Mutagenesis and Mapping of the Gene for a Sporulation-Specific Penicillin-Binding Protein in *Bacillus subtilis*

CHRISTINE E. BUCHANAN* AND ANN GUSTAFSON

Department of Biological Sciences, Southern Methodist University, Dallas, Texas 75275

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Penicillin-binding protein (PBP) 5* is produced by *Bacillus subtilis* only during sporulation and is believed to be required for synthesis of the peptidoglycan-like cortex layer of the spore. The structural gene (*dacB*) for PBP 5* was insertionally mutagenized by integration of a plasmid bearing an internal fragment of the gene, and the phenotype of the null mutant was characterized. The mutant had no apparent vegetative growth or germination defect, but it produced extremely heat-sensitive spores. This property is consistent with a defect in the amount or assembly of the cortex and supports the hypothesis that PBP 5* is required for synthesis of this structure. Analysis of the progeny after spontaneous excision of the integrated plasmid led to the conclusion that expression of the *dacB* gene was required only in the mother cell compartment during sporulation, which is also consistent with a role for PBP 5* in cortex synthesis and with its location in the outer forespore membrane. Genetic mapping located *dacB* midway between *aroC* (206°) and *lys* (210°) on the *B. subtilis* chromosome. This is a region where there are no other known *spo*, *ger*, or PBP genes. In related studies, we found that a null mutant of *dacA*, the structural gene for vegetative PBP 5, produced normal heat-resistant spores, which suggests that this PBP is not essential for cortex synthesis. In addition, a candidate for another sporulation-specific PBP was revealed on gels at approximately the same position as PBP 5*. The two PBPs could be distinguished by immunoassays.

During stage IV of sporulation, *Bacillus subtilis* synthesizes a peptidoglycan-like material known as cortex. This material is assembled in concentric layers between the inner and outer membranes that surround the developing forespore. The cortex is made from the same amino acids and sugars that are found in the vegetative peptidoglycan, but there is no teichoic acid associated with it. There is still a controversy about whether the cortical peptidoglycan is more or less cross-linked than the vegetative form (23, 43). Nevertheless, although the mechanism is not clear, it is generally agreed that both the structure and amount of cortex are important for maintenance of fully heat-resistant spores.

The penicillin-binding proteins (PBPs) are membrane-bound enzymes required for peptidoglycan metabolism. They belong to a superfamily of D,D-peptidases (carboxypeptidases, transpeptidases, and endopeptidases) that have an active-site serine, which binds to penicillin (15, 16). There are at least six PBPs produced by vegetative cells of *B. subtilis*, and some of them are also synthesized during sporulation (30, 35). However, the specific focus of this report is PBP 5*, which is synthesized exclusively during sporulation beginning at approximately stage III. The timing of its appearance and its location in the outer forespore membrane led to the hypothesis that PBP 5* is required for synthesis of the cortex (9, 30). This hypothesis is supported by the observation that *gerJ* mutants, which are delayed in their production of PBP 5* during sporulation, are late in acquiring heat resistance and have a visibly defective cortex (40). Recently *dacB*, the structural gene for PBP 5*, was cloned and sequenced (8). From an analysis of the derived amino acid sequence, it was concluded that sporulation-specific PBP 5* is related to other low-molecular-weight membrane-bound PBPs from *Escherichia coli* and *B. sub-*

tilis, all of which have D,D-carboxypeptidase activity in vitro (16, 32). There is preliminary evidence that PBP 5* may also be a carboxypeptidase (34).

In the study described here we directly tested the hypothesis that PBP 5* is required for cortex synthesis by constructing and characterizing a mutant that could not produce this PBP. The most striking phenotypic property of the null mutant was the extreme heat sensitivity of its spores, which is fully consistent with the proposed role for PBP 5* in normal cortex synthesis. We also mapped the PBP 5* structural gene. It was located between *aroC* and *lys* on the *B. subtilis* chromosome in a region that has no other known markers of related function.

In the course of these studies unexpected information was obtained about two other PBPs in *B. subtilis*. First, we learned that the reported heat sensitivity of spores formed by a *dacA* null mutant (37), which produces no detectable PBP 5, could not be reproduced. This result suggests that this major vegetative PBP does not have a critical role in cortex synthesis after all. Second, a candidate for another sporulation-specific PBP was discovered. Although it appeared almost at the same position as PBP 5* on gels, it did not cross-react with anti-PBP 5*. It weakly cross-reacted with anti-PBP 5, but several considerations lead us to conclude that this is a new PBP and not a derivative of PBP 5.

MATERIALS AND METHODS

PBP assays. Membranes were prepared as described previously (30) from 250-ml samples of cultures at various stages of growth or sporulation. Detection of the membrane-bound PBPs was usually based on their ability to react specifically with radiolabeled penicillin. [³H]penicillin was provided by P. J. Cassidy of Merck Sharp & Dohme Laboratories, West Point, Pa. The labeled proteins were separated from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently visualized on

* Corresponding author.

X-ray film after fluorography (3, 11, 30). Enzyme-linked immunoassays for specific PBPs were done on Western blots (immunoblots) prepared from polyacrylamide gels of membrane samples (7, 8, 38). The secondary antibody was alkaline phosphatase-linked goat anti-rabbit immunoglobulin G (Fc; Promega Corp., Madison, Wis.).

Construction of a *dacB* mutant. A *dacB* mutant was constructed by insertional mutagenesis of the chromosomal gene with a plasmid vector bearing an internal fragment of *dacB*. A 328-bp *Bgl*III-*Rsa*I restriction fragment from the middle of the sequence coding for the active-site domain of PBP 5* (8) was gel purified and then ligated to the *Bam*HI and *Eco*RV sites of the integrative plasmid pCP115 (28). The recombinant plasmid was named pCB3. After amplification in *E. coli* HB101 (4), pCB3 was used to transform *B. subtilis* *trpC2* (18, 31) with selection for the chloramphenicol resistance marker on the plasmid. A similar transformation was performed with pCB1, which carries the entire *dacB* gene and flanking sequences (8).

Genetic techniques and strains used for mapping. Preliminary mapping of the *dacB* gene was done by transduction with bacteriophage PBS1 and the Dedonder kit of reference strains (12), which was obtained from the *Bacillus* Genetic Stock Center, The Ohio State University, Columbus, Ohio. Additional strains from the *Bacillus* Genetic Stock Center that were used were as follows: 1A83 *aroB2 hisH2*, 1A132 *aroC7 trpC2*, and 1A208 *hisH2*. Competent cells for transformation were prepared by the method of Anagnostopoulos and Spizizen (1).

Sporulation conditions. When samples were to be harvested at various times during sporulation, the cells were induced to sporulate by the nutrient exhaustion technique in Schaeffer's medium (29). The maximum amount of PBP 5* was usually present in *dacB*⁺ cells after 4 h of sporulation (*t*₄) under these conditions. For large-scale production of mature spores, the cells were allowed to sporulate for 24 h in 2XSG medium (22). Except where noted otherwise, the chloramphenicol-resistant strains were always grown in the presence of 5 µg of chloramphenicol per ml.

Preparation of spores. Initially, 1-ml cultures of spores were purified with multiple water rinses and lysozyme treatment exactly as described by Todd et al. (37). When larger batches of spores were needed for the germination studies, the spores from 1-liter cultures were subjected to a more rigorous protocol that included washing them with 0.1% sodium dodecyl sulfate and adding 0.01% (vol/vol) Tween 80 to subsequent rinses to reduce clumping (21).

Germination conditions. Purified spores were diluted into hot sodium phosphate buffer and incubated at 65°C for 30 min. This temperature was lower than that used previously (25), because the mutant spores were extremely heat sensitive. An aliquot of the heat-activated spores was immediately added to the germinant and shaken at 37°C. The germinants were 10 mM L-alanine in 10 mM Tris-Cl buffer, pH 8.4, at 22°C and Penassay broth (41). A Coleman Junior II spectrophotometer was used to follow the change in absorbance.

Assays for resistance properties of spores. Spores were harvested from 5 ml of a culture grown for 24 h in 2XSG medium, and washed three times with cold water. The pellet of spores was resuspended in 1 ml of 0.05 M Tris-Cl buffer, pH 7.5, and the number of viable cells was determined. The spores were then incubated at 37°C for 60 min with 50 µg of lysozyme per ml. Samples were plated to determine the number of survivors.

Chloroform resistance was measured with the unwashed

FIG. 1. Western blot of a preparative gel loaded with a mixture of PBPs 5 and 5* that were copurified by covalent affinity chromatography (2, 8). Lanes: a and d, duplicate strips from the blot assayed with immune serum; b, strip assayed with blot-purified anti-PBP 5*; c, strip assayed with blot-purified anti-PBP 5. The reactions were detected with alkaline phosphatase-linked secondary antibody.

spores. After plating samples to determine the viable count, 1 ml of chloroform was added to 5 ml of a culture grown for 24 h in 2XSG medium (39). The sample was vortexed vigorously over a period of 25 min and after allowing the chloroform to settle out for about 5 min, dilutions of the sample were plated to determine the number of survivors.

Heat resistance was measured on purified spores as described previously (37, 41), except for some studies in which the temperature was raised to 90°C and longer incubation times were used.

RESULTS

Specificity of blot-purified antibody. Antibody that was highly specific for either PBP 5 or PBP 5* was prepared by blot affinity purification as described previously (7, 8, 26). There was no detectable reaction of anti-PBP 5 or anti-PBP 5* with the pure heterologous protein (Fig. 1). This is a key control experiment, because subsequent studies (see below) revealed that anti-PBP 5 cross-reacted with a protein in crude membrane samples that could easily be confused with PBP 5*.

Examination of the *dacB::cat* and *dacB*⁺*::cat* transformants for production of PBP 5*. The original vector pCP115 cannot replicate as a plasmid in *B. subtilis*, and since it has no homology with the *B. subtilis* chromosome it also does not integrate. In contrast, the recombinant plasmid pCB3 has homology with part of the *dacB* gene and should integrate within the gene by a Campbell-type recombination, thereby interrupting the gene as well as conferring chloramphenicol resistance (*Cm*^r) on the host. Several independently isolated *Cm*^r transformants created with pCB3 were examined at *t*₄ and, as predicted, none of them had any detectable PBP 5*, although each one produced a small amount of a "new" PBP

FIG. 2. Lanes: a through f, fluorograph of [^3H]penicillin-labeled membrane samples; d' through f', Western blot of membrane samples assayed with anti-PBP 5*. Lanes a through e, are t_0 , t_1 , t_2 , t_3 , and t_4 samples, respectively, from a *dacB::cat* mutant of *B. subtilis*; lane f, t_4 sample from a *dacB⁺::cat* strain. Lanes d', e', and f' contain the same samples found in lanes d, e, and f, respectively. The PBPs are numbered in descending order of their molecular weights. PBP 2 is actually two distinct proteins that are referred to as PBP 2A and PBP 2B. The newly discovered sporulation-specific PBP is indicated with an x.

that ran close to the position of authentic PBP 5* on gels (Fig. 2). This PBP did not cross-react with antibody to PBP 5* (Fig. 2, lanes d' and e').

When the same *B. subtilis* strain was transformed with pCB1, which carries the entire *dacB* gene (8), the cells produced a typical level of PBP 5* during sporulation (Fig. 2, lanes f and f'). Subsequent studies (see below) confirmed that pCB1 and pCB3 integrated within the same region of the chromosome.

Identification of a candidate for another sporulation-specific PBP. Absence of PBP 5* from the membranes of cells that were not blocked in sporulation revealed for the first time the presence of another PBP of similar electrophoretic mobility (Fig. 2, band x). We excluded the possibility that this was an example of a nonspecific reaction with the radiolabeled penicillin by boiling the membrane samples prior to the penicillin-binding assay. This treatment eliminated the activity of the PBP in question as well as that of all the other PBPs (data not shown). An analysis of the time course of production of this protein suggested that it was produced specifically during sporulation, since it was never detectable at t_0 or t_1 (Fig. 2, lanes a and b), nor in any samples from *spo0* mutants (data not shown). Initially it seemed most likely that the protein was a derivative of one of the higher-molecular-weight PBPs. This notion was supported by the fact that the newly detected PBP cross-reacted with antibody specific for PBP 5 (Fig. 3), which is the most abundant vegetative PBP and one which undergoes sporulation-related turnover (10). This cross-reacting material was also produced by *dacB⁺* cells (Fig. 3, lane f), but it was never previously considered because its appearance on fluorographs is usually masked by the presence of PBP 5*.

Although the newly discovered PBP was produced only by

FIG. 3. Western blot of *B. subtilis* membrane samples assayed with anti-PBP 5. Lanes a through f contain the same samples described in the legend to Fig. 2.

sporulating cells, it could not be detected in every sporulating culture of a given strain. It was not clear whether this was due to the limitations of the assays or the true absence of the protein. Also, there was not a stoichiometric relationship between the amount of PBP 5 that disappeared and the amount of the cross-reacting protein that appeared. It is tempting to conclude from these observations that PBP 5 is a relatively nonspecific substrate for one or more of the proteases that are produced by sporulating cells. However, there are two compelling reasons to believe that this cross-reacting material is not a derivative of PBP 5 at all. First, the cross-reacting PBP is approximately 5,000 Da smaller than authentic PBP 5, which suggests that it has at least 42 fewer amino acids. PBP 5 cannot be proteolytically trimmed at its amino terminus by this amount without losing the active-site serine that binds to penicillin, and it cannot be trimmed at its carboxy terminus without losing its membrane anchor (44, 45). Second, a *dacA* null mutant, which does not produce even a trace of PBP 5, did produce this cross-reacting protein during sporulation in two of three cultures that were examined (Fig. 4).

Genetic mapping of *dacB*. The readily selectable marker for chloramphenicol resistance (*cat*), which was inserted within the *dacB* gene by transformation with pCB3 and adjacent to an intact copy of *dacB* by transformation with pCB1, was used for mapping *dacB*. Transductional mapping with bacteriophage PBS1 that was grown on either CB36 (*dacB::cat*) or CB11 (*dacB⁺::cat*) gave essentially the same percent linkage of *cat* to the unselected markers (Table 1). The gene order suggested by these results is *lys dacB::cat aroC aroB hisH*. The location of *dacB* midway between *lys* and *aroC* was confirmed by transformational crosses, in which it was found that *dacB* (Cm^r) was 24% cotransferred with *lys* (100 Lys^+ among 414 Cm^r) and 23% cotransferred with *aroC* (103 Aro^+ among 450 Cm^r).

Characterization of the *dacB* mutant phenotype. Vegetative

FIG. 4. Western blot of membrane samples assayed with anti-PBP 5. Lanes: a and b, t_4 and t_5 samples, respectively, of *B. subtilis* JT175 *dacA::cat* (37); c, t_4 sample of *B. subtilis* 168 *trpC2*. The cross-reacting PBP (band x) is present in all three lanes, although it is less apparent in lane c.

TABLE 1. Mapping of the *dacB* gene by transduction with PBS1

No. of Cm ^r transductants	No. with unselected marker	Linkage (%)
31	24, <i>lys</i> ⁺	77
127	114, <i>aroC</i> ⁺	90
72	34, <i>aroB</i> ⁺	47
139	63, <i>hisH</i> ⁺	45

growth of *B. subtilis* CB36 was not remarkably affected by inactivation of the *dacB* gene, nor was there any morphological change that could be seen with the phase contrast microscope. In addition, the cells could sporulate and produce refractile spores that were similar to spores of the parent strain in their resistance to such chemicals as lysozyme and chloroform. However, the spores of CB36 were quite sensitive to heat. Approximately 99.9% of the spores of CB36 were killed during a 10-min incubation at 90°C, whereas spores of CB11 suffered only a 50% drop in viability after 1 h at the same temperature (Fig. 5). The heat sensitivity of the *dacB* mutant spores was also apparent at 80°C, although the difference was not so dramatic. There was approximately 3% survival of the mutant after 15 min at 80°C compared with 75% survival of the *dacB*⁺ strain.

For comparison, we also included the *dacA* null mutant JT175 in our examination of heat resistance. This strain, which lacks the major vegetative PBP (PBP 5), was reported to produce heat-sensitive spores that had only 8% survival after 15 min at 80°C (37). Our results clearly do not confirm that report (Fig. 5). Despite the higher incubation temperature used here, spores of JT175 were no less heat resistant than spores of CB11, which has the full complement of PBPs. We examined three different preparations of JT175 spores with the same outcome, and we verified by penicillin-binding assays and immunoassays that our sample of JT175 was indeed missing PBP 5.

Germination, the degradative phase preceding outgrowth of heat-activated spores, was evaluated with two different germinants, L-alanine and Penassay broth. The *dacB* mutant spores appeared to germinate fully in either germinant with an average loss of 59 and 71% of their absorbance after 90 min in Penassay broth and L-alanine, respectively. These values were not significantly different from those obtained with spores of the parent strain or CB11.

Location of *dacB* expression in the sporulating cell. Previous studies of the putative promoter sequence of *dacB* and the dependence of PBP 5* production on certain *spo* genes led to the proposal that *dacB* was probably a member of the σ^E regulon, which is composed of mother cell-specific genes (8, 19). The availability of CB36, which has its *dacB* gene insertionally inactivated by a plasmid, allowed us to test whether expression of *dacB* was required in the mother cell but not the forespore according to the following rationale. If *dacB* expression in the forespore is required for development of a heat-resistant spore, then only those spores that have regained *dacB* function by loss of the integrated plasmid, a relatively low-frequency event, will survive heat treatment. On the other hand, if *dacB* expression is only required from the genome copy present in the mother cell compartment, then a high frequency of heat-resistant progeny will still be *dacB::cat* (Cm^r) mutants. This approach for determining the cellular location of *spo* gene expression was devised and validated by Illing et al. (20), and we followed their protocol exactly.

FIG. 5. Heat resistance of purified spores from *B. subtilis* CB11 (*dacB*⁺::*cat*; empty circles), CB36 (*dacB*::*cat*; filled circles), and JT175 (*dacA*::*cat*; triangles).

Four separate cultures of CB36 were examined after sporulation in a medium that lacked chloramphenicol, and the results were averaged. The viable count at *t*₁₀ was essentially the same on plates with or without chloramphenicol. The average frequency of pCB3 excision, measured as the frequency of heat-resistant spores in the culture, was 2.2×10^{-5} , which is very similar to the frequency of plasmid excision obtained by others (20, 33). Of these heat-resistant spores, 63.5% were chloramphenicol resistant. Since a significant fraction of the heat-resistant spores were still *dacB::cat*, one may tentatively conclude that *dacB* expression is required only in the mother cell for the development of heat resistance. However, it remains a formal possibility

that these results could be due to the oligosporogenous phenotype of the mutant.

DISCUSSION

Null mutants of the *dacB* gene, which encodes sporulation-specific PBP 5*, produced refractile spores that were resistant to chemicals but extremely sensitive to heat. This phenotype is consistent with the hypothesis that PBP 5* is required for cortex synthesis, but the precise structural defect in these mutant spores is not known. Assays of a partially purified sample of PBP 5* revealed a barely detectable amount of D,D-carboxypeptidase activity (34), which is the same activity attributed to the low-molecular-weight vegetative PBPs from a variety of species. If carboxypeptidase or a related activity is indeed the PBP's function in vivo, then it is likely that the *dacB* mutant spores have an abnormal amount of peptide cross-links, which should affect the physical properties of the cortex (see below). Alternatively or additionally, the PBP defect may somehow impair overall cortex synthesis. In any case, the consequence is formation of a heat-sensitive spore.

The mechanism by which the cortex, a highly conserved structure among members of the genus *Bacillus* (42), contributes to the heat resistance of spores is not understood. Most models are based on the premise that the cortex is loosely cross-linked and therefore capable of either expansion against a strong inflexible coat, anisotropic swelling in the radial direction, or contraction (42, 43). Any of these actions would have the net effect of compressing the contents in the core and helping to maintain the dehydrated state that is essential for heat resistance (17). Recently these models have been challenged by the finding that the intact cortex has very low conductivity, which is more consistent with a compact, uncharged, and highly cross-linked structure (23) that can act as a barrier to prevent osmotic swelling of the spore protoplast. Perhaps elucidation of the structural changes in the heat-sensitive *dacB* mutant spores will help resolve this controversy.

In contrast to the phenotype of the *dacB* mutant, a *dacA* mutant produced fully heat-resistant spores, which suggests that PBP 5 is not essential for this event. We have no explanation for an earlier report to the contrary (37), because to our knowledge we worked with the same strain and used the same protocol for growing and purifying spores. Our conclusion is consistent with other observations that have been made about this PBP. PBP 5 is the most abundant vegetative PBP, but the amount of PBP 5 declines to approximately 60% of its vegetative level early in sporulation. There is no evidence that any synthesis of PBP 5 occurs in sporulating cells (10), which implies that the residual amount of this protein is sufficient, if it is needed at all. It is difficult to rationalize how the preexisting PBP 5, which is in the mother cell membrane and inner forespore membrane that is partially derived from the vegetative membrane, could find its way to the newly synthesized outer forespore membrane where it would have to be located if it participated in cortex synthesis.

The *dacB* gene was mapped by both transduction and transformation to the region on the *B. subtilis* chromosome between *aroC* (206° [27]) and *lys* (210° [27]). The linkage data obtained by cotransduction (Table 1) are similar to data once published for the *gerC* and *gerJ50* markers (24, 41), but additional transductional mapping with more distant markers and subsequent transformation results now place these two germination genes much closer to *aroB* (203°), which is on

the other side of *aroC* from where we have located the *dacB* gene (40, 41, 47). Thus, there are no known *spo* or *ger* genes in the immediate vicinity of *dacB*. Only two other PBP genes have been mapped in *B. subtilis*, and neither of them is nearby either. The *dacA* gene, which encodes PBP 5, is located near 0° (7), and the *pbpB* gene, which encodes PBP 2B, is located next to *spoVD* at 133° (6).

The location of *dacB*, which was determined by genetic mapping, is supported by independent analysis of the DNA sequence for this region. Ehrlich and colleagues (13) recently sequenced the *lys-aroC* portion of the chromosome and found the open reading frame that corresponded to the *dacB* sequence (8). The gene was approximately 5 kb from *ribB* (209°) and 9.9 kb from *aroC*.

The discovery of what appears to be another sporulation-specific PBP was not altogether unanticipated. There are two *spo* genes whose sequences have significant homology to the PBPs, particularly to their active-site motifs (14, 46). The protein product of one of them, the *spoIIA*-PBP gene, would be only slightly larger than PBP 5* after removal of its presumptive signal sequence (8, 46), and thus it may correspond to the new PBP we have described in this report. However, the homology of the *spoIIA*-PBP sequence with that of PBP 5 is not much greater than its homology with PBP 5*, and yet our new PBP only cross-reacts with antibody to PBP 5. Since this cross-reaction is weak at best, it may be due to a single immunodominant region that the two proteins, but not PBP 5*, have in common. It is also quite possible that there are other genes that code for PBPs of this size, which have not yet been identified.

Identification of additional sporulation-specific PBPs is hampered by at least two technical difficulties. One is that the penicillin-binding assay may not be optimum for these novel PBPs. The new PBP described here, for example, may have significantly less affinity for penicillin or form a less stable complex with the antibiotic (5). This latter explanation is apparently why efforts to purify the sporulation-specific PBP 5a from *Bacillus megaterium* have not succeeded (36). The second potential difficulty arises from the fact that small amounts of a new PBP may be produced and sequestered in the forespore at a late stage in sporulation when it is very difficult to achieve good recovery of the spore membranes (9).

Although the identity of the new PBP remains uncertain, it does provide a note of caution for these types of studies. One could easily confuse this PBP with PBP 5*, if one were relying only on the penicillin-binding assay. In turn, this could lead to mistaken conclusions regarding the dependence of PBP 5* production on various conditions, if the second PBP is not regulated in the same manner as PBP 5*. Immunoassays with highly specific antibody preparations can be helpful in avoiding such confusion.

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