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Identification and Characterization of *pbpC*, the Gene Encoding *Bacillus subtilis* Penicillin-Binding Protein 3

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Penicillin-binding proteins (PBPs) are enzymes involved in the synthesis of peptidoglycan structures in *Bacillus subtilis* such as the vegetative cell wall and the spore cortex. The *B. subtilis* sequencing project has identified a gene (*orf16*, EMBL accession number D38161) which exhibits significant sequence similarity to genes encoding class B high-molecular-weight PBPs. We have found that *orf16* encodes PBP3 and have renamed this locus *pbpC*. Transcriptional fusions to *lacZ* were used to demonstrate that *pbpC* is transcribed primarily during log-phase growth, with lower amounts expressed during sporulation. During spore germination and outgrowth, *pbpC* expression resumes coincident with an increase in the optical density of the culture. The major promoter for *pbpC* is located just upstream of the gene; a low level of expression during sporulation appears to originate from much further upstream. Loss of PBP3 does not produce any detectable change in phenotype with respect to cell morphology, growth, sporulation, spore heat resistance, or spore germination and outgrowth. This was also true when the *pbpC* mutation was combined with mutations affecting other PBP-encoding genes to produce double mutants. These findings are consistent with previous evidence that many PBPs of *B. subtilis* have redundant functions within the cell.

Peptidoglycan synthesis is required for the elongation of the vegetative cell wall, septation, and formation of the spore cortex in gram-positive bacteria such as *Bacillus subtilis*. A class of proteins known as penicillin-binding proteins (PBPs) have been shown to exhibit the enzymatic activities involved in the polymerization and cross-linking of peptidoglycan (11). The penicillin-binding domain of PBPs has three highly conserved protein motifs, including SxxK, which contains the active-site serine that covalently binds penicillin and other β-lactam antibiotics (11). As this covalent interaction inhibits the activity of these enzymes, it is not surprising that modification of these proteins has been responsible for one type of β-lactam antibiotic resistance. One example is the synthesis of the novel low-affinity PBP2′ by methicillin-resistant *Staphylococcus aureus* (12).

We have undertaken the characterization of the genes encoding PBPs involved in the synthesis and modification of peptidoglycan in *B. subtilis*. To date, 11 different PBPs have been identified biochemically, and the genes encoding 9 of these PBPs are known (5, 6, 8, 9, 14, 26–29, 35, 37, 38, 40). However, the genes encoding two relatively abundant PBPs, PBP2a and PBP3, have yet to be identified. In addition, three genes, *dacF* (39) and two genes identified by the *B. subtilis* genome sequencing project (EMBL accession numbers Z34883 and D38161), appear to encode PBPs, but the protein products of these genes remain to be elucidated. The product of one of the genes identified by the *B. subtilis* genome sequencing project (*orf16* [2], EMBL accession number D38161, entered as the *ycsM* gene in *SubtiList* [18]) has significant amino acid sequence similarity to *Enterococcus faecium* PBP5 (39.6% identity in 639 residues), *S. aureus* PBP2′ (35.7% identity in 672 residues), and *Escherichia coli* PBP2 (28% in 446 residues), as determined by using tfastA software (10). The *orf16* gene maps to approximately 39° on the *B. subtilis* chromosome and encodes a putative 74.2-kDa PBP of 668 amino acids. Here we report the identification of the product of *orf16* as PBP3. In keeping with the nomenclature proposed by Buchanan et al. (7), we have renamed the *orf16* locus *pbpC*. The phenotypes of single *pbpC* mutant strains as well as strains in which mutations in other PBP-encoding genes were combined with the *pbpC* mutation have been examined during growth, sporulation, and spore germination. Transcriptional fusions of *pbpC* to *lacZ* were used to study expression of *pbpC* during growth, sporulation, and spore germination and outgrowth. Primer extension analysis was performed to determine the transcription start site of *pbpC*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains used were derived from strain 168 and are listed in Table 1. *B. subtilis* was routinely grown and sporulated in 2× SG medium (16). Spores were purified by repeated washing in distilled water as described previously (20). *B. subtilis* was transformed as previously described (3), and transformants were selected on the basis of resistance to the antibiotics chloramphenicol (3 μg/ml) and spectinomycin (100 μg/ml) or a combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml). Growth of *B. subtilis* for membrane preparations was at 37°C in either 2× SG or LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of distilled H_2O). Bacterial endospores were heat shocked for 30 min at 70°C in H_2O and germinated at 37°C in 2× YT medium (16 g of tryptone, 10 g of yeast extract, and 4 g of NaCl per liter of distilled H_2O) with 4 mM L-alanine as described previously (24). Spore heat resistance was measured as previously described (24) by heating spores in water at 90°C for 15 min. *E. coli* was grown at 37° in 2× YT with ampicillin (50 μg/ml).

Enzyme assays, membrane preparations, and peptide sequencing. β-Galactosidase assays of cells (20) and dormant spores (20, 25) by using the substrate *o*-nitrophenyl-β-D-galactopyranoside, labeling of PBPs with fluorescein-hexanoic-6-amino-penicillanic acid (FLU-C_6-APA) (30), and their separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (30) were done as described elsewhere.

For peptide sequencing total PBPs were isolated by penicillin affinity chromatography as described previously (26). Individual PBPs were separated by SDS-PAGE and transferred overnight to a polyvinylidene difluoride membrane (Immobilon-P^{sq}; Millipore) at 30 V in 25 mM Tris–192 mM glycine–0.1% SDS. Amino acid analysis of a tryptic fragment of PBP3 was obtained as described previously (21).

PCR amplification and cloning of *pbpC* and upstream regions. Primers were designed to PCR amplify two fragments, the first an 819-bp fragment which included 412 upstream bp and the first 407 bp of *orf16* (*pbpC*). The upstream

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TABLE 1. Bacterial strains used

B. subtilis strain	Genotype	Construction[a]	Source or reference
PS832	Derived from 168		Laboratory stock
PS1869	Δ*pbpF*::Erm	pDPC89→PS832	27
PS2022	Δ*pbpD*::Erm	pDPC186→PS832	28
PS2062	Δ*ponA*::Sp^r	pDPC197→PS832	29
PS2326	*pbpC-lacZ* at *pbpC*	pTMM2→PS832	This work
PS2327	*pbpC-lacZ* at *amyE*	pTMM3→PS832	This work
PS2328	*pbpC*::pTMM4 Sp^r	pTMM4→PS832	This work
PS2352	*pbpC*::Cm^r	pTMM5→PS832	This work
PS2363	*pbpC*::Cm^r Δ*ponA*::Sp^r	PS2062→PS2352	This work
PS2364	*pbpC*::Cm^r Δ*pbpF*::Erm	PS1869→PS2352	This work
PS2365	*pbpC*::Cm^r Δ*pbpD*::Erm	PS2022→PS2352	This work
PS2399	*pbpC-lacZ* at *amyE*	pTMM7→PS832	This work

[a] The arrow indicates transformation of plasmid or chromosomal DNA into the indicated strain.

primer for this product, M1 (33-mer 5′GGCGAATTCCGTCGGGAACGGGGTATAGAACCG3′), included an *Eco*RI site and flanking residues added to the 5′ end (underlined residues). The downstream primer, M2 (33-mer 5′GCCGGATCCCAATCTATATTCCATGACTCCTCG3′), included a *Bam*HI site as well as flanking residues at the 5′ end for cloning purposes (underlined residues). The second PCR product amplified (402 bp) was generated by using the M1 upstream primer and a different downstream primer, M3 (27-mer 5′CGGGATCCTTCCACTTGTTATTTTCTC3′), which also contained a *Bam*HI site and flanking residues at the 5′ end (underlined residues). This fragment ends 10 bp upstream of the *pbpC* coding sequence.

The 819-bp PCR product was cloned in plasmids pUC19, pJF751a (36), and pDG268 (4), generating plasmids pTMM1, pTMM2, and pTMM3, respectively (Fig. 1). pTMM1 was used for sequencing, while pTMM2 and pTMM3 were transformed into *B. subtilis* to generate transcriptional fusions at the *pbpC* (strain PS2326) and *amyE* (strain PS2327) loci, respectively (Table 1). The plasmid with the 402-bp insert ligated into pUC19 was termed pTMM6 (Fig. 1), and the insert in this plasmid, as well as in pTMM1, was sequenced either by the chain termination procedure (31) with a Sequenase kit (U.S. Biochemicals) or with an automated DNA sequencer (373 DNA Sequence System; Applied Biosystems), or by both procedures. The 402-bp PCR product was cloned in pDG268 (4) to generate pTMM7, which was transformed into *B. subtilis* to generate PS2399, containing a transcriptional *pbpC-lacZ* fusion at the *amyE* locus (Fig. 1).

pTMM1 was cut with *Spe*I and *Bam*HI, releasing a 387-bp fragment within the *pbpC* coding region. This fragment was ligated into pJL73 (15) also cut with *Spe*I and *Bam*HI to generate plasmid pTMM4 (Fig. 1), which was transformed into *B. subtilis* PS832 to produce a *pbpC*::pTMM4 (Sp^r) mutant (strain PS2328) (Table 1). The expected chromosomal structures of all constructs were confirmed by Southern blot analysis of appropriately digested chromosomal DNAs (data not shown) (34).

To generate a Cm^r *pbpC* mutant, the chloramphenicol cassette was cut out of pDG268 (4) and cloned in pTMM1 to generate pTMM5. This plasmid was transformed into *B. subtilis* PS832, resulting in integration into the chromosome at the *pbpC* locus via a double crossover event. The expected genomic structure of this strain, PS2352, was confirmed by Southern blot analysis (data not shown) (34), and this strain was used in subsequent generation of strains containing additional PBP-encoding gene mutations.

Primer extension. RNA from *B. subtilis* PS832 was extracted as previously described (26) or by a hot phenol extraction procedure (17). A 50-ml culture of *B. subtilis* PS832 was grown in 2× SG to an optical density at 600 nm of 3 and harvested for RNA extraction. Twenty-five and 50 μg of RNA were used in various primer extension reactions. Two oligonucleotides, PEC1 (complementary to positions 94 to 113 [Fig. 2]; 5′GCTTCCATGCGATCCTCTGG3′) and PEC2 (complementary to positions 52 to 71 [Fig. 2]; 5′TAACCCAATCAATCCGACGC3′), were ^{32}P end labeled and used in primer extension reactions as described previously (26). pTMM1 was used as the template for sequencing by the chain termination method with a Sequenase kit (U.S. Biochemicals). The sequencing reactions and the primer extension reactions were run on a denaturing 6% polyacrylamide gel, which was dried and exposed to X-ray film at −80°C with an intensifying screen for 14 days.

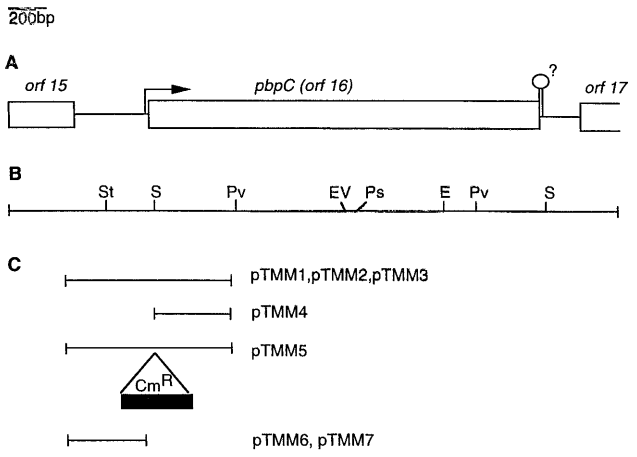

FIG. 1. Map of the *pbpC* (*orf16*) locus. (A) Open reading frames are represented by open boxes, and the arrow shows the direction of transcription. A putative transcription terminator is indicated at the end of *pbpC*. (B) Abbreviations for restriction endonuclease cleavage sites: St, *Stu*I; S, *Spe*I; Pv, *Pvu*II; EV, *Eco*RV; Ps, *Pst*I; E, *Eco*RI. (C) Map of plasmid constructs used in the generation of *pbpC-lacZ* fusions and *pbpC* mutant strains. The chloramphenicol cassette is not drawn to scale.

RESULTS

Identification and mutagenesis of *orf16* (*pbpC*). We used PCR amplification of DNA from the upstream region and beginning of *orf16* to construct plasmids for mutagenesis and *lacZ* fusions (Fig. 1). Two different PCR products were subjected to DNA sequence analysis, and in each case, the sequence was found to be identical to that previously reported

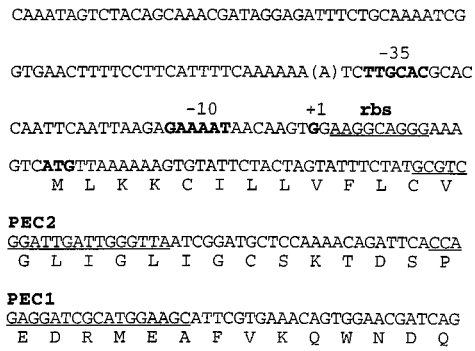

FIG. 2. Map of the *pbpC* N terminus and upstream regions (positions 14300 to 14548 in EMBL accession number D13168). The (A) is the extra residue that we found in sequencing; rbs is the putative ribosome binding site for *pbpC*. The potential promoter recognition sites are in boldface type, as is the putative transcription start site and the translation initiation codon. PEC1 and PEC2 are the two primers used in primer extension analysis.

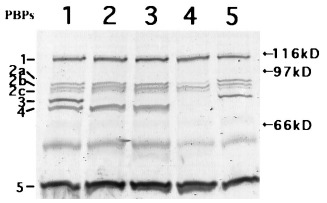

FIG. 3. Identification of the *pbpC* (*orf16*) product. Membrane preparations from 50-ml cultures of vegetative cells harvested at an optical density of 2.5 were incubated with FLU-C_6-APA and run on an SDS–10% polyacrylamide gel for 4 h at 100 V. Approximately 5 to 10 μg of protein was loaded in each lane. The FLU-C_6-APA-labeled PBPs were detected with a FluorimagerSI (Vistra) at a voltage of 500 V and a resolution of 100 μm during the scan. Lane 1, wild-type strain PS832; lane 2, *pbpC*::pTMM4 (Sp^r), PS2328; lane 3, *pbpC*::Cm^r, PS2352; lane 4, *pbpC*::Cm^r Δ*pbpD*::Erm, PS2365; lane 5, Δ*pbpD*::Erm, PS2022.

except that seven A residues, instead of the published six, were present at positions −44 to −38 (Fig. 2), 56 bases upstream from the open reading frame of *orf16*. This may be due to a sequencing error or may simply be due to strain differences.

Two different *orf16* mutants were constructed, one by Campbell insertion of pTMM4 (strain PS2328) and one by insertion of a Cm^r cassette through a double crossover (strain PS2352) (Table 1). Membranes were extracted from cultures of these mutant strains, and PBPs in the membranes were labeled with FLU-C_6-APA, separated via SDS-PAGE, and visualized with a fluorimager. PBP3 was not present in either mutant strain PS2328 or mutant strain PS2352 (Fig. 3, lanes 2 and 3). These results, along with the fact that *pbpC* encodes a putative protein of 74.2 kDa and PBP3 runs with an apparent mass of about 75 kDa on an SDS–10% polyacrylamide gel, strongly suggested that *orf16* encodes PBP3. In addition, sequence analysis of a purified tryptic peptide of PBP3 gave TFGFSEDVPYE, which corresponds to amino acids 492 to 502 of the predicted *pbpC* gene product. We have therefore renamed the *orf16* locus *pbpC* in accordance with nomenclature proposed by Buchanan et al. (7).

The mutant strains PS2328 and PS2352 displayed no observable change in cell morphology when examined under the light microscope (data not shown). Both *pbpC* mutant strains grew at rates comparable to those of the wild-type strain, and the mutant cells sporulated with high efficiency; spore germination, outgrowth, and heat resistance of mutant spores were also similar to those of wild-type spores (data not shown).

The lack of a detectable phenotypic change of the *pbpC* mutant is consistent with the idea that PBP3 performs a redundant function in peptidoglycan synthesis. To further explore this possibility, mutations in *ponA*, *pbpD*, and *pbpF* were combined with the *pbpC* mutation to generate double mutants (Table 1). The addition of a *pbpC* mutation to these single-mutant strains did not change the observed phenotypes in terms of growth rate, cell morphology, sporulation, spore germination or outgrowth, or spore heat resistance. All mutants remained motile, as determined by examination under the microscope. Analysis of the double mutants confirmed the absence of the expected PBPs (Fig. 3, lane 4, and data not shown).

Expression of the *pbpC* locus. To study expression of *pbpC*, transcriptional *lacZ* fusions were constructed and placed at both the *amyE* (strain PS2327) and *pbpC* (strain PS2326) loci (Table 1; Fig. 1). Analysis of the expression of the fusion at the *pbpC* locus demonstrated that transcription takes place predominately during vegetative growth, with much lower activity present during sporulation (Fig. 4A). The specific activity of β-galactosidase was consistently highest at an optical density of around 3 and dropped shortly after entry into stationary phase. The fusion at the *amyE* locus produced similar levels of expression during vegetative growth. However, expression of the *pbpC-lacZ* fusion during sporulation was lower at *amyE* than at the *pbpC* locus (Fig. 4A). This finding is consistent with the major promoter for the *pbpC* gene being within the 400 bases upstream of *pbpC* (present in the *pbpC-lacZ* transcriptional fusion at the *amyE* locus), while a low level of expression during sporulation may be from an additional promoter much further upstream. To determine whether this low level of *pbpC* expression during sporulation contributed a nonredundant function to spore cortex formation, reversed-phase high-pressure liquid chromatography was used to compare the cortical peptidoglycan structures from both wild-type and *pbpC* mutant spores (23). It was found that the cortex peptidoglycan was identical in both the mutant and wild-type spores (data not shown), suggesting that any involvement of PBP3 in spore cortex formation is minimal or can be compensated for in its absence by another enzyme.

β-Galactosidase from the *pbpC-lacZ* fusion was not observed in the dormant spores of either strain PS2326 or strain PS2327 (<1 Miller unit [data not shown]). However, during spore germination and outgrowth, β-galactosidase activity began to increase significantly at around 70 to 80 min. The large

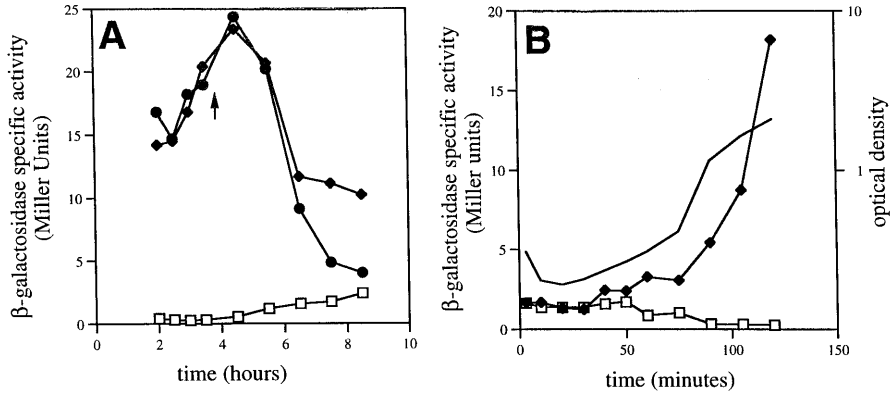

FIG. 4. Expression of *pbpC-lacZ* fusions. (A) Expression of transcriptional *lacZ* fusions during vegetative growth and sporulation in 2× SG at 37°C. The end of vegetative growth is designated by the arrow. (B) Expression of *lacZ* fusions during spore germination and outgrowth in 2× YT medium with 4 mM L-alanine. The optical density decreased by about 38% during spore germination, and the lowest optical density obtained was used to calculate Miller units for all samples during the first 30 min of germination. Symbols denoting the strains and type of fusion present: □, PS832 (wild type); ◆, PS2326 (*pbpC-lacZ* fusion at the *pbpC* locus); ●, PS2327 (*pbpC-lacZ* fusion at the *amyE* locus). −, optical density at 600 nm.

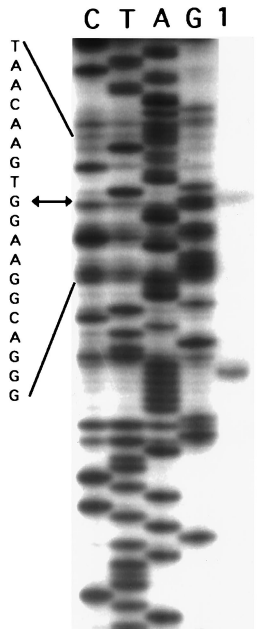

FIG. 5. Identification of the transcription start site of *pbpC*. RNA was isolated from cells as described in Materials and Methods. Primer extension mapping was done with a ^{32}P-labeled oligonucleotide complementary to positions 96 to 115 (Fig. 2), and this same oligonucleotide was used to generate DNA sequence. The primer extension product is in lane 1. The sequence lanes are reversed so that the top-strand sequence is shown on the left. The smaller primer extension product we believe to be either an early termination of the reverse transcriptase or an artifact of the hot phenol RNA extraction, as expression measured from a *pbpC-lacZ* fusion to the region upstream of this transcript produced levels of β-galactosidase similar to those for all other *pbpC-lacZ* fusions. This image was generated by using Adobe Photoshop software.

increase in β-galactosidase activity was associated with an increase in the optical density of the culture (Fig. 4B). This was true with the *pbpC-lacZ* transcriptional fusion at both the *pbpC* and *amyE* loci (Fig. 4B and data not shown), suggesting that *pbpC*'s primary promoter during spore germination and outgrowth is just upstream of *pbpC*. These results further indicate that during spore outgrowth, *pbpC* appears to be transcribed most strongly as cells begin to reenter vegetative growth. The latter results with the transcriptional *pbpC-lacZ* fusions are consistent with a previous report of the timing of the increase in levels of PBP3 during outgrowth (19).

Primer extension mapping. The transcription start site of *pbpC* was elucidated by primer extension. Reverse transcription of RNA from cells harvested during vegetative growth using two different primers revealed a band at the G residue +1 (Fig. 5 and data not shown). We predict that the gene's ribosome binding site is AAGGCAGGG, positions 3 to 11 (Fig. 2). Candidate sequences for the −35 and −10 recognition sites are TTGCAC and GAAAAT, respectively. These sequences are separated by 18 bp and, given the rather low level of *pbpC* expression, are reasonable matches to the consensus sites for a σ^A promoter: TTGACA for −35 and TATAAT for −10. Another primer extension product was detected at the A residue at position +24 (Fig. 5), which is in the putative open reading frame of *pbpC*. Only RNA extracted via the hot phenol method gave this product; RNA extracted with guanidine isothiocyanate did not (data not shown). To ensure that this was not an actual transcription start site, a *lacZ* fusion to DNA completely upstream of the open reading frame (pTMM7 [Fig. 1]) was constructed and placed at *amyE* in *B. subtilis*, creating strain PS2399. Assays of β-galactosidase from this strain revealed that expression was comparable to that for all other previously constructed *lacZ* fusions, indicating that transcription started upstream of the predicted *pbpC* open reading frame (data not shown). On one occasion when using the primer PEC1, we detected a band at the A of position −3 (Fig. 5). However, analysis of upstream DNA regions did not reveal any reasonable matches to −10 and −35 recognition sites for σ^A. We do not have an explanation for why this band was observed.

DISCUSSION

The identification of *orf16* as *pbpC* encoding PBP3 brings to 10 the number of *B. subtilis* PBP-encoding genes for which protein products have been identified. The N-terminal region of *pbpC* is identical to a sequence previously identified in a screen for functional signal sequences in *B. subtilis* (32). This identity at the DNA level encompasses the first 29 codons of the open reading frame of *pbpC*, after which the two sequences are very different. One possible explanation for this is that the clone obtained in the signal sequence screen contained multiple *Sau*3AI fragments of the *B. subtilis* chromosome (33), as a *Sau*3AI site exists where the DNA sequences diverge. However, the presence of a functional signal sequence in *pbpC* suggests that the protein is likely to be inserted into the cell membrane. Sequence similarities indicate that PBP3 of *B. subtilis* is a class B high-molecular-weight PBP, joining PBP2b as the other identified *B. subtilis* class B PBP expressed during vegetative growth. On the basis of the proteins to which PBP3 is similar, we hypothesize that PBP3 possesses a transpeptidase activity. In *E. coli*, the transpeptidase activity associated with the class B PBP2 is lost upon mutagenesis of specific residues predicted to be within the catalytic site (1). The PBPs with highest sequence homology to PBP3 are notable for their low affinity for penicillin (22). In *B. subtilis*, PBP3 requires the largest amount of FLU-C_6-APA to reach 50% saturation, and the same has been shown to be true for penicillin G (13, 30).

pbpC is clearly transcribed most strongly during vegetative growth, and its expression drops significantly after the start of sporulation. PBP3 is present in the cell membrane in detectable amounts during vegetative growth (5). Sowell and Buchanan previously found large increases in PBP3 during sporulation (35). However, analysis of the level of PBP3 in sporulating cells has been complicated by the fact that the *spoVD* product, a sporulation-specific PBP, comigrates with PBP3 on gel electrophoresis (9). We may now be able to directly observe the presence of the *spoVD* gene product in membrane preparations of sporulating strains which lack PBP3. Our data indicate that a small amount of *pbpC* transcription continues during sporulation, suggesting that PBP3 may have a minor role in the synthesis of peptidoglycan structures during sporulation. However, our data do not directly address PBP3 levels during sporulation. An alternative explanation for the lower levels of β-galactosidase from *pbpC-lacZ* observed during sporulation could be an increase in the rate of degradation of β-galactosidase.

Late in sporulation, a slightly higher level of β-galactosidase expression was observed from *pbpC-lacZ* fusions at the *pbpC* locus than at *amyE*. To explore the possibility of a sporulation-specific promoter, the *pbpC-lacZ* fusion was moved into mutant strains lacking σ^E, σ^F, σ^G, and σ^H. However, analysis of levels of β-galactosidase in these mutant strains did not allow us to determine which sigma factor was responsible for the small amount of sporulation-specific *pbpC* expression. Analysis of the DNA sequence at the 3′ end of *pbpC* suggests the

possibility of a hairpin transcription terminator, between positions 16405 and 16439 in the EMBL database (accession number D13168). However, part of this putative terminator is within the open reading frame of *pbpC*, and so we are unsure of its effectiveness as a terminator. Thus, we are unable to conclude definitively whether *pbpC* is monocistronic or polycistronic, although it is not clear why cotranscription of *pbpC* with an open reading frame encoding an aryl alcohol dehydrogenase, the next putative open reading frame, would be advantageous.

The lack of a phenotype associated with the loss of PBP3 is not surprising, as this has been the case with several other single PBP mutants in *B. subtilis* (26–28, 39). This observation is consistent with the idea that many PBPs have partially overlapping functions in the cell and that in the absence of one, others can compensate. PBP2b is the notable exception to this redundancy of function, as *pbpB* is an essential gene (40). Further evidence of this redundancy with respect to PBP3 is also seen by the lack of phenotypic changes in double mutants lacking PBP3 and class A high-molecular-weight PBP1, -2c, or -4. Alternatively, the role of PBP3 in peptidoglycan synthesis under laboratory conditions may be minimal, and its absence may have no effect. To further understand the roles that different PBPs play in peptidoglycan synthesis, we are continuing efforts to identify the remaining genes encoding PBPs.

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