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Cloning, Nucleotide Sequence, and Regulation of the *Bacillus subtilis pbpF* Gene, Which Codes for a Putative Class A High-Molecular-Weight Penicillin-Binding Protein

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The partial nucleotide sequence of a gene encoding a *Bacillus subtilis* homolog to the *Escherichia coli ponA* gene, encoding penicillin-binding protein 1A, was previously reported. The remaining part of this gene, termed *pbpF*, was isolated, and its nucleotide sequence was completed. Deletion of this gene did not alter the profile of *B. subtilis* penicillin-binding proteins observed after gel electrophoresis and resulted in no observable phenotype. A transcriptional *pbpF-lacZ* fusion was weakly expressed during vegetative growth. Expression diminished during the first hours of sporulation but was slightly induced in the forespore compartment during late sporulation. This sporulation expression was dependent on *spoIIIG*, which encodes the forespore-specific transcription factor σ^G. A single transcription start site which was apparently directly dependent on $E\sigma^A$ was detected in vegetative cells.

Sporulation of *Bacillus* spp. is a simple model of cellular differentiation. The regulatory mechanisms governing gene expression throughout this process have been the subject of intense investigation (reviewed in references 18 and 19), and a recurring topic of interest has been the coordination of alterations of gene expression with the progression of the sporulating cell's morphological changes. Two of the major changes in cell structure involved in formation of the spore, septation and cortex synthesis, require peptidoglycan synthesis (3, 9). A detailed understanding of the factors involved in the various facets of peptidoglycan synthesis in *Bacillus subtilis* might therefore be extremely valuable in unraveling the communication between the genomes enclosed in the two compartments of the sporangium and the cell structural elements. While much work has been devoted to characterizing some of the proteins involved in *B. subtilis* peptidoglycan synthesis in general (3, 16, 28), it is not at all clear which of these proteins function at different cellular locations and stages of the life cycle. We have begun work to isolate and characterize the genes encoding proteins involved in peptidoglycan metabolism in order to further study of their biochemical roles.

Many enzymes involved in polymerization of peptidoglycan subunits are penicillin-binding proteins (PBPs), so named because of their affinity for penicillins and other β-lactam antibiotics which mimic a peptide bond found in peptidoglycan and its precursors (12). The PBPs fall into three broad classes: the class A high-molecular-weight PBPs, which are generally believed to possess transglycosylase and transpeptidase activities; the class B high-molecular-weight PBPs, which possess transpeptidase and possibly other activities; and the low-molecular-weight PBPs, which are generally DD-carboxypeptidases and in some cases transpeptidases (12). In *B. subtilis* at least five high-molecular-weight PBPs and four low-molecular-weight PBPs have been identified by biochemical methods (3, 16, 28), but for only four of these have genes been identified (6, 8, 25, 34). Three additional sequenced or partially sequenced genes are predicted to encode PBPs which have not been identified biochemically (6, 13, 37). We report here the completion of the nucleotide sequence of one of these genes as well as studies of its expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains were derived from strain 168. The *spoIIIG* mutation used was *spoIIIGΔ1* (15). Growth for studies of gene expression, for preparation of membranes, and for sporulation by the nutrient exhaustion method was in 2× SG medium (17). Induction of sporulation by the resuspension method was done as previously described (31). Growth on minimal medium was tested with Spizizen's minimal medium (29). *B. subtilis* was transformed as previously described (2).

Enzyme assays. β-Galactosidase was assayed by using the substrate 4-methylumbelliferyl-β-D-galactosidase as previously described (22). Coat proteins were removed from forespores and spores prior to β-galactosidase assay by using 8 M urea, 1% (wt/vol) sodium dodecyl sulfate, and 50 mM dithiothreitol as previously described (22). Glucose dehydrogenase was assayed as previously described (22). Cell membranes were prepared and binding of [^{3}H]benzylpenicillin (New England Nuclear) was assayed as previously described (28).

Cloning of *pbpF*. A restriction fragment containing the distal portion of *pbpF* (supplied by M. Hansson and L. Hederstedt) was inserted into *Eco*RI-*Eco*RV-digested pJH101 to create pDPC51. This plasmid was transformed into strain 168 with chloramphenicol selection for integration of the plasmid into the *pbpF* locus on the chromosome. Chromosomal DNA was purified from one transformant, digested with *Bam*HI, and then ligated at a low DNA concentration. Transformation of the ligated DNA into *Escherichia coli* with selection for ampicillin resistance resulted in the isolation of pDPC52, which contained approximately 7 kbp of DNA adjacent to the pDPC51 insert.

Gene fusion and mutant construction. In order to create a fusion of *pbpF* to *lacZ*, the 1,900-bp *Pst*I-*Pvu*II fragment of pDPC52 containing the beginning of *pbpF* was inserted into

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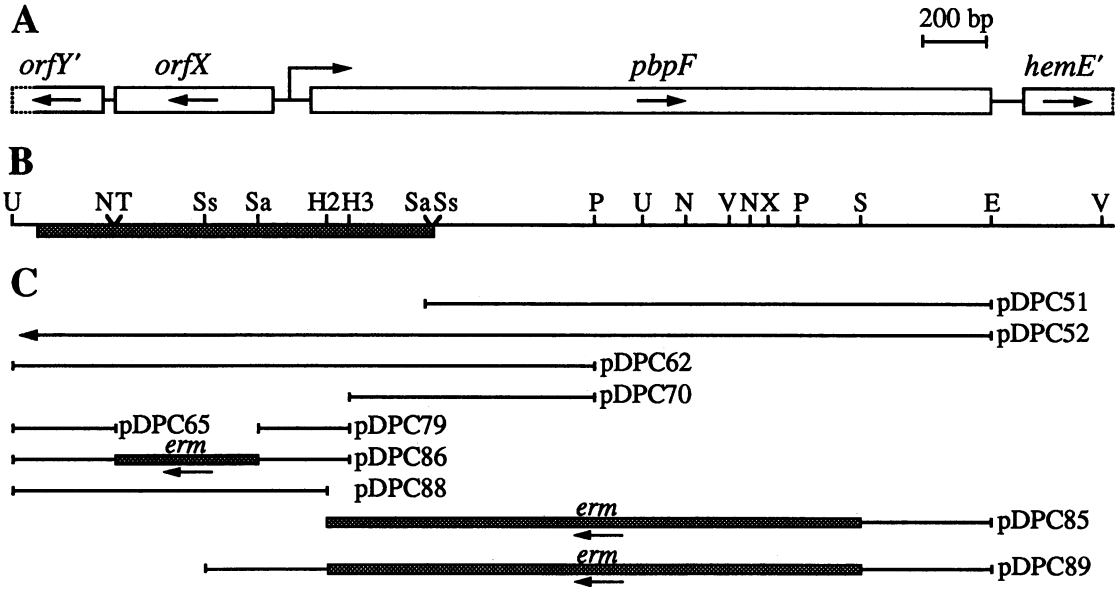

FIG. 1. Map of the *pbpF* locus. (A) Open reading frames are represented by boxes. Arrows within the boxes indicate the directions of transcription. The raised arrow indicates the start site of vegetative *pbpF* transcription. (B) The shaded box indicates the nucleotide sequence reported in Fig. 2. Restriction endonuclease cleavage sites indicated are as follows: E, *Eco*RI; H2, *Hin*cII; H3, *Hin*dIII; N, *Nae*I; P, *Pst*I; S, *Sac*I; Sa, *Sau*3AI; Ss, *Ssp*I; T, *Taq*I; U, *Pvu*II; V, *Eco*RV; X, *Xho*I. Only those *Sau*3AI and *Taq*I sites used in plasmid constructions are shown. (C) The bars represent the inserts in the indicated plasmids. The left end of the pDPC52 insert is not shown. The shaded regions representing the *ermC* gene (orientation indicated by arrows) are not drawn to scale.

*Pst*I-*Sma*I-digested pUC19 to create pDPC62. The 1,000-bp *Eco*RI-*Hin*cII fragment of pDPC62 was then inserted into *Eco*RI-*Sma*I-digested pUC19. The resulting 1,000-bp *Eco*RI-*Bam*HI fragment was cloned in *Eco*RI-*Bam*HI-digested pJM783 (24) to create a *pbpF-lacZ* transcriptional fusion in plasmid pDPC88 (Fig. 1).

The 770-bp *Hin*dIII-*Pst*I fragment of pDPC52 was inserted into *Hin*dIII-*Pst*I-digested pUC19. The resulting 780-bp *Hin*dIII-*Bam*HI fragment was inserted into *Hin*dIII-*Bam*HI-digested pJH101 to create pDPC70. This plasmid contains an internal fragment of *pbpF* and thus will interrupt the gene when introduced into the chromosome via a single crossover recombination event.

pDPC70 and pDPC88 were introduced into the *pbpF* locus on the chromosome via Campbell recombinations by transformation of strain 168 with selection for chloramphenicol resistance. The nature of each recombination event was verified by Southern hybridization (27).

Deletion of *orfX* involved insertion of the 290-bp *Hin*dIII-*Sau*3AI and 320-bp *Taq*I fragments of pDPC62 carrying the beginning and end of *orfX* into *Hin*dIII-*Bam*HI- and *Acc*I-digested pUC19 to create pDPC79 and pDPC65, respectively (Fig. 1; the *Taq*I site at the left end of the pDPC65 insert was in the pDPC62 vector sequence). pDPC79 was then digested with *Eco*RI and *Sac*I, and the small *Eco*RI-*Pst*I fragment of pDPC65 and an *erm*-containing *Pst*I-*Sac*I fragment were inserted to create pDPC86 (Fig. 1). Construction of a *pbpF* deletion began with digestion of pDPC51 with *Sac*I and *Bam*HI, which cut in the *pbpF* sequence and in the vector sequence, respectively, and replacement of most of the *pbpF* sequence with the *Sac*I-*Bam*HI *erm* fragment of pPS937 (24) to produce pDPC85. The *Ssp*I-*Hin*cII fragment containing the beginning of *pbpF* was subcloned such that it could be inserted into the *Bam*HI site of pDPC85. The resulting plasmid, pDPC89, contained the *erm* gene flanked by the two ends of *pbpF*. pDPC86 and pDPC89 were linearized and transformed into strain 168 with selection for macrolide-lincosamide-streptogramin B resistance. Replacement of the *orfX* and *pbpF* coding sequences by the *erm* gene via double crossover recombination events in transformants was verified by Southern hybridization (27).

DNA sequencing and primer extension. The DNA sequence was determined by the chain termination method (26), using a Sequenase kit (U.S. Biochemical) as previously described (25). The sequence reported was determined from both strands, and all restriction sites used for cloning were sequenced across.

RNA was purified from *B. subtilis* 168 as previously described (21), and 50 μg was used in each primer extension reaction. Two oligonucleotides, complementary to positions 855 through 877 and 924 through 945 (see Fig. 2), were ^{32}P end labeled and used as previously described (21) except they were purified by ethanol precipitation three times after labeling. DNA sequence from the *pbpF* region was generated by the chain termination method, using the same end-labeled oligonucleotides as primers and supercoiled pDPC79 (Fig. 1) as the template.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession number L10630.

RESULTS

We used a DNA fragment containing the distal portion of an apparent *B. subtilis* homolog to the *E. coli ponA* gene (13) to isolate the remainder of the gene by a chromosomal walking technique (Fig. 1). DNA sequence analysis indicated that the complete gene, which we have designated *pbpF*, contains 714 codons and encodes a protein with a

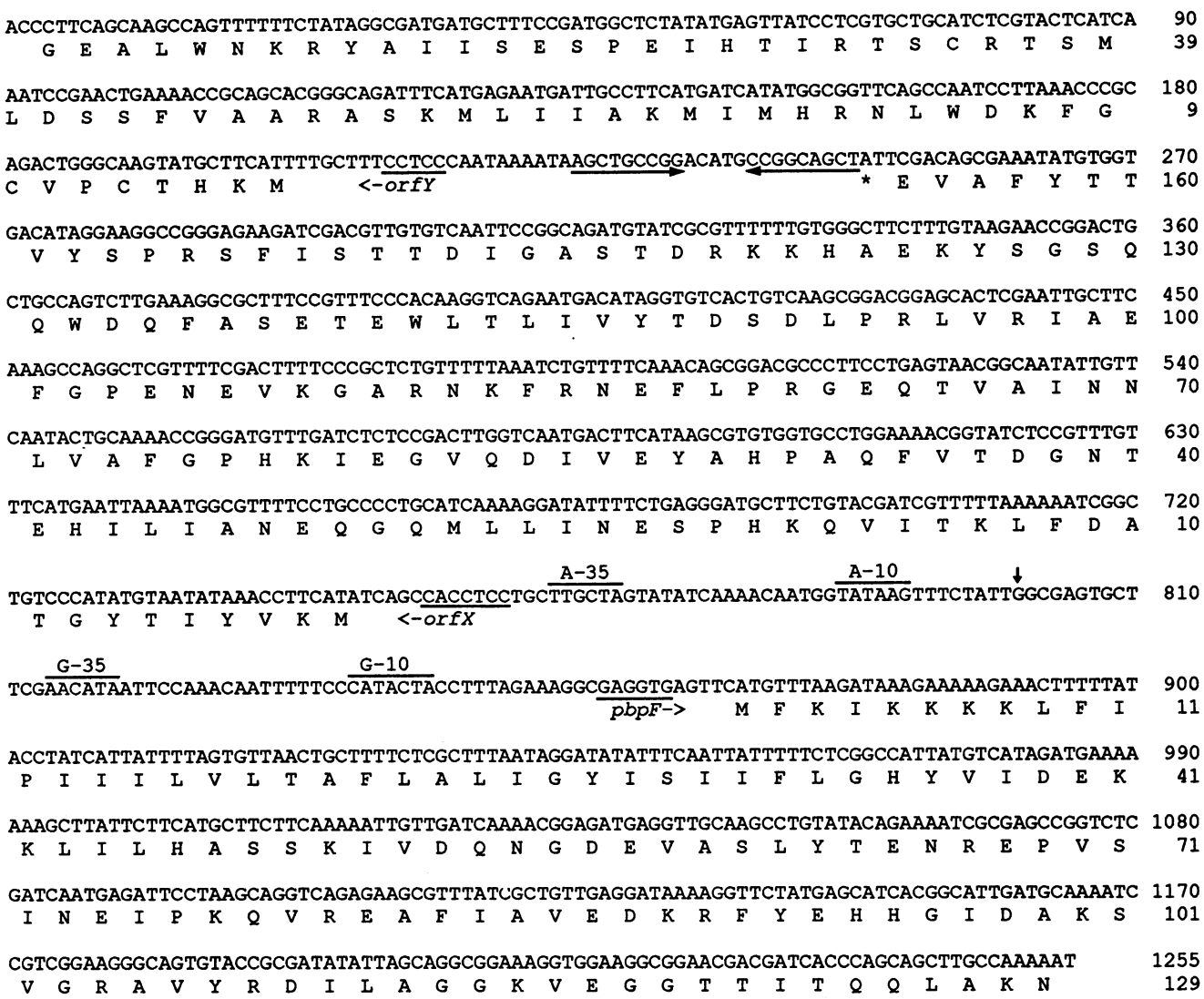

FIG. 2. Nucleotide sequence of the proximal portion of *pbpF*. The numbers on the right designate the nucleotide or amino acid at the right end of each row. Amino acids are numbered separately for each open reading frame. Underlined sequences are the predicted ribosome-binding sites for each open reading frame. Horizontal arrows indicate the region of dyad symmetry predicted to be a transcription terminator downstream of *orfX*. The vegetative transcription start site for *pbpF* determined by primer extension is indicated by the vertical arrow at position 801. The predicted −35 and −10 recognition sequences for RNA polymerase containing σ^A and σ^G are overlined and designated A-35, A-10 and G-35, G-10, respectively. Nucleotides 1221 through 1255 are identical to positions 1 through 25 in reference 13, which reports the remaining part of the *pbpF* sequence.

calculated molecular weight of 79,278 (Fig. 2) (13). The predicted initiation codon is preceded by a good match to the 3′ end of *B. subtilis* 16S rRNA (Fig. 2). The predicted product of this gene is 33, 28, 31, 33, and 31% identical to the *E. coli ponA* and *ponB* genes and to *Haemophilus influenzae*, *Streptococcus oralis*, and *Streptococcus pneumoniae ponA* homologs, respectively (Fig. 3). This places the product of *pbpF* in class A of the high-molecular-weight PBPs as defined by Ghuysen (12).

Upstream of *pbpF* we found an open reading frame of 166 codons oriented in the opposite direction, which we termed *orfX*. It was preceded by a strong ribosome-binding site and followed by a potential terminator structure and the beginning of another gene, termed *orfY* (Fig. 1 and 2). The predicted products of these two open reading frames exhibit no significant sequence similarity to any found in the GenBank and EMBL DNA sequence data bases. Deletion of much of *orfX* (Fig. 1) produced no obvious phenotype.

We created a disruption of the *pbpF* gene by a Campbell-type insertion of pDPC70 into the *B. subtilis* chromosome. We prepared membranes from vegetative cells of both the mutant and wild-type strains and used radiolabeled benzylpenicillin to examine the PBP contents. We observed no difference between the mutant and wild-type preparations (Fig. 4).

We also constructed a strain in which a large part of *pbpF* was deleted. This strain grew normally on rich and minimal media and sporulated at wild-type levels when induced by either the nutrient exhaustion or resuspension method. The spores produced by the mutant strain were as heat resistant

FIG. 3. Amino acid sequence alignment of class A high-molecular-weight PBPs. The protein sequences were aligned by using the PILEUP program (11). Gaps introduced into the sequences are indicated by periods. Positions at which identical or highly conserved residues are found in at least five of the proteins are indicated by * below the sequences. Sequences motifs which are highly conserved among all penicillin-recognizing proteins (12) are boxed. The numbers at the right indicate the last residue in that row for each protein. The first 123 residues of EcPONB are not shown. The sequences are as follows: BsPBPF, *B. subtilis* PBPF; EcPONA, *E. coli* PBP 1A (4); HiPONA, *H. influenzae* PBP (35); StrPONA, *S. oralis* PONA (20); StrPONAa, *S. pneumoniae* PONA (20); EcPONB, *E. coli* PBP 1B (4).

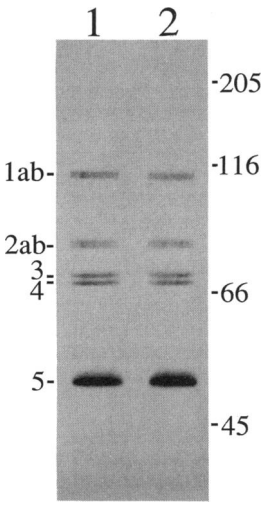

FIG. 4. PBP profiles of wild-type and *pbpF* mutant strains of *B. subtilis*. Membrane preparations from vegetative cells of strains PS1838 (*pbpF*::pDPC70) (lane 1) and 168 (wild type) (lane 2) were incubated with [^{3}H]benzylpenicillin, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% slab gel. The PBPs were detected by fluorography and are numbered on the left as in references 3 and 16. Molecular mass markers indicated on the right were myosin (205 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

as wild-type spores and germinated and went through outgrowth with normal kinetics (data not shown).

Construction of a transcriptional fusion of *pbpF* to *lacZ* allowed us to examine the timing and level of *pbpF* expression. We detected β-galactosidase activity during vegetative growth; this activity began to disappear upon the start of sporulation (Fig. 5). The maximum amount of β-galactosidase activity produced by the *pbpF-lacZ* fusion during vegetative growth was <4 Miller units when assayed with *o*-nitrophenyl-β-D-galactopyranoside. We therefore measured hydrolysis of 4-methylumbelliferyl-β-D-galactoside by the more sensitive fluorescence assay. A small amount of *pbpF-lacZ*-dependent β-galactosidase activity reappeared during the later stages of sporulation (Fig. 5), at the same time that glucose dehydrogenase activity appeared (data not shown). This late *pbpF* expression could be clearly differentiated from the endogenous β-galactosidase activity seen in a strain lacking a *lacZ* fusion (Fig. 5) by the fact that 88% of the total β-galactosidase activity present in the *pbpF-lacZ* strain could be found in the mature spores after removal of the coat proteins (Fig. 5), whereas essentially no activity was present in the wild-type spores (data not shown). This result also indicated that the sporulation-specific *pbpF* expression was confined to the forespore compartment. To further examine this phenomenon, we moved the *pbpF-lacZ* fusion into a *spoIIIG* mutant strain which lacks the forespore-specific RNA polymerase sigma factor σ^G, and we found that this mutation abolished the sporulation-specific *pbpF* expression (Fig. 5).

Using primer extension mapping to determine the start sites for *pbpF* transcripts, we detected a single transcript in RNA samples prepared from vegetative cells (Fig. 6). This transcript disappeared during the first hours of sporulation (Fig. 6), and we observed no other transcript during the remainder of the sporulation process (data not shown). Upstream of the vegetative transcript start site there is a sequence (TATAAG) which resembles the consensus −10

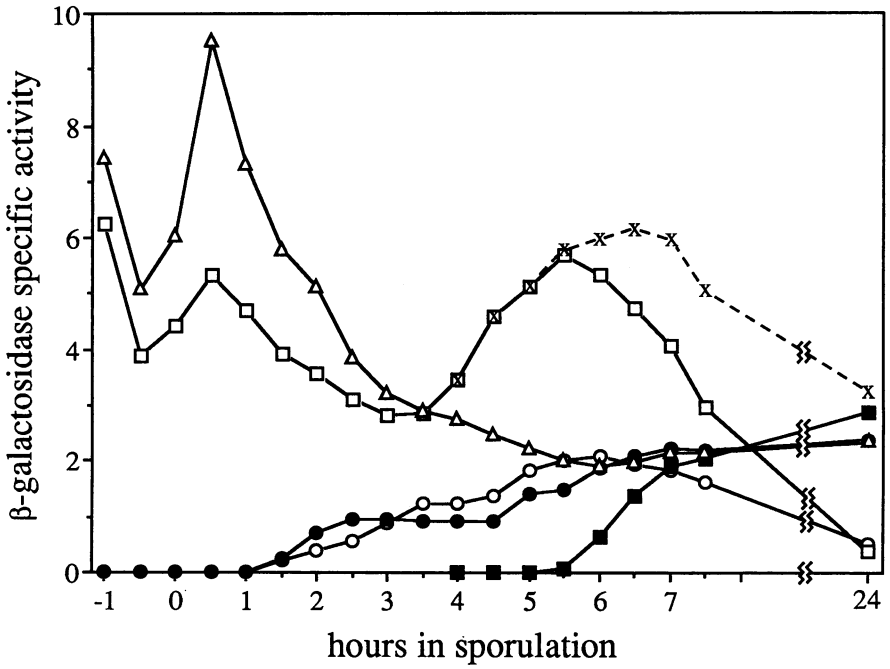

FIG. 5. Expression of a *pbpF-lacZ* fusion during growth and sporulation. Strains were cultivated in 2× SG medium at 37°C and assayed at the times indicated. β-Galactosidase specific activity is expressed in arbitrary units of fluorescence/optical density at 600 nm. Relevant genotypes of the strains (and symbols for specific activities) are as follows: 168, wild type (○); PS1868, *pbpF-lacZ* (□, before removal of coat proteins; ■, after removal of coat proteins from forespores or spores; x, sum of activities detected before and after removal of coat proteins); PS683, *spoIIIGΔ1* (●); PS1877, *pbpF-lacZ spoIIIGΔ1* (△). The data shown are from a single experiment. Similar results were obtained in two additional experiments.

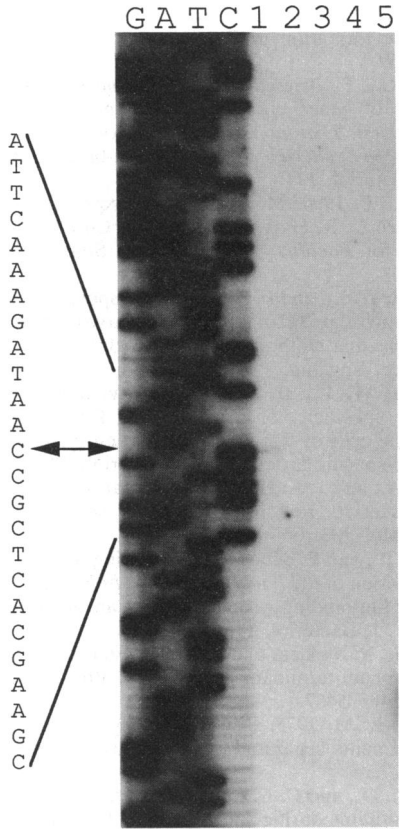

FIG. 6. Mapping of a *pbpF* transcription start site. RNA was purified from strain 168 at various stages of growth and sporulation in 2× SG medium and used for primer extension mapping with an oligonucleotide complementary to positions 855 through 877 in Fig. 2. DNA sequence lanes (G, A, T, and C) were produced by using the same oligonucleotide as a primer. The reaction in lane 5 contained no RNA. The reactions in lanes 1 through 4 contained RNA from 0.5 h before and 0.5, 1.5, and 2.5 h after the start of sporulation, respectively. The DNA sequence shown on the left is the complement of positions 789 to 813 in Fig. 2. The arrow indicates the transcription start site.

recognition sequence (TATAAT) for σ^A-dependent promoters, and spaced 17 bp further upstream is an excellent match (TTGCTA) to the consensus −35 recognition sequence (TTGACA) (Fig. 2). In the region upstream of the predicted *pbpF* ribosome-binding site we also observed reasonable matches (AACATA and CATACTA, respectively) to the consensus −35 (TGAATA) and −10 (CATACTA) recognition sequences for σ^G-dependent promoters (23) (Fig. 2).

DISCUSSION

Hansson and Hederstedt determined much of the nucleotide sequence of an open reading frame upstream of the *hemEHY* locus at 94° on the *B. subtilis* chromosome (13). The strong sequence similarity between the predicted product of this gene and the product of the *E. coli ponA* gene, which codes for PBP 1A, led these authors to designate this gene *ponA* (13). We have isolated the remainder of this gene and determined its nucleotide sequence. Our results demonstrate that this gene does not encode a previously identified PBP. (The possibility remains that *pbpF* encodes the PBP 2c tentatively identified in one report [16], but we have not observed this protein in wild-type membrane preparations, and we agree with the authors of the previous report [16] that PBP 2c may have been a proteolytic product of a higher-molecular-weight PBP.) We therefore suggest that in accordance with a nomenclature system suggested by C. E. Buchanan (5), the *pon* designation be reserved for the gene(s) encoding PBP 1 and that the gene described herein be named *pbpF*.

All species of eubacteria examined produce a number of PBPs which are involved in synthesis of cell wall peptidoglycan (12). In several cases a significant degree of overlap in function of these proteins within an organism has been observed. This can result in a failure to produce an obvious phenotype upon mutation of a single PBP-encoding gene. This has been the case in several studies of *B. subtilis* PBP-encoding genes. Mutation of the *pbpE* (25) and *dacF* (37) genes, encoding PBP 4* and an unidentified PBP, respectively, resulted in no obvious phenotype. A *dacA* mutation which eliminated PBP 5 was reported to produce a minor change in spore heat resistance (34), but this has recently been disputed (7). In the present study of the *B. subtilis pbpF* gene, we have again found no phenotype associated with loss of the gene. This might be expected because of the sequence similarity between *pbpF* and the *ponA* and *ponB* genes of *E. coli*. Either *ponA* or *ponB* can be mutated in *E. coli* without serious consequences for the cell; however, mutation of both of the genes is lethal, indicating that their activities are to a significant degree redundant (30, 32, 38). We therefore expect that another gene encoding this class of protein will eventually be found in *B. subtilis* and that mutation of this gene in combination with *pbpF* will produce a phenotype. Our inability to detect the product of *pbpF* by using radiolabeled penicillin could be the result of a number of factors. This protein may comigrate with another PBP during sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and thus the disappearance of the protein in a *pbpF* mutant would be masked, especially if the cell compensates for the loss of one PBP by elevating the production of another. Alternatively, the *pbpF* product may be labeled poorly because of low affinity for benzylpenicillin or high turnover of the penicillin after binding. Finally, this PBP may be present in extremely small amounts because of low expression or instability. Consistent with the latter hypothesis is the extremely weak signal we observed in our transcript mapping experiments. This signal was at least 10-fold weaker than that we obtained in studies of the gene encoding another PBP (25). Our failure to observe any signal corresponding to the σ^G-dependent transcript may have been due to a problem in isolating RNA from cells in a latter stage of sporulation or because the transcript is even less abundant than the vegetative transcript but more rapidly translated.

The product of *pbpF* initiates with a basic, lysine-rich region followed by a long hydrophobic stretch which we predict to be a signal sequence for membrane insertion. There is, however, no signal peptidase recognition sequence (36). We therefore expect that this is an uncleaved signal sequence which functions as a membrane anchor. This method of membrane attachment has been observed for a number of other high-molecular-weight PBPs (1, 10, 14).

The expression of *pbpF* in the forespore during the later stages of sporulation raises intriguing possibilities. It suggests that this protein could be involved in outgrowth of the germinated spore. Alternatively, it could function in the synthesis of the germ cell wall. It has been demonstrated in another species of *Bacillus* that the peptidoglycan precursors

of this structure are synthesized in the forespore (33). Whatever the function of this PBP, it is clear that it is redundant and that elucidation of its role(s) by genetic methods will require the isolation and mutation of additional PBP-encoding genes.

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