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A Mother Cell-Specific Class B Penicillin-Binding Protein, PBP4b, in *Bacillus subtilis*

Yuping Wei,† Derrell C. McPherson,‡ and David L. Popham*

Department of Biology, Virginia Tech, Blacksburg, Virginia 24061

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The *Bacillus subtilis* genome encodes 16 penicillin-binding proteins (PBPs), some of which are involved in synthesis of the spore peptidoglycan. The *pbpI* (*yrrR*) gene encodes a class B PBP, PBP4b, and is transcribed in the mother cell by RNA polymerase containing σ^E. Loss of PBP4b, alone and in combination with other sporulation-specific PBPs, had no effect on spore peptidoglycan structure.

During bacterial endospore formation, two cells cooperate to produce a single dormant spore. Engulfment of the smaller cell, the forespore, by the larger mother cell results in the forespore being surrounded by two opposed membranes. A specialized peptidoglycan (PG) cell wall is synthesized in the intermembrane space (reviewed in reference 17), and this wall plays a key role in maintaining spore dormancy and heat resistance. Synthesis of the innermost PG layer, the germ cell wall, involves forespore-produced enzymes (12), while synthesis of the outer 80 to 90% of the spore PG, the cortex, is carried out by mother cell-expressed enzymes (5). The germ cell wall appears to serve as a template for synthesis of the cortex (12) and serves as the initial cell wall of a germinating spore (3), whereas the cortex is rapidly degraded during spore germination. The proteins involved in PG polymerization, the penicillin-binding proteins (PBPs), can be divided into three classes based upon domain structures and enzymatic activities (8). *Bacillus subtilis* possesses six genes that encode class B PBPs (7), proteins that frequently play roles in determining specific PG morphology, such as the rod shape or septum production (reviewed in reference 8). A class B PBP encoded by *spoVD* is mother cell specific and is required for cortex PG synthesis (5). We present here evidence that the product of *yrrR* is another mother cell-specific class B PBP, but that this protein plays no clear role in spore PG synthesis.

Identification of the *yrrR* product. A sequence alignment of the *yrrR* product using the tBLASTN software (1) revealed that the most similar proteins are class B PBPs, including *B. subtilis* SpoVD (27% identical and 42% similar) and *Escherichia coli* PBP3 (22% identical and 38% similar). SpoVD is transcribed in the mother cell and is required for synthesis of the spore cortex (5), while *E. coli* PBP3, the product of *pbpB* (*ftsI*), is essential for synthesis of septal PG during cell division (23). The gene names *pbpA* through *pbpH* have been assigned to other *B. subtilis* PBP-encoding genes, so we will refer to *yrrR* as *pbpI* from this point on.

We PCR amplified the coding sequence of *pbpI* and inserted it into the plasmid pSWEET (4) to produce pDPV146 (Tables 1 to 3), which contains a xylose-inducible expression system and can integrate into the *B. subtilis* chromosome at the *amyE* locus. Radioactively labeled penicillin was used to visualize the PBPs present in membranes prepared from xylose-induced DPVB210 (*amyE*::*xylAp-pbpI*), DPVB213 (*amyE*::*xylAp-bgaB* as a control), and PS832 (wild type) (Fig. 1). In DPVB210, we identified a new PBP with an apparent mass of 65 kDa, which matches the predicted molecular mass of the *pbpI* product (64.8 kDa). To follow the convention of naming PBPs based upon their migration during denaturing polyacrylamide gel electrophoresis, we will refer to this protein as PBP4b, since it runs slightly faster than PBP4. PBP4a, which runs in a similar position but is not visible under these growth conditions, is encoded by the *dacC* gene (15).

Expression of *pbpI*. A *pbpI-lacZ* transcriptional fusion was constructed in pDPV126 (Table 1) and inserted into the *B. subtilis* chromosome via a single-crossover recombination. No β-galactosidase was detected in vegetative cells and outgrowing spores (data not shown). Expression of *pbpI* began 1 to 2 h after the initiation of sporulation (Fig. 2), and the level of expression was very low compared to those of several other PBP-encoding genes (20–22). Based on this timing of expression, we predicted that *pbpI* was transcribed under the control of σ^E or σ^F. Mutations in *spoIIAC* (encoding σ^F) and *spoIIGB* (encoding σ^E) completely abolished *pbpI-lacZ* expression, while a null mutation in *spoIIIG* (encoding σ^G) had no effect on the timing and level of expression (Fig. 2). This pattern is consistent with transcription by σ^E RNA polymerase holoenzyme. The *pbpI* (*yrrR*) gene was also recently identified in a transcription-profiling search for σ^E-dependent genes, and putative σ^E recognition sequences were located 50 bp upstream of the *pbpI* start codon (6). Active σ^E also drives expression of a gene starting 64 bp downstream of the *pbpI* start codon, *yrrS* (6), and these genes may constitute an operon; however, cotranscription has not been demonstrated.

* Corresponding author. Mailing address: Department of Biology, Virginia Tech, 2119 Derring Hall MC0406, Blacksburg, VA 24061. Phone: (540) 231-2529. Fax: (540) 231-9307. E-mail: dpopham@vt.edu.

† Present address: Department of Microbiology, Cornell University, Ithaca, NY 14851.

‡ Present address: Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL 60153.

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype[b]	Construction[c]	Source or reference
DPVB45	Δ*pbpG*::Kn		12
DPVB56	Δ*pbpF*::Erm Δ*pbpG*::Kn		12
DPVB64	*spoVD*::Kn		Laboratory stock (5)
DPVB160	Δ*pbpI*::Erm	pDPV114→PS832	This work
DPVB169	*pbpI*-lacZ	pDPV126→PS832	This work
DPVB176	Δ*pbpI*::Erm *spoVD*::Kn	DPVB160→DPVB64	This work
DPVB183	*pbpI-lacZ*	DPVB169→PY79	This work
DPVB184	*pbpI-lacZ spoIIAC1*	DPVB169→SC1159	This work
DPVB185	*pbpI-lacZ spoIIGB*::Tn*917*Ωnv325	DPVB169→SC137	This work
DPVB186	*pbpI-lacZ spoIIIG*Δ1	DPVB169→SC500	This work
DPVB198	Δ*pbpG*::Kn Δ*pbpI*::Erm	DPVB45→DPVB160	This work
DPVB199	*pbpF*::Cm Δ*pbpI*::Erm	PS1838→DPVB160	This work
DPVB200	*pbpF*::Cm Δ*pbpG*::Kn Δ*pbpI*::Erm	PS1838→DPVB199	This work
DPVB210	*xylAp-pbpl* at *amyE*	pDPV146→PS832	This work
DPVB213	*xylAp-bgaB* at *amyE*	pSWEET-*bgaB*→PS832	This work
PS832	Wild-type, Trp^+ revertant of 168		Laboratory stock
PS1838	*pbpF*::Cm		21
PY79[a]	Wild type		16, 24
SC137[a]	*spoIIGB*::Tn917Ωnv325		S. Cutting (16)
SC500[a]	*spoIII*GΔ1		S. Cutting (16)
SC1159[a]	*spoIIAC1*		S. Cutting (16)

[a] The genetic background is PY79. The other strains' genetic background is PS832.
[b] Antibiotic resistance abbreviations: Cm, chloramphenicol; Erm, lincomycin and erythromycin; Kn, kanamycin.
[c] The designation preceding the arrow indicates the source of donor DNA in a natural transformation of the recipient strain following the arrow.

Phenotypic properties of *pbpI* mutant strains. We constructed a mutant strain in which 89% of the *pbpI* coding sequence (codons 4 to 525 out of 584, including the conserved penicillin-binding active site) was deleted and replaced with an erythromycin resistance gene cassette (DPVB160, Table 1). This mutation may have a polar effect on expression of *yrrS*, if *pbpI* and *yrrS* constitute an operon. PBPs of the same class frequently exhibit functional redundancy, so we also constructed a double-mutant strain lacking *pbpI* and *spoVD*, the only other class B PBP-encoding gene specifically expressed during sporulation (5). Two genes encoding class A PBPs, *pbpF* and *pbpG*, are expressed specifically within the forespore, and a *pbpF pbpG* double mutant produces defective spore PG. We constructed double and triple mutants lacking *pbpF*, *pbpG*, and *pbpI* to examine the effects on spore PG synthesis. Phenotypic properties, including growth rate, cell morphology, sporulation efficiency, PG structures of both the vegetative cell and spore cortex, spore heat resistance, spore germination rate, and the rate of spore outgrowth, were studied.

There were no significant differences between the growth rates and vegetative cell morphologies of any of the mutant strains and the wild type. The *pbpI*, *pbpF pbpI*, and *pbpG pbpI* strains produced as many chloroform-resistant (10% chloroform, 10 min) and heat-resistant spores (80°C, 10 min) per ml of culture as the wild type. To measure spore heat resistance precisely, spores were purified, heated in water at 80°C for various times, and plated to determine the number of surviving CFU per milliliter. There was no significant difference among the spore killing rates of these strains. In addition, the germination and outgrowth kinetics of the mutant spores were indistinguishable from those of the wild type (data not shown). PG was purified from each of the spore preparations, and muropeptides obtained from the PG were analyzed by reverse-phase high-performance liquid chromatography (18). The overall structures of the spore PGs of the *pbpI*, *pbpI pbpF*, and *pbpI pbpG* mutant strains were indistinguishable from those of the wild-type and single-mutant strains (Table 4, 48-h samples).

The *spoVD* and *pbpF pbpG* strains produce extremely few spores, so no difference in sporulation efficiency could be seen when the *pbpI* mutation was introduced into these backgrounds, and spore phenotypic properties could not be as-

TABLE 2. Plasmids used in this study

Plasmid	Construction	Reference
pDG646	Vector carrying Erm cassette	9
pDPC87	Transcriptional *lacZ* fusion vector	21
pDPV107	PCR fragment using primers pbpI1 and pbpI2 inserted into pGEM-T (Promega)	
pDPV114	*Sma*I-*Hin*dIII fragment of pDG646 inserted into *Eco*RV-*Hin*dIII-digested pDPV107, replacing 89% of *pbpI* with Erm cassette	
pDPV126	PCR fragment using primers pbpIa and pbpI2 cut with *Eco*RI and *Hin*dIII, with 975-bp fragment inserted into *Eco*RI-*Hin*dIII-digested pDPC87	
pDPV146	PCR fragment using primers pbpI3 and pbpI5 inserted into *Pac*I-*Bam*HI-digested pSWEET-*bgaB*	
pSWEET-*bgaB*		4

TABLE 3. Primers used in this study

Primer	Sequence[a]	Characteristic
pbpI1	5′AGAGGGCCGCGTGACGACTCTTG	Anneals 345 bp upstream of *pbpI*
pbpI2	5′ATCAGAGTCAGAAGACTTCTCAG	Anneals 355 bp downstream of *pbpI*
pbpI3	5′CGGGATCCTTAACATGTGCTGAGAAGTTG	Places a *Bam*HI site 26 bp downstream of *pbpI*
pbpI5	5′GCGCTTAATTAACACAATGTGGGTGAGGTGTTT	Places a *Pac*I site 25 bp upstream of *pbpI*
pbpIa	5′CGGAATTCAGAGGGCCGCGTGACGACTCTTG	Places an *Eco*RI site 345 bp upstream of *pbpI*

[a] Underlined bases are restriction sites.

sessed. However, forespore PG synthesis during sporulation was analyzed (13) in cultures of a *pbpI* single mutant (DPVB160), a *pbpI spoVD* double mutant (DPVB176), and a *pbpI pbpF pbpG* triple mutant (DPVB200) and compared to that of the wild-type (PS832), *spoVD* (DPVB64), and *pbpF pbpG* (DPVB56) strains, respectively. The amount of spore PG produced during sporulation was assayed by determination of the muramic acid content of culture samples. There were no significant differences between the strains in each pair (data not shown). The PG structural analyses demonstrated that throughout sporulation (Table 4, 8-h samples) (data not shown), the *pbpI* strain produced spore PG with structural parameters similar to those found in the wild type. The *pbpI spoVD* strain produced spore PG with structural parameters similar to those found in the *spoVD* strain—essentially a small amount of germ cell wall PG. The *pbpI pbpF pbpG* triple mutant produced spore PG with structural parameters similar to those found in the *pbpF pbpG* strain (12).

The *pbpI* gene encodes a previously unidentified sporulation-specific PBP. PBP4b is expressed in the mother cell during sporulation, under the control of σ^E. We could find no reproducible structural differences between the spore PG produced by a *pbpI* mutant and that produced by a wild-type strain. In addition, we found no effects of *pbpI* on the limited amount of abnormal spore PG produced in *pbpF pbpG* and *spoVD* strains. We conclude that either PBP4b plays no significant role in spore PG synthesis or other PBPs carry out redundant functions, masking any effects of the loss of PBP4b.

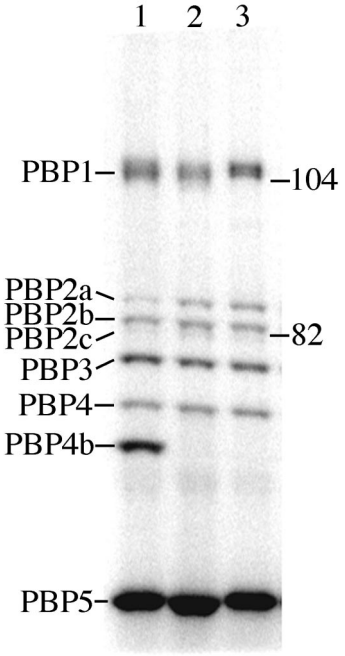

FIG. 1. Identification of PbpI (PBP4b). Strains were grown in 2× SG medium (10) at 37°C to an optical density at 600 nm of 0.1. Xylose was then added to a final concentration of 2%, and incubation was continued until the optical density reached 1.0. Cell membranes were prepared as previously described (19). PBPs were detected with ^{125}I-labeled penicillin X as previously described (11, 12). Proteins were separated on a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, and PBPs were detected with a STORM 860 PhosphorImager (Molecular Dynamics). Lanes: 1, DPVB210 (over-expressed *pbpI*); 2, DPVB213 (overexpressed *bgaB*); 3, PS832 (wild-type). PBPs are indicated on the left and are numbered as previously described (2). The migration positions of molecular mass markers (Bio-Rad low-range, prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards) are indicated on the right in kilodaltons.

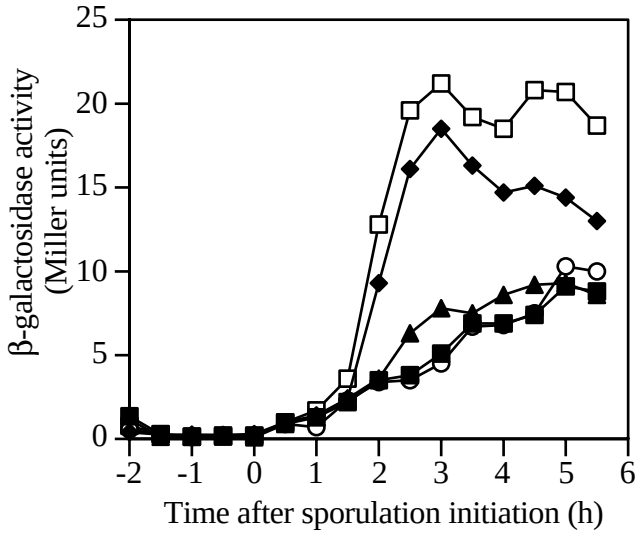

FIG. 2. Expression of *pbpI*. Growth and sporulation were in 2× SG medium (■) at 37°C. Strain PY79 (■) contained no *lacZ* fusion and revealed the background activity. The expression of *pbpI-lacZ* was assayed with *o*-nitrophenyl-β-D-galactopyranoside as described previously (14) in the wild-type background (♦, DPVB183) and in isogenic *spoIIAC* (*sigF*) (○, DPVB184), *spoIIGB* (*sigE*) (▲, DPVB185), and *spoIIIG* (*sigG*) (□, DPVB186) mutants.

TABLE 4. Structural parameters of dormant spore and forespore PG

Strain	Genotype	Time in sporulation (h)	% Muramic acid with side chain:				
			Lactam	Alanine	Tripeptide	Tetrapeptide	Cross-linked
PS832	Wild type	48	49.4	20.4	1.4	28.9	3.1
PS1838	Δ*pbpF*::Cm	48	49.8	19.4	0.8	29.9	3.2
DPVB45	Δ*pbpG*::Kn	48	49.1	20.3	1.7	28.9	3.3
DPVB160	Δ*pbpI*::Erm	48	49.3	16.7	1.3	32.7	3.6
DPVB198	Δ*pbpG*::Kn Δ*pbpI*::Erm	48	48.6	18.1	1.3	32.0	3.4
DPVB199	Δ*pbpF*::Cm Δ*pbpI*::Erm	48	49.5	18.1	1.0	31.4	3.3
PS832	Wild type	8	45.4	27.5	4.0	23.1	2.8
DPVB160	Δ*pbpI*::Erm	8	44.7	29.7	4.4	21.1	2.7
DPVB64	*spoVD*::Kn	8	8.6	20.8	50.1	20.5	2.2
DPVB176	Δ*pbpI*::Erm *spoVD*::Kn	8	9.1	24.0	43.3	23.6	2.2
DPVB56	Δ*pbpF*::Cm Δ*pbpG*::Kn	8	14.4	5.4	12.1	68.1	11.0
DPVB200	Δ*pbpF*::Cm Δ*pbpG*::Kn Δ*pbpI*::Erm	8	12.9	4.6	11.1	71.4	11.5

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