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Cloning and Sequencing of the Cell Division Gene *pbpB*, Which Encodes Penicillin-Binding Protein 2B in *Bacillus subtilis*

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The *pbpB* gene, which encodes penicillin-binding protein (PBP) 2B of *Bacillus subtilis*, has been cloned, sequenced, mapped, and mutagenized. The sequence of PBP 2B places it among the class B high-molecular-weight PBPs. It appears to contain three functional domains: an N-terminal domain homologous to the corresponding domain of other class B PBPs, a penicillin-binding domain, and a lengthy carboxy extension. The PBP has a noncleaved signal sequence at its N terminus that presumably serves as its anchor in the cell membrane. Previous studies led to the hypothesis that PBP 2B is required for both vegetative cell division and sporulation septation. Its sequence, map site, and mutant phenotype support this hypothesis. PBP 2B is homologous to PBP 3, the cell division protein encoded by *pbpB* of *Escherichia coli*. Moreover, both *pbpB* genes are located in the same relative position within a cluster of cell division and cell wall genes on their respective chromosomes. However, immediately adjacent to the *B. subtilis pbpB* gene is *spoVD*, which appears to be a sporulation-specific homolog of *pbpB*. Inactivation of SpoVD blocked synthesis of the cortical peptidoglycan in the spore, whereas carboxy truncation of PBP 2B caused cells to grow as filaments. Thus, it appears that a gene duplication has occurred in *B. subtilis* and that one PBP has evolved to serve a common role in septation during both vegetative growth and sporulation, whereas the other PBP serves a specialized role in sporulation.

Bacillus subtilis is attractive as a model organism for studies of cell division because it undergoes two different types of division. During vegetative growth, it divides precisely in half by the inward growth of a septum or cross wall that is composed of membrane and peptidoglycan. During sporulation, it divides asymmetrically to form a small cell at one pole that will become the spore. Other than having a thinner layer of peptidoglycan, the asymmetric septum does not appear to be chemically or structurally different from the vegetative septum. Thus, by studying enzymatic and regulatory proteins that are common to the formation of both structures, we can learn about the basic mechanism of septum formation, and by examining proteins that are uniquely required for one process or the other, we should ultimately be able to explain the differences in their subcellular location and the timing of their appearance. The specific subject of this report is penicillin-binding protein (PBP) 2B, an enzyme that appears to be needed for both types of division and whose expression may be under dual control.

The PBPs are membrane-bound enzymes involved in the extracellular phase of peptidoglycan metabolism during vegetative growth and sporulation. Most of the low-molecular-weight (MW) PBPs (MW under 50,000) have DD-carboxypeptidase activity in vitro, whereas at least some of the high-MW PBPs are transpeptidases. Penicillin inhibits these enzymes by binding covalently to a serine residue in their active site, thereby interrupting normal cell wall synthesis (24). Despite their different catalytic activities, all the PBPs have certain conserved structural features that constitute their penicillin-binding domain. On the basis of this conserved structure, the PBPs and the class A, C, and D β-lactamases are grouped together as a family of evolutionarily related penicilloyl serine transferases (25). In addition, the high-MW PBPs have a second conserved domain of greater than 200 amino acid residues on the amino side of the penicillin-binding domain. This N-terminal domain is believed to catalyze a penicillin-insensitive reaction (24). In vitro studies on PBP 1B of *Escherichia coli* attributed a weak transglycosylase activity to this domain, but whether transglycosylation is one of its functions in vivo and whether the other high-MW PBPs also are transglycosylase-transpeptidase bifunctional enzymes is not yet known (24, 74).

There are multiple PBPs in every eubacterium that has been studied, except the mycoplasmas. They are designated by number in order of descending MW, and as additional ones are identified a letter may be added to the name; hence, we have PBPs 1A and 1B in *E. coli* and PBPs 2A, 2B, and 2C in *B. subtilis*. These are distinct proteins encoded by separate genes. Because the nomenclature was established before the functions of any PBPs were known, one cannot assume that a PBP of a given name from one organism is the homolog of a similarly named PBP from another source.

The roles that the individual PBPs play in normal wall metabolism are best understood for the *E. coli* proteins, and even with this organism there are still some questions. It appears that the low-MW PBPs are dispensable, at least under laboratory conditions, and that some of the essential high-MW ones have overlapping functions. For example, cells can survive the loss of either PBP 1A or PBP 1B, but lysis occurs if both PBPs are inactivated at the same time (69). In contrast, some PBPs appear to have a specialized role: PBP 2 is required for maintenance of the rod shape of *E. coli* cells, and PBP 3 is required for cell division. Inactivation of these PBPs by specific β-lactam antibiotics or by mutation of their structural genes leads to formation of round cells or filaments, respectively (65, 66).

The scarcity of useful mutants and absence of antibiotics that target only one of the PBPs have limited the progress made in identifying the roles of the PBPs in *B. subtilis*.

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TABLE 1. Plasmid constructions

Plasmid	Construction
pAY19	Original 3,492-bp fragment with *Eco*RI linkers cloned into the unique *Eco*RI site of pTZ19R
pAY18	2,884-bp *Mro*I-*Bcl*I fragment cloned into pTZ18R digested with *Xma*I and *Bam*HI
pAY115	2,648-bp *Sph*I-*Bcl*I fragment cloned into pCP115 digested with *Bam*HI and *Sph*I
pAY197	Removal of 198-bp *Sph*I-*Bst*EII fragment from pAY115, followed by treatment with mung bean nuclease and blunt end ligation
pAY215	Ligation of 159-bp *Sph*I-*Hin*dIII fragment from pAY115 to pCP115 digested with *Sph*I and *Hin*dIII
pAY315	Ligation of 398-bp *Hin*dIII-*Hin*cII fragment from pAY115 to pCP115 digested with *Hin*dIII and *Eco*RV
pAY352	Ligation of 352-bp *Eco*RI-*Hin*dIII fragment from pAY19 to pCP115 digested with *Eco*RI and *Hin*dIII
pER923	Ligation of 924-bp *Mro*I-*Eco*RV fragment from pAY19 to pAZ106 digested with *Eco*RI, mung bean nuclease, and then *Xma*I
pER670	Ligation of 671-bp *Sph*I-*Hae*III fragment from pAY19 to pAZ106 digested with *Sph*I and *Sma*I
pAY1009	Ligation of 1,010-bp *Hin*dIII fragment from pAY115 to the unique *Hin*dIII site of pCP115
pL92	Digestion of pSG1304 with *Sal*I and *Kpn*I, whose recognition sites are on the vector downstream from *spoVD*, followed by partial digestion with exonuclease III from the *Kpn*I site through *spoVD* and into the *pbpB* fragment and then treatment with nuclease S1

Nevertheless, evaluation of the PBP profiles of normal cells during vegetative growth, sporulation, germination, and outgrowth has enabled us to assign tentative roles to some of them. Two PBPs, PBP 5* and SpoVD, are specialized for synthesis of the cortical peptidoglycan; they are made only by sporulating cells, and inactivation of either of their genes, which have recently been cloned, leads to heat-sensitive or cortex-deficient spores (11, 13, 16, 64). PBP 5* resembles other low-MW PBPs and is believed to act as a carboxypeptidase (70). The SpoVD protein is a high-MW PBP of unknown catalytic activity. Loss of vegetative PBP 1 results in viable cells that have a reduced diameter, which suggests a requirement for this PBP in synthesis of the normal-length glycan strand of the peptidoglycan (8, 39). Vegetative PBP 2A has been implicated in synthesis of the cell's side walls on the basis of its dramatic decline early in sporulation, when side wall synthesis is no longer a significant activity, and its restoration very early in germinating cells immediately before the resumption of elongation (50, 64). It has been proposed that PBP 2A of *B. subtilis* may be the functional homolog of PBP 2 in *E. coli* (12).

PBP 2B of *B. subtilis*, the focus of our currect work, is of particular interest because it has been implicated not only in the process of vegetative cell division but also in formation of the spore-specific septum (50, 64). PBP 2B is a high-MW PBP (MW of approximately 76,500 ± 1,100), which is synthesized throughout vegetative growth. It achieves a steady state in early stationary phase, and then if conditions are appropriate for sporulation, there is a period of enhanced synthesis of PBP 2B during stage II of sporulation (64). This is the stage when the asymmetric septum is formed. The rate of turnover of this PBP increases in the later stages of sporulation, so that it is the only vegetative PBP that is undetectable in the mature spore (14). It also cannot be detected until more than 30 min after the spores have begun to germinate, which suggests that it has no role in the early outgrowth phase of germination (50). However, the synthesis of PBP 2B resumes at an exponential rate before the first vegetative cell division occurs. Thus, unlike for any of the other PBPs in this strain, net synthesis of PBP 2B of *B. subtilis* seems to occur only in cells that are dividing; for this reason, we have proposed that it may be the functional homolog of PBP 3 in *E. coli* (12).

So far, the only evidence for the involvement of PBP 2B in cell division has been circumstantial. To address its role more directly, we have cloned and sequenced *pbpB*, the structural gene for this PBP, and its neighboring genes. From its sequence alone we have obtained additional support for the hypothesis that PBP 2B is involved in cell division. Not only is the sequence of PBP 2B from *B. subtilis* similar to the sequence of PBP 3 from *E. coli*, but also their structural genes are each located at the same relative position within a cluster of genes involved in cell division and cell wall metabolism, which are highly conserved between the two species. Additional studies that extend the comparison between these PBPs, and highlight some of the differences as well, are described. One intriguing difference was that *B. subtilis* had another gene (*spoVD*), adjacent to *pbpB*, which encoded a sporulation-specific homolog of PBP 2B. Finally, we describe our analysis of two *B. subtilis* mutants, each with an altered form of PBP 2B. Both of them had abnormal morphology, but only in one case could the phenotype be attributed to the PBP defect. Cells that had a carboxy-truncated version of PBP 2B were viable, but they tended to grow as filaments. This phenotype provides further support for the proposed role of PBP 2B in cell division.

MATERIALS AND METHODS

Bacterial strains and phage. The *E. coli* strains used for propagating λgt11 and the plasmids have been described previously (13). *E. coli* MCI23 *ftsI23* was obtained from J. Lutkenhaus (2, 15). The mutant strain JE7925 (W311 *prc-7304*) and its isogenic wild-type strain JE7924 were from H. Hara (31). *B. subtilis* 168 *trpC2*, BR151, and SG38 are among our standard laboratory strains (8, 13, 23, 77); JT175 was acquired from D. J. Ellar (71); UB8524 was from I. Chopra (61); BGSC1A6 was supplied by the *Bacillus* Genetic Stock Center, Columbus, Ohio; and ATCC 23857 (7) was purchased from the American Type Culture Collection, Rockville, Md. The PBP 1-deficient strains (see Fig. 1) were constructed by transformation of BR151 with DNA from the original PBP 1 mutant as described previously (8).

Plasmids. Plasmids pTZ18R and pTZ19R were obtained from U.S. Biochemical Corp., Cleveland, Ohio. Plasmid pSG1304, which carries a chromosomal fragment of *B. subtilis* that begins within the *pbpB* gene and extends almost to the distal end of *spoVD*, has been described previously (16). Plasmid pCP115 (59) was acquired from the *Bacillus* Genetic Stock Center, and plasmid pAZ106 was obtained from A. Moir (38). These plasmids were used to construct various subclones of the *pbpB* region as described in Table 1.

Preparation of membranes and PBP assays. The details of membrane preparation and PBP assays have been described previously (13, 64). The PBPs were routinely detected in membrane samples by radiolabelling with [^{3}H]benzylpenicillin (Merck Sharpe & Dohme Laboratories, West Point, Pa.) or

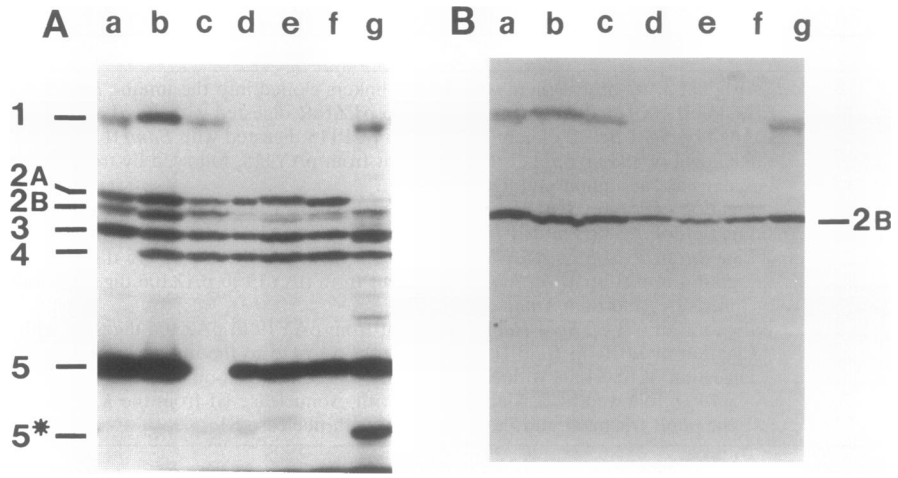

FIG. 1. (A) Fluorograph of an SDS-polyacrylamide gel containing the [^{3}H]penicillin-labelled membrane proteins from various *B. subtilis* 168 strains. All cultures except the sample in lane g were harvested in late log phase. The PBPs are indicated by number in the left margin. (B) Western blot of the same samples assayed with anti-PBP 2B and detected with enzyme-linked secondary antibody. The unnamed cross-reacting protein is believed to be PBP 1 (see text). Lanes: a, ATCC 23857, a mutant that is missing PBP 4; b, UB8524, a mutant whose PBP 2B has slightly faster electrophoretic mobility than the wild type; c, JT175, a mutant that is missing PBP 5; d, e, and f, mutants that are missing PBP 1 and have a lower-than-average amount of PBP 2B; g, the 168 *trpC2* strain harvested 4 h after the beginning of sporulation. PBP 5* is a sporulation-specific PBP (64).

immunoassays of Western blots with enzyme-linked secondary antibody (Promega Corp., Madison, Wis.).

Preparation of antibodies. The high-MW vegetative PBPs were copurified by penicillin affinity chromatography (6) from the membranes of *B. subtilis* JT175. This bacterial strain has an insertionally interrupted *dacA* gene and produces no PBP 5, which is normally the major vegetative PBP (5, 71). By eliminating PBP 5 from the mixture, we were able to load more of the other PBPs per sample without overloading the preparative gels. The vegetative PBPs were separated from one another by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide preparative slab gels and then electroblotted to nitrocellulose (72). After being stained with India ink (32), the protein band corresponding to PBP 2B was excised from the dry blot with a clean razor blade. The nitrocellulose strip (approximately 12 cm by 3 mm) was cut into tiny fragments and dissolved in 0.5 ml of dimethyl sulfoxide (40). This highly viscous solution was passed through a 21-gauge hypodermic needle to ensure its homogenization. Immunization of a rabbit with the sample was performed by Berkeley Antibody Company, Richmond, Calif. The initial immunization and first boost each contained the equivalent of two nitrocellulose strips of PBP 2B (roughly equivalent to 50 μg of protein [total]) mixed with an equal volume of Freund's complete adjuvant (initially) or incomplete adjuvant (all boosts); the second and third boosts each contained the equivalent of one nitrocellulose strip of PBP 2B.

Monospecific antibodies were prepared from the immune serum by blot affinity purification as described previously (13, 52).

DNA sequencing. Both strands of the 3.48-kb cloned fragment were sequenced by using a Sequenase version 2.0 kit from U.S. Biochemical Corp. and α-^{35}S-dATP from Amersham Corp., Arlington Heights, Ill. The universal and reverse primers purchased from U.S. Biochemical Corp. were supplemented with custom-synthesized oligonucleotides prepared by Operon Technologies, Alameda, Calif.

Sequence analysis was performed with the aid of the software package from the Genetics Computer Group, Madison, Wis. (program manual for the GCG package, version 7, April 1991).

Nucleotide sequence accession number. The sequence reported here is available in the GenBank data base under accession no. L09703.

RESULTS

Specificity of anti-PBP 2B antibodies and immunoassays of various strains. The purified antibodies reacted strongly with PBP 2B in membrane samples from a variety of *B. subtilis* 168 mutants (Fig. 1). In addition, they cross-reacted with a higher-MW membrane protein, which appears to be PBP 1 (Fig. 1, lanes a, b, c, and g). The proposed identity of the cross-reacting material with PBP 1 is supported not only by the fact that it had the same electrophoretic mobility as PBP 1 but also by the fact that there was no cross-reaction with *B. subtilis* membranes that lacked PBP 1 (Fig. 1, lanes d, e, and f). Arguments against the possibility that this higher-MW material was another form of PBP 2B include the observations that the cross-reacting protein was not present in any of the *E. coli* samples that contained PBP 2B produced from the cloned *B. subtilis* gene (results not shown) and that the mobility of this protein was not changed when a truncated version of PBP 2B was created in *B. subtilis* (see Fig. 6B).

Among the samples examined were three derivatives of BR151, a strain that was previously reported to have much less PBP 2B than other mutants of the *B. subtilis* 168 parent strain (8). This feature is readily apparent in Fig. 1A (lanes d, e, and f). However, the immunoassays revealed that there was relatively more PBP 2B in BR151 (Fig. 1B, lanes d, e, and f) than could be determined by the usual penicillin-binding assay (Fig. 1A) or by various modified versions of the assay that were tested (8).

Also examined was the *B. subtilis* mutant UB8524, which was reported to have a PBP 2B with altered electrophoretic mobility (61). Immunoassay with the purified antibody con-

firmed that this altered protein was indeed PBP 2B (Fig. 1, lane b).

Cloning of *pbpB*, the gene for PBP 2B. The monospecific polyclonal anti-PBP 2B was used to screen two *B. subtilis* genomic libraries prepared with the expression vector λgt11 by C. Price (68). The library constructed with *Eco*RI-digested DNA gave no clear-cut positive results, whereas numerous positive plaques were obtained from the "random-cut" library that contained a mixture of DNA fragments, which were generated by separate partial digestions with *Alu*I, *Hae*III, or *Rsa*I and then ligated to the vector by means of *Eco*RI linkers (68). The phage from three positive plaques were purified and used to plaque purify anti-PBP 2B from immune rabbit serum (63). This confirmed that plaques of each recombinant phage were indeed reacting specifically with anti-PBP 2B.

To determine which, if any, of the phage encoded the entire PBP, it was necessary to lysogenize *E. coli* Y1089 with each phage. An extract of each induced lysogen was then examined on a Western blot of a polyacrylamide gel. Two of the three recombinant phage produced a protein with the same mobility as authentic PBP 2B, and the third phage produced a much larger cross-reacting protein (results not shown). A subsequent blot, which was reacted with anti-β-galactosidase, confirmed that this larger protein was a β-galactosidase fusion protein. The full-length PBP produced by two of the phage clones was also detectable in the cell extracts by the penicillin-binding assay, but the fusion protein apparently did not bind penicillin (not shown).

DNA from one of the recombinant phage that carried the intact *pbpB* gene was digested with *Eco*RI to release the cloned fragment. The cloned DNA was recovered in three pieces; the largest piece, which was 3.48 kb, had been incompletely digested and still contained an *Eco*RI restriction site. This fragment was subcloned into pTZ19R (43) and used for subsequent studies.

DNA sequence of the *pbpB* region. A *B. subtilis* DNA fragment of 3,478 bp that contained the entire *pbpB* gene and flanking regions was sequenced (Fig. 2). The *Eco*RI linkers are not included in the data presented in Fig. 2. Each of the natural ends of the fragment had one half of an *Alu*I recognition site, which indicates that the original recombinant phage carried an *Alu*I-generated fragment from the "random-cut" library. It is unlikely that the clone contained noncontiguous *Alu*I fragments, because the organization of its open reading frames (ORFs) was similar to that found in the corresponding region on the *E. coli* chromosome (see below), and this same sequence was also obtained from strain 168 of *B. subtilis* by chromosome walking from the downstream *spoVD* region (18).

There were four ORFs on the same strand of DNA; these were located at nucleotides (nt) 1 through 612, 616 through 1005, 984 through 3152, and 3257 through 3478 (Fig. 2). The first ORF appeared incomplete at its 5′ end, the last ORF was incomplete at its 3′ end, and the middle two ORFs overlapped by 22 bp. A possible start codon for the second ORF was located at nt 655, which was 13 bp downstream from the center of a very strong ribosome binding site ($\Delta G = -88.7$ kJ mol^{-1}) at nt 637 through 648. This putative gene has been designated *orfA*, and the incomplete one preceding it is *orfB*.

The longest ORF corresponded to *pbpB*, which encodes PBP 2B. This conclusion is based not only on its derived amino acid sequence but also on the observed production of PBP 2B in *E. coli* from cloned fragments that lacked complete copies of any other gene except this one (see below). Two possible start codons (ATG) were located at nt 1005 and 1014. The first of these codons overlapped the stop codon (TGA) for *orfA* in the sequence ATGA, which raises the possibility that initiation of translation of *pbpB* is coupled to translation of *orfA*. Sprengel et al. (67) determined that ribosomes reinitiate translation within this particular arrangement of overlapping start and stop codons with up to 10% efficiency. However, the downstream ATG codon may also be accessible to ribosomes because it was the proper distance from a strong ribosome binding site ($\Delta G = -56.9$ kJ mol^{-1}) that began at nt 997. Following the stop codon for *pbpB* was a sequence that could form a stem-loop structure and might serve as a transcription termination site.

The ORF at the 3′ end of the cloned fragment was identified as the beginning of the *spoVD* gene. Its start codon TTG was located at nt 3272. The complete sequence for *spoVD* has been described elsewhere (16). The *spoVD* gene is located at 133° on the *B. subtilis* chromosome (53). Because it is so closely linked to *spoVD*, we place *pbpB* at 133° also. This conclusion is consistent with earlier observations that *pbpB* is linked by PBS1 transduction to *pyrD*, which is located at 139° (9).

We note that there are potential promoter sequences TGG ACA (nt 944 through 949) and TTGAAT (nt 967 through 972) upstream of *pbpB* and AAGACA (nt 416 through 421) and TTTAAA (nt 439 through 444) upstream of *orfA* that may be recognized by vegetative sigma factor A (46), but no transcription start sites have been identified with certainty.

Expression of *pbpB* from various subclones. Four recombinant plasmids that carried the entire *pbpB* gene were constructed, but each plasmid carried a different length of the gene's 5′-flanking region (Fig. 3). Initiation of transcription from any nearby promoters on the vector was in the opposite direction from the cloned insert for each plasmid except pAY18. PBP 2B was produced in an *E. coli* host from three of the four plasmids (pAY19, pAY18, and pAY115) but not from pAY197 (Fig. 4; other results not shown). This result suggests that the sequence between the *Sph*I site at nt 698 (the 5′ end of pAY115) and the *Bst*EII site at nt 896 (the 5′ end of pAY197) is essential for expression of *pbpB* in *E. coli*. However, plasmid integration studies (see below) indicate that this promoter, if active in *B. subtilis*, cannot by itself provide sufficient PBP 2B for cell viability.

PBP 2B produced from the cloned gene in *E. coli* cells was detectable in the inner membrane fraction. This is where the *E. coli* PBPs are also found, which suggests that the foreign protein's signal sequence was properly recognized by the heterologous host. Moreover, the protein produced in *E. coli* appeared to be the same size as it was in *B. subtilis* membranes (Fig. 4, compare lane b with lane a). Figure 4 also illustrates a phenomenon that we regularly observed with *E. coli* samples that had a resident plasmid: there was less of the *E. coli* PBPs detectable in their membranes by the penicillin-binding assay (Fig. 4, compare lanes b and c with lane d). This characteristic was observed in cells with plasmids derived from pBR322 or the pUC series.

Three integrative plasmids (pER923, pER670, and pAY1009) that carried various DNA fragments whose sequences began upstream of the *pbpB* gene and extended into the gene itself were used to determine the general location for transcription initiation of *pbpB* in *B. subtilis*. Each of these plasmids could undergo a Campbell-type recombination and thereby integrate into the *B. subtilis* chromosome in the region homologous to the DNA fragment carried by the plasmid. As a consequence of plasmid integration, an intact *pbpB* gene would be contiguous with the upstream sequence of the cloned fragment and separated from the more distant 5′ sequences by the integrated vector.

No cells with integrated pER670 or pAY1009 could be recovered, despite repeated attempts and various efforts to

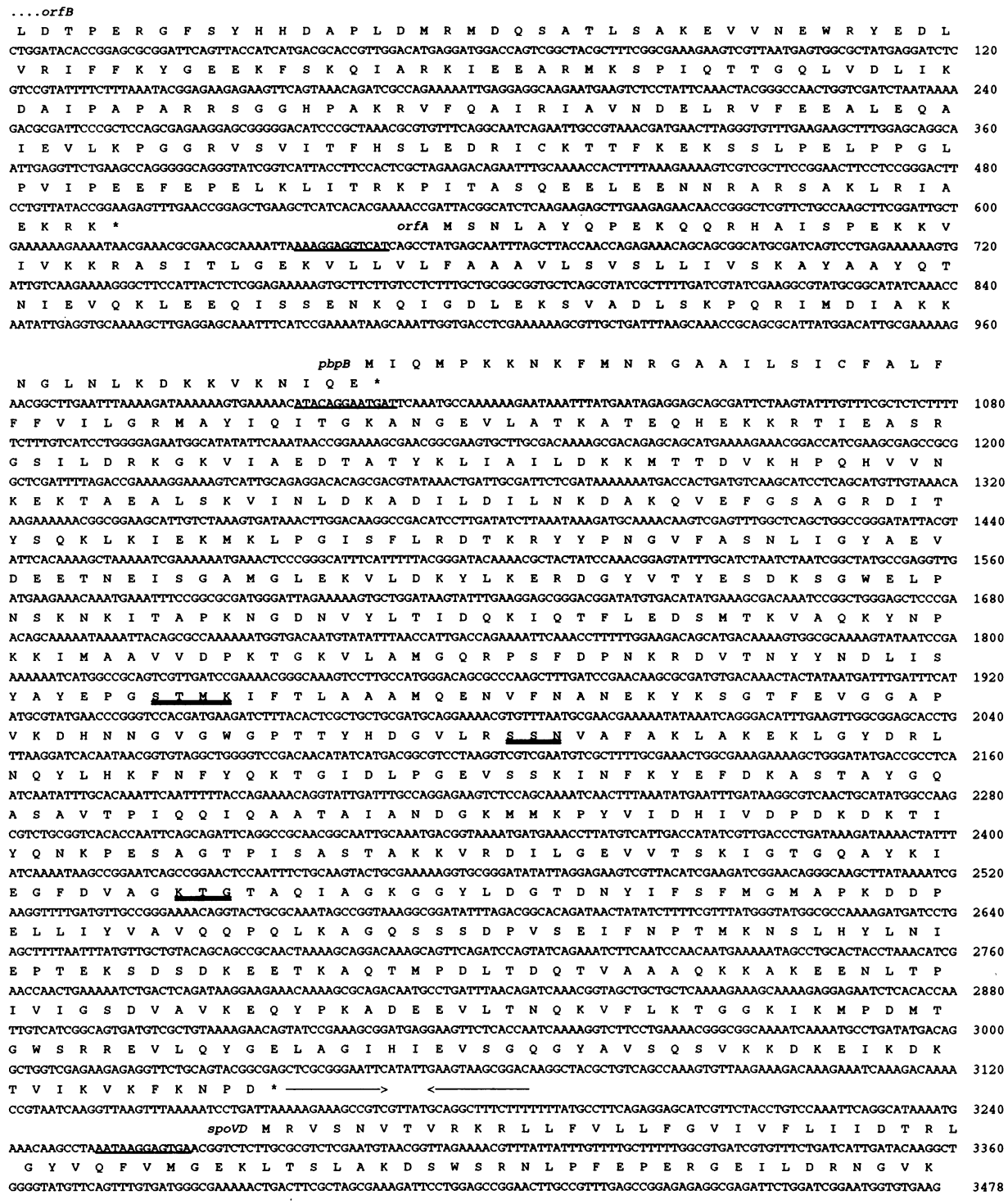

FIG. 2. Nucleotide sequence of the *pbpB* region from *B. subtilis* and deduced amino acid sequences of the four ORFs named, in the order of their appearance, *orfB*, *orfA*, *pbpB*, and *spoVD*. The putative ribosome binding sites (28) for *orfA*, *pbpB*, and *spoVD* are underlined. The three active-site motifs of PBP 2B are doubly underlined. Stop codons are marked by an asterisk, and sequences that have the potential to form a stem-loop structure are overlined with arrows.

enrich for a rare recombinant. The tentative conclusion is that integration of either of these plasmids separated the *pbpB* gene from the sequence essential for its expression in *B. subtilis* and thus was a lethal event. In contrast, there was no difficulty in successfully integrating pER923. Its integration at the expected location was confirmed by Southern blotting (not shown). The production of PBP 2B in the recombinant was also confirmed (not shown). These results indicate that *B. subtilis* can initiate

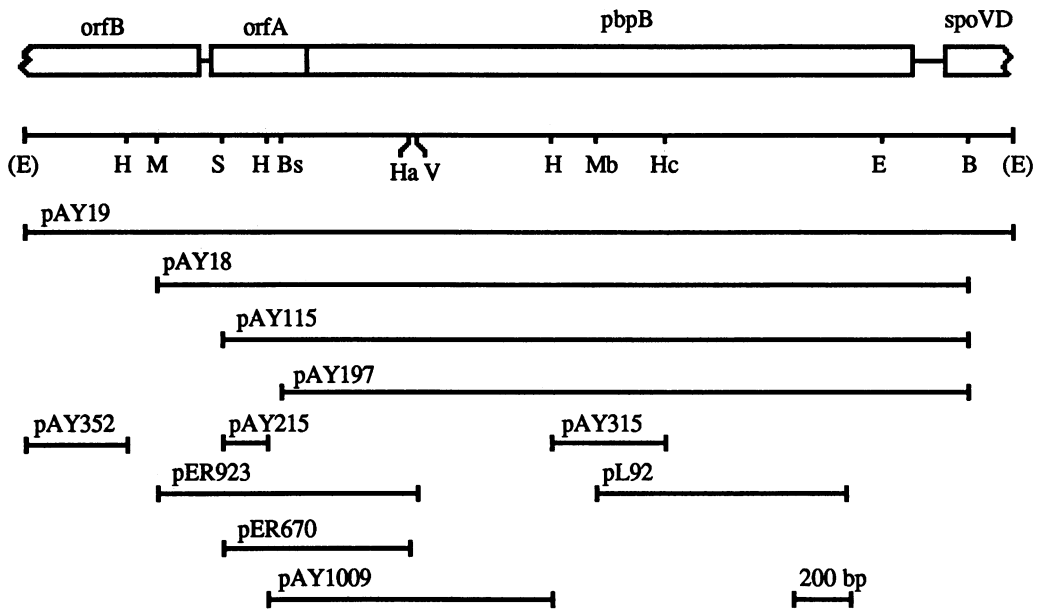

FIG. 3. Physical map of the *pbpB* region. The top part shows the relative positions of the four genes. The direction of transcription is from left to right for each one. The stop codon for *orfA* and the putative start codon for *pbpB* overlap. Below the genes is a partial restriction map of the region. Only the restriction sites referred to in the text or Table 1 are labelled: B, *Bcl*I; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae*III; Hc, *Hin*cII; M, *Mro*I; Mb, *Mbo*I; S, *Sph*I; V, *Eco*RV. The *Eco*RI sites in parentheses correspond to the linkers attached to the blunt ends of the original DNA fragment (68). Below the restriction map are the DNA subclones of the region carried by the various plasmids described in Table 1.

transcription of *pbpB* at some point downstream of the *Mro*I site at nt 460 (the 5′ end of the fragment carried by pER923).

Although *B. subtilis* with integrated pER923 was viable and could efficiently form heat-resistant spores, we observed that this strain grew more slowly in 2× YT medium (59a) at 37°C than the parent strain; its generation time was approximately 48 min, whereas cells without the plasmid had a generation time of approximately 32 min. In addition, its colonies on tryptose blood agar base medium were clearly distinguishable from the wild type by their glisteny appearance. Whether these characteristics reflect an abnormality in the expression of *pbpB* or the fact that the plasmid also carried the *lacZ* gene in a transcriptional fusion to the cloned fragment remains to be determined.

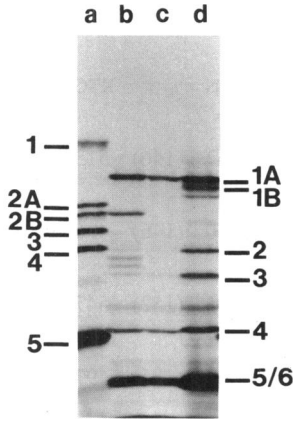

FIG. 4. Production of PBP 2B from a recombinant plasmid in *E. coli*. Lanes: a, *B. subtilis* membranes; b, *E. coli* with the recombinant plasmid pAY115; c, *E. coli* with the nonrecombinant plasmid pCP115; d, *E. coli* with no plasmid. Only the inner membrane fraction from each *E. coli* sample was loaded on the gel. The [^{3}H]penicillin-labelled PBPs were detected by fluorography. The positions of the *B. subtilis* and *E. coli* PBPs are indicated on the left and right sides of the figure, respectively.

Features of the *pbpB* gene product and comparison with other PBPs. Translation of *pbpB* beginning with the first available start codon at nt 1005 would give a product that is 716 amino acids long and has a calculated mass of 79.2 kDa, which is very close to that predicted from the relative mobility of PBP 2B on SDS gels (8). Unfortunately, efforts to determine the actual NH_2-terminal sequence of purified PBP 2B were unsuccessful, but the nearby location of the only candidate sequence for the membrane anchor of this protein supports the idea that the codon at nt 1005 is very close, if not identical, to the actual start site for translation. We shall refer to this first methionine as residue 1 of PBP 2B, while keeping in mind that this identification is tentative.

In contrast to the low-MW PBPs, which have a cleavable signal sequence and a carboxy-terminal membrane anchor, all the high-MW ones are anchored in the plasma membrane by a hydrophobic sequence at their amino terminus (24). PBP 2B appeared to be typical in this regard; the only region of the protein that could potentially serve as the transmembrane anchor was the signal sequence located near the amino terminus. This sequence included a stretch of 18 hydrophobic amino acids (Gly-14 through Gly-31) preceded by a positively charged region, which is especially typical of signal peptides in *Bacillus* spp. and suggests that the protein's N terminus is on the negatively charged cytoplasmic side of the membrane (47, 75). Within the putative membrane-spanning sequence was the only cysteine residue of the entire protein. Although the amino acid sequence ILSIC is very similar to the consensus modification and cleavage site found in lipoproteins (41, 47), we have no evidence that any of PBP 2B is lipid modified.

TABLE 2. Percent identities of the amino acid sequence of PBP 2B to those of other high-MW PBPs[a]

Protein	% identity with PBP 2B		
	Full-length protein	Penicillin-binding domain[b]	N-terminal domain
B. subtilis SpoVD	35.2	42.1	29.6
S. pneumoniae PBP 2X	33.3	37.5	33.7
N. meningitidis PBP 2	28.4	28.8	28.5
E. coli PBP 3	28.1	28.5	27.9

[a] Comparisons were done by the Bestfit program (gap weight = 3) with the Genetics Computer Group sequence analysis software package (19).

[b] The penicillin-binding domain begins 60 residues upstream of the SXXK motif (homology box 6) and extends for 60 residues downstream of the KTG motif (homology box 8) (36).

The derived amino acid sequence of PBP 2B was used to search the data bases for related proteins. Not surprisingly, the sequence was most similar to those of various high-MW PBPs that belong to class B (Table 2). PBP 2B had the nine sequence motifs or boxes (Fig. 5) that are conserved in all PBPs of its class (21, 25, 55). Three of the motifs in the C-terminal domain are common not just to this class but to all the penicilloyl serine transferases (24, 25, 36). The active-site serine residue that binds to penicillin is typically part of the motif SXXK (box 6); this was located at residue 312 in PBP 2B. The SXN and KTG motifs (boxes 7, and 8, respectively) that are also present in the active site of every penicillin-binding domain were located at residues 367 and 513, respectively. The spacing between these active-site motifs was highly conserved, as was the spacing between the other regions of homology (Fig. 5).

The penicillin-binding domain of a PBP has been defined as the amino acid sequence that begins 60 residues upstream from box 6 and ends 60 residues downstream from box 8 (36). An integrative plasmid (pAY315 [Fig. 3]) carrying a 398-bp fragment of *pbpB*, whose ends were both within this domain, was used in an effort to insertionally inactivate the *pbpB* gene in *B. subtilis*. All attempts to integrate this plasmid failed. However, pL92, a plasmid with a fragment of *pbpB* whose 3′ end extended beyond the penicillin-binding domain, was successfully integrated into the chromosomal gene (see below). These results are consistent with the prediction that the penicillin-binding domain of PBP 2B cannot be interrupted without loss of an essential function.

Construction and properties of a mutant with a carboxy-truncated PBP 2B. On the carboxy side of its penicillin-binding domain PBP 2B had a peptide whose length accounts for the difference in size between this PBP and some other high-MW PBPs (Fig. 5). This carboxy extension is clearly the most variable feature among these proteins. For at least some of them, it is not essential for their function, although part of it may be required for stability (30, 37). This also appeared to be the case for PBP 2B. A truncated version of the *pbpB* gene was generated in *B. subtilis* SG38 by integration of the plasmid pL92 (Fig. 3). This plasmid carried an internal fragment of the gene that was created by partial digestion with exonuclease III (Table 1). It was determined by DNA sequencing that the fragment terminated with nt 2900, which corresponded to the last base of the codon for Val-632 (Fig. 2). Thus, upon integration of the plasmid into the homologous region on the *B. subtilis* chromosome, the upstream portion of *pbpB*, coding for residues 1 through 632, was in frame with five sense codons (AGC TTT TGT TCC CTT) of the vector followed by two stop codons. After taking into account the loss of the carboxy-terminal 84 residues from PBP 2B and the addition of 5 residues of a foreign peptide, the size of the modified PBP 2B that was encoded by SG38::pL92 was calculated to be 70.29 kDa.

Membrane samples from SG38 and SG38::pL92 were examined by both the penicillin-binding assay and immunoassay (Fig. 6). There was no trace of PBP 2B in its usual position between PBPs 2A and 2C on gels of SG38::pL92. There was also no trace of PBP 2B at the position predicted for a 70.29-kDa protein. Instead, the truncated version of PBP 2B was detected below PBP 4 at a position that roughly corresponded to a protein of 62 kDa. A second, slightly larger derivative of PBP 2B was also present in membrane samples from cells grown in Penassay (Difco antibiotic medium 3) or LB broth but not in samples from Schaeffer's medium (60). At this time we can only speculate that there is either degradation or posttranslational processing of the modified PBP, which is incomplete under certain cultural conditions. There was no evidence that the wild-type PBP 2B from SG38 cells grown in

	H_2N	box1		box2		box3		box4		box5		SXXK		SXN		KTG		box9		COOH
PBP 2B, *B. subtilis*	65	(103)	168	(26)	194	(43)	237	(40)	277	(35)	312	(55)	367	(146)	513	(28)	541	(175)	716
SpoVD, *B. subtilis*	59	(92)	151	(21)	172	(43)	215	(40)	255	(39)	294	(56)	350	(146)	496	(26)	522	(123)	645
PBP 2X, *S. pneumoniae*	76	(110)	186	(29)	215	(43)	258	(40)	298	(39)	337	(58)	395	(152)	547	(30)	577	(173)	750
PBP 2, *N. meningitidis*	75	(92)	167	(21)	188	(44)	232	(40)	272	(38)	310	(52)	362	(135)	497	(26)	523	(58)	581
PBP 3, *E. coli*	71	(96)	167	(21)	188	(41)	229	(40)	269	(38)	307	(52)	359	(135)	494	(26)	520	(68)	588

FIG. 5. Alignment of the nine regions or boxes of sequence homology in PBP 2B with the corresponding regions in the PBPs most closely related to it. Boxes 1 through 4 are in the N-terminal domain, and boxes 5 through 9 are in the C-terminal domain of each high-MW PBP (21, 55). The active-site motifs SXXK, SXN, and KTG correspond to boxes 6, 7, and 8, respectively. The number in each column for a box is the position of the first amino acid residue of the box in that protein; the numbers in parentheses are the number of residues in the interval from the beginning of one box to the beginning of the next box. References for the published sequences are 16 (SpoVD), 42 (PBP 2X), 79 (PBP 2), and 49 (PBP 3).

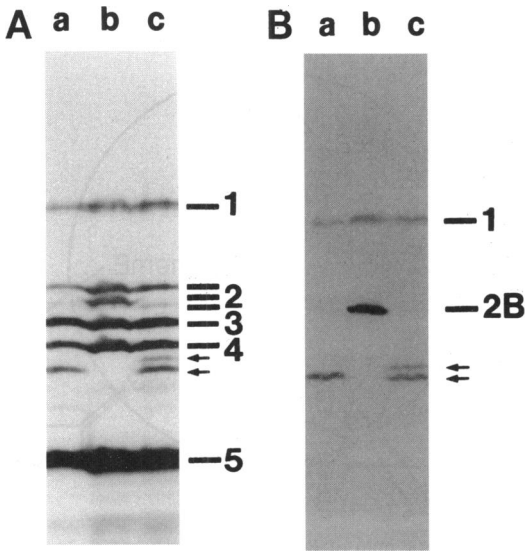

FIG. 6. Assays for a carboxy-truncated version of PBP 2B. (A) Fluorograph of [^{3}H]penicillin-labelled membrane proteins. Lanes: a, SG38::pL92 grown to t_0 (i.e., the end of exponential growth) in Schaeffer's medium; b, SG38 grown to late log phase in Penassay broth; c, SG38::pL92 grown to late log phase in Penassay broth. PBP 2 in lane b is a triplet composed of three distinct PBPs (2A, 2B, and 2C). (B) Western blot of the same three samples assayed with anti-PBP 2B. Authentic PBP 2B is present in lane b; the antibody cross-reacts with PBP 1 in every sample. Arrows, truncated versions of PBP 2B present in lanes a and c.

the same medium was a doublet, so either processing of the normal PBP was complete or only the truncated PBP was subject to processing.

The doublet nature of truncated PBP 2B was reminiscent of the doublet form of PBP 3 of *E. coli*, whose appearance was dependent on the presence of glucose in the medium and a temperature of 37°C or above (58). We excluded the possibility that this was also the case in *B. subtilis*. Truncated PBP 2B appeared as a doublet in SG38::pL92 cells grown in LB medium with 0.4% glucose, 0.4% lactose, or no supplement at 30 as well as at 37°C (data not shown).

A small amount of a doublet of PBP 2B located at approximately the same position on gels as that produced by SG38::pL92 (Fig. 6, lanes c) was observed in *E. coli* samples in which the normal-length PBP 2B was being produced from a plasmid-borne gene (Fig. 4, lane b; other results not shown). This raises the possibility that both abnormal PBP 2B in *B. subtilis* and normal PBP 2B in a foreign host decay in similar discrete steps in a manner that may be intrinsic to the protein. A similar observation has been made for PBP 3 of *E. coli*. Both in vivo degradation of overproduced PBP 3 and in vitro degradation of purified PBP 3 from *E. coli* yield discrete products, some of which retain their penicillin-binding activity (48, 51).

Vegetative cells of *B. subtilis* with the truncated version of PBP 2B were viable and motile. There was virtually no difference in the growth rates of SG38 and SG38::pL92 at 30, 37, or 42°C in Penassay broth or a minimal salts medium (data not shown). The mutant was able to form refractile, chloroform-resistant, and heat-resistant spores, and the sporulation-specific increase in PBP 2B that occurs in the wild-type strain (64) also occurred with the truncated version of the PBP (not shown). However, the cells with a truncated PBP 2B tended to be 2- to 10-fold longer than the average length of SG38 cells, and in every microscopic field examined by phase-contrast optics, there were some extremely long filaments of greater than 100 cell lengths. After 24 h in sporulation medium, there were mostly free refractile spores, but some long filaments persisted. Usually there was a single refractile endospore at one pole of the filament. The number of spores in these cultures was <20% of the wild-type number, which suggests that the average filament produced no more than one spore. The germination properties of the mutant spores have not been examined.

Characterization of upstream ORFs. Translation of *orfA* gave a sequence that had 117 amino acids and a calculated mass of 13 kDa. The identity of this putative protein is unknown, and a computer search (TFASTA program) did not reveal any significant homology with sequences in the GenBank and EMBL data bases. Its sequence was only 18% identical to that of the 121-amino-acid product of the *orfA* sequence that is located immediately upstream of the *pbpB* gene in *E. coli* (49; as corrected by J. Ayala [GenBank accession no. X55034]). However, the sequences were similar in that the *B. subtilis orfA* product had a stretch of 19 hydrophobic or uncharged amino acids (residues 36 through 54) that roughly aligned with a membrane-spanning sequence of 20 amino acids in the *E. coli* protein. The essential nature of the *E. coli orfA* (also referred to as *mraR* or *ftsL*) has been reported (27, 73). All efforts to insertionally interrupt the *B. subtilis orfA* with integrative plasmid pAY215 (Fig. 3) were unsuccessful, which suggests that the *orfA* product may also be essential in *B. subtilis* and/or that expression of the downstream *pbpB* gene was impaired. However, we have not excluded the possibility that the small size of the insert on this plasmid accounts for its failure to integrate.

Translation of the incomplete *orfB* sequence gave a product of 204 amino acids. A computer search with the TFASTA program revealed that this sequence was 44% identical to that of the product of the *orfB* sequence in the corresponding position upstream of *E. coli pbpB* (26; as corrected by J. Ayala [GenBank accession no. X55034]). We could not transform cells with the integrative plasmid pAY352, which carried an internal fragment of *orfB* (Fig. 3). The most likely interpretation is that the *orfB* product is essential for viability. We do not believe that an intact *orfB* DNA sequence must be physically contiguous with essential genes downstream (e.g., *pbpB*), because cells in which pER923 was integrated (see above) survived with a separation of *pbpB* from all but 157 bp at the 3′ end of *orfB*. The function of the *orfB* product in either *B. subtilis* or *E. coli* is not known.

Complementation studies with *E. coli*. The results described above suggested that *B. subtilis* PBP 2B could be the functional homolog of PBP 3 of *E. coli*, which is encoded by the *pbpB* (*ftsI*) gene (Fig. 5 and Table 2). Because the *B. subtilis pbpB* gene was readily expressed in *E. coli* and produced an active product (as determined by its ability to react with penicillin) that was localized to the appropriate membrane, we decided to test whether *B. subtilis* PBP 2B could function in lieu of *E. coli* PBP 3 and thereby restore temperature-resistant growth to an *ftsI*(Ts) mutant. *E. coli* MCI23 (*ftsI23*) has only a trace of PBP 3 detectable in its membranes, even after the cells have been grown at the permissive temperature (3); it can be complemented by the *E. coli ftsI*$^+$ gene (2). Plasmids pAY115, pAY18, and pAY19 were introduced into the mutant with selection for ampicillin resistance. The transformants were grown at 30°C and then plated at both 30 and 42°C. None of them could form colonies at 42°C. We confirmed the presence of PBP 2B in the membranes of each transformant. Compared with the amount

of *E. coli* PBP 3 normally produced by the wild-type strain HB101 (Fig. 4, lane d), the MCI23 transformants with plasmids pAY115, pAY19, and pAY18 produced approximately one-half, twofold more, and at least eightfold more PBP 2B, respectively, than PBP 3.

Production of PBP 2B in a *prc* mutant of *E. coli*. PBP 3 produced by *E. coli* runs faster on an SDS gel than the in vitro-synthesized protein, which led to the suggestion that perhaps it is processed in vivo (49). This suggestion was later confirmed after isolation of a *prc* (also referred to as *tsp*) mutant that fails to process PBP 3 (31, 62). The processing was found to be at the carboxy terminus, which was not unexpected because cleavage of the N terminus would have released the protein from the membrane. Although the carboxy extension of PBP 3 of *E. coli* is much shorter than that of PBP 2B (Fig. 5) and there was no significant sequence homology between them, PBP 2B did have a potential recognition site (Val-Ile) for the tail-specific endopeptidase nine residues from its carboxy terminus. In addition, we already had evidence that cells could survive a much greater truncation at the carboxy terminus of PBP 2B (see above). Thus, it seemed possible that the *B. subtilis* protein might be susceptible to cleavage by the same enzyme that normally removes the carboxy-terminal 11 amino acids from its *E. coli* counterpart. However, this was found not to be the case. PBP 2B had the same electrophoretic mobility when assayed in membranes from the *E. coli prc* mutant, its isogenic wild-type strain, or the *B. subtilis* parent.

Characterization of a mutant with abnormal morphology and an altered PBP 2B. There are only two reports in the literature of *B. subtilis* strains with a PBP 2B phenotype that is distinct from what is typically found in strain 168 *trpC2* and most of its derivatives. One of these reports describes reproducible quantitative differences in the amount of active PBP 2B present in vegetative cells of three strains, but no phenotypic differences among the strains could be attributed to this phenomenon (8). In contrast, the other report attributes a major defect in the shape of mutant strain UB8524 to an altered PBP 2B that moves faster on SDS gels than the wild-type PBP 2B (61). We confirmed that PBP 2B from this strain did indeed move slightly faster than the normal protein (Fig. 1, compare lanes a and b) and that the mutant's shape was abnormal. During growth at various temperatures the cells looked like bloated rods, and none of them appeared to have straight side walls when observed with a phase-contrast microscope.

The above-described mutant was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis and did not revert (61), which suggested to us that its phenotype could be due to more than one genetic defect. We took advantage of our knowledge of the chromosomal location of *pbpB* to move the mutant's *pbpB* gene to a different genetic background. Transducing phage PBS1 was grown on UB8524 and used to transduce BGSC1A6 to *pyrD*$^+$. Thirty-two transductants were screened for their PBP phenotype; 17 of them had a PBP 2B that moved faster than the recipient's PBP 2B. Thus, the alteration in PBP 2B was 53% linked to *pyrD* by transduction. This placed the mutation within or very near *pbpB*, the structural gene for PBP 2B.

The phenotypes of two *pyrD*$^+$ transductants, one with a PBP 2B like that of the donor and the other with a PBP 2B like that of the recipient, were examined further. There was virtually no difference in the morphology, or growth properties of the two transductants; they both resembled those of the recipient strain. The transductants and the recipient all grew as normal rods, and at temperatures of 30, 37, or 42°C their generation times were less than half of those of the original mutant strain UB8524. Thus, the altered PBP 2B cannot alone be responsible for the abnormal shape and slow growth of UB8524.

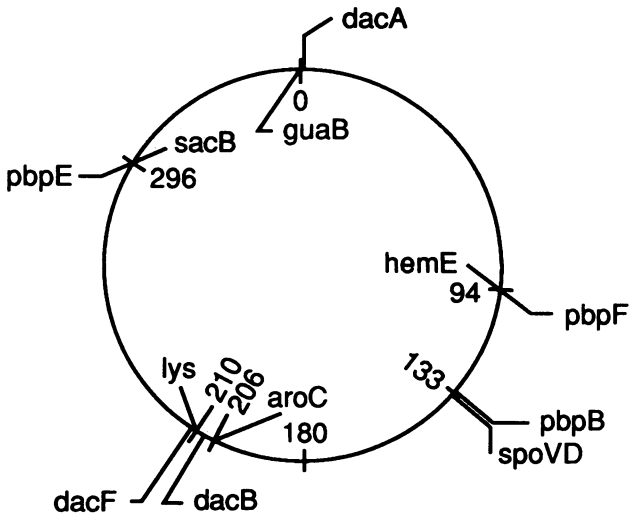

FIG. 7. Genetic map of *B. subtilis* with the positions of the genes that code for authentic and putative PBPs indicated on the outside of the ring and nearby markers indicated on the inside of the ring. The *pbpF* locus corresponds to the locus formerly named *ponA* (29, 57). The numbers indicate the position on a scale of 360°.

DISCUSSION

The gene for PBP 2B, a high-MW PBP from *B. subtilis*, has been cloned, sequenced, and located on the genetic map. It was named *pbpB* (*pbpB*$_B$) to highlight the functional and sequence similarities of its product to that of PBP 3, which is encoded by the *pbpB* gene (*pbpB*$_E$) in *E. coli*. Analysis of its primary structure suggests that PBP 2B is a typical high-MW PBP of class B. High-MW PBPs of both classes A and B have a modular structure composed of at least two domains: an amino-terminal domain of >200 amino acids and a penicillin-binding domain (24). A membrane-spanning hydrophobic sequence at the amino terminus serves as an uncleaved signal sequence and membrane anchor for these proteins. They are essentially extracellular with very little, if any, of their structure on the cytoplasmic side of the membrane. The feature that places PBP 2B among the PBPs of class B is the presence of all nine conserved boxes in its sequence (Fig. 5) (55); the class A proteins do not have boxes 2, 3, 5, and 9 (21, 25). A feature that distinguishes PBP 2B from some of the class B proteins, particularly those of gram-negative origin, is the length of its carboxy-terminal extension. This region, which begins 60 residues beyond box 8, may correspond to a third functional domain.

The genes for five PBPs of *B. subtilis* have now been sequenced and mapped (Fig. 7). PBP 5 is a vegetative low-MW PBP encoded by the *dacA* gene (10, 71). It accounts for about 95% of the DD-carboxypeptidase activity in vegetative cells (4). PBPs 4* and 5* are low-MW sporulation-specific PBPs encoded by the *pbpE* and *dacB* genes, respectively (11, 13, 56, 64). PBP 4* has no known catalytic activity, and it appears to be dispensable, whereas PBP 5* is required for cortex synthesis (11, 56). PBP 5* may also be a DD-carboxypeptidase (70). SpoVD, encoded by the *spoVD* gene, is also a sporulation-specific PBP that is required for cortex synthesis (16). Unlike PBP 5*, however, the SpoVD protein is a high-MW PBP, and

it bears a striking degree of similarity to vegetative PBP 2B (Table 2).

In addition to the genes for five authentic PBPs, the genes for two putative PBPs have been sequenced. The *dacF* gene is expressed in the forespore relatively late during sporulation, and its putative product, which has not been identified, has significant homology to the other low-MW PBPs (76). For this reason, it is assumed that DacF may also be a DD-carboxypeptidase. The gene for what appears to be another high-MW PBP is located near *hemE* on the *B. subtilis* chromosome (29) (Fig. 7). Its product has the most sequence homology with PBP 1A of *E. coli*, and consequently, the gene was initially named *ponA*. However, more-recent studies indicate that this gene does not encode PBP 1 of *B. subtilis* (57). The gene linked to *hemE* has been renamed *pbpF*, so the *pon* designation can be reserved for the PBP 1 gene.

The sequence of PBP 2B of *B. subtilis* is most similar to the sequences of those PBPs that are essential to their host cells (Table 2). PBP 2X of *Streptococcus pneumoniae* R6 and PBP 2 of *Neisseria meningitidis* (and *Neisseria gonorrhoeae*) are believed to be the primary targets for β-lactam antibiotics in their respective hosts (20, 42, 78). The homology with PBP 2X is particularly noteworthy, because until now, PBP 2X has been compared primarily with the high-MW PBPs from gram-negative organisms (42). Both its N-terminal domain and its penicillin-binding domain are more homologous to the corresponding regions in PBP 2B of *B. subtilis* than to any other PBPs that have been sequenced. Whether this reflects a subtle difference between the high-MW PBPs of gram-negative and gram-positive cells or a closer evolutionary relationship between these two particular PBPs is not known. PBP 3 of *E. coli*, another homolog of PBP 2B, is also an essential PBP; when this PBP is inactivated, the cells form filaments and die (65). Only conditional *pbpB* mutants can be isolated (66). PBP 3 is believed to be required specifically for cell division in *E. coli*.

The similarity of PBP 2B with *E. coli* PBP 3 supports our hypothesis that PBP 2B serves a comparable role in cell division in *B. subtilis*. Further support comes from the discovery that $pbpB_B$ was located in a large cluster of genes at 133° to 135° on the *B. subtilis* chromosome that are involved in cell division and cell wall metabolism. With a few exceptions, products of the genes in this cluster have sequence and presumably functional homology with proteins encoded by a similar cluster of genes located at 2 min (the *mra* region) on the *E. coli* chromosome (12, 17, 22, 33). The order of the corresponding genes in the two clusters is also highly conserved, so that the genes upstream and downstream of $pbpB_E$ are homologous to the genes that flank $pbpB_B$, although there is one intriguing difference.

Immediately downstream of the $pbpB_E$ gene and overlapping it by 14 bp is *murE*, which encodes UDP-MurNac-tripeptide synthetase (44). The homolog of *murE* in *B. subtilis* (17) is also downstream of $pbpB_B$, but between the two genes is *spoVD*. The SpoVD protein is 29% identical in sequence to PBP 3 from *E. coli* and 35% identical to PBP 2B of *B. subtilis* (16) (Table 2). Thus, it appears as if duplication of the *pbpB* gene occurred during evolution of the sporulating organism, so that one PBP can now serve a function that is required by both vegetative and sporulating cells (i.e., septum formation) and the other can serve a function that is unique to sporulation (i.e., cortex synthesis). The penicillin-binding domains of SpoVD and PBP 2B are much more homologous than their N-terminal domains (Table 2). This suggests that the functional differences of these two PBPs are most likely to be reflected in the catalytic differences of their N-terminal domains.

In *E. coli* the activity of PBP 2 depends on the presence of another membrane protein, RodA; their joint role is to support or participate in peptidoglycan synthesis during elongation of the cell (35). RodA is 31.9% identical to FtsW, which also appears to be a membrane protein. Because *ftsW* mutants of *E. coli* are defective in cell division and the FtsW protein is so similar to RodA, it has been proposed that FtsW may interact with PBP 3 to promote peptidoglycan synthesis during septation (34). Thus, the two pairs, RodA-PBP 2 and FtsW-PBP 3, are participants in different morphogenetic events. In *B. subtilis* the only known FtsW homolog is SpoVE; the two proteins are 40% identical to one another (34). The *spoVE* gene is located in the same gene cluster as $pbpB_B$ and *spoVD*, and its position in this cluster relative to those of these two PBP genes is the same as the position of *ftsW* relative to that of $pbpB_E$ in *E. coli*. The question that arises is whether either or both of the *B. subtilis* PBPs, PBP 2B and SpoVD, interact with the SpoVE protein. We and others have suggested that it is most likely that SpoVE interacts with SpoVD because both of them are required for synthesis of the spore cortex (16, 33). Although this does not exclude an additional interaction with PBP 2B, *spoVE* mutants are not blocked in asymmetric septation and engulfment (54). Thus, the stage in sporulation when PBP 2B is most prominent does not depend on the activity of SpoVE. We predict that there is a vegetative homolog of SpoVE that acts in conjunction with PBP 2B during vegetative growth and perhaps also in formation of the spore septum.

The putative start codon of the $pbpB_B$ gene overlaps the stop codon for the upstream gene, *orfA* (Fig. 2). This arrangement is different from that found in the *E. coli* gene cluster, in which the upstream gene ends 15 bp before the start codon of $pbpB_E$ (27). The upstream gene for *E. coli* has recently been named *ftsL* and *mraR* by two groups who independently demonstrated a requirement for the gene product in cell division (27, 73). The FtsL protein is a membrane protein with 37 amino acid residues as its N terminus exposed to the cytoplasm. The periplasmic carboxy-terminal domain of the protein has a sequence that could constitute a leucine zipper, which raises the possibility that this protein can form dimers with itself or other proteins (27). The FtsL protein had only limited sequence similarity to the derived product of *orfA* from *B. subtilis*, although they were nearly the same length. In addition, there was no apparent leucine zipper motif in the *B. subtilis* protein. However, they both had similarly positioned hydrophobic domains, and our unsuccessful attempts to insertionally interrupt *orfA* suggest that it too may encode an essential membrane protein. It is also possible that interruption of *orfA* would prevent expression of the essential $pbpB_B$ gene downstream. The likelihood that *orfA* and $pbpB_B$ are cotranscribed from a promoter upstream of *orfA* is supported by our failure to obtain integration of the plasmid pER670. The proximity of the *ftsL* and $pbpB_E$ genes in *E. coli* also suggests the possibility of cotranscription, but the presence of a promoter for $pbpB_E$ within *ftsL* has not been ruled out (27).

Although PBP 2B was an active membrane protein in *E. coli*, in the sense that it could bind penicillin, we have no unambiguous evidence that it can perform a function or interfere with normal *E. coli* functions. The presence of *B. subtilis* PBP 2B in the *E. coli* inner membrane had no apparent effect on normal cells, with one exception: cells containing pAY18 had a much slower growth rate. The level of expression of the *pbpB* gene on this plasmid was substantially greater than for the other plasmids (not shown), which was probably due to transcription from the *lac* promoter on the vector. However, the effect of this expression on the growth rate may not be due to the activity of PBP 2B but rather to the presence of an excessive amount of

this foreign protein in the cell membrane. Another approach we tried was to test whether the $pbpB_B$ gene could complement an *E. coli pbpB* (*ftsI*) mutant. Three different plasmids from which PBP 2B was synthesized in *E. coli* were tested, but no complementation of the temperature-sensitive phenotype was observed. The negative results are consistent with the outcome of other efforts to demonstrate complementation between these two organisms with various homologous genes involved in cell division (1).

We have established the precise chromosomal location of the *B. subtilis pbpB* gene to be 133° by DNA sequencing and identification of the neighboring genes. Its presence in this general area had been suspected since it was discovered that the PBP 2B phenotype of BR151 was cotransducible with *pyrD*, which maps at 139° (9). Subsequently, the PBP 2B phenotype of UB8524 was found to be 53% cotransducible with *pyrD*. This linkage value is similar to the 54% cotransduction of the *ts31* mutation with *pyrD* (45). Despite this coincidence, there is no evidence that *ts31* is actually located within *pbpB*. In fact, an examination of the PBPs of the *ts31* mutant after growth at 30 or 42°C did not reveal any abnormalities in its PBP profile (9). It is possible that the *ts31* mutation is located in the *orfA* gene upstream of *pbpB*. The temperature-sensitive cell division phenotype of this *B. subtilis* mutant is similar to that of the *fts-36* mutant of *E. coli*, whose defect was recently mapped to *orfA* (*mraR*, *ftsL*) (73).

It would be helpful to know what function, if any, is served by the carboxy-terminal extension on PBP 2B, because this region is long enough that it could be a separate catalytic domain (21, 55). The fact that both PBP 2B and SpoVD have a lengthy carboxy extension, which is mostly absent from the structure of their *E. coli* homolog PBP 3, is intriguing (Fig. 5). Our preliminary results with cells that produced a carboxy-truncated version of PBP 2B suggest that at least part of the carboxy extension is dispensable (Fig. 6). The mutant cells were able to divide, but apparently at a reduced frequency. This defect led to the formation of relatively short filaments (typically, no more than 10 cell lengths). A similar phenotype was obtained when PBP 3 of *E. coli* was shortened to 560 residues, which brought its carboxy terminus to within 10 residues of its penicillin-binding domain (30). The mutant cells grew as a mixture of rods and filaments, and the truncated PBP 3 was unstable in vivo (30). The fact that the observed size of truncated PBP 2B on SDS gels of *B. subtilis* membranes was substantially less than what we expected suggests that it too is unstable. We have not yet examined more severe truncations of the carboxy terminus that approach as close to the penicillin-binding domain of PBP 2B as the truncation described above for *E. coli* PBP 3. Nevertheless, the phenotype of this one *B. subtilis* mutant with a shortened PBP 2B supports our hypothesis that this PBP has an essential role in cell division.

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