as previously described (Todd & Ellar, 1982; Todd *et al.*, 1983a).

synthetic role during this unique period of peptidoglycan formation.

We have shown previously (Todd *et al.*, 1983b) that PBPs 3a and 3b are sensitive to concentrations of the β -lactam nocardicin A that specifically inhibit cell elongation and induce a spherical morphology in exponentially growing *B. megaterium*. When spores were germinated in the presence of 100 μ g of nocardicin A/ml they resembled untreated cells until t_{45} , when outgrowth and elongation were not observed. The cells were osmotically stable and rounded, and swelling continued to a limited extent until about t_{130} (results not shown). This is consistent with the hypothesis (Todd *et al.*, 1983b) that PBP 3a and 3b, which re-appear just before spore outgrowth, are involved specifically in cell elongation.

A recent analysis of *B. subtilis* PBPs during germination suggested specific roles for PBPs 2A and 2B in cell elongation and septum formation respectively (Neyman & Buchanan, 1985). The data shown in Fig. 1 for *B. megaterium* KM, however, do not provide clear evidence of specific synthesis of any particular PBP before or during either cell division.

Extensive studies (Hitchins & Sadoff, 1972a,b; Yan & Hitchins, 1980) on the temperature-sensitive filamentous mutant TH14 of *B. megaterium* A.T.C.C. 19213 have shown that the mutation responsible for filamentation at 39°C is probably in a gene coding for a protein that functions in protein synthesis. In order to test their original proposal (Yan & Hitchins, 1980) that this protein-synthesis limitation may be restricted to the synthesis of a PBP associated with septum formation, we assayed the PBPs of the membranes isolated from exponentially growing cells of the parent strain *B. megaterium* A.T.C.C. 19213 and the mutant strain TH14 grown at both temperatures. After 3–6 h at 39°C the mutant had formed long filaments, although a small proportion of the cells formed septa after transfer to the higher temperature (Hitchins & Sadoff, 1972b). PBP analysis revealed no significant differences between the profiles of actively dividing cells at 30°C and filamentous cells at 39°C (results not shown). Further, no differences were apparent between the latter and the parent at both temperatures (results not shown). If, however, the PBPs of *B. megaterium* are relatively stable *in vivo*, as found for *E. coli* (Buchanan, 1980), then it is possible that a decrease in a PBP would not be detected. This problem was avoided by examining the PBPs of germinated spores of the mutant at the permissive and restrictive temperatures.

Under the same conditions as used for *B.*

Fig. 2. Penicillin-binding profiles of dormant and germinated spores of *B. megaterium* A.T.C.C. 19213 TH14. The Figure is a fluorogram showing the PBPs of membranes isolated from dormant (0 min) and germinated spores of *B. megaterium* A.T.C.C. 19213 TH14. Spores were germinated at 30°C (a) and 39°C (b), and the PBPs were identified as previously described (Todd & Ellar, 1982; Todd *et al.*, 1983a).

megaterium KM, *B. megaterium* A.T.C.C. 19213 TH14 spores germinated more slowly at 30°C than did strain KM. The sequence of visible morphological events was identical, however, with the first cell division at t_{225} – t_{285} . At 39°C swelling and elongation (from t_{60}) of the mutant appeared to be normal, but even after 360 min no septa were observed in the mutant filaments. In contrast, by t_{360} the cells germinated at 30°C had divided twice. Fig. 2(a) shows that the re-establishment of the vegetative PBP profile at 30°C is essentially the same as demonstrated in Fig. 1. Interestingly, the only major difference between the profiles at the permissive and restrictive temperatures was in the concentrations of PBP 1. At 39°C (Fig. 2b) the re-appearance of this protein at t_{180} was not as pronounced as found at the lower temperature (Fig. 2a). PBP 1 is probably the major transpeptidase of *B. megaterium* (Reynolds *et al.*, 1978) during vegetative growth, and it is possible that the enzymes involved in septum formation require a specific peptidoglycan substrate whose synthesis is dependent on cell elongation catalysed by PBP 1.

We conclude that the failure to synthesize a septum during germination of mutant TH14 may be due to a failure to synthesize sufficient amounts of PBP 1, and that the initiation of cell division during germination is not associated with the synthesis of any particular PBP. It seems likely therefore that periodic synthesis of *B. megaterium* PBPs is not responsible for the control of peptidoglycan synthesis during elongation and septum formation of exponentially growing cells, and some proteins such as PBP 1 may have essential roles in both these phases of the cell cycle. PBPs 3a and 3b may be partly responsible for transpeptidation during cell elongation.

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