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Cloning, Nucleotide Sequence, and Mutagenesis of the *Bacillus subtilis ponA* Operon, Which Codes for Penicillin-Binding Protein (PBP) 1 and a PBP-Related Factor

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An oligonucleotide probe designed to hybridize to genes encoding class A high-molecular-weight penicillin-binding proteins (PBPs) was used to identify the *ponA* gene encoding PBP1a and -1b (PBP1) of *Bacillus subtilis*. The identity of the *ponA* product was established by (i) the presence of a sequence coding for a peptide generated from PBP1 and (ii) the disappearance of PBP1 in a *ponA* mutant. DNA sequence analysis revealed that the amino acid sequence of PBP1 was similar to those of other class A high-molecular-weight PBPs and that *ponA* appeared to be cotranscribed with an upstream gene (termed *prfA*) of unknown function. Null mutations in *ponA* resulted in a slight decrease in growth rate and a change in colony morphology but had no significant effect on cell morphology, cell division, sporulation, spore heat resistance, or spore germination. Mutations in *prfA* which did not effect *ponA* expression produced a more significant decrease in growth rate but had no other significant phenotypic effects. Deletion of both *prfA* and *ponA* resulted in extremely slow growth and a reduction in sporulation efficiency. Studies of expression of transcriptional fusions of *ponA* and *prfA* to *lacZ* demonstrated that these two genes constitute an operon. Expression of these genes was relatively constant during growth, decreased during sporulation, and was induced approximately 15 min into spore germination. The *ponA* locus was mapped to the 200° region of the chromosomal physical map.

Elongation and septation of the peptidoglycan sacculus are mandatory processes for the growth and division of eubacteria. Synthesis of peptidoglycan on the outer surface of the cytoplasmic membrane requires two enzymatic activities, a transglycosylase which polymerizes the glycan strands and a transpeptidase which cross-links these strands via their peptide side chains (reviewed in reference 16). DD-Carboxypeptidase activity is required for maturation of the peptidoglycan and may be involved in regulation of the degree of cross-linking. The penicillin-binding proteins (PBPs) which possess these activities have been grouped into three classes on the basis of protein sequence similarities, the class A and class B high-molecular-weight PBPs and the low-molecular-weight PBPs (16). Transglycosylase and transpeptidase activities have been clearly demonstrated for *Escherichia coli* class A high-molecular-weight PBP1a and -1b (20, 29, 38). Class B high-molecular-weight PBPs possess transpeptidase activity, and the low-molecular-weight PBPs have generally been found to have DD-carboxypeptidase activity (16). It is often the case that a species will possess multiple PBPs of a particular class which exhibit fully or partially redundant functions. A simplified model of sacculus synthesis is that the class A high-molecular-weight PBPs polymerize and to some degree cross-link the peptidoglycan strands, while the class B high-molecular-weight PBPs modify the basic structure to maintain cell shape and produce septation.

Our recent work has involved identification of 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. Eleven PBPs have been identified in *B. subtilis* by biochemical methods (6, 9, 12, 23, 36, 40). The genes encoding seven of these proteins have now been identified (10, 12, 18, 33–35, 41, 45). Two additional genes which are predicted to encode PBPs based on sequence similarities have been identified, but the protein products of these genes have not (44) (GenBank accession number Z34883). We have used a general method for isolating genes encoding class A high-molecular-weight PBPs on the basis of a conserved sequence within these proteins (35). We report here the use of this technique to isolate the *B. subtilis ponA* gene encoding PBP1. We have determined the nucleotide sequence of this gene and a gene, *prfA*, which is in the same operon. We have carried out studies of the expression of these genes, determined their positions on the *B. subtilis* genetic map, and examined the effects of *ponA* and *prfA* mutations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains were derivatives of strain 168. Growth for sporulation, membrane preparation, and studies of gene expression was in 2xSG medium (25) at 37°C. Minimal growth medium was Spizizen's minimal medium (37). Spores were purified by water washing as previously described (32). Spore heat resistance was determined by measuring the number of CFU in a spore suspension before and after heating at 85°C for 15 min. Spores were heat activated at 70°C for 30 min prior to germination at 37°C in 2xYT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) medium containing 4 mM L-alanine (32). *B. subtilis* was transformed as previously described (4).

Cloning of *ponA*. A λEMBL3 library of random *Sau*3AI digestion products of *B. subtilis* 168 chromosomal DNA was screened with a degenerate oligonucleotide probe encoding all possible combinations of a 10-amino-acid consensus sequence derived from class A high-molecular-weight PBPs as previously described (35). DNA was prepared from hybridizing λ phages and subjected to restriction digestion and Southern hybridization. Hybridizing 2-kb *Hin*dIII and 1.9-kb *Pvu*II fragments from one of these phages were subcloned into *Hin*dIII- and *Hin*cII-digested pUC19 to produce pDPC110 and pDPC136, respectively (Fig. 1). In both plasmids, the *ponA* gene was in the same orientation as *lacZ*. The 1,048-bp *Hin*dIII-*Pvu*II fragment of pDPC110 was inserted into *Hin*dIII-*Hin*cII-digested pUC19 to produce pDPC114, and the 1,081-bp *Eco*RI-*Hin*dIII fragment of pDPC114 was inserted in *Eco*RI-*Hin*dIII-digested pJH101 (14) to produce pDPC126 (Fig. 1). Transformation of *B. subtilis* with pDPC126 led to integration of the plasmid into the *ponA* locus by a Campbell-type recombina-

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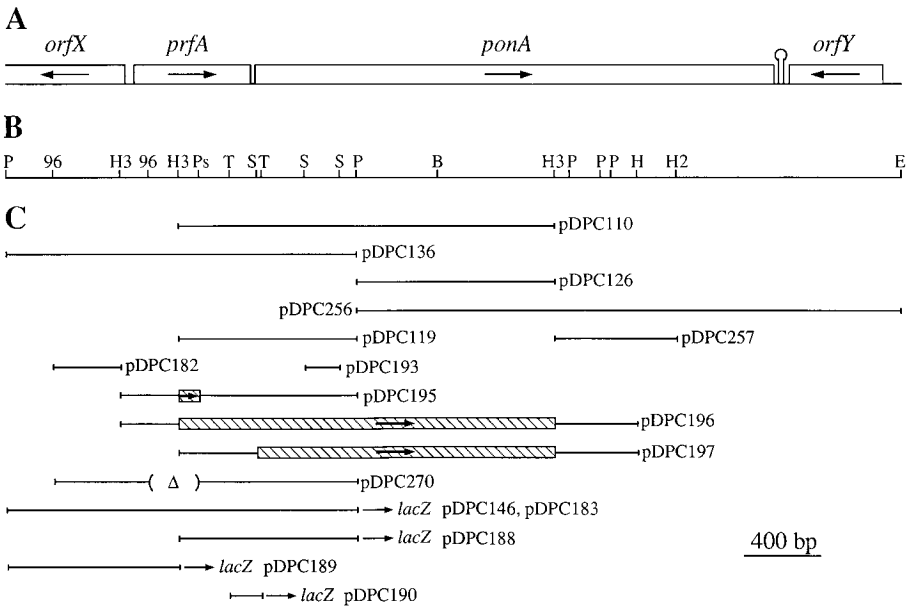

FIG. 1. Map of the *ponA* locus. (A) Open reading frames are represented by open boxes. Arrows within the boxes represent the direction of transcription. The stem-loop structure indicates the position of two predicted transcription terminators. (B) Restriction endonuclease cleavage sites are represented as follows: B, *Bgl*II; E, *Eco*RV; H, *Hae*III; H2, *Hin*cII; H3, *Hin*dIII; P, *Pvu*II; Ps, *Pst*I; S, *Sau*3AI; T, *Taq*I; 96, *Sau*96I. Only those *Taq*I, *Sau*3AI, and *Sau*96I sites used in plasmid constructions are shown. (C) Bars represent the inserts in the indicated plasmids. The hatched boxes representing Sp^r cassettes in pDPC195, pDPC196, and pDPC197 are not drawn to scale. Arrows within the boxes indicate the orientation of the Sp^r gene. The gap and Δ in pDPC270 represent an in-frame deletion of *prfA*. Arrows pointing to *lacZ* indicate the direction of transcription in *lacZ* fusion constructs.

tion. A chromosomal walking step was carried out as previously described (34). Digestion of the chromosomal DNA containing pDPC126 with *Eco*RV followed by ligation led to the production of pDPC256 (Fig. 1). Additional subclones from pDPC110, pDPC136, and pDPC256 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 504-bp *Sau*96I fragment of pDPC136 was treated with T4 DNA polymerase and deoxynucleoside triphosphates to render the ends blunt and was inserted into *Hin*cII-digested pUC19 to produce pDPC176. The 391-bp *Eco*RI-*Hin*dIII fragment of pDPC176, containing part of *orfX*, was inserted into *Eco*RI-*Hin*dIII-digested pJH101 to produce pDPC182 (Fig. 1). The 943-bp *Hin*dIII-*Pvu*II fragment of pDPC110 was inserted into *Hin*dIII-*Hin*cII-digested pUC19 to produce pDPC113. The smaller *Eco*RI-*Hin*dIII fragment of pDPC113, containing parts of *prfA* and *ponA*, was inserted into *Eco*RI-*Hin*dIII-digested pJH101 to produce pDPC119 (Fig. 1). The 189-bp *Sau*3AI fragment of pDPC110 was inserted into *Bam*HI-digested pUC19 to produce pDPC172. The small *Eco*RI-*Hin*dIII fragment of pDPC172, containing a fragment of *ponA*, was inserted into *Eco*RI-*Hin*dIII-digested pJH101 to produce pDPC193 (Fig. 1). The 641-bp *Hin*dIII-*Hin*cII fragment of pDPC256 was inserted into *Hin*dIII-*Hin*cII-digested pUC19 to produce pDPC253. The small *Eco*RI-*Hin*dIII fragment of pDPC253, containing a fragment of *ponA*, was inserted into *Eco*RI-*Hin*dIII-digested pJH101 to produce pDPC257 (Fig. 1). Plasmids pDPC182, pDPC119, pDPC193, and pDPC257 were transformed into *B. subtilis* with selection for chloramphenicol resistance (Cm^r) in order to isolate strains in which the plasmids had integrated into the chromosome via a Campbell-type mechanism. Insertions of pDPC182 and pDPC193 produced mutations of *orfX* and of *ponA* in strains PS2021 and PS2031, respectively. Insertion of pDPC119 in strain PS1936 interrupted the *ponA* operon structure. Insertion of pDPC257 in strain PS2096 truncated the *ponA* gene.

The 308-bp *Hin*dIII fragment of pDPC136 was inserted into *Hin*dIII-digested pUC19 to produce pDPC140, in which the *prfA* gene was in the same orientation as *lacZ*. pDPC136 was digested with *Pst*I and ligated to produce pDPC143, containing the 835-bp *Pst*I-*Pvu*II fragment of the *ponA* operon. The smaller *Eco*RI-*Pst*I fragment of pDPC143 and the *Bam*HI-*Pst*I spectinomycin resistance (Sp^r) cassette from pJL73 (24) were inserted into *Eco*RI-*Bam*HI-digested pDPC140 to produce pDPC195, in which part of *prfA* is replaced by the Sp^r cassette (Fig. 1). The 434-bp *Hin*dIII-*Hae*III fragment of pDPC256 was inserted into *Hin*dIII-*Hin*cII-digested pUC19 to produce pDPC192. The smaller *Eco*RI-*Hin*dIII fragment of pDPC192 and the *Bam*HI-*Hin*dIII Sp^r cassette from pJL73 were inserted into *Eco*RI-*Bam*HI-digested pDPC140 to produce pDPC196, in which parts of *prfA* and *ponA* are replaced by the Sp^r cassette (Fig. 1). The 307-bp *Pst*I-*Sau*3AI fragment of pDPC110 was inserted into *Pst*I-*Bam*HI-digested pUC19 to produce pDPC161. The smaller *Eco*RI-*Hin*dIII fragment of pDPC192 and the *Sac*I-*Hin*dIII Sp^r cassette from pJL73 were inserted into *Eco*RI-*Sac*I-digested pDPC161 to produce pDPC197, in which part of *ponA* is replaced by the Sp^r cassette (Fig. 1). Plasmids pDPC195, pDPC196, and pDPC197 were linearized with *Sca*I and transformed into *B. subtilis* with selection for Sp^r to produce strains PS2061, PS2067, and PS2062, respectively.

The 550-bp *Eco*RI-*Pst*I fragment from pDPC176 and the 840-bp *Pst*I-*Bam*HI fragment from pDPC113 were inserted into *Eco*RI-*Bam*HI-digested pJH101 to produce pDPC270, which contains a 261-bp in-frame deletion and a 3-bp insertion in *prfA* (Fig. 1). The precise nature of this deletion was verified by DNA sequencing. This plasmid was transformed into *B. subtilis* with selection for Cm^r to produce strains containing two copies of *prfA*. Transformants producing two different colony sizes were obtained. Southern blotting revealed that the larger colonies contained one wild-type copy and one deleted copy of *prfA* and that in the smaller colonies, a gene conversion event had resulted in the deletion being present in both copies of *prfA*. One of the small transformants was grown nonselectively through >20 generations and then plated for single colonies. Again, colonies of two different sizes were obtained. Colonies were screened for Cm^s to identify strains in which pDPC270 had exigrated from the chromosome and been lost. In all cases, Cm^s coincided with the production of a larger colony size. Cm^s strains were then screened by Southern blotting to verify that the *prfA* deletion was present in the chromosome, and one isolate was saved as strain PS2123.

pDPC137 contains the same insert as pDPC136 but in the opposite orientation. The 2-kb *Eco*RI-*Sph*I fragment of pDPC137 was inserted into *Eco*RI-*Sph*I-digested pDPC87 (35) to produce a transcriptional fusion of *ponA* to *lacZ* in pDPC146 (Fig. 1). This plasmid was transformed into *B. subtilis* with selection for Cm^r in order to produce strain PS1957, containing a Campbell-type insertion at the *ponA* locus. The 2.8-kb *Eco*RI-*Cla*I fragment of pDPC146 was inserted into *Eco*RI-*Cla*I-digested pDG268 (5) to produce pDPC183 (Fig. 1). The 850-bp *Bam*HI-*Hin*dIII fragment of pDPC143 was inserted into *Bam*HI-*Hin*dIII-digested pDG268 to produce pDPC188 (Fig. 1). Digestion of pDPC144 with *Pst*I, followed by treatment with T4 DNA polymerase and digestion with *Eco*RI allowed the isolation of an 1,050-bp fragment in which the *Pst*I-digested end was blunt. This fragment was inserted into pDG268 which had been digested with *Bam*HI, treated with T4 DNA polymerase, and digested with *Eco*RI to produce pDPC189 (Fig. 1). The 171-bp *Taq*I fragment of pDPC113 was inserted into *Acc*I-digested-pUC19 to produce pDPC164, in which *ponA* is in the same orientation as *lacZ*. The 199-bp *Hin*dIII-*Bam*HI fragment of pDPC164 was inserted into *Hin*dIII-*Bam*HI-digested pDG268 to produce pDPC190 (Fig. 1). The derivatives of pDG268, pDPC183, pDPC188, pDPC189, and pDPC190, were linearized with *Sca*I and transformed into *B. subtilis* with selection for Cm^r. The resulting strains contained transcriptional fusions of the beginning of *prfA* and *ponA* to *lacZ* in the chromosome at the *amyE* locus. The correct chromosome structures in the *ponA* operon and *amyE* regions of all mutant and fusion strains

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212  Q K G L R I S E E A K S F R K V S I G Y F F G N S S P F I V T N F H F W D S L Y
  1 CTGCTTTCCCAGCCTTATGCTTTCCTCCGCCTTTGAAAAGCGTTTCACTGAAATGCCGTAAAAAAACCCATTTGAGGAAGGAAAAATAACGGTGTTAAAATGAAACCAGTCACTTAAATA

172  F P I T D F V N K K F S P N Q I I P T Q I T N Q E N I I L A Y M L R E T N R E H
120 AAAAGGAATCGTGTCAAAAACATTCTTTTTAAACGAAGGATTTTGTATAATAGGTGTTTGGATAGTGTTTTGTTCATTTATAATGAGGGCATACATTAGCCGCTCCGTATTTCGCTCATG

132  W F R T W E E S M F Q S V G F V H L L H F L P K G V R K S W H Y L L L Q P Y A D
240 CCAAAAACGGGTCCATTCTTCCGACATAAATTGTGAGACGCCAAACACATGCAGCAGATGAAACAGCGGTTTCCCAACTCTTTTTGACCAGTGGTATAAAAGAAGCTGCGGATAGGCATC

 92  S F I L W N A R E Y A L F F W K Q Q R E R L G L Q F L S G K L D T M S W G A N R
360 CGAAAAAATAAGCCAGTTCGCTCTTTCATAGGCTAAAAAGAACCATTTTTGCTGTCTCTCACGGAGTCCAAGCTGAAACAGACTGCCTTTTAAGTCAGTCATTGACCAGCCGGCATTACG

 52  S V F S A L L S W K I E P H R D Y F A K Y A N T R S I N D A N K M K T Q R E I R
480 AGAAACGAAAGAAGCCAAAAGCGACCATTTGATTTCCGGATGACGGTCGTAAAATGCTTTATAAGCATTTGTTCTTGAAATGTTATCCGCGTTTTTCATTTTTGTCTGCCGCTCAATCCG

 12  S L L K R I A Q Q E Q M <orfX                                    prfA> M I R Y P N G K T F Q P K
600 GCTCAGAAGCTTTCTGATGGCCTGTTGTTCTTGCACGTTCATTCACTTCCGTCATGATTAGTTTAATAAGGAGGATGAGAAAGTGATTCGGTATCCTAATGGAAAAACATTTCAGCCGAA

 14  H S V S S Q N S Q K R A P S Y S N R G M T L E D D L N E T N K Y Y L T N Q I A V
720 ACATTCGGTTTCATCCCAAAACAGTCAGAAAAGGGCCCCGTCTTACAGTAATCGCGGAATGACCCTCGAAGATGACTTAAACGAAACGAATAAGTATTATCTGACAAACCAAATTGCCGT

 54  I H K K P T P V Q I V N V H Y P K R S A A V I K E A Y F K Q S S T T D Y N G I Y
840 TATACACAAAAAGCCGACACCTGTTCAAATTGTAAATGTCCATTATCCAAAAAGAAGTGCCGCAGTGATTAAAGAAGCTTACTTTAAACAATCATCGACAACAGACTACAATGGGATTTA

 94  K G R Y I D F E A K E T K N K T S F P L Q N F H D H Q I E H M K Q V K A Q D G I
960 CAAAGGGCGGTATATTGATTTTGAGGCGAAAGAAACGAAAAACAAGACCTCTTTCCCCCTGCAGAATTTTCATGACCATCAAATCGAGCATATGAAGCAGGTAAAGGCTCAAGACGGTAT

134  C F V I I S A F D Q V Y F L E A D K L F Y F W D R K E K N G R K S I R K D E L E
1080 TTGTTTTGTTATTATATCCGCTTTCGACCAGGTTTATTTTTTGGAAGCCGATAAGCTGTTTTATTTCTGGGACAGAAAAGAAAAAAACGGCAGAAAATCAATTCGAAAAGATGAGTTGGA

174  E T A Y P I S L G Y A P R I D Y I S I I E Q L Y F S P S S G A K G *                ponA>
1200 AGAAACAGCTTATCCGATTTCTCTTGGATACGCACCCAGAATTGATTATATTAGTATTATTGAACAGCTTTATTTTTCGCCATCATCTGGTGCGAAAGGTTGATTAACGAAAGGTTGAGA

  1  M S D Q F N S R E A R R K A N S K S S P S P K K G K K R K K G G L F K K T L F
1320 TGTTATGTCAGATCAATTTAACAGCCGTGAAGCTCGACGAAAAGCGAACAGCAAATCGAGTCCTTCACCGAAAAAAGGCAAGAAACGAAAAAAGGGCGGATTGTTTAAAAAGACCCTTTT

 40  T L L I L F V L G V V G G A V T F A V M V S D A P S L D E S K L K T P Y S S T I
1440 CACTTTACTCATTCTGTTTGTTTTAGGCGTAGTCGGCGGTGCTGTTACATTTGCCGTCATGGTTTCTGATGCGCCGTCCCTTGATGAAAGCAAATTGAAGACGCCTTATTCATCAACGAT

 80  Y D K N G K E I A E V G A E K R T Y V S I D E I P D V V K E A F I A T E D A R F
1560 TTATGATAAAAATGGAAAAGAGATCGCCGAAGTCGGCGCCGAAAAACGGACCTACGTCTCGATAGATGAAATTCCCGATGTTGTAAAAGAAGCCTTTATCGCGACAGAAGACGCTCGTTT

120  Y E H H G I D P V R I G G A L V A N F K D G F G A E G G S T I T Q Q V V K N S L
1680 TTATGAGCACCACGGGATTGACCCTGTCCGTATCGGCGGTGCATTAGTTGCCAACTTTAAAGACGGCTTCGGGGCTGAAGGCGGAAGTACGATCACCCAGCAGGTCGTCAAAAACTCCCT

160  L S H Q K T L K R K V Q E V W L S I Q L E R N Y S K D E I L E M Y L N R I Y F S
1800 TCTTTCACATCAGAAGACGTTGAAACGGAAGGTGCAGGAAGTATGGCTTTCGATTCAGCTGGAGCGCAATTACTCTAAAGATGAAATTTTAGAAATGTATTTAAACCGGATTTATTTTTC

200  P R A Y G I G K A A E E F F G V T D L S K L T V E Q A A T L A G M P Q S P T A Y
1920 TCCTAGAGCATACGGAATCGGAAAAGCGGCGGAAGAATTTTTCGGCGTTACTGATTTAAGCAAATTGACTGTCGAACAGGCTGCGACGCTTGCAGGAATGCCGCAGAGCCCAACAGCGTA

240  N P V K N P D K A E K R R N I V L S L M K K Q G F I S D S Q Y N K A K K V A V K
2040 CAACCCGGTCAAAAACCCGGATAAAGCGGAAAAACGACGGAACATCGTACTCAGTCTGATGAAAAAGCAAGGATTTATTTCTGATTCTCAATATAACAAAGCGAAAAAAGTGGCAGTGAA

280  D E G V V S Q K E Y E K A S T N K Y S A F V E E V M K E I D E K S D V D P S A D
2160 AGACGAAGGCGTCGTGTCACAGAAGGAATATGAAAAAGCAAGTACAAACAAATACAGCGCATTTGTTGAAGAGGTTATGAAGGAAATTGATGAAAAATCTGATGTCGATCCATCTGCTGA

320  G L K I Y T T L D T K A Q D K L D E L M D G D T V G F T E G M Q G G V T L L D T
2280 CGGATTAAAGATCTATACGACATTAGATACAAAAGCACAAGATAAACTAGATGAATTAATGGACGGAGACACCGTCGGATTTACTGAAGGCATGCAGGGCGGTGTGACGCTTCTCGATAC

360  K N G E V R A I G A G R N Q P V G G F N Y A T Q T K A Q P G S T I K P I L D Y G
2400 CAAAAACGGAGAAGTCCGAGCGATTGGTGCGGGACGCAATCAGCCTGTCGGAGGCTTTAACTATGCTACTCAAACTAAGGCACAGCCTGGTTCGACCATAAAACCGATTTTGGACTACGG

400  P V I E N K K W S T Y E Q I D D S A Y T Y S N G K P I R D W D R K Y L G P I S M
2520 ACCAGTTATTGAAAATAAGAAATGGTCCACATACGAACAAATTGATGACTCAGCTTATACGTATTCTAACGGAAAACCGATTCGTGATTGGGACCGCAAATATCTAGGGCCTATCTCAAT

440  R Y A L A Q S R N I P A L K A F Q A V G K D T A V D F A N G L G L G L T K D N V
2640 GCGTTACGCTCTGGCCCAATCAAGAAATATACCTGCTTTAAAAGCATTCCAGGCAGTCGGTAAAGATACTGCTGTAGACTTTGCAAATGGACTCGGGCTTGGTTTAACAAAAGATAATGT

480  T E A Y S I G G F G G N D G V S P L T M A G A Y S A F G N N G T Y N E P H F V K
2760 AACAGAGGCCTATTCTATTGGCGGTTTCGGTGGGAACGATGGTGTTTCTCCTCTGACAATGGCAGGTGCATACAGCGCGTTTGGAAATAACGGAACGTATAATGAACCGCATTTTGTAAA

520  S I E F N D G T K L D L T P K S K S A M S D Y T A F M I T D M L K T A V K T G T
2880 ATCTATCGAATTTAACGATGGCACGAAGCTTGACTTAACACCAAAATCAAAATCAGCCATGAGTGATTATACTGCGTTTATGATTACAGATATGCTGAAAACAGCTGTGAAGACAGGTAC

560  G Q L A Q V P G V E V A G K T G T T N F D D N E V K R Y N I A S G G A R D S W F
3000 TGGACAGCTTGCACAAGTACCTGGTGTAGAAGTTGCAGGAAAAACAGGAACGACTAACTTTGATGATAATGAAGTCAAAAGGTACAATATCGCTAGCGGTGGCGCCCGAGATTCTTGGTT

600  V G Y T P Q Y T A A V W T G M G E N E A G K K S L S A E E Q K V A K R I F A Q L
3120 CGTTGGCTACACACCGCAATATACAGCTGCCGTCTGGACGGGAATGGGAGAAAACGAGGCTGGAAAGAAATCACTTTCAGCTGAAGAGCAAAAAGTTGCAAAGCGCATCTTCGCCCAGCT

640  I A D V D D G S G S F E K P D S V V E A T V E K G S N P A K L A G P N T P S D K
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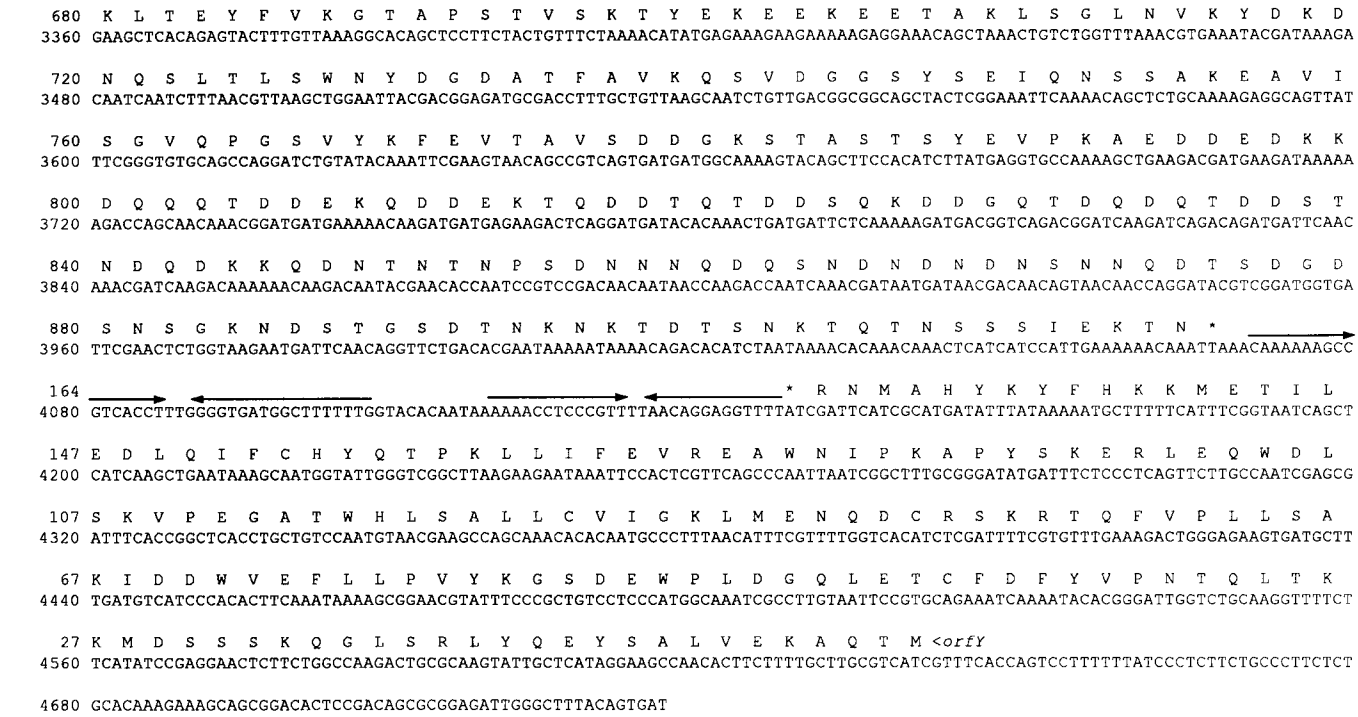

FIG. 2. Nucleotide sequence of the *ponA* locus. The numbers on the left designate the nucleotide or amino acid at the left end of each row. Amino acids are numbered independently for each open reading frame. Underlined nucleotide sequences are predicted ribosome binding sites. Horizontal opposed arrows indicate the regions of dyad symmetry predicted to function as transcription terminators. The underlined amino acid sequence is the class A high-molecular-weight PBPs consensus sequence described in the cloning strategy. The amino acid sequence in boldface is that determined from a PBP1 cyanogen bromide digestion product. The boxed amino acid sequences are those conserved in penicillin-binding active sites (16).

were verified by Southern hybridizations. β-Galactosidase was assayed by using *o*-nitrophenyl β-D-galactopyranoside as previously described (32).

Membrane preparation, PBP purification, and PBP assays. Membranes were prepared on a large scale, PBPs were purified by penicillin affinity chromatography and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and a cyanogen bromide digestion product of PBP1 (a mixture of PBP1a and -1b) was purified and sequenced as described previously (35). For small-scale membrane preparations, strains were grown at 37°C to an optical density at 600 nm of 1.5 in 100 ml of 2xSG medium. All steps in the membrane purification were carried out at 4°C. Cells were harvested by centrifugation at 4°C, resuspended in 25 ml of cold 50 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$–1 M KCl–0.1 mM phenylmethylsulfonyl fluoride (PMSF), and centrifuged again. The cell pellets were frozen in a dry ice-ethanol bath and stored at −80°C. Cell pellets were thawed and resuspended in 2 ml of buffer A (100 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM PMSF). The cells were broken by sonication in the presence of 200 mg of glass beads for two 30-s bursts. The suspensions were then diluted with 8 ml of buffer A and centrifuged at 8,000 × *g* for 10 min to remove unbroken cells and large debris. Membranes were pelleted from the supernatant by centrifugation at 48,000 × *g* for 1 h. The pellet was resuspended in 5 ml of buffer B (50 mM Tris-HCl [pH 8.0], 1 mM β-mercaptoethanol, 0.1 mM PMSF) and centrifuged again to pellet the membranes. The membrane pellets were suspended in 0.2 ml of buffer B, aliquoted to several tubes, frozen in a dry ice-ethanol bath, and stored at −80°C. The protein concentrations of membrane samples were determined by the method of Lowry et al. (26), with the addition of 1% SDS to the reaction mixture. Samples for protein assay were first lyophilized and resuspended in H_2O to avoid interference from β-mercaptoethanol.

PBPs were detected using [^{3}H]benzylpenicillin (19 Ci/mmol; Amersham). Fifty micrograms of membrane proteins in buffer B was incubated with 20 μM [^{3}H]benzylpenicillin in a total volume of 20 μl for 10 min at room temperature. The reaction was stopped by the addition of 20 μl of 2× SDS-PAGE sample buffer and heating at 90°C for 3 min. The entire reactions were separated by SDS-PAGE on a 7.5% polyacrylamide gel. The gel was stained, impregnated with En^{3}Hance (Amersham), dried, and subjected to fluorography at −80°C for 1 to 3 weeks.

Digoxigenin-labeled ampicillin (DIG-ampicillin) was prepared and purified by column chromatography as previously described (43). The concentration of DIG-ampicillin was estimated on the basis of a total digoxigenin recovery in usable column fractions of 50%. Reactions of PBPs with DIG-ampicillin and SDS-PAGE were exactly as for [^{3}H]benzylpenicillin except that only 25 μg of protein was in each reaction mixture and 1.4 mM (1.25 mg/ml) DIG-ampicillin was substituted for [^{3}H]benzylpenicillin. The separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore), and bound DIG-ampicillin was detected by using antidigoxigenin antibody-alkaline phosphatase conjugate (Boehringer Mannheim) and Lumiphos 530 (Boehringer Mannheim) as suggested by the manufacturer.

GenBank accession number. The DNA sequence reported here has been deposited in the GenBank sequence database under accession number U11883.

RESULTS

Cloning and nucleotide sequence of the *ponA* locus. A degenerate oligonucleotide encoding a highly conserved peptide sequence found in the amino-terminal domains of class A high-molecular-weight PBPs was used to screen a λEMBL3 library of *B. subtilis* DNA (35). Phages containing three distinct hybridizing sequences were obtained. Two of these hybridizing sequences were found to be parts of the previously identified *pbpD* and *pbpF* genes (18, 34, 35). Part of the insert from the third class of hybridizing phage was subcloned (Fig. 1), and the DNA sequence was determined. The sequence made it clear that the hybridizing sequence was part of a previously unidentified gene coding for a class A high-molecular-weight PBP. The remainder of this gene was obtained by using a chromosome walking technique (Fig. 1), and the nucleotide sequence of the entire locus was determined (Fig. 2).

The gene containing the hybridizing sequence codes for a 914-amino-acid protein with a molecular weight of 99,562. This open reading frame is preceded by a sequence with limited similarity to the 3′ end of 16S rRNA that presumably functions as a ribosome binding site and is followed by a region of dyad symmetry which we believe to be a transcription terminator. We found a peptide sequence in this protein which we had previously obtained by microsequencing a cyanogen bromide

digestion product of *B. subtilis* PBP1 (Fig. 2, boldface residues). Further evidence that this gene encodes PBP1 was provided by the disappearance of both PBP1a and PBP1b in a strain in which this gene was interrupted and the truncation of both proteins produced by an insertion near the distal end of the gene (see below). We have named this gene *ponA* (penicillin-binding protein one), consistent with the nomenclature used for *E. coli* (39). It must be noted that this name was previously used for the gene which is now referred to as *pbpF* (18, 34). The predicted *ponA* product exhibits a high degree of sequence similarity with class A high-molecular-weight PBPs of other organisms. When sequences are aligned by using the GAP program (15), we find 37% identical residues in the *Streptococcus pneumoniae* PBP1a (27) and 30% identical residues in the *E. coli* PBP1a (7). In its amino terminus, PBP1 contains a signal sequence composed of a positively charged N region of 36 residues and an H region of 25 hydrophobic residues. There are potential signal peptidase cleavage sites following residues 58 and 64 (Fig. 2). We attempted to determine the amino-terminal sequence of PBP1 and found that it was blocked to Edman degradation. As in other class A high-molecular-weight PBPs, the highly conserved amino acid motifs which make up the penicillin-binding active site are found in the carboxyl-terminal half of PBP1 (Fig. 2). The penicillin-binding domain in PBPs is suggested to end approximately 60 amino acids beyond the conserved KTG motif (16). The *B. subtilis ponA* product has 279 amino acids beyond this domain. This region of the protein contains 33% acidic and basic residues and has a total charge of −33.

Upstream of *ponA*, we found an open reading frame encoding a 206-amino-acid protein with a molecular weight of 23,927 (Fig. 2). A strong predicted ribosome binding site is found upstream of this open reading frame. This gene is in an operon with *ponA* (see below), and we refer to it as *prfA* (PBP-related factor). The predicted product of *prfA* has 50% identical residues with a gene found in the same position upstream of the *S. pneumoniae* and *S. oralis ponA* genes (27). Upstream of *prfA* and in the opposite orientation is the beginning of an open reading frame which we refer to as *orfX* (Fig. 2). This gene is preceded by a reasonable ribosome binding site and encodes a protein of at least 212 amino acids. The predicted partial product of this gene exhibits no significant sequence similarity to any found in DNA and protein sequence databases. A Campbell-type insertion disrupting this gene had no effect on growth in rich or minimal medium, on sporulation, or on spore heat resistance. Downstream of *ponA* and in the opposite orientation is a gene, referred to as *orfY*, predicted to encode a protein of 170 amino acids (Fig. 2). This gene is identical to the *jooC* gene identified in the sequence recently entered into the GenBank database under accession number U11289. There is not a strong match to the 3′ end of 16S rRNA preceding *orfY*, but sequence data (GenBank accession number U11289) indicate that *orfY* may be translationally coupled to an upstream gene. A region of dyad symmetry predicted to be a transcription terminator is found immediately downstream of *orfY*.

Expression of the *ponA* operon. We measured transcription of *prfA* and *ponA* by using transcriptional fusions to *lacZ*. Expression of a *ponA-lacZ* fusion (pDPC146; Fig. 1) was relatively constant during vegetative growth and decreased throughout sporulation to a level equivalent to the background found in a strain containing no fusion (Fig. 3). There was essentially no β-galactosidase activity in the purified spores of the fusion strain. Activity reappeared approximately 15 min after exposure of the spores to germinants and rose to approximately the specific activity found in vegetative cells within 60 min. The patterns of transcription of *ponA* were similar when the fusion construct was placed at the *ponA* locus (pDPC146), so that all normal upstream sequences were present, and at the unlinked *amyE* locus (pDPC183), so that 1.3 kb of the normal sequence upstream of *ponA*, including *prfA* and 682 bp upstream of *prfA*, was present (Fig. 3). The higher level of expression at the *amyE* locus we attribute to its proximity to the chromosomal origin of replication, whereas *ponA* is closer to the replication terminus (see below). The *amyE* locus is thus at a higher copy number than *ponA* when chromosome replication is taking place. The fact that levels of expression at the two loci are identical during the first 60 min of germination (Fig. 3), prior to the initiation of DNA replication, supports this theory. (DNA replication was previously found to initiate 120 min after exposure to germinants in minimal medium, 90 min prior to cell division [46]. In rich medium, we did not observe cell division until 100 min after exposure to germinants. We infer that DNA replication was not initiated until after the first 50 to 60 min.) In all further studies, fusions were placed in the chromosome at the *amyE* locus. When we fused *prfA* transcription to *lacZ* (pDPC189), we observed approximately the same specific activity and pattern of expression as we had found for the *ponA-lacZ* fusion (Fig. 3). When the sequences preceding *prfA* and the first 55% of *prfA* were removed from the original *ponA-lacZ* fusion construct (pDPC188), 75% of the β-galactosidase activity was lost (Fig. 3). The remaining 25% of *ponA* transcription was detected when only the 141 bp preceding the *ponA* start codon was fused to *lacZ* (pDPC190) (Fig. 3). Further experiments indicated that there are at least two promoters upstream of *prfA*. However, attempts to determine the positions of these promoters by primer extension mapping were inconclusive.

Mutations in the *ponA* operon. We examined the effects of four different mutations in the *ponA* gene on growth in rich and minimal media, sporulation, spore heat resistance, and spore germination. Strain PS2096, carrying a Campbell-type insertion in the carboxyl-terminal region of the gene (pDPC257) which removed the last 173 codons and added 26 codons (encoding DSRGSPGTELEFLKTKGPRDTPIFIG), was indistinguishable from the wild type for growth on solid rich medium, but a very slight decrease in growth rate was found in rich and minimal liquid media (Table 1). This strain produced as many spores as the wild type (Table 1). PBP1a and -1b in this strain were both truncated to form a single band with an apparent molecular weight of 73,000 (Fig. 4). The predicted molecular weight of this truncated protein is 83,576. A Campbell insertion (pDPC119 in strain PS1936) which interrupted the *prfA-ponA* operon but left both genes intact produced a similar slight decrease in growth rate in liquid media (Table 1), but again this change was not obvious on solid rich medium. Dissociation of *ponA* from the promoter activity upstream of *prfA* in this strain resulted in a 5- to 10-fold decrease in the amount of PBP1 in the cell (Fig. 5, lane 2). This mutation also had no effect on the number of spores produced (Table 1). Strains in which *ponA* was interrupted early in the coding sequence (pDPC193 in strain PS2031) or in which part of *ponA* was deleted and replaced by an antibiotic resistance marker (pDPC197 in strain PS2062) exhibited identical phenotypes. Both strains had no PBP1 in their membranes (Fig. 4 and 5), and relative to a wild-type strain, both had a significant decrease in growth rate in rich and minimal media (Table 1). The slow-growth phenotype of these two strains was also obvious on solid rich medium, and they exhibited an abnormal colony phenotype (Fig. 6). During the later stages of colony development, the outer edges of the colonies raised to form a "crown," whereas the center of the colony raised in the wild type. Microscopic observation indicated that relative to the wild-type

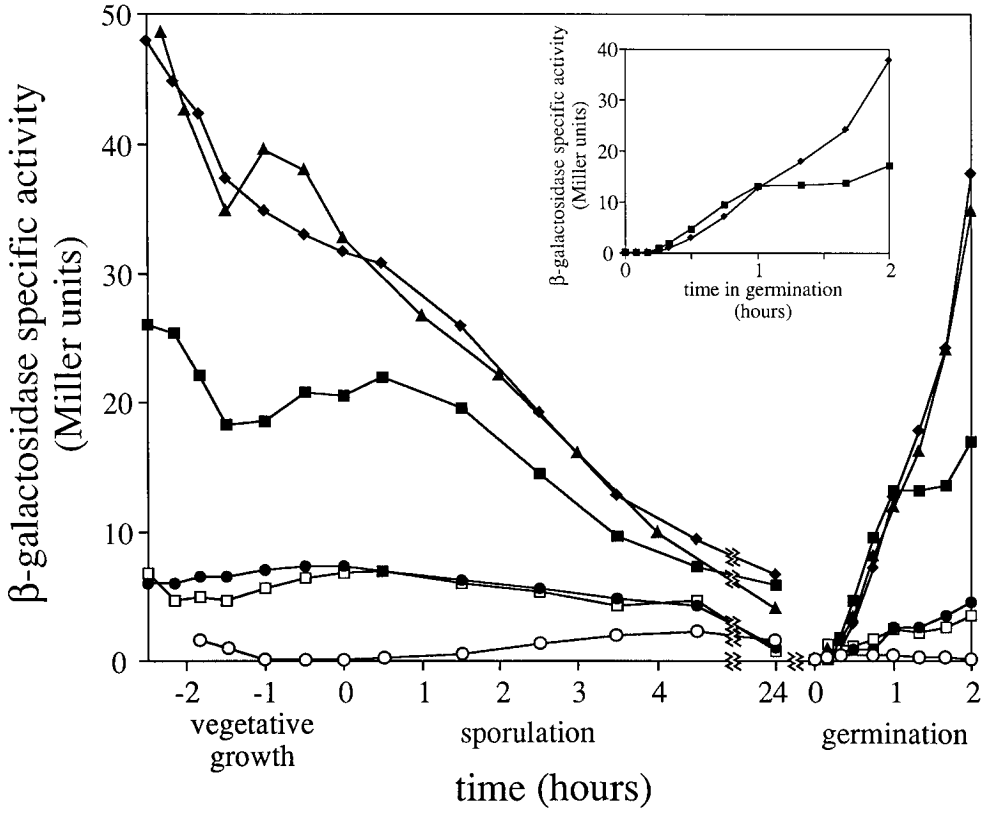

FIG. 3. Expression of *lacZ* fusions. Strains were grown and sporulated in 2xSG medium at 37°C. Purified spores were germinated in 2xYT medium containing 4 mM L-alanine. The left and right points labeled 0 on the *x* axis indicate the time of initiation of sporulation and the time of exposure of spores to germinants, respectively. Optical density decreased by approximately 50% 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. The inset graph is an enlargement of the germination period for two fusion strains to clarify the time of initiation of expression. Symbols used for the strains and the fusion plasmids that they contain: ■, PS1957, pDPC146, *ponA-lacZ* at the *ponA* locus; ◆, PS2014, pDPC183, 1.3 kb preceding *ponA-lacZ* at the *amyE* locus; ●, PS2024, pDPC188, 410 bp preceding *ponA-lacZ* at the *amyE* locus; ▲, PS2025, pDPC189, 682 bp preceding *prfA-lacZ* at the *amyE* locus; □, PS2026, pDPC190, 141 bp preceding *ponA-lacZ* at the *amyE* locus; ○, PS 832, no fusion.

strain, a lower percentage of the mutant cells produced spores, though the number of spores produced per milliliter of culture was equal to or greater than that produced by the wild type. These data, in conjunction with the fact that the cultures achieved equal optical densities, suggest that the *ponA* cells may be smaller than the wild-type cells. None of the *ponA* mutations resulted in an obvious change in cell morphology or motility when examined during logarithmic growth. The spores produced by all of the different types of *ponA* mutant strains were fully heat resistant (Table 1) and underwent germination and outgrowth with wild-type kinetics (data not shown).

The phenotypic effects of two types of mutations in *prfA* were also examined. Strain PS2123, containing an in-frame deletion of residues 26 to 112 of the *prfA* coding sequence (42% of the gene), produced a normal amount of PBP1 (Fig. 5, lane 7). Surprisingly, a strain in which much of *prfA* was deleted and replaced with an Sp^r cassette (pDPC195 in strain PS2061) also produced a normal amount of PBP1 (Fig. 5, lane

TABLE 1.

Strain (genotype)	Doubling time (min) in:		No. of spores/ml[a]	% of cells producing spores[b]	Heat resistance (% survival)[c]
	2xSG medium	Minimal medium			
PS832 (wild type)	20	48	6.8×10^8	≥80	39
PS1936 (*ponA*::pDPC119)	22	49	6.0×10^8	≥80	35
PS2096 (*ponA*::pDPC257)	22	50	4.2×10^8	≥80	36
PS2031 (*ponA*::pDPC193)	28	62	9.6×10^8	≥50	47
PS2062 (Δ*ponA*::Sp^r)	28	58	1.1×10^9	≥50	55
PS2061 (Δ*prfA*::Sp^r)	36	62	3.8×10^8	≥80	30
PS2123 (Δ*prfA* in frame)	35	63	5.8×10^8	≥80	33
PS2067 [Δ(*prfA-ponA*)::Sp^r]	≥80	≥120	1.2×10^8	10–20	ND[d]

[a] The number of CFU surviving 15 min at 85°C was determined 48 h after the end of exponential growth.
[b] The percentage of cells which had produced spores 24 h after the end of exponential growth was estimated by microscopic observation.
[c] Purified spores which exhibited >30% survival after 15 min at 85°C were considered to have normal heat resistance.
[d] ND, not determined. The low percentage of strain PS2067 cells which produced spores made purification of these spores impractical.

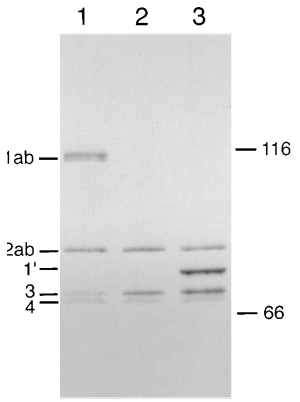

FIG. 4. PBP profiles of wild-type and *ponA* mutant strains. Membrane preparations from vegetative cells were incubated with [^{3}H]benzylpenicillin and separated by SDS-PAGE on a 7.5% polyacrylamide gel at 40 mA for 4 h. Samples were from strains PS832 (wild type; lane 1), PS2031 (*ponA*::pDPC193; lane 2), and PS2096 (*ponA*::pDPC257; lane 3). PBPs were detected by fluorography and are numbered on the left as described in references 6 and 23 (PBP5 has run off the bottom of the gel). The truncated form of PBP1 produced by strain PS2096 is indicated by 1′. Molecular mass markers indicated on the right were β-galactosidase (116 kDa) and bovine serum albumin (66 kDa). The film image was digitally scanned and reproduced by using Adobe Photoshop software.

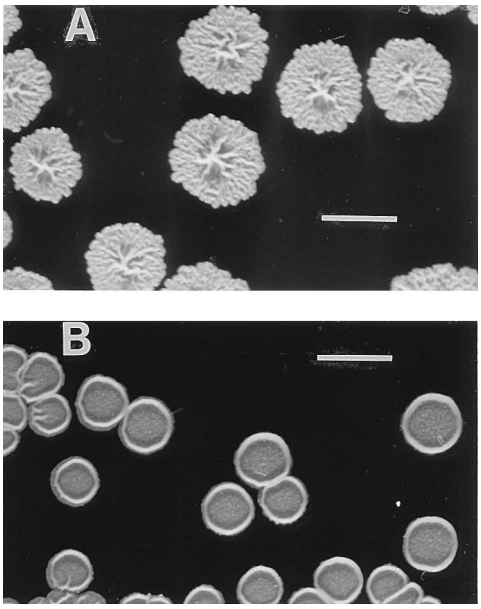

FIG. 6. Colony morphologies of wild-type and *ponA* mutant strains. Strains PS832 (wild type; A) and PS2062 (Δ*ponA*::Sp^r; B) were incubated on solid 2xSG medium at 37°C for 3 days until sporulation was complete. Bar, 3 mm.

6). We believe that *ponA* expression in this strain may have resulted from transcription readthrough from the Sp^r gene. These two mutations resulted in identical phenotypes. On rich solid medium, the mutant strains produced slowly growing colonies of normal morphology. When inoculated into rich or minimal liquid media, these strains entered a long lag phase, relative to wild-type and *ponA* strains, before beginning to grow exponentially at a greatly decreased rate (Table 1). A normal number of spores was produced by the *prfA* mutants, and these spores had normal heat resistance (Table 1). The response of these mutant spores to germinants was somewhat slower than that of the wild type, though once germination was initiated, germination and outgrowth appeared to proceed normally (data not shown).

We attempted to construct a strain in which much of the coding sequences of both *prfA* and *ponA* were deleted and replaced by an Sp^r marker. A plasmid containing this construction (pDPC196) was linearized and transformed into a wild-type strain. The transformed cells were plated on rich medium containing spectinomycin and incubated at 37°C. We observed no colonies until day 3 of incubation. The colonies which arose grew extremely slowly when streaked onto fresh medium and gave rise to a few faster-growing colonies which we presumed to contain additional mutations. We cannot rule out the possibility that deletion of *prfA* and *ponA* is lethal and that even the slowly growing strains contain suppressor mutations. Southern blot analysis of chromosomal DNA from the slowly growing and faster-growing strains indicated that all had the expected deletion of *prfA* and *ponA*. To verify this result, we attempted to transform a strain carrying a Campbell-type insertion early in *ponA* (pDPC193, Cm^r) with chromosomal DNA from a strain carrying a deletion mutation in *prfA* (pDPC195, Sp^r) (Fig. 1). When we selected only Sp^r, we obtained many transformants, all of which were Cm^s, indicating that they had lost the *ponA* mutation. When we selected both Sp^r and Cm^r, we obtained only very small colonies, similar to those containing the deletion of both genes. These suspected results were confirmed by Southern blotting (data not shown). We obtained identical results when we attempted the reverse transformation. Growth of the *prfA-ponA* deletion strain (PS2067) was very slow in liquid media (Table 1), but no change in cell morphology was evident. This strain sporulated at a slightly reduced level relative to the wild type (Table 1). Though the poor sporulation of this strain made purification of the spores impractical, the fact that 1.2×10^8 spores per ml survived heat treatment (Table 1) suggests that they possessed normal heat resistance.

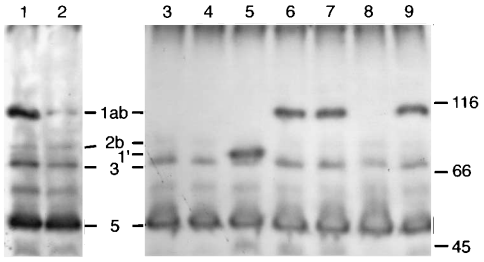

FIG. 5. Levels of PBP1 in *ponA* and *prfA* mutants. Membrane preparations from vegetative cells were incubated with DIG-ampicillin and separated by SDS-PAGE on a 7.5% polyacrylamide gel. PBPs were detected with an anti-digoxigenin antibody-alkaline phosphatase conjugate and a chemiluminescent phosphatase substrate. PBPs are numbered between the two panels as described in references 6 and 23. Control experiments using mutant strains lacking individual PBPs demonstrated that PBP2a and -4 are not detected by this method (data not shown). Molecular weight markers indicated on the right were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa). Membrane preparations were from strains PS832 (wild type; lanes 1 and 9), PS1936 (*ponA*::pDPC119; lane 2), PS2031 (*ponA*::pDPC193; lane 3), PS2062 (Δ*ponA*::Sp^r; lane 4), PS2096 (*ponA*::pDPC257; lane 5), PS2061 (Δ*prfA*::Sp^r; lane 6), PS2123 (Δ*prfA* in frame; lane 7), and PS2067 [Δ(*prfA-ponA*)::Sp^r; lane 8]. Film images were digitally scanned and reproduced by using Adobe Photoshop software.

Mapping of the chromosomal location of the *ponA* operon. The location of the *prfA-ponA* operon on the physical map of the *B. subtilis* chromosome (21) was determined by hybridization of a *ponA*-specific probe to a Southern blot of *Not*I and *Sfi*I chromosomal restriction fragments which had been separated by pulsed-field gel electrophoresis. The probe hybridized

to the 265-kb 1N *Not*I fragment and the 160-kb KS *Sfi*I fragment (data not shown). These data demonstrate that this locus is found within a 47-kb region located at approximately 200°C on the map of the chromosome. Our mapping data were recently verified when the sequence encoding *orfY*, found downstream of *ponA*, was entered into GenBank as a gene of unidentified function called *jooC* (GenBank accession number U11289). This gene is found near *dnaD*, which has been placed at 199° on the genetic map of the chromosome (3).

DISCUSSION

We have isolated and characterized the single gene encoding the class A high-molecular-weight PBP1a and -1b of *B. subtilis*. (This differs from the situation in *E. coli*, in which PBP1a and -1b are the products of distinct genes.) The *B. subtilis ponA* gene encoding the PBPs is the distal gene in a two-gene operon. The proximal gene, termed *prfA*, is very similar to a gene found immediately upstream of the *ponA* gene of *S. pneumoniae*, which also encodes a class A high-molecular-weight PBP (27). The protein product of *prfA* does not contain a signal sequence, indicating that it is probably found in the cytoplasm. We suspect that the function of this protein is related to that of PBP1 (or other PBPs) because the combined effects of *ponA* and *prfA* mutations are much more dramatic than the effects of either individual mutation. Further genetic and biochemical experiments will be required to verify this.

The amino acid sequence of PBP1 predicted from the *ponA* gene sequence indicates that PBP1 possesses all of the normal characteristics of a class A high-molecular-weight PBP. It has a signal sequence which contains a relatively long basic N region (36 amino acids versus the usual 5 to 20 [42]) (Fig. 7). A similar extended N region is found in the *Staphylococcus aureus* PBP2 (GenBank accession number L25426) (Fig. 7). Whether this region of the protein is important for interaction with a cytoplasmic component is unknown. We were unable to determine if the signal sequence was cleaved or remains on the protein as a membrane anchor as has been demonstrated or suggested for a number of other class A high-molecular-weight PBPs (1, 13, 22, 34, 35). Cleavage of the signal peptide would result in a requirement for a different membrane anchor such as that observed in PBP1b of *E. coli* (13, 31). The putative transglycosylase domain of PBP1 shows a great degree of similarity to those of other species (Fig. 7).

We find that the sequence conservation among class A high-molecular-weight PBPs ends at approximately the point suggested to be the end of the penicillin-binding domain (16). The previously sequenced class A high-molecular-weight PBPs generally have carboxyl-terminal extensions of 72 to 115 residues beyond the predicted penicillin-binding domain (16) (one exception is the *B. subtilis* PBP4, which has no extension [35]). The *B. subtilis* PBP1 carboxyl-terminal extension of 279 amino acids is thus relatively large. This region of the protein is rich in asparagine and glutamine and is highly charged, containing 29 positively charged and 62 negatively charged amino acids. The carboxyl-terminal extensions of the *E. coli* PBP1a and -1b have high negative charge-to-mass ratios similar to that found in *B. subtilis* PBP1, and a high level of glutamine residues is also found in this region of *E. coli* PBP1b (7). The *S. pneumoniae* PBP1a (27) and the *Staphylococcus aureus* PBP2 (GenBank accession number L25426) do not have high charge-to-mass ratios but do have high levels of asparagine and glutamine residues in their carboxyl-terminal extensions. Finally, the carboxyl-terminal extension of the *B. subtilis pbpF* product (34) has a low charge-to-mass ratio and little asparagine or glutamine, but it does have a high percentage of charged residues. The function of this protein domain is unknown. Whereas there does seem to be some selective pressure for maintenance of a particular amino acid composition, this domain does not seem to be essential for the function of the protein. Removal of 61% of the *B. subtilis* PBP1 carboxyl-terminal extension had little effect on growth of the cells. Removal of these amino acids did result in the production of a single truncated PBP1 band rather than the doublet of PBP1a and -1b, suggesting that these two protein species normally differ in their carboxyl termini. We note that the *E. coli* PBP3 is normally processed at its carboxyl terminus (19, 28); however, we see no sequence similarity between the *E. coli* PBP3 processing site and the carboxyl-terminal extension of *B. subtilis* PBP1. An effect of the carboxyl-terminal extension on the electrophoretic mobility of PBP1 was also suggested by examination of the truncated protein. Whereas PBP1 normally has an apparent molecular weight of >100,000 when analyzed by SDS-PAGE (Fig. 4 and 5) (8), consistent with the 99,562 predicted from the DNA sequence, truncation of the protein resulted in an apparent molecular weight of 73,000, significantly less than the predicted 83,576. Possibly the odd structure of the carboxyl-terminal extension has a significant effect on the mobility of the normal protein. Alternatively, truncation of the protein may render it sensitive to proteolysis to produce a species with an apparent molecular weight of 73,000. We note that removal of the entire 279-amino-acid carboxyl-terminal extension would produce a protein of molecular weight 69,321.

A 5- to 10-fold reduction in the amount of PBP1 had very little effect on growth, suggesting that an excess of this protein is present in the cell. The complete loss of PBP1 resulted in a significant growth defect and the production of an abnormal colony morphology. This growth phenotype is similar to that resulting from the loss of a class A high-molecular-weight PBPs in *E. coli*. Strains lacking PBP1b had a slight decrease in growth rate, but there was no reported change in cell or colony morphology (47). Loss of both of the *E. coli* class A high-molecular-weight PBP1a and -1b was lethal (39, 47). We suspect that a similar pattern will emerge when we combine a *ponA* mutation with mutations in the other two genes encoding class A high-molecular-weight PBPs in *B. subtilis*, *pbpD* and *pbpF* (18, 34, 35). No phenotype was previously reported for the loss of PBP1 in *B. subtilis* (8, 11). The original *ponA* mutation was isolated as one step in a series of mutational events leading to high-level cloxacillin resistance, which affected a number of PBPs (11). DNA from this highly resistant strain was then transformed into a cloxacillin-sensitive strain, with selection for low-level resistance (8). Whereas a number of the transformants appeared to be affected only in PBP1, we feel it possible that these transformants also received a linked suppressor mutation that alleviated the growth and colony morphology phenotypes that we observe. We do not feel confident to comment on whether the cell diameter of strains lacking PBP1 is altered as has been previously found (8). We found it difficult to judge a 16% decrease in diameter (23) by using only phase-contrast microscopy. However, the fact that these strains produced as many spores per milliliter of culture as the wild type did, despite the fact that a lower percentage of the cells produced spores in cultures of equal optical density, suggests that the mutant cells may be smaller than the wild-type cells. Because of the errors inherent in these types of measurements, further experiments will be required to verify this change in cell size.

Expression of *prfA* and *ponA* is driven by at least one promoter upstream of *prfA* and at least one promoter within *prfA*. Transcription of the operon decreased and essentially ceased during sporulation, consistent with previous observations that the amount of PBP1 decreases during sporulation (36, 40). Tran-

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         1                                                  50                                                 100
EcPBP1a                                     MKFVKYF LILAVCCILL GAGSIYGLYR YIEPQLPDV. .......... ATLKDVRLQI PMQIYSADGE
 PssPBP                                     MIRLLKFF WWSLVAVFCS LLLGLSGAFL YLSPTLPSV. .......... EALRSIQLQI PLRVFSSDGK
 HiPONA                                     MRIAKLI LNTLLTLCIL GLVAGGMLYF HLKSELPSV. .......... ETLKTVELQQ PMQIYTADGK
 PsaPBP                                     MRLLKFL WWTCVTLICG VLLSFSGAYL YLSPSLPSV. .......... EALRNVQLQI PLKVYSEDGK
  AePBP                                     MKRLWSAF IKLLVLAVIG GALLAAIAIL AANRQLPSL. .......... DALTAF.... ..........
BsPBP2c                                     MFKIKKKK LFIPIIILVL TAFLALIGYI SIIFLGHYV. .......... IDEKKLILHA SSKIVDQNGD
 BsPBP4                                 M TMLRKIIGWI LLLCIIPLFA FTVIASGKEV KQMKSLDQVL .......DKN IDLKDISLVQ NSYMYDRDGS
SpPBP1a                                 MNK PTILRLIKYL SISFLSLVIA AIVLGGGVFF YYVSKAPSL. .......... .SESKLVATT SSKIYDNKNQ
SoPBP1a                                 MNK QTILRIAKYI SICFLTVFIA AIMLGGGLFL YYVSNAPAL. .......... .SESKLVATT SSKIYDNNDE
 BsPBP1     MSDQFNS REARRKANSK SSPSPKKGKK RKKGGLFKKT LFTLLILFVL GVVGGAVTFA VMVSDAPSL. .......... .DESKLKTPY SSTIYDKNGK
 SaPBP2   MTENKGSSQ PKKNGNNGGK SNSKKNRNVK RTIIKIIGFM IIAFFVVLLL GILL....FA YYAWKAPAF. .......... .TEAKLQDPI PAKIYDKNGE
EcPBP1b  QLAAAVYGRM VNLEPDMTIS KNEMVKLLEA TQYRQVSKMT RPGEFTVQAN SIEMIRRPFD FPDSKEGQVR ARLTFDGDHL ATIVNMENNR QFGFFRLDPR
                                           ** * *     * ***   *     *       P *                 *       *Y

                                                  150                                                 200
EcPBP1a  LIAQYGEKR. RIP..VTLDQ IPPEMVKAFI ATEDSRFYEH HGVDPVGIFR AASVALFSGH ASQGASTITQ QLARNFFLSP ...ERTLMRK IKEVFLAIRI
 PssPBP  LIAEFGEMR. RTP..IRFAE IPPNFINALL SAEDDNFANH YGVDPSSIMR AATQLVKSGH IQSGGSTITM QVAKNFFLTS ...ERSFSRK TTEILLALQI
 HiPONA  LIGEVGEQR. RIP..VKLAD VPQRLIDAFL ATEDSRFYDH HGLDPIGIAR ALFVAVSNGG ASQGASTITQ QLARNFFLTS ...EKTIIRK AREAVLAVEI
 PsaPBP  LISEFGEMR. RTP..IRFAD IPQDFIHALL SAEDDNFANH YGVDVKSLMR AAAQLLKSGH IQTGGSTITM QVAKNYFLTN ...ERSFSRK INEILLALQI
  AePBP  ........R. HTPDYVPIGK IPRELTGAVV AIEDERFYVH DGIDYIGVVR AGVANL.SDE LSQGASTITM QVARNFYLSR ...DKTYTRK LYEVLLSYRI
BsPBP2c  EVASLYTEN. REP..VSINE IPKQVREAFI AVEDKRFYEH HGIDAKSVGR AVYRDILAGG KVEGGTTITQ QLAKNIFLTH ...DKTFLRK TKEVIIAINL
 BsPBP4  LVSEIVSDH. ENRVLVPFNK IPEEVKQIFL TSEDRHFYEH KGFDFMGMVP PTASNVKDKK IDQGASTITQ QLSRNLYLSH ...ERSFSRK LTELAYSYQL
SpPBP1a  LIADLG.SE. R.RVNAQAND IPTDLVKAIV SIEDHRFFDH RGIDTIRILG AFLRNLQ.SN SLQGGSALTQ QLIKLTYFST STSDQTISRK AQEAWLAIQL
SoPBP1a  LIADLG.SE. R.RVNAQANE IPTDLVNAIV SIEDHRFFNH RGIDTIRILG ATLRNLRGGG GLQGASTLTQ QLIKLTYFST STSDQTLSRK AQEAWLAVQL
 BsPBP1  EIAEVG.AE. K.RTYVSIDE IPDVVKEAFI ATEDARFYEH HGIDPVRIGG ALVANFKDGF GAEGGSTITQ QVVKNSLLSH ...QKTLKRK VQEVWLSIQL
 SaPBP2  LVKTLDNGQ. R.HEHVNLKD VPKSMKDAVL ATEDNRFYEH GALDYKRLFG AIGKNLTGGF GSEGASTLTQ QVVKDAFLSQ ...HKSIGRK AQEAYLSYRL
EcPBP1b  LITMISSPNG EQRLFVPRSG FPDLLVDTLL ATEDRHFYEH DGISLYSIGR AVLANLTAGR TVQGASTLTQ QLVKNLFLSS ...ERSYWRK ANEAYMALIM
         L*  *      *    * *   IP  *  A**   ED *F* H  G*D   *   A    *  G     G*ST*TQ Q* *N *L*     **** RK   E  L * *

                                                  250                                                 300
EcPBP1a  EQLLTKDEIL ELYLNKIYLG YRAY....GV GAAAQVYFG. KTVDQLTLNE MAVIAGLPKA PSTFNPLYSM DRAVARRNVV LSRMLDEGYI TQQQFDQTRT
 PssPBP  ERQLTKDEIL ELYVNKIYLG NRAY....GI EAAAQVYYG. KSIRDVSLAQ MAMIAGLPKA PSRFNPLANP TRAKERRDWI
 HiPONA  ENTLNKQEIL ELYLNKIFLG YRSY....GV AAAAQTYFG. KSLNELTLSE MAIIAGLPKA PSTMNPLYSL KRSEERRNVV LSRMLDEKYI SKEEYDAALK
 PsaPBP  ERQLTKDEIL ELYVNKI
  AePBP  EKALTKDEIL ELYMNKIYLG QGAY....GF ADAARTYFG. KRLDQLTLAE CAMLAGLGKA PSANNPVANP RRARQRQVYI LQ
BsPBP2c  ERDYSKDKLL EMYLNQLYFG HGVY....GI QAASHYYFN. KEVKDLTVSE GAVLAAIPKA PSTYSPILHP DKNKERRDTI LGMMNDQGYI SAKEAVTAQG
 BsPBP4  EKKYTKNEIL EAYLNTIYFN NGVY....GV GSAAQFYFS. KPLKSLTVGE MAFICAIPNN PTLYDPLKHF DYTKSRQERL LKGLKDAGVI TDKELKKAVK
SpPBP1a  EQKATKQEIL TYYINKVYMS NGNY....GM QTAAQNYYG. KDLNNLSLPQ LALLAGMPQA PNQYDPYSHP EAAQDRRNLV LSEMKNQGYI SAEQYEKAVN
SoPBP1a  EQKATKQEIL TYYINKVYMS NGNY....GM QTAAQSYYG. KDLKDLSIPQ LALLAGMPQA PNQYDPYSHP EAAQERRNLV LSEMKGQGYI TAEQYEKAIN
 BsPBP1  ERNYSKDEIL EMYLNRIYFS PRAY....GI GKAAEEFFGV TDLSKLTVEQ AATLAGMPQS PTAYNPVKNP DKAEKRRNIV LSLMKKQGFI SDSQYNKAKK
 SaPBP2  EQEYSKDDIF QVYLNKIYYS DGVT....GI KAAAKYYFN. KDLKDLNLAE EAYLAGLPQV PNNYNIYDHP KAAEDRKNTV LYLMHYHKRI TDKQWEDAKK
EcPBP1b  DARYSKDRIL ELYMNEVYLG QSGDNEIRGF PLASLYYFG. RPVEELSLDQ QALLVGMVKG ASIYNPWRNP KLALERRNLV LRLLQQQQII DQELYDMLSA
         E   *K*EIL E*Y*N**Y*     Y    G*   AA  YFG  K *  L**    A**AG*P * P* * P   P    A  RR  * L  M    *I *   *  A

                                                  350                 380
EcPBP1a  EAINANYHAP EIAFSA.... ..PYLSEMVR QEMYNRYGES A......... ..........  .YEDGYRIYT TITRKVQQAA
 HiPONA  EPIVASYHGA KFEFRA.... ..DYVTEMVR QEMVRRFGEE NA........ ..........  .YTSGYKVFT TVLSKDQAEA
BsPBP2c  RTL..... ......GLHV KKQSETPWFD SYIDLVIKEA EDKYSISGEQ L.........  .LQGGYTIKV PLDSKLQKTA
 BsPBP4  QKIKLDVEKR EDKYPDYVSY VNDEFTQLVS ESEGFDKRLQ KASGKQKEKI ENELSARVST LMKDGVKIYT ALDPYMQNQV
SpPBP1a  TPITDGLQS. ..LKSASNYP AYMDNYLKEV INQVEEETGY NL........ ..........  .LTTGMDVYT NVDQEAQKHL
SoPBP1a  TPITDGLQS. ..LKSANSYP PYMDNYLKEV IDQVEQETGY NL........ ..........  .LTTGMEVYT NVDSKVQQRL
 BsPBP1  VAVKD.EGVV SQKEYEKAST NKYSAFVEEV MKEIDEKSDV DP........ ..........  .SADGLKIYT TLDTKAQDKL
 SaPBP2  IDLKANLVNR TAEERQNIDT NQDSEYNSYV NFVKSELMNN KEFKDENLGN V.........  .LQSGIKIYT NMDKDVQKTL
EcPBP1b  RPLGVQPRGG VISPQPAFMQ LVRQELQAKL GDKVK..... ..........  .DLSGVKIFT TFDSVAQDAA
           *                                                      G* **T  *D   Q
```

FIG. 7. Alignment of the amino-terminal putative transglycosylase domains of class A high-molecular-weight PBPs. Sequence conservation at each position is indicated under the aligned sequences. Universally conserved amino acids are in boldface, amino acids conserved in 75% of the proteins are in standard type, and the presence of chemically similar amino acids in 75% of the proteins is indicated with an asterisk. Chemically similar groups of amino acids were considered to be (A,G), (S,T), (N,Q) (D,E), (H,K,R), (F,W,Y), and (F,I,L,M,V). Initial sequence similarities were detected by using the TBLASTN program (2). Complete and partial PBP sequences were extracted from the databases and aligned by using the PILEUP program (15). The lack of significant sequence conservation beyond position 300 prevented the PILEUP program from recognizing the box 4 conserved region recently defined by Ghuysen and Dive (17). We therefore modified the alignment beyond position 300 by hand in order to reveal box 4. Box 1 and box 4 defined by Ghuysen and Dive (17) are at positions 136 to 142 and 364 to 377, respectively, in our alignment. Examination of our alignment indicates that a box 4 consensus sequence for class A high-molecular-weight PBPs is G(X4)TXXD(X3)Q, whereas that for class B high-molecular-weight PBPs is G(X5)TXD(X3)Q (17). The highly conserved 10-amino-acid sequence used for identification of clones containing class A high-molecular-weight PBPs genes is found at positions 163 to 172 in this alignment. Species abbreviations used and the DNA sequence accession numbers: Bs, *B. subtilis* (PBP4, U11882; PBP2c, L10630; PBP1, U11883); Sa, *Staphylococcus aureus* (L25426); So, *S. oralis* (M90528); Sp, *S. pneumoniae* R6 (M90527); Ec, *E. coli* (PBP1a, X02164; PBP1b, X02163); Hi, *Haemophilus influenzae* (M62809); Ae, *Alcaligenes eutrophus* (Z22737); Pss, *Pseudomonas syringae* (L28837); Psa, *P. aeruginosa* (L13867). The first 100 amino acids of *E. coli* PBP1b are not shown. The *A. eutrophus*, *P. syringae*, and *P. aeruginosa* sequences are incomplete at their carboxy termini.

scription was reinitiated approximately 15 min after the initiation of spore germination. An increase in the amount of PBP1 early during germination had been previously observed (30).

The large number of class A high-molecular-weight PBPs for which sequence data are now available allows the identification of the most highly conserved regions in the amino-terminal putative transglycosylase domain (Fig. 7). These conserved sequences are potentially part of the active site for this enzymatic activity. In light of the considerable problem caused by pathogenic bacteria which have acquired resistance to the β-lactam antibiotics which inhibit the transpeptidase activity of the class A high-molecular-weight PBPs, further study of the transglycosylase activity of these proteins may make them an attractive target for rational antibacterial drug design.

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