JOURNAL OF BACTERIOLOGY, Feb. 1986, p. 498–503
0021-9193/86/020498-06$02.00/0

Vol. 165, No. 2

Correlation of Penicillin-Binding Protein Composition with Different Functions of Two Membranes in *Bacillus subtilis* Forespores

CHRISTINE E. BUCHANAN* AND SHERRY L. NEYMAN
Department of Biology, Southern Methodist University, Dallas, Texas 75275

Received 30 August 1985/Accepted 11 November 1985

The distribution of penicillin-binding proteins (PBPs) within different membranes of sporulating cells of *Bacillus subtilis* was examined in an effort to correlate the location of individual PBPs with their proposed involvement in either cortical or vegetative peptidoglycan synthesis. The PBP composition of forespores was determined by two methods: examination of isolated forespore membranes and assay of the in vivo accessibility of the PBPs to penicillin. In both cases, it was apparent that PBP 5*, the major PBP synthesized during sporulation, was present primarily, but not exclusively, in the forespore. The membranes from mature dormant spores were prepared by either chemically stripping the integument layers of the spores, followed by lysozyme digestion, or lysozyme digestion alone of coat-defective *gerE* spores. PBP 5* was detected in membranes from unstripped spores but was never found in stripped ones, which suggests that the primary location of this PBP is the outer forespore membrane. This is consistent with a role for PBP 5* exclusively in cortex synthesis. In contrast, vegetative PBPs 1 and 2A were only observed in stripped spore preparations that were greatly enriched for the inner forespore membrane, which supports the proposed requirement for these PBPs early in germination. The apparent presence of PBP 3 in both membranes of the spore reinforces the suggestion that it catalyzes a step common to both cortical and vegetative peptidoglycan synthesis.

The six membrane-bound penicillin-binding proteins (PBPs) in vegetative cells of *Bacillus subtilis* are believed to be involved in the synthesis and modification of the peptidoglycan layer of the cell wall, although specific functions have only tentatively been assigned to the individual proteins (2, 17, 19, 24). To account for the effect of penicillin on growing cells, the activities of some of the PBPs must be indispensable, whereas others are clearly not required under at least some growth conditions (7, 30). Sporulating cells are also inhibited by penicillin (10, 21, 40), but it cannot be assumed that the antibiotic has the same PBP target(s) as in the vegetative cell. Previous reports have shown that synthesis of the unique cortical peptidoglycan of the spore is preceded by the appearance of two new PBPs (PBPs 4* and 5*) that are never detected in vegetative cell membranes (30, 38). In addition, there is enhanced synthesis of vegetative PBPs 2B and 3 during sporulation, suggesting a requirement for these two proteins during both sporulation and vegetative growth.

Analysis of the changing profile of PBPs or any other membrane proteins in sporulating cells is complicated by the existence of three separate membranes: the cytoplasmic membrane surrounding both the mother cell and developing spore, the inner forespore membrane (IFM) enclosing the core of the spore, and the outer forespore membrane (OFM). The OFM is formed when the membrane of the large mother cell proliferates and engulfs the smaller forespore during stage III of sporulation. As a consequence of this event, the outer or wall-synthesizing surfaces of the IFM and OFM face one another, creating what is essentially an extracellular space between them (42, 43). This space is later filled with the cortex, although a thin layer of germ cell wall indistinguishable from vegetative peptidoglycan exists immediately adjacent to the IFM. There is evidence that the two membranes are subject to different modifications during sporulation, which presumably reflect the different functions that have been proposed for them (12, 34, 36, 43).

Our studies until now have not distinguished between the different membranes of the sporulating cell. In an effort to relate structure (in this case, location of a PBP in a specific membrane) to function (metabolism of the cortical versus the vegetative peptidoglycan), we determined the PBP composition of membranes from purified stage IV forespores, mature dormant spores, and dormant spores that have been stripped of their OFM. Our results confirm an earlier report (38) that sporulation-specific PBP 5* is located primarily, but not exclusively, in the forespore membrane and are most consistent with an OFM rather than an IFM location. This lends support to the contention that PBP 5* is required specifically for synthesis or modification of the cortex, functions that are usually attributed to enzymes in the OFM and mother cell cytoplasm (29, 30, 34). In addition, we demonstrate that PBPs 1 and 2A, whose functions are believed to be strictly vegetative (24), are detectable only in the IFM, whereas PBP 3, which is probably required for both cortical and vegetative peptidoglycan synthesis, appears to be located in both membranes of the forespore.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* 168 *trp* was used as the wild type for these studies. It was obtained from J. H. Hageman, New Mexico State University, Las Cruces. Mutant SB-23, a *trp spoIV* derivative of *B. subtilis* 168, was isolated by and obtained from R. C. Goldman, Abbott Laboratories, North Chicago, Ill. Stable stage IV spores are released by this strain (12, 13). The *B. subtilis* 168 germination mutant *gerE36*, isolated and characterized by Moir (23), was obtained as the *leu* strain 1G12 from the Bacillus Genetic Stock Center. This mutant forms fully refractile and heat-resistant spores that are susceptible to lysozyme.

Preparation of spores. Sporulation of the wild-type strain was by the nutrient exhaustion method in supplemented nutrient broth (2× SG) at 37°C (22, 28). The spores were

* Corresponding author.

harvested by centrifugation and subjected to a series of treatments (lysozyme digestion, 0.1% sodium dodecyl sulfate, 1 M NaCl, 0.14 M NaCl, and rinses with distilled water) as described previously (24, 31). The cleaned spores were stored at 4°C in distilled water.

Sporulation of the *gerE36* mutant was also by the nutrient exhaustion method at 37°C in 2× SG. Because its spores have a coat defect that renders them susceptible to lysozyme and sodium dodecyl sulfate, they were simply washed many times with large volumes of distilled water. Five rinses were usually sufficient to eliminate any remaining rod-shaped structures in the spore preparation, as determined by phase-contrast microscopy.

Isolation of forespores. Sporulation of SB-23 was induced by nutrient exhaustion at 30°C in 2× SG. Growth and sporulation were monitored with a Coleman Junior II spectrophotometer at 600 nm. Although this strain grows well at higher temperatures, the best yield of stable forespores is obtained at 30°C (R. C. Goldman, personal communication). Samples (200 ml) were collected at the end of exponential growth (t_0) and 6 h later (t_6). The bulk of the culture (five Fernbach flasks containing 500 ml of medium each) was harvested at t_{24}. The forespores were washed and suspended in approximately 8 ml of buffer A (12). Any residual sporangia (mother cells) were disrupted by sonication in the cold (150 s in five 30-s pulses) as described previously (30). The samples were then incubated for 20 min at 37°C with 0.1 mg of DNase per ml and 0.01 M $MgCl_2$ (13). Low-speed centrifugation (2,700 × *g*, 12 min) resulted in the formation of three layers. The upper supernatant fraction was removed and centrifuged at high speed (100,000 × *g*, 45 min, 4°C) to collect mother cell membranes. The lower layer of the supernatant was discarded. The forespore pellet was suspended in a final volume of 6 ml of cold buffer A and diluted with an equal volume of 30% Renografin (E. R. Squibb & Sons, Princeton, N.J.). Samples (2 ml) of the diluted forespores were layered onto chilled, linear 30 to 50% Renografin gradients (12). The forespores banded in the middle of the gradient after centrifugation (32,000 × *g*, 45 min) in a Beckman Instruments, Inc. (Fullerton, Calif.), SW25.1 rotor at 4°C. These were collected with a syringe with a bent needle, diluted with an equal volume of buffer A, and then pelleted by centrifugation (30,000 × *g*, 30 min, 4°C). The forespores were stored at −20°C.

Membrane preparations. Membranes from vegetative and sporulating cells were routinely prepared by differential centrifugation after disruption of the cells by 150 s of sonication (1, 3, 30).

Membranes from purified SB-23 forespores were prepared by lysozyme digestion essentially as described by Goldman (12). The forespores were thawed and suspended in 0.05 M Tris hydrochloride (pH 7.5) containing 1 mM $MgCl_2$ and then incubated with 0.5 mg of lysozyme per ml at 30°C for 30 min with occasional mixing. This was followed by another 30 min of incubation after doubling the lysozyme concentration. Cells of SB-23 harvested at t_0 and t_6 received only one 30-min incubation with lysozyme, which was followed by a 15-min incubation with 0.1 mg of DNase per ml. The membranes were then collected and stored as previously described (30). To identify any lysozyme-induced artifacts, other samples of SB-23 cells harvested at t_0 and t_6 were disrupted by the usual sonication procedure. The PBP profiles of SB-23 samples collected during sporulation were not affected by the technique used to prepare the membranes (data not shown).

Membranes were prepared from dormant spores of the *gerE36* mutant by first suspending the spores to a final concentration of 1.5 g (wet weight)/ml of sucrose-salts buffer (11) and then digesting them at 37°C with 0.5 mg of lysozyme per ml for 30 min. The osmotically sensitive spores were collected by centrifugation and suspended in 0.05 M Tris hydrochloride (pH 7.5) containing 1 mM $MgCl_2$ at room temperature. After vigorous vortexing, osmotic lysis of the sample was apparent by visual inspection. Usually 1 mg of DNase was added at this time. The sample was centrifuged (5,000 × *g*, 5 min), the supernatant was saved, and the pellet was suspended again in Tris-Mg buffer to lyse any residual spores. The supernatant recovered from the second round of osmotic lysis was combined with the first, and the membranes were collected by ultracentrifugation as usual (1, 3).

Sensitization of wild-type spores to lysozyme. The coat proteins of cleaned wild-type spores were dissolved by treatment with 50 mM dithiothreitol–8 M urea at pH 3 for 1 h at 37°C, followed by treatment with cold 0.1 M NaOH for 15 min, exactly as described by Jenkinson (18). After the final wash with water, the stripped spores were suspended in sucrose-salts solution (11) and digested with lysozyme by the same procedure used for the *gerE* mutant (see above).

Assay for PBPs. PBPs can be detected in membranes by their ability to covalently bind [^{3}H]penicillin (2). The [^{3}H]penicillin-PBP complexes were separated from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the solubilized membranes and quantitated by fluorography (7, 30).

Chemicals. Renografin-76 (Squibb) is actually a 76% solution, but it is considered to have a concentration of 100% when used to prepare dilutions of Renografin for gradients (25, 33). [^{3}H]benzylpenicillin (ethylpiperidine salt, 25 Ci/mmol) was synthesized by Avery Rosegay and obtained from P. J. Cassidy of Merck & Co., Inc., Rahway, N.J. Urea and all reagents for polyacrylamide gels were purchased from Bio-Rad Laboratories, Richmond, Calif. All reagents for fluorography were purchased from Fisher Scientific Co., Pittsburgh, Pa. Nutrient broth was purchased from Difco Laboratories, Detroit, Mich. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

PBP content of forespores. The rationale for examining the membranes of forespores at stage IV was that by this time engulfment is complete, the OFM and IFM are in place, and cortex synthesis has begun, so it was reasonable to expect that those PBPs involved in the process would be at their peak levels in the membranes. Moreover, stage IV cells were still easy to disrupt with lysozyme, because an intact coat had not yet been assembled. To increase the proportion of forespores in the harvested sample that were in stage IV and to avoid the problem of achieving synchrony in large batches of *B. subtilis*, a mutant that does not develop beyond stage IV was used. SB-23, a derivative of *B. subtilis* 168, is an SpoIV oligosporogenic mutant that releases stable forespores (12). Its forespores were collected at t_{24} and purified. Sporulation-specific PBP 5* was substantially more abundant in the forespore membranes than in the stage IV sporangial (mother cell) membrane (compare lanes C and D in Fig. 1). In fact, it was the only PBP that was clearly enriched in the forespores at this stage.

Accessibility of the PBPs to penicillin in intact sporulating cells. To confirm the results described above, the PBP composition of forespores was also examined by a second method. This method was designed with the expectation that PBPs located in forespore membranes would be less acces-

sible to penicillin than those PBPs on the surface of the mother cell (16). It was not practical to treat the cells directly with radiolabeled penicillin because of the large amount of label required for a 250-ml culture. Thus, sporulating cells of the wild type were treated with 0.05 mg of unlabeled penicillin per ml for 15 min prior to harvesting. Later, their membranes were assayed to determine which of the PBPs had not bound to the cold penicillin in vivo and thus were available for in vitro labeling with the tritiated antibiotic. At least 50% of PBP 5* was inaccessible during the 15-min exposure of cells to penicillin at t_4 (compare lanes E and F in Fig. 2). In contrast, most of the vegetative PBPs were still accessible to the antibiotic at t_4 (compare lanes B and F in Fig. 2). It is likely that some of the vegetative PBPs did become part of the forespore membranes, because the OFM and IFM both originate at least partly from the cytoplasmic membrane. However, unless a lot of additional synthesis of these PBPs occurred during sporulation, their levels in the forespore would be too low to be detected by this method. Thus, the fact that there is enhanced synthesis of PBP 3 during stage III (6, 30) probably accounts for our ability to detect the fraction of PBP 3 that became inaccessible to penicillin by t_4 (Fig. 2).

Release of bound penicillin. One explanation for the observation that only PBP 5* was substantially available to bind [^{3}H]penicillin in vitro after exposure to the unlabeled form in vivo is that it was indeed accessible to the cold penicillin and reacted with it but then released it more rapidly than did any of the other PBPs. Waxman and Strominger (41) measured the rates at which the PBPs of *B. subtilis* catalyze release of covalently bound penicillin, but they did not examine PBP 5*, and they used a mixture of the PBPs purified by affinity chromatography rather than the membrane-bound forms. We repeated the penicillin release assays with membrane preparations of *B. subtilis* cells harvested either in their exponential phase or at t_4 of sporulation. The half-life of the penicilloyl-PBP 1 complex in membranes was about 10 min at 37°C, whereas the PBP 4 and PBP 5* complexes had half-lives of about 30 min each. The remainder of the PBPs formed more stable penicilloyl complexes with half-lives equal to or greater than 60 min.

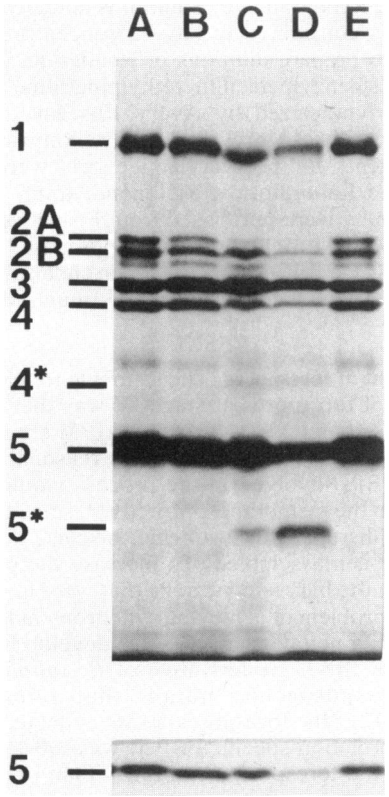

FIG. 1. Fluorograph of [^{3}H]penicillin-labeled PBPs in membranes from sporulating cells of *B. subtilis* mutant SB-23. Each lane contains approximately the same amount of protein. Lanes: A and E, duplicates of the vegetative (t_0) sample; B, membranes from a sample harvested at t_6; C, sporangial (mother cell) membranes at stage IV; D, membranes of the forespores at stage IV. The upper section is a 24-h exposure of the X-ray film to the gel, and the lower section is a 3-h exposure.

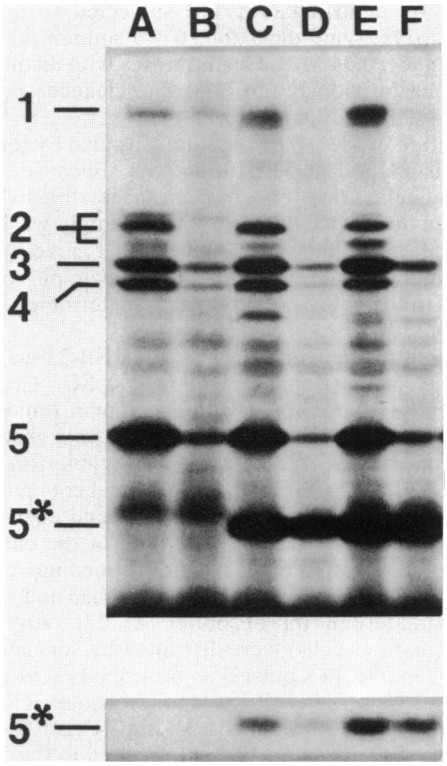

FIG. 2. Fluorograph of membranes prepared from sporulating cells of *B. subtilis* 168 *trp*. Lanes: A, normal PBP profile of membranes at t_0; B, PBPs detectable in membranes from t_0 cells that were treated with cold penicillin in vivo; C and D, untreated and treated t_2 samples, respectively; E and F, untreated and treated t_4 samples, respectively. The three components of PBP 2 are designated PBPs 2A, 2B, and 2C, in descending order. The upper section is a 3-day exposure of the X-ray film to the gel, and as a consequence the degradative products of the PBPs are very obvious.

PBP content of dormant spores. When mature spores that have an intact integument are disrupted by physical rather than chemical means, the yield of membranes is poor (26), and those membranes that are recovered are very likely to be so damaged as to cast doubt on any conclusions derived from them. Thus, we chose to examine the spore membranes of a germination mutant, *gerE36*, because they could be obtained by the relatively gentle process of lysozyme digestion followed by osmotic disruption of the spores. Mutant spores, but not those of the wild type, are susceptible to this treatment because they have a defective coat (23). Membranes prepared in this manner had both sporulation-specific

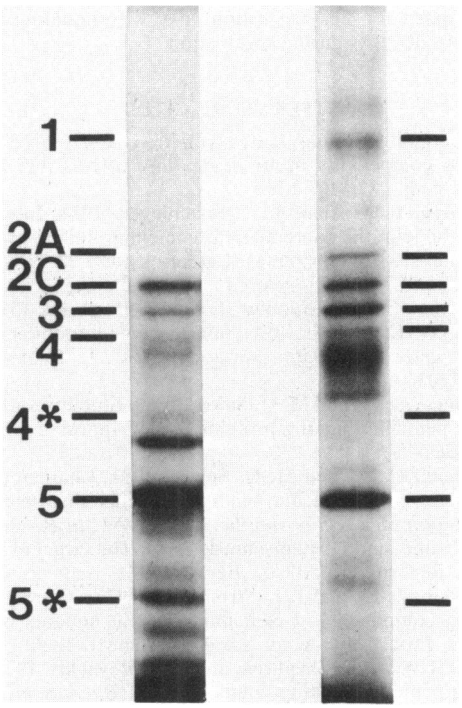

FIG. 3. Fluorographs of membranes from unstripped *gerE36* (left lane) and stripped 168 *trp* spores (right lane). The dark band below PBP 4* was reproducible. It has also been seen in samples from vegetative cells (24), but its relationship to the other PBPs is not clear.

PBPs 4* and 5*, as well as PBPs 2C, 3, 4 and 5 (Fig. 3, left lane). No PBP 1, PBP 2A, or PBP 2B was ever detected in the six or more samples of *gerE36* spore membranes prepared by the above method without prior stripping (see below).

Dormant spores of the wild type could be rendered susceptible to lysozyme by chemically stripping them of their outer or integument layers. Membranes from stripped spores did not contain detectable levels of the sporulation-specific PBPs or vegetative PBP 2B. The other vegetative PBPs were always present, but some deterioration of PBP 4 was evident (Fig. 3, right lane). Chemically stripping *gerE36* spores prior to lysozyme digestion resulted in a PBP profile (data not shown) which was indistinguishable from that of the wild-type spore membranes illustrated in Fig. 3. That is, PBPs 1 and 2A could be detected, and PBPs 4* and 5* could not. The results were not affected by the presence of chloramphenicol and phenylmethylsulfonyl fluoride (inhibitors of protein synthesis and protease activity, respectively) during stripping and subsequent treatment with lysozyme.

DISCUSSION

It was established by two different methods that PBP 5*, the major PBP synthesized during sporulation, is located primarily, but not exclusively, in the forespore (Fig. 1 and 2). This supports an earlier report of the distribution of the protein in *B. subtilis* but contrasts with the exclusive forespore location described for the analogous sporulation-specific PBP in *B. megaterium* (38, 39). Other differences in the activities and various properties of the PBPs from these two species are known to exist, so the apparent difference described above may be real (1, 7, 8, 27).

The more interesting question with respect to a structure-function relationship is where in the forespore, the IFM or OFM, is PBP 5* located. We assume that PBP 5* serves a function that is unique to sporulation, because its synthesis has always been observed by stage IV in sporulating cells, and the protein is never detected during germination or vegetative growth (4, 24, 30). Because of the timing of its synthesis and the relatively large amount that is made, and by analogy with the putative vegetative functions of the other PBPs, we have proposed that PBP 5* is a penicillin-sensitive enzyme involved in the terminal stages of cortex synthesis (30). This proposal is supported by the recent demonstration that the protein has DD-carboxypeptidase activity in vitro (37). The results reported here are most consistent with its location in the OFM. This conclusion is based on a comparison of PBPs in membranes from stripped and unstripped spores (Fig. 3). Preparations from unstripped spores should contain both the OFM and IFM, whereas stripped spores have lost most, if not all, of their OFM and coat proteins, and are therefore enriched for the IFM (20, 26, 32). PBP 5* was detectable only in the former and never in the latter.

The location of PBP 5* in the OFM is consistent with the hypothesis (34) that is based on the orientation and location of the two membranes, enzymes within the core and IFM of the spore are probably responsible for germ cell wall synthesis, whereas enzymes required exclusively for cortex synthesis should be in the OFM and sporangial cytoplasm. Tipper and Linnett (35) have demonstrated that diaminopimelate ligase, a soluble enzyme required strictly for cortex synthesis in *B. sphaericus*, is detectable only in the sporangial cytoplasm and have proposed (15) that D-glu-meso-diaminopimelate endopeptidase, a membrane-bound enzyme uniquely involved in cortex synthesis, is located in the OFM. It follows that the synthesis of these two enzymes would involve the sporangial, not the forespore, genome. That PBP 5* is also probably synthesized in the sporangial cytoplasm may account for our observation that some of this protein was inserted into the sporangial membrane (Fig. 1). The mechanism by which most of it apparently finds its way into the OFM is unknown.

Todd and Ellar (39) reported that PBP 5a, the sporulation-specific PBP of *B. megaterium* that is similar in many respects to PBP 5* described here, is located primarily in the IFM. Apparently some of the protein was also detected in the OFM, although the data were not shown. This would mean that the PBP was being synthesized in both compartments of the sporulating cell. It is more likely that their preparation of IFM still contained a significant amount of OFM, because it has subsequently been demonstrated that differential centrifugation alone does not adequately remove integument material from the IFM fraction (32).

It could be argued that stripping the spores triggers their subsequent germination during lysozyme treatment (14, 26, 32). The PBP profile of membranes from stripped spores (Fig. 3, right lane) was indeed very similar to the PBP profile of cells early in germination (24). However, in the studies cited above, the lysozyme treatment did not include sucrose. When sucrose is present, lysozyme digestion of stripped spores of *B. megaterium* does not affect their dormancy for up to 3 h (20). Nevertheless, there is no unambiguous method to distinguish between some of the earliest events in germination and chemical treatments that essentially have the same effect. In this case, the distinction is moot; deterioration and loss of the integument, known to occur early in germination, probably account for the absence of PBPs 4* and 5* in germinating samples. The possibility of their loss

from an IFM location early in germination is particularly remote, since recent studies strongly suggest that there is very little degradation of IFM proteins during germination (32).

The amount of PBP 2A in the membranes drops very low during sporulation of *B. subtilis*, and in some strains the PBP seems to disappear altogether (6, 30, 38). In contrast, it is readily detected in the membranes of the earliest samples collected during germination (24). Since there was no evidence to suggest that PBP 2A is synthesized immediately upon initiation of germination, we proposed earlier that some PBP 2A must be inherited from the dormant spore. The results presented here demonstrate that PBP 2A is indeed present in the membranes of the dormant spore, specifically in the IFM (Fig. 3). This location is fully consistent with the putative involvement of the protein in vegetative, but not cortical, peptidoglycan synthesis. The fact that PBP 2A seems to disappear from sporulating cells can be attributed to the loss of this unstable protein from the mother cell membrane (5); some late synthesis in the forespore must occur, but this cannot normally be detected unless efforts are specifically made to isolate the forespore membranes. Our previous method of preparing membranes from sporulating cells by sonication recovered only a small fraction of the forespore membranes from the later stages of sporulation (30). A similar argument can also be made for PBP 1. That neither PBP 1 or PBP 2A was detected in the membranes from unstripped spores (Fig. 3, left lane) can be explained by the small amount of IFM protein relative to the amount of integument, particularly coat proteins, present in these preparations. The likelihood that PBPs 1 and 2A are located only in the IFM, and not in the OFM, raises the possibility that they could be useful as specific markers for the inner membrane (9).

PBP 2B, whose synthesis is induced during stage II of sporulation, was not detectable in dormant (Fig. 3) or germinating spores (24). Furthermore, only a relatively small amount was found in forespore membranes at stage IV (Fig. 1 and 2). This PBP is believed to play a role in both the vegetative and sporulation-specific (stage II) septations. It, too, is an unstable protein (5), so when its synthesis eventually stops, the concentration of PBP 2B in the membranes drops (5, 6, 30, 38). Unlike what was proposed above for PBPs 1 and 2A, probably no synthesis of PBP 2B occurs at all in the forespore. This is consistent with the notion that PBP 2B is not required for early germination events or even for construction of the spore's germ layer of vegetative cell wall.

We have suggested that PBP 3 is required for a common step in both cortical and vegetative peptidoglycan synthesis (24, 30). Here we showed by two different methods (Fig. 1 and 2) that some PBP 3 was incorporated into the forespore membranes. In support of its putative dual role, the results indicate that PBP 3 was present in both the IFM and OFM (Fig. 3).

In conclusion, we identified the membrane location for each of the PBPs synthesized by sporulating cells of *B. subtilis*, and in every case the location is consistent with the proposed involvement of the protein in either cortex synthesis, vegetative peptidoglycan synthesis, or both.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-19829 from the National Institute of Allergy and Infectious Diseases.

We thank Deanne Simmons for her technical assistance and Donald Tipper for his suggestion that we should examine the accessibility of PBPs during sporulation.

LITERATURE CITED

1. **Blumberg, P. M., and J. L. Strominger.** 1972. Five penicillin-binding components occur in *Bacillus subtilis* membranes. J. Biol. Chem. **247**:8107–8113.
2. **Blumberg, P. M., and J. L. Strominger.** 1974. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol. Rev. **38**:291–335.
3. **Buchanan, C. E.** 1979. Altered membrane proteins in a minicell-producing mutant of *Bacillus subtilis*. J. Bacteriol. **139**:305–307.
4. **Buchanan, C. E.** 1985. Induction of penicillin-binding proteins under catabolite-repressed conditions. J. Bacteriol. **162**: 1302–1303.
5. **Buchanan, C. E., and M. O. Sowell.** 1983. Stability and synthesis of the penicillin-binding proteins during sporulation. J. Bacteriol. **156**:545–551.
6. **Buchanan, C. E., and M. O. Sowell.** 1983. Characterization of the changes in penicillin-binding proteins that occur during sporulation of *Bacillus subtilis*, p. 451–457. *In* R. Hakenbeck, J.-V. Holtje, and H. Labischinski (ed.), The target of penicillin. Walter de Gruyter and Co., Berlin.
7. **Buchanan, C. E., and J. L. Strominger.** 1976. Altered penicillin-binding components in penicillin-resistant mutants of *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **73**:1816–1820.
8. **Chase, H. A., S. T. Shepherd, and P. E. Reynolds.** 1977. Studies on the penicillin-binding components of *Bacillus megaterium*. FEBS Lett. **76**:199–203.
9. **Crafts-Lighty, A., and D. J. Ellar.** 1980. The structure and function of the spore outer membrane in dormant and germinating spores of *Bacillus megaterium*. J. Appl. Bacteriol. **48**: 135–145.
10. **Dancer, B. N.** 1979. Requirement for peptidoglycan synthesis during sporulation of *Bacillus subtilis*. J. Bacteriol. **140**:786–797.
11. **Ellar, D. J., and J. A. Posgate.** 1973. Characterization of forespores isolated from *Bacillus megaterium* at different stages of development into mature spores, p. 21–40. *In* A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research 1973. Academic Press, Inc., New York.
12. **Goldman, R. C.** 1973. Protein composition of cell and forespore membranes of *Bacillus subtilis*. J. Supramol. Struct. **1**:185–193.
13. **Goldman, R. C.** 1976. Membrane protein alterations during the early stages of sporulation of *Bacillus subtilis*. J. Supramol. Struct. **5**:457–473.
14. **Gould, G. W.** 1969. Germination, p. 397–444. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press, Inc., New York.
15. **Guinand, M., G. Michel, and D. J. Tipper.** 1974. Appearance of a γ-D-glutamyl-(L)-*meso*-diaminopimelate peptidoglycan hydrolase during sporulation in *Bacillus sphaericus*. J. Bacteriol. **120**:173–184.
16. **Hamilton, T. E., and P. J. Lawrence.** 1975. Accessibility of the [^{14}C]benzylpenicillin binding proteins in membranes of sporulating bacilli. Antimicrob. Agents Chemother. **8**:38–44.
17. **Jackson, G. E. D., and J. L. Strominger.** 1984. Synthesis of peptidoglycan by high molecular weight penicillin-binding proteins of *Bacillus subtilis* and *Bacillus stearothermophilus*. J. Biol. Chem. **259**:1483–1490.
18. **Jenkinson, H. F.** 1983. Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. J. Gen. Microbiol. **129**:1945–1958.
19. **Kleppe, G., and J. L. Strominger.** 1979. Studies of the high molecular weight penicillin-binding proteins of *Bacillus subtilis*. J. Biol. Chem. **254**:4856–4862.
20. **Koshikawa, T., T. C. Beaman, H. S. Pankratz, S. Nakashio, T. R. Corner, and P. Gerhardt.** 1984. Resistance, germination, and permeability correlates of *Bacillus megaterium* spores successively divested of integument layers. J. Bacteriol. **159**:624–632.
21. **Lawrence, P. J.** 1974. Penicillin: reversible inhibition of forespore septum development in *Bacillus megaterium* cells.

Antimicrob. Agents Chemother. **6**:815–820.
22. **Leighton, T. J., and R. H. Doi.** 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J. Biol. Chem. **246**:3189–3195.
23. **Moir, A.** 1981. Germination properties of a spore coat-defective mutant of *Bacillus subtilis*. J. Bacteriol. **146**:1106–1116.
24. **Neyman, S. L., and C. E. Buchanan.** 1985. Restoration of vegetative penicillin-binding proteins during germination and outgrowth of *Bacillus subtilis* spores: relationship of individual proteins to specific cell cycle events. J. Bacteriol. **161**:164–168.
25. **Prentice, G. A., F. H. Wolfe, and L. F. L. Clegg.** 1972. The use of density gradient centrifugation for the separation of germinated from nongerminated spores. J. Appl. Bacteriol. **35**: 345–349.
26. **Racine, F. M., and J. C. Vary.** 1980. Isolation and properties of membranes from *Bacillus megaterium* spores. J. Bacteriol. **143**:1208–1214.
27. **Reynolds, P. E., S. T. Shepherd, and H. A. Chase.** 1978. Identification of the binding protein which may be the target of penicillin action in *Bacillus megaterium*. Nature (London) **271**:568–570.
28. **Schaeffer, P., J. Millet, and J.-P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54**:704–711.
29. **Setlow, P.** 1981. Biochemistry of bacterial forespore development and spore germination, p. 13–28. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
30. **Sowell, M. O., and C. E. Buchanan.** 1983. Changes in penicillin-binding proteins during sporulation of *Bacillus subtilis*. J. Bacteriol. **153**:1331–1337.
31. **Spudich, J. A., and A. Kornberg.** 1968. Biochemical studies of bacterial sporulation and germination. VI. Origin of spore core and coat proteins. J. Biol. Chem. **243**:4588–4599.
32. **Swerdlow, R. D., and P. Setlow.** 1984. Isolation and characterization of two distinct fractions from the inner membrane of dormant *Bacillus megaterium* spores. J. Bacteriol. **158**:9–15.
33. **Tamir, H., and C. Gilvarg.** 1966. Density gradient centrifugation for the separation of sporulating forms of bacteria. J. Biol. Chem. **241**:1085–1090.
34. **Tipper, D. J., and J. J. Gauthier.** 1972. Structure of the bacterial endospore, p. 3–12. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
35. **Tipper, D. J., and P. E. Linnett.** 1976. Distribution of peptidoglycan synthetase activities between sporangia and forespores in sporulating cells of *Bacillus sphaericus*. J. Bacteriol. **126**:213–221.
36. **Tipper, D. J., I. Pratt, M. Guinand, S. C. Holt, and P. E. Linnett.** 1977. Control of peptidoglycan synthesis during sporulation in *Bacillus sphaericus*, p. 50–68. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
37. **Todd, J. A., E. J. Bone, and D. J. Ellar.** 1985. The sporulation-specific penicillin-binding protein 5a from *Bacillus subtilis* is a DD-carboxypeptidase in vitro. Biochem. J. **230**:825–828.
38. **Todd, J. A., E. J. Bone, P. J. Piggot, and D. J. Ellar.** 1983. Differential expression of penicillin-binding protein structural genes during *Bacillus subtilis* sporulation. FEMS Microbiol. Lett. **18**:197–202.
39. **Todd, J. A., and D. J. Ellar.** 1982. Alteration in the penicillin-binding profile of *Bacillus megaterium* during sporulation. Nature (London) **300**:640–643.
40. **Vinter, V.** 1964. Spores of microorganisms. XIV. Late stages of intrasporangial development of bacterial spores: their sensitivity to antibiotics. Folia Microbiol. **8**:58–71.
41. **Waxman, D. J., and J. L. Strominger.** 1979. Cephalosporin-sensitive penicillin-binding proteins of *Staphylococcus aureus* and *Bacillus subtilis* active in the conversion of [^{14}C]penicillin G to [^{14}C]phenylacetylglycine. J. Biol. Chem. **254**:12056–12061.
42. **Wilkinson, B. J., J. A. Deans, and D. J. Ellar.** 1975. Biochemical evidence for the reversed polarity of the outer membrane of the bacterial forespore. Biochem. J. **152**:561–569.
43. **Young, M., and J. Mandelstam.** 1979. Early events during bacterial endospore formation. Adv. Microb. Physiol. **20**: 103–162.