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Restoration of Vegetative Penicillin-Binding Proteins During Germination and Outgrowth of *Bacillus subtilis* Spores: Relationship of Individual Proteins to Specific Cell Cycle Events

SHERRY L. NEYMAN AND CHRISTINE E. BUCHANAN*

Department of Biology, Southern Methodist University, Dallas, Texas 75275

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The order in which the vegetative penicillin-binding proteins (PBPs) are first synthesized and the rate of their return to normal levels during germination and outgrowth of *Bacillus subtilis* spores were determined. The rate of synthesis of most of the PBPs was much faster than that of the majority of other membrane proteins, which is consistent with the involvement of PBPs in biosynthesis of the rapidly expanding peptidoglycan. The pattern of PBP changes that occurred during the cell cycle, including sporulation, suggests a likely role for PBP 2A in cell elongation and a unique requirement for PBP 2B during both symmetric and asymmetric septum formation. PBP 3 is the only PBP that appears to be equally necessary for vegetative and cortical peptidoglycan synthesis.

The transition of a bacterial cell from vegetative growth through sporulation, germination, and back to the vegetative state involves a number of biochemical and morphological changes. The mechanisms that control this unicellular differentiation are complex and still relatively unclear. Because the cell wall is vital during all stages of the cell cycle and its precise structure varies from one stage to the next (20, 36), a study of its biosynthesis should reveal information pertaining to regulation of differentiation in addition to providing insight into the methods by which the shape of a cell changes from rod to round and back again and by which a septum forms during vegetative- and sporulation-specific divisions.

Our work focuses on a set of membrane proteins that covalently bind penicillin and are believed to be enzymes involved in the last steps of peptidoglycan synthesis (2). Detailed studies of the gram-negative organism *Escherichia coli* have determined that the various penicillin-binding proteins (PBPs) play different enzymatic roles in peptidoglycan metabolism and that individual ones may be required specifically for wall growth, septum formation, and shape determination (21, 27, 28). Less specific information is available about most of the PBPs in *Bacillus subtilis*. By in vitro assays it has been found that the major PBP (PBP 5) of *B. subtilis* is a D-alanine carboxypeptidase and that at least one of the higher-molecular-weight PBPs has transglycosylase activity (1, 16). All of the PBPs, of course, react with penicillin enzymatically, and most, if not all of them, will slowly degrade the antibiotic (2, 37). As yet, no transpeptidase activity has been detected with preparations from this gram-positive organism (2, 16, 18). An examination of the changes in the PBPs that occur during sporulation of *B. subtilis* (26, 32) revealed that synthesis of one of the vegetative proteins, PBP 2B, increases shortly before stage II septation, which suggests that this protein is required for formation of the forespore septum. Enhanced synthesis of PBP 3 at a later time implicates it in assembly of the cortical peptidoglycan. In addition to these two vegetative PBPs, two sporulation-specific PBPs (4* and 5*) are synthesized at particular times during sporulation. A role for PBP 5* in cortex synthesis, perhaps as a carboxypeptidase, has been proposed (26, 34).

This report provides the first detailed account of the PBP changes that occur during development of a vegetative cell from a mature dormant spore. It is a logical extension of our analysis of sporulation-related PBP changes (5, 26) and adds to the number of changes in the PBPs that can be correlated with specific cell cycle events. We found it useful to divide the transition period into two stages, germination and outgrowth. Germination has been characterized as primarily degradative in nature, involving breakdown of the cortex, the multiple layers of the spore coat, and some specific proteins in the core (8, 25, 35). This is accompanied by a loss of spore refractility, swelling from uptake of water, and a decrease in turbidity of the culture (12, 24). Outgrowth is the period when net macromolecular synthesis begins; this is recognized by an increase in turbidity and elongation of the cells (10, 22, 30). Outgrowth culminates with the first vegetative cell division. An earlier examination of germinating *Bacillus megaterium* indicated that the typical vegetative PBP profile is fully restored before this cell division occurs (33). Here, we present a quantitative analysis of the steps involved in this restoration in *B. subtilis*, the specific order in which the individual vegetative PBPs reappear, and their differing rates of synthesis. We conclude that PBP 2B is almost certainly involved in vegetative- as well as sporulation-specific septation, whereas PBP 2A is more likely to be required specifically for cell elongation. PBPs 3, 4, and 5 seem to be necessary for all vegetative peptidoglycan synthesis, although PBP 3 is also implicated in formation of the cortex during sporulation. The two PBPs (4* and 5*) synthesized exclusively during sporulation have no role in germination, outgrowth, or vegetative peptidoglycan synthesis.

MATERIALS AND METHODS

Bacterial strain. *B. subtilis* 168 *trp* was obtained from J. H. Hageman, New Mexico State University, Las Cruces, N. Mex.

* Corresponding author.

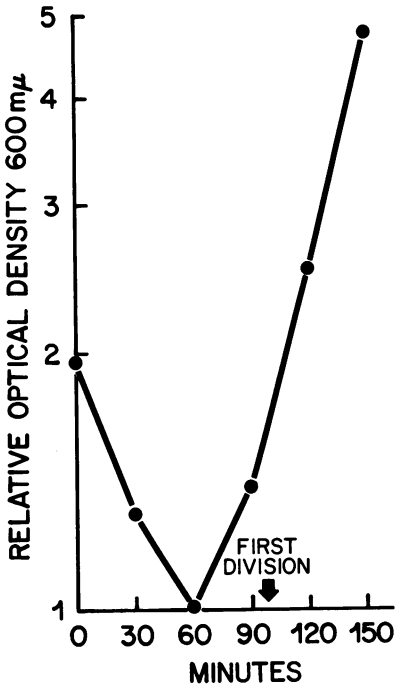

FIG. 1. Growth of heat-activated spores in Penassay broth at 37°C. All values are expressed relative to the 60-min-postactivation value and represent the average of results from at least five different cultures.

Preparation of spores. Sporulation was induced by nutrient exhaustion in supplemented nutrient broth (23, 26). The spores were harvested by centrifugation 19 to 25 h after exponential growth had ceased and were resuspended to a final concentration of 0.02 g (wet weight) per ml of 0.05 M sodium phosphate buffer (pH 7.0). The suspension was then incubated at 37°C for 15 min with 0.1 mg of lysozyme per ml, followed by a low-speed centrifugation (2,300 × *g*, 10 min). Cell debris was discarded with the supernatant. The spores were washed with 0.1% sodium dodecyl sulfate, 1 M NaCl, and 0.14 M NaCl, respectively, and rinsed six times with distilled water (11, 29). Both salt solutions contained 0.01% Tween 80 (vol/vol) to reduce clumping (17). Washed spores were stored at 4°C in distilled water (0.5 g [wet weight] per ml, final concentration).

Activation, germination, and outgrowth conditions. Washed spores that were at least 2 weeks old were brought to room temperature and diluted to 0.13 g/ml with distilled water. The suspension was diluted with an equal volume of hot sodium phosphate buffer and incubated at 80°C for 10 min with occasional swirling.

The spores were added to Penassay broth immediately after the heat shock. A sufficient amount of spores was used to inoculate 500 ml of warm medium in each of five 2.8-liter baffled Fernbach flasks to a final optical density at 600 nm of between 0.5 and 1.0. Cultures were incubated at 37°C with vigorous shaking (400 rpm) in a New Brunswick Gyrotory shaker. In some studies the protein synthesis inhibitor chloramphenicol (CAM; 100 μg/ml, final concentration) was added to the medium before inoculation with the heat-activated spores. Samples were periodically harvested and stored at −20°C. The optical density was monitored with a Coleman Junior II spectrophotometer.

Membrane preparation and PBP assay. Membranes were prepared by differential centrifugation after sonic disruption of the cells as described previously (4, 26). The membrane-bound PBPs were detected and quantitated by incubating the membranes with a saturating concentration of either [^{3}H]benzylpenicillin or [^{14}C]benzylpenicillin, followed by separation of the proteins from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of the dried gel (7, 26). In the present work, all solutions routinely contained 2mM phenylmethysulfonyl fluoride. The values depicted in Fig. 3 represent the average of multiple assays with a minimum of five samples for every time point.

Chemicals. All prepared bacterial media were purchased from Difco Laboratories, Detroit, Mich. Reagents for electrophoresis, including sodium dodecyl sulfate, were purchased from Bio-Rad Laboratories, Richmond, Calif. Lysozyme, phenylmethylsulfonyl fluoride, CAM, Tween 80, and the sodium dodecyl sulfate used in the spore washes were purchased from Sigma Chemical Co., St. Louis, Mo. [^{3}H]benzylpenicillin (ethylpiperidine salt, 25 Ci/mmol) was prepared by Merck Sharp & Dohme Laboratories, West Point, Pa. [^{14}C]benzylpenicillin (53 mCi/mmol) was purchased from the Amersham Corp., Arlington Heights, Ill.

RESULTS

Response to heat activation. Although the turbidometric and morphological changes that occur in spores after heat-shock have been described previously (12, 19, 30), it was necessary to repeat these observations to determine the timing of events under large-scale conditions and with the particular strain used in the current work. The pattern of germination (0 to 60 min) and outgrowth (60 to 90+ min) presented in Fig. 1 is comparable to previous results (3, 22). Within 60 min after inoculation into Penassay broth, the spores were no longer refractile and had become rounded, slightly swollen, and phase gray. Elongating forms were observed soon after the increase in turbidity (optical density at 600 nm) began. Motile cells were first noted at about 75 min post-inoculation. The time at which the initial vegetative cell division occurred (Fig. 1) was identified by the appearance of chains of two cells (3). Chains of four cells (the second vegetative cell division) were observed approximately 30 min later.

Changes in the PBPs during germination and outgrowth. All of the vegetative PBPs, except PBP 2B, were present in samples harvested after only 15 min of germination, and there was no trace of sporulation-specific PBPs 4* and 5* (Fig. 2, lane A). The most abundant high-molecular-weight PBP early in germination was a previously uncharacterized protein, designated here as PBP 2C, with a molecular weight of approximately 75,000. Treatment with CAM during this time interval (0 to 15 min) had little effect on the PBP profile (Table 1).

The amount of every vegetative PBP increased before the first cell division occurred. By analyzing an equivalent amount of membrane protein at each time point, we corrected for the relatively nonspecific increase in these proteins that might accompany growth of the membrane. Therefore, the PBP changes reported here (Fig. 3) are those that occurred above the level of the bulk of the membrane proteins at a given time. Increased synthesis of all of the vegetative PBPs did not begin simultaneously but occurred in a particular order, with the high-molecular-weight PBPs being synthesized earlier than the lower-molecular-weight proteins. Furthermore, the individual PBPs were synthesized at different rates.

Synthesis of PBPs 1 and 2A began within the first 30 min of germination and was sensitive to inhibition with CAM (Table 1). The increase in PBP 2A was linear for about 1 h, after which time a steady-state level was apparently achieved. An exponential increase in PBP 2B began between 30 and 60 min postactivation, which was long before the cells were growing exponentially (Fig. 1 and 3). The appearance of PBP 2B was completely blocked by addition of CAM to the germinating culture (data not shown). Also late in germination there was a significant loss of PBP 2C. The membrane concentrations of PBPs 3, 4, and 5 began to increase at roughly the same time as outgrowth began (60 min) and continued to increase at a rate faster than that of the majority of membrane proteins throughout vegetative growth (Fig. 3). The level of PBP 2C continued to drop until this protein was the most minor PBP in the vegetative cell membrane (Fig. 2).

TABLE 1. Comparison of PBPs from germinating samples with those from parallel samples inhibited by CAM

PBP	Ratio of germinating cells/inhibited cells at min after activation:	
	15	30
1	1.66 ± 0.44[a]	5.42 ± 1.89
2A	0.99 ± 0.30	3.00 ± 0.94
2B	—[b]	—[b]
2C	0.80 ± 0.15	0.93 ± 0.22
3	0.85 ± 0.17	1.02 ± 0.27
4	1.33 ± 0.22	2.02 ± 0.70
5	0.82 ± 0.22	1.36 ± 0.22

[a] Average of seven values ± one standard deviation.
[b] PBP 2B was not detected in germinating cells until after 30 min.

DISCUSSION

The early uncoordinated synthesis of the vegetative PBPs during germination and outgrowth of *B. subtilis* spores is consistent with the proposal that these proteins have different functions in peptidoglycan metabolism. The pattern of their restoration to vegetative levels and the changes they undergo during sporulation together provide some important clues about when in the cell cycle they are individually most important. For example, we suggested earlier that PBP 2B is probably uniquely involved in synthesis of the asymmetric forespore septum because there was a significant amount of renewed synthesis of this protein just before stage II in sporulation (26). However, it is not likely that a protein with a highly specialized role in sporulation would continue to be synthesized as PBP 2B was throughout vegetative growth. This protein was not detectable in membranes for at least 30 min after heat activation of spores (Fig. 2), which eliminates the possibility of any function for PBP 2B during germination. The protein appeared and began to increase exponentially before initiation of the first vegetative cell division (Fig. 3). Because its appearance was completely blocked by CAM, de novo synthesis of PBP 2B rather than activation of preexisting enzyme must have occurred. From these observations we conclude that PBP 2B is probably involved in vegetative cell division as well as sporulation septation. However, it apparently is not responsible for determining the site of septum formation, since it has now been implicated in both symmetric and asymmetric septations.

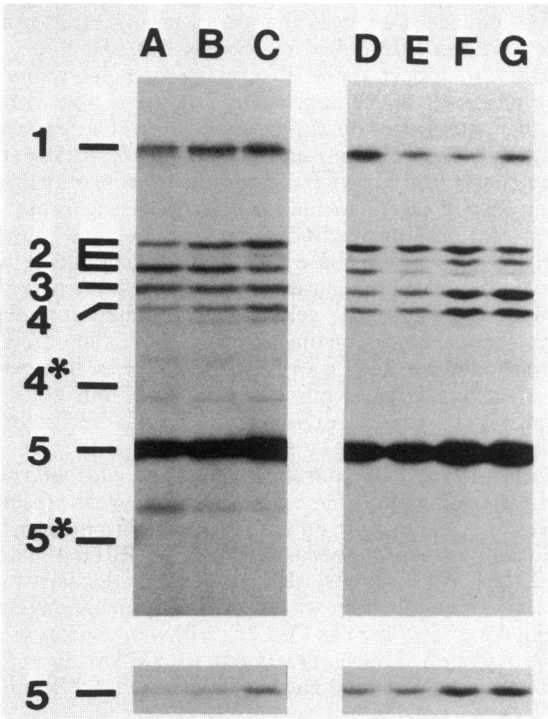

FIG. 2. Fluorographs of two gels which illustrate the changes that occur in the PBP profile during germination and outgrowth of *B. subtilis* 168 *trp*$^-$ spores. The upper sections are 24-h exposures of the gels to X-ray film, and the lower sections are 2-h exposures. The three bands labeled PBP 2 are referred to in descending order as PBPs 2A, 2B, and 2C, respectively. The locations of PBPs 4* and 5* are indicated, although neither protein has ever been detected in germinating cells. An equal amount of membrane protein was run in each lane. Lanes (time postactivation): A, 15 min; B, 30 min; C and D, 60 min; E, 90 min; F, 120 min; G, 150 min. The unlabeled PBP that is visible below PBP 5 in lane A was present in various amounts in all samples harvested at 15 min postactivation. It most likely was formed by partial breakdown of one of the higher-molecular-weight PBPs.

PBP 2A was one of the first PBPs to be synthesized during germination (Fig. 3). The amount detectable at 15 min postactivation may have been inherited from the dormant spore, but it is clear that by 30 min de novo synthesis was occurring (Table 1). That this protein was produced before initiation of net macromolecular synthesis implies a function for it very early in outgrowth. For two reasons we propose that this function is likely to be participation in the synthesis of the sidewalls of the cell. First, the biosynthetic enzymes involved in elongation of the cell are required earlier than those specifically needed for formation of the septum. Second, the membrane concentration of PBP 2A rapidly declines to 20% or less of its vegetative level at roughly the same time that cells cease to elongate (5, 26). Although it appears in Fig. 3 as though the rate of PBP 2A synthesis slowed down so that it eventually was no greater than synthesis of most other membrane proteins, this is probably not the case. Continued synthesis of PBP 2A is masked by simultaneous turnover which becomes evident when CAM or tetracycline is added to the vegetative culture (5, 6). The linear increase in PBP 2A between 30 and 90 min postactivation may actually be due to an exponential rate of synthesis.

PBP 2C is an extremely minor PBP in vegetative cells (Fig. 2, lane G) that has also been detected in other strains (18, 26, 32). In some strains of *B. subtilis*, it seems to increase slightly during sporulation (26; unpublished data), but its variability in sporulating cells has so far precluded a meaningful analysis. The prominence of this protein in

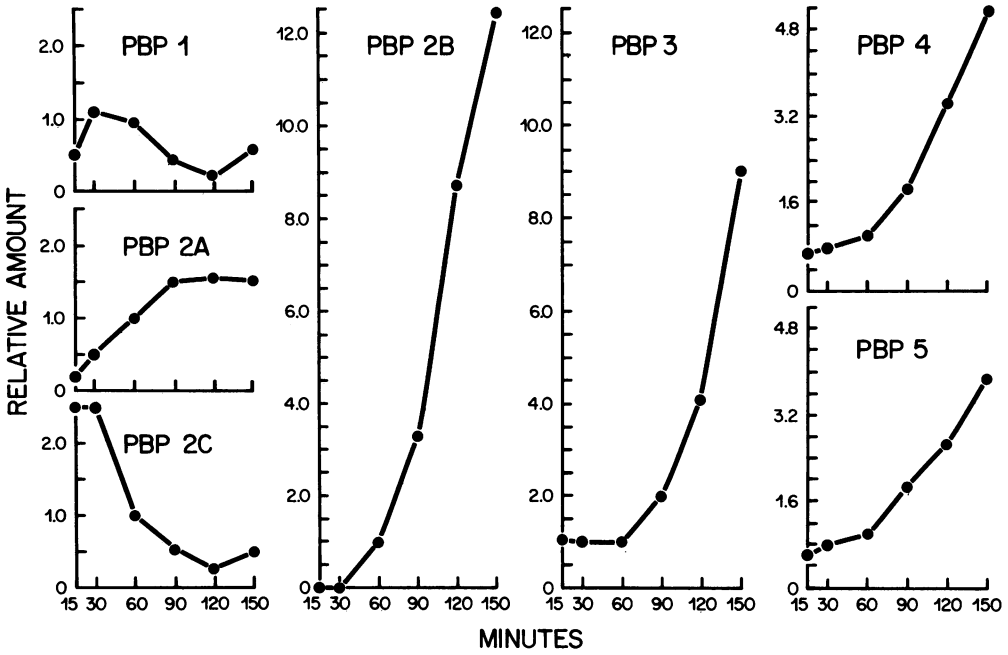

FIG. 3. Quantitative summary of the PBP changes that occur during germination and outgrowth. All amounts are given relative to the 60-min-postactivation value for each PBP.

germinating cells (Fig. 2, lane A) is apparently not dependent on early protein synthesis (Table 1) nor is it likely to be the consequence of proteolysis during germination. PBP 2C is readily detectable in membranes prepared from mature spores (C. E. Buchanan, unpublished data). Because of uncertainty about the origin of this protein (it may be derived from one of the higher-molecular-weight PBPs, or it may be related to one of the lower-molecular-weight PBPs, not necessarily as a precursor), any speculation as to its significance in vivo would be premature.

Sporulation-specific PBPs 4* and 5* were not present in the germinating spores at 15 min postactivation (Fig. 2). However, some PBP 5* is found in mature spores, most probably in the outer forespore membrane (32; manuscript in preparation). Thus, it is likely that at least this PBP was lost upon deterioration of the outer layers of the spore during the early phase of germination.

The remaining vegetative PBPs, 3, 4, and 5, all began to increase in amount during outgrowth (Fig. 2 and 3). The more rapid synthesis of these PBPs (and PBPs 2A and 2B) compared with the bulk of the membrane proteins is consistent with the involvement of these putative biosynthetic enzymes in the rapid expansion of the peptidoglycan that occurs during exponential growth. It is also consistent with the results of Tipper et al. (31), who analyzed some of the soluble enzymes required for peptidoglycan synthesis in *Bacillus sphaericus*. They found that the activity of these biosynthetic enzymes increases faster than total cell protein during the first 30 min of germination. Once balanced growth is achieved, the soluble enzymes of *B. sphaericus* and the membrane-bound PBPs of *B. subtilis* are produced in constant proportion to the increase in total protein (13, 31). However, the present results suggest that most of the vegetative PBPs continue to increase faster than total membrane protein throughout the exponential phase (Fig. 3). This rate of increase probably reflects their more rapid synthesis rather than the accumulation of stable proteins, because with the exception of PBPs 3 and 5, the PBPs are not unusually stable in vivo (5, 6). Their continued high rate of synthesis may be necessary because of the relative immobility of these proteins and the creation of new cell wall sites at which they must act.

In summary, an examination of the PBP changes that occur during the relatively synchronous processes of sporulation and germination in *B. subtilis* has provided some useful clues about what the functions of some of these proteins might be. The results are consistent with the long-held belief that the PBPs are involved in the final assembly and modification of peptidoglycan (2). The pattern of synthesis of the various PBPs during the different phases of the cell cycle suggests that PBP 2A may play a role specifically in cell elongation, which is strictly a vegetative function. In contrast, at least two PBPs appear to be involved in both vegetative growth and sporulation. For example, it seems likely that PBP 2B may participate in synthesis of the symmetric septum that forms during normal cell divisions as well as the asymmetric one that precedes forespore development. The structures of these two septa are believed to be the same, except that there is less peptidoglycan in the forespore septum (9, 14, 15, 20). PBP 3 evidently is necessary for a common step in the synthesis of two different peptidoglycan structures, the cortex and the vegetative cell wall. The other vegetative PBPs are clearly most active during synthesis of vegetative peptidoglycan, whereas PBPs 4* and 5* can only be involved in cortex synthesis.

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