Cloning, Nucleotide Sequence, Mutagenesis, and Mapping of the *Bacillus subtilis* *pbpD* Gene, Which Codes for Penicillin-Binding Protein 4

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The gene encoding penicillin-binding protein 4 (*PBP 4*) of *Bacillus subtilis*, *pbpD*, was cloned by two independent methods. *PBP 4* was purified, and the amino acid sequence of a cyanogen bromide digestion product was used to design an oligonucleotide probe for identification of the gene. An oligonucleotide probe designed to hybridize to genes encoding class A high-molecular-weight PBPs also identified this gene. DNA sequence analysis of the cloned DNA revealed that (i) the amino acid sequence of *PBP 4* was similar to those of other class A high-molecular-weight PBPs and (ii) *pbpD* appeared to be cotranscribed with a downstream gene (termed *orf2*) of unknown function. The *orf2* gene is followed by an apparent non-protein-coding region which exhibits nucleotide sequence similarity with at least two other regions of the chromosome and which has a high potential for secondary structure formation. Mutations in *pbpD* resulted in the disappearance of *PBP 4* but had no obvious effect on growth, cell division, sporulation, spore heat resistance, or spore germination. Expression of a transcriptional fusion of *pbpD* to *lacZ* increased throughout growth, decreased during sporulation, and was induced approximately 45 min into spore germination. A single transcription start site was detected just upstream of *pbpD*. The *pbpD* locus was mapped to the 275 to 280° region of the chromosomal genetic map.

Peptidoglycan is the major structural element in eubacteria. The structures of the peptidoglycans of a wide variety of species have been determined in general terms, but the finer details of the structures and their syntheses are not well understood. A general picture has emerged in which transglycosylase activities polymerize the glycan strands and transpeptidase activities cross-link these strands via their peptide side chains (reviewed in reference 17). These two enzymatic activities have been found to reside in the family of penicillin-binding proteins (PBPs). The PBPs can be grouped into three classes based on protein sequence similarities, the class A and class B high-molecular-weight PBPs and the low-molecular-weight PBPs. Transglycosylase activity has been clearly demonstrated for a class A high-molecular-weight PBP. Both class A and class B high-molecular-weight PBPs have been shown to possess transpeptidase activity. The low-molecular-weight class of PBPs has generally been found to have d-d-carboxypeptidase activity. It has often been found that a species will possess multiple PBPs of a particular class which exhibit fully or partially redundant functions.

We have begun work to identify the genes encoding the PBPs of *Bacillus subtilis* in order to facilitate understanding of the roles that the individual PBPs play in growth and sporulation of this organism. Seven high-molecular-weight PBPs and four low-molecular-weight PBPs have been identified in *B. subtilis* by biochemical methods (6, 8, 13, 27, 37, 43). The genes encoding six of these proteins have now been identified (10, 13, 33–35, 44, 49). In addition, one gene which is predicted to encode a PBP on the basis of sequence similarities has been identified, but the protein product of this gene has not been identified (48). We have developed a method for isolating genes encoding class A high-molecular-weight PBPs which may be generally applicable to a wide range of species. We report here the use of this technique to isolate the *B. subtilis* *pbpD* gene encoding *PBP 4*. We have determined the nucleotide sequence of this gene, carried out studies of its expression, determined its position on the *B. subtilis* genetic map, and examined the effects of a *pbpD* mutation.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. All *B. subtilis* strains were derivatives of strain 168. The *sigD* mutation was *sigD::plM5* (22). Growth for sporulation, membrane preparation, and studies of gene expression was in 2×SG medium (28) at 37°C. Minimal growth medium was Spizizen's minimal medium (38). Spores were purified by water washing as previously described (32). Spores were heat activated at 70°C for 30 min prior to germination at 37°C in 2×YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) containing 4 mM L-alanine (32). *B. subtilis* was transformed as previously described (4).

Purification and amino acid sequence analysis of PBP 4. Membranes were prepared from *B. subtilis*, and PBPs were purified by penicillin affinity chromatography as previously described (33). PBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide gels were electrophoresed at 40 mA for 4 h). PBP 4 was electroeluted from gels by using an Elutrap apparatus (Schleicher & Schuell), concentrated by using Centricon-30 units (Amicon) and trichloroacetic acid precipitation, and digested overnight at 22°C with cyanogen bromide in 70% formic acid. Cyanogen bromide digestion products were dried under vacuum and separated by reverse-phase high-pressure liquid chromatography as previously described (11). One purified peptide was subjected to automated sequencing as previously described (11).

Cloning of *pbpD*. A degenerate oligonucleotide (29-mer;
5'SARGGNN5WSNACHTNACRCARCRYT 3', where H = A, C, or T; N = A, C, G, or T; R = A or G; S = C or G; W = A or T; and Y = C or T) encoding all possible combinations of a 10-amino acid consensus sequence derived from class A high-molecular-weight PBPs (Q/E-G-A/G-S/T-T/A-I/L-T-Q-Q-L) was synthesized. The oligonucleotide was 3' end labeled with digoxigenin (Genius kit 6; Boehringer Mannheim) and used to screen a AEMBL3 library of random 10 to 20-kb Sau3AI partial digestion products of B. subtilis 168 chromosomal DNA. DNA was prepared from hybridizing ϕ phages and subjected to restriction digestion and Southern hybridization. A hybridizing 1.4-kb HindIII fragment from one of these ϕ phages was subcloned into HindIII-digested pUC19 to produce pDPC111 (Fig. 1). The 543-bp HindIII-Rsal fragment of pDPC111 was inserted into HindIII-HindIII-digested pUC19 to produce pDPC112. The small EcoRI-HindIII fragment of pDPC112 was inserted into EcoRI-HindIII-digested pJH101 (15) to produce pDPC127 (Fig. 1). Transformation of B. subtilis with pDPC127 led to integration of the plasmid into the pbpD locus by a Campbell-type recombination. Chromosomal walking steps were carried out as previously described (34). Digestion of the chromosome with SalI and with Stul led to the production of pDPC141 and pDPC142, respectively (Fig. 1). Additional subclones from pDPC111, pDPC141, and pDPC142 were subjected to DNA sequencing using a Sequenase kit (U.S. Biochemical). The sequence reported was determined on both strands, and all restriction sites used for subcloning were sequenced across.

Construction of mutants and lacZ fusion strains. The 365-bp HindIII-EcoRV fragment of pDPC111 was inserted into HindIII-Smal-digested pUC19 to produce pDPC115. The small EcoRI-HindIII fragment of pDPC115 was inserted into EcoRI-HindIII-digested pJH101 to produce pDPC147 (Fig. 1). Digestion of pDPC111 with HindII followed by ligation led to the production of pDPC129. The small EcoRI-HindIII fragment of pDPC129 was inserted into EcoRI-HindIII-digested pJH101 to produce pDPC163 (Fig. 1). The 515-bp HindII-Sphi fragment of pDPC142 was inserted into HindII-digested pUC19 to produce pDPC158. The 879-bp EcoRI-Sau3AI fragment of pDPC142 was inserted into EcoRI-BamHI-digested pUC19 to produce pDPC169. The small EcoRI-HindIII fragments of pDPC158 and pDPC169 were inserted into EcoRI-HindIII-digested pJH101 to produce pDPC178 and pDPC179, respectively (Fig. 1). Plasmids pDPC163, pDPC147, pDPC178, and pDPC179 were transformed into B. subtilis with selection for chloramphenicol resistance in order to isolate strains in which the plasmids had integrated into the chromosome via a Campbell-type mechanism. Insertions of pDPC163 and pDPC147 produced mutations of the msbB homolog gene and of pbpD, respectively. Insertions of pDPC178 and pDPC179 interrupt the pbpD operon structure. The ermC cassette was extracted from pPS937 (33) with Smal and HindII and inserted into EcoRV-HindIII-digested pDPC141 to produce pDPC186 (Fig. 1). This plasmid was linearized with ScaI and transformed into B. subtilis with selection for erythromycin and lincomycin resistance in order to produce strain PS2022, in which a part of pbpD is replaced with the ermC gene.

The pUC19 multiple cloning site was inserted into EcoRI-HindIII-digested pJF751A (41) to produce pDPC87. The 1.2-kb HindII-BamHI fragment of pDPC111 was inserted into Smal-BamHI-digested pDC87 to produce a transcription fusion of pbpD to lacZ in pDPC145 (Fig. 1). This plasmid was transformed into B. subtilis with selection for chloramphenicol resistance in order to produce strain PS1956 containing a Campbell-type insertion at the pbpD locus. The 1.2-kb EcoRI-BamHI fragment of pDPC145 was inserted into EcoRI-BamHI-digested pDG268 (5) to produce pDPC181 (Fig. 1). Digestion of pDPC181 with NsiI followed by treatment with T4 DNA polymerase and digestion with BamHI allowed the isolation of an 850-bp fragment in which the NsiI-digested end was blunt. This fragment was inserted into pDG268 which had been digested with EcoRI, treated with T4 DNA polymerase, and digested with BamHI to produce pDPC187 (Fig. 1). The two derivatives of pDG268, pDPC181 and pDPC187, were linearized with ScaI and transformed into B. subtilis with selection for chloramphenicol resistance. The resulting strains contained transcriptional fusions of the beginning of pbpD to lacZ in the chromosome at the amyE locus. The correct chromosome structures in the pbpD operon and amyE regions of all mutant and fusion strains were verified by Southern hybridizations.

The antibiotic resistance associated with pDPC145 inserted in the pbpD locus was switched from chloramphenicol to spectinomycin by transformation with linear pJL62 (pJL62, constructed by J. Ledeaux, has a spectinomycin resistance gene inserted into the Ncol site of pJH101). The sigD::pLM5 mutation was moved into this strain by transformation with limiting chromosomal DNA from strain CB100 (22) and selection for chloramphenicol and spectinomycin resistance. The resulting strain retained the pbpD-lacZ fusion at the pbpD locus and had obtained two phenotypes associated with the sigD mutation, loss of motility and the formation of long chains of cells (22).

Enzyme assays. β-Galactosidase was assayed by using o-nitrophenyl-β-galactopyranoside as previously described (32). PBPs were detected by using [3H]benzylpenicillin (Amersham) as previously described (37).

Primer extension transcript mapping. RNA was purified from B. subtilis, and primer extension was carried out as previously described (33); 40 μg of RNA was used in each reaction. Two oligonucleotides, complementary to positions...
888 to 909 and 971 to 988, were $^{32}$P end labeled and used as previously described (33). DNA sequence for determination of the primer extension product sizes was generated by using these same end-labeled primers and supercoiled pDPC111 as the template.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in the GenBank sequence database under accession number U11882.

RESULTS

Cloning of the pbpD locus. The gene encoding PBP 4 of B. subtilis was isolated by two independent methods. The first method involved purification of PBP 4 and determination of the amino acid sequence of a cyanogen bromide digestion product of this protein. The sequence (M)KDGVKITYALD PYM was found. A degenerate oligonucleotide encoding the first 10 amino acids of this sequence was used to screen a λEMBL3 library of B. subtilis DNA. Two recombinant phages which contained sequences exhibiting strong hybridization to this oligonucleotide probe were isolated. Subcloning and partial DNA sequencing of the insert from one of these phages revealed a coding sequence containing the amino acid sequence determined from PBP 4 as well as sequences similar to those found in the carboxy-terminal regions of other high-molecular-weight PBPs.

The pbpD gene was also isolated concurrently by screening the same λEMBL3 library for a coding sequence that is conserved in the amino-terminal regions of class A high-molecular-weight PBPs. Previous analysis of such coding sequences had revealed the very highly conserved 10 amino acid sequence Q/E-G-A/G-S/T-T/A-L/I/T-Q-Q-L (34). A degenerate oligonucleotide containing all possible coding sequences for this consensus peptide was initially used to probe a Southern blot of B. subtilis chromosomal DNA. Four strongly hybridizing HindIII fragments were detected, one of which was of the size expected for the previously characterized pbpF gene (34). Screening of the λEMBL3 library with this probe resulted in the isolation of recombinant phages carrying three of the strongly hybridizing sequences. One of these sequences was found to be part of the pbpF gene, and a second was found to be in the ponA gene encoding PBPs 1a and 1b (35). Partial DNA sequencing of the insert from the third hybridizing phage revealed coding sequences similar to those found in the amino-terminal regions of class A high-molecular-weight PBPs. A subclone from this phage was used to perform a chromosomal walking procedure to isolate the remainder of the gene. The chromosomal fragment obtained overlapped that found in the λ phage described above that contains PBP 4-encoding sequences. Subclones from the λ phage containing the amino-terminal part of the gene and from the chromosomal walking step were used for the determination of the DNA sequence of the complete gene.

Organization of the pbpD locus. Examination of the complete DNA sequence of this region revealed an apparent two-gene operon (Fig. 1). On the basis of sequence similarities, the first gene is predicted to encode a class A high-molecular-weight PBP. There are two possible start codons for this gene (Fig. 2), of which we favor the proximal one because of its spacing from a potential ribosome binding site. We attempted to determine the amino-terminal sequence of PBP 4, but the amino terminus of the protein was blocked. Translation initiation at the proximal start codon would result in a protein of 624 residues with a molecular weight of 70,593, which agrees well with the PBP 4 molecular weight of 67,000 predicted from the results of SDS-PAGE. We have named this gene pbpD in accordance with the nomenclature recommended by Buchanan et al. (9). A 137-codon open reading frame, referred to here as orf2, encoding a protein with a molecular weight of 15,696 is found immediately downstream of and apparently cotranscribed with pbpD (Fig. 1 and 2). The predicted protein product of orf2 exhibits no significant sequence similarity to any entry found in DNA and protein sequence databases. Upstream of pbpD and in the same orientation is a 256-codon partial open reading frame (Fig. 1 and 2) encoding a protein exhibiting sequence similarity with the msyB gene of Escherichia coli (45).

Though there is no obvious transcription terminator between this gene and pbpD, we suspect that this gene is not in an operon with pbpD because of the distance between the two genes and the results of our studies on pbpD expression (see below). Disruption of this upstream gene by a Campbell-type insertion of pDPC163 (Fig. 1) had no effect on sporulation or on growth in rich or minimal medium (data not shown). Disruption of the E. coli msyB gene also had no effect on growth (45).

Downstream of the proposed pbpD operon we found an apparently untranslated region of over 240 bp. There does not appear to be the end of any gene originating outside of the sequenced region, there are no obvious translation initiation signals, and there is no open reading frame over 47 codons in either orientation. In this region there are four regions of significant dyad symmetry (Fig. 2 and 3), one of which may serve as a transcription terminator for the pbpD operon. A 78-bp sequence containing one of these symmetrical sequences as well as a flanking region is >85% conserved (allowing for the introduction of a single small gap in each sequence) in two other sequenced regions of the B. subtilis chromosome (Fig. 3), one upstream of the sfp gene (12, 20) and another in the 325 to 333° region of the chromosome (19). The sequence conservation between the latter two regions extends over a larger region, an additional 97 bp with 86% sequence identity, than that found downstream of the pbpD operon (Fig. 3). Each of these sequence elements contains several regions of dyad symmetry, some of which are indicated in Fig. 3. The significance of this repeated sequence element is unknown.

We examined the effects of several mutations in the pbpD operon, all of which probably resulted in the loss of expression of orf2. Neither a Campbell-type plasmid insertion in pbpD (pDPC147; Fig. 1) nor a deletion of part of pbpD in which the deleted region was replaced with an antibiotic resistance cassette (pDPC186; Fig. 1) resulted in an observable phenotype when tested for growth on rich or minimal medium or for sporulation and spore germination. However, the deletion mutation did result in the disappearance of PBP 4 from the cell membranes (Fig. 4). The apparent overproduction of PBP 3 in the pbpD mutant (Fig. 4) was not reproducible. Two Campbell-type insertions, one in which the downstream endpoint is 6 bp beyond the pbpD stop codon (pDPC179; Fig. 1) and one in which the downstream end is inside orf2 (pDPC178; Fig. 1), had no effect on the size or amount of PBP 4 (data not shown), indicating that these are truly two genes and not simply a single gene divided by a sequencing error.

Expression of pbpD. We examined the expression of pbpD by using a transcriptional fusion of pbpD to lacZ (pDPC145) which was integrated at the pbpD locus in such a way that pbpD expression was not affected. Expression of pbpD increased during vegetative growth and began to decrease soon after the entry into sporulation (Fig. 5). The slight increase in β-galactosidase activity in late sporulation and the small amount of activity found 21 h into sporulation were equivalent to the endogenous activities found in strains lacking a lacZ fusion. No β-galactosidase activity was found in the spores of the fusion
strain. Expression resumed approximately 45 min after exposure of the spores to germinants. The same pattern and level of expression were observed when the predicted pbpD promoter region was fused to lacZ (pDP1C181 and pDP1C187; Fig. 1) and placed in the chromosome at the amyE locus (data not shown). This result indicates that all sequences required for full expression of pbpD are found in the 330 bp preceding the translational start codon in pDP1C187 and that pbpD is not in an operon with the upstream gene.

Primer extension mapping was used to determine the pbpD transcription start site during growth, sporulation, and germination. At all times, a single start site was observed 20 bp upstream of the proposed pbpD start codon (Fig. 6). The same start site was found when a second oligonucleotide primer was used (data not shown). There are no strong matches to the consensus σ^A promoter recognition sequences upstream of the transcription start site. The best match to the σ^A promoter −10 consensus (TATAAT) is TACGAT, and the best match to the −35 consensus (TTGACA) is TTCTCG (Fig. 2). These two potential promoter recognition sequences are separated by the consensus 17 bp. There is, however, a good match (TAC GATAT) to the consensus σD −10 recognition element (GC CGATAT) (21) and a match (TAAT) to the σD −35 consensus sequence (TAAA) (21). The spacing between these two elements is only 13 bp as opposed to the consensus 15 bp (21). This match to the σD promoter recognition consensus and the fact that pbpD expression increased throughout vegetative growth, a characteristic of σD-controlled genes, led us to examine the possibility that transcription of pbpD was dependent on σD. However, expression of a pbpD-lacZ fusion was normal in a sigD (the gene encoding σD) mutant background (data not shown), indicating that this σ factor is not involved in pbpD transcription.

Chromosomal location of pbpD. The B. subtilis chromosome was digested with restriction enzymes NorI and SfiI. The resulting fragments were separated by pulsed-field gel electrophoresis, transferred to a filter membrane, and hybridized with a pbpD-specific probe. The probe hybridized to a 143-kb SfiI fragment and to a NorI fragment of approximately 60 kb. These data allowed us to limit the possible locations of pbpD to three NorI fragments on the B. subtilis physical genome map (25). Linkage of a chromaphilin restriction marker in the pbpD gene (pDP1C127) to these three regions of the chromosome was tested by PBS1 transduction. It was found that pbpD was 5, 6, 13, 89, and 97% cotransduced with the argGH, sspA, aroG, alaA::Tn917 (46) and ala-1 loci, respectively. These data place pbpD on the 27N NorI fragment and the MS SfiI fragment on the physical map of the B. subtilis genome (25) and in the 275 to 280° region on the genetic map (3).

DISCUSSION

We report here the characterization of the B. subtilis pbpD gene encoding PBP 4. The gene was isolated both by a conventional reverse genetic technique utilizing a PBP 4 peptide sequence to design an oligonucleotide probe as well as by a novel technique designed to identify genes encoding class A high-molecular-weight PBPs. This technique, utilizing a degenerate oligonucleotide encoding a peptide sequence conserved among these proteins, identified three of these genes in B. subtilis: pbpD, pbpF (34), and ponA (35). This technique should be applicable for a wide variety of bacterial genera, as the peptide sequence involved is well conserved in the genes of both gram-negative and gram-positive species (Fig. 7). However, the consensus sequence that we used will have to be expanded in order to make the technique universally applicable. Recently published sequences containing genes or partial genes of this class reveal deviations from our consensus, most notably the presence of a methionine at position 8 and a valine at position 10 (Fig. 7). Other species may have different deviations from our consensus, but this might not preclude the success of this technique in identifying PBP-encoding genes. A B. subtilis gene which differed from our consensus at position 10 was still identified by this technique despite this coding difference (35).

The amino terminus of the pbpD product contains two basic residues followed by a hydrophobic stretch of 22 residues that is predicted to be a signal peptide. There is a possible signal peptidase cleavage site after residue 28. The -1, −3 rule (47) is satisfied by the presence of glycine and alanine, respectively, at these positions with respect to the potential cleavage site. Verification of this signal peptidase cleavage site will require further analysis of PBP 4, since we found the amino terminus to be blocked to Edman degradation. The molecular weight of 67,000 predicted from the migration of PBP 4 in SDS-PAGE (9) (Fig. 4) is consistent with the molecular weights of the cleaved (67,008) and uncleaved (70,593) proteins predicted from the nucleotide sequence. PBP 4 may therefore have an uncleaved signal peptide which serves to anchor the PBP to the outer surface of the cytoplasmic membrane, as has been shown or suggested for a number of other PBPs (1, 14, 26, 34), or it may have a cleaved signal peptide, in which case another mechanism for anchoring the protein to the membrane would have to be imagined. A precedent for such an alternative membrane anchor was found in the E. coli PBP 1B when the signal peptide of this protein was removed (14, 31).

Examination of the DNA sequence downstream of pbpD and orf2 revealed an extragenic sequence element which is highly conserved in two other regions of the chromosome. We refer to this sequence element as Bs-rep. This 78-bp sequence is ≥85% conserved and includes a perfect dyad of ±17 bp. Two of the three examples of Bs-rep are flanked by an additional 97-bp conserved region of 86% sequence identity which contains part of a second large dyad. The fact that part of this second large dyad is within Bs-rep suggests that the additional 97-bp conserved region may actually be part of the Bs-rep element and that the element downstream of pbpD is partially deleted. This repetitive sequence element shares no sequence similarity with the repetitive extragenic palindromic (REP) (18, 23, 40) or enterobacterial repetitive intergenomic consensus (ERIC) (24, 36) sequences that have been found in enteric and a number of related bacteria (reviewed in reference 29). Bs-rep does, however, share at least two characteristics with these other elements. (i) Each contains palindromic sequences;
FIG. 3. Nucleotide sequence alignment of the conserved B. subtilis sequence element downstream of the pbpD operon. The sequence labeled ipa29 is the complement of positions 30879 to 31106 in GenBank accession number X73124. The sequence labeled sfp is found upstream of the sfp gene in GenBank accession numbers L17438 and X70356. The boxed nucleotides are termination codons for the unidentified gene ipa29 (19), for an unidentified gene upstream of sfp (29), and for oref2 of the pbdD operon. Asterisks indicate positions at which nucleotides are identical in the sequences above and below. Arrows above and below the sequences indicate regions of dyad symmetry. Only the most significant dyads are indicated; there are a large number of additional symmetrical features in each of these sequences.

multiple secondary structures can be predicted for single-stranded ERIC and Bs-rep sequences. (ii) Each is found in extragenic regions in either orientation with respect to transcription of flanking genes. Transcription of the genes flanking the Bs-rep elements labeled ipa29 and sfp in Fig. 3 is from right to left and from left to right, respectively. DNA replication is from right to left for both of these Bs-rep elements; the orientation of the pbdD region on the chromosome is unknown. Further experimentation is required to determine if the Bs-rep elements are transcribed as is the case for some REP and ERIC elements. Bs-rep resembles the ERIC sequence more than the REP sequence in size and predicted frequency. REP sequences are 38 nucleotides, and over 500 copies are present in E. coli. The ERIC sequence is 126 nucleotides, and approximately 30 copies are found in E. coli (possibly 150 copies are present in Salmonella typhimurium). We estimate that ≥15% of the B. subtilis genome has been sequenced and deposited in sequence databases. The presence of three Bs-rep sequences in the databases suggests that 15 to 20 copies may be present in the genome and that it is not widely spread among eubacteria.

Disruption of the pbdD gene leading to the loss of PBP 4,

FIG. 4. PBP profiles of B. subtilis strains. Membrane preparations from vegetative cells of strains PS2022 (pBpdDEermC; lane 1) and PS832 (wild type; lane 2) were incubated with [3H]penicillin and subjected to SDS-PAGE on a 7.5% polyacrylamide gel at 40 mA for 2 h. PBPs were detected by fluorography and are numbered on the left as described in references 6 and 27. Molecular mass markers indicated on the right were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

FIG. 5. Expression of a pbdD-lacZ fusion. Strains PS832 (wild type; ○) and PS1956 (pBpdD lacZ, insertion of pDPC145 at the pbdD locus; △) were grown and sporulated in 2×SG medium at 37°C. Purified spores were germinated in 2×YT medium containing 4 mM L-alanine. The left and right points labeled 0 on the x axis indicate the time of initiation of sporulation and the time of exposure of spores to germinants, respectively. Optical density decreased from 0 to 30 min during germination. The optical density at 30 min was used for the calculation of specific activity for points between 0 and 30 min during germination.
and probably to the loss of the orf2 product, resulted in no obvious phenotype. This is consistent with an earlier report of apparently normal B. subtilis strains which lack PBP 4 (7). This is the second case of dispensability of a class A high-molecular-weight PBP in B. subtilis. Disruption of pbpF, leading to the loss of PBP 2c (35), also resulted in no phenotype (34). A similar situation was observed in E. coli: either ponA or ponB could be disrupted without extreme consequence for the cells (39, 42). Loss of both genes in E. coli was lethal, suggesting that they had somewhat redundant functions (42, 50). Whether this holds true for the B. subtilis genes awaits construction of a multiple mutant. Such analysis is further complicated by the presence of a third gene encoding a class A high-molecular-weight PBP in B. subtilis (35). Possession of multiple PBPs with redundant functions might be a strategy for resistance to β-lactam antibiotics in the soil. Differences in the substrate binding sites of the proteins might allow them to carry out the same reactions in synthesis of the peptidoglycan and yet have differing sensitivities to the various classes of β-lactams. However, this explanation for the presence of multiple class A high-molecular-weight PBPs may be an oversimplification, since these different PBP genes exhibit different expression patterns during the B. subtilis life cycle. Expression of pbpF, measured by using a transcriptional fusion to lacZ, was relatively constant during vegetative growth, decreased during early sporulation, and was induced in the forespore during late sporulation (34). Expression of a pbpD-lacZ fusion increased throughout vegetative growth and decreased throughout sporulation. Production of β-galactosidase activity from this fusion was only reinitiated 45 min into spore germination. A very similar pattern of expression of the PBP 4 protein during sporulation (37, 43) and germination (30) was previously observed.

Analysis of transcripts indicated that a single promoter is used for pbpD expression throughout germination and growth. Examination of the sequence upstream of the transcription start site revealed a weak match to the consensus promoter sequences recognized by σ^A and a relatively strong match to the consensus promoter sequences recognized by σ^P. Expression of pbpD was, however, found to be independent of σ^P. It was previously observed that PBP 4 expression during spore germination initiated somewhat later than that of several other PBPs (30). We have found this to be true also at the transcriptional level (Fig. 5) (35). This transcriptional regulation could be simply the result of a very weak σ^A-dependent promoter, the presence of a promoter recognized by another sigma factor that is not active early in germination, or the activity of an unidentified transcriptional regulatory factor.

We now have in hand a significant number of the genes encoding PBPs in B. subtilis (10, 13, 33, 34, 35, 44, 48, 49). While null mutations in these genes often result in no phenotype, we expect that multiple mutations in genes encoding redundant functions will affect cell morphology and viability. The ability to manipulate the PBP complement of B. subtilis cells should allow us to discern more precisely the roles played.
by these proteins in the morphological changes that take place during growth and sporulation.

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