

## Two Class A High-Molecular-Weight Penicillin-Binding Proteins of *Bacillus subtilis* Play Redundant Roles in Sporulation

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Received 14 June 2001/Accepted 19 July 2001

**The four class A penicillin-binding proteins (PBPs) of *Bacillus subtilis* appear to play functionally redundant roles in polymerizing the peptidoglycan (PG) strands of the vegetative-cell and spore walls. The *ywhE* product was shown to bind penicillin, so the gene and gene product were renamed *pbpG* and PBP2d, respectively. Construction of mutant strains lacking multiple class A PBPs revealed that, while PBP2d plays no obvious role in vegetative-wall synthesis, it does play a role in spore PG synthesis. A *pbpG* null mutant produced spore PG structurally similar to that of the wild type; however, electron microscopy revealed that in a significant number of these spores the PG did not completely surround the spore core. In a *pbpF pbpG* double mutant this spore PG defect was apparent in every spore produced, indicating that these two gene products play partially redundant roles. A normal amount of spore PG was produced in the double mutant, but it was frequently produced in large masses on either side of the forespore. The double-mutant spore PG had structural alterations indicative of improper cortex PG synthesis, including twofold decreases in production of muramic  $\delta$ -lactam and L-alanine side chains and a slight increase in cross-linking. Sporulation gene expression in the *pbpF pbpG* double mutant was normal, but the double-mutant spores failed to reach dormancy and subsequently degraded their spore PG. We suggest that these two forespore-synthesized PBPs are required for synthesis of the spore germ cell wall, the first layer of spore PG synthesized on the surface of the inner forespore membrane, and that in the absence of the germ cell wall the cells lack a template needed for proper synthesis of the spore cortex, the outer layers of spore PG, by proteins on the outer forespore membrane.**

Peptidoglycan (PG) is the essential structural element that provides shape and stability to most bacterial cells. In the dormant endospore PG is required for the maintenance of spore core dehydration and therefore for spore heat resistance. Both vegetative-cell and spore PGs are composed of glycan strands of repeating *N*-acetylglucosamine and *N*-acetyl muramic acid residues cross-linked by peptide side chains (reviewed in reference 2). Polymerization of PG involves the addition of disaccharide pentapeptide subunits onto a growing glycan strand by a glycosyltransferase. The peptide side chains are then utilized by a transpeptidase to cross-link the glycan strands. Side chains that are not utilized for cross-linking are cleaved to tripeptides or tetrapeptides.

Spore PG consists of two layers that can be distinguished structurally and functionally. The germ cell wall is adjacent to the inner forespore membrane and serves as the initial cell wall during spore germination and outgrowth. It is surrounded by the cortex, which comprises the outer 70 to 90% of the spore PG (28) and which is rapidly degraded during spore germination (4, 14). Cortex PG is more loosely cross-linked than vegetative PG, and 50% of the *N*-acetyl muramic acid residues have had their peptide side chains removed and have been converted to muramic  $\delta$ -lactam (3, 37, 54, 55). The structure of the germ cell wall appears to be more similar to that of vegetative PG in that most of the peptide side chains are tripeptides and there is little or no muramic  $\delta$ -lactam (4, 28, 53). Despite

the differences between the spore and vegetative PG structure, the mechanisms of PG polymerization in the two situations appear to be similar.

The glycosyltransferase and transpeptidase activities required for PG synthesis are found in the penicillin-binding proteins (PBPs) (15). The PBPs can be placed into three classes based on amino acid sequence similarities (15, 16). Class A high-molecular-weight PBPs are bifunctional PBPs that contain an N-terminal glycosyltransferase domain and a C-terminal transpeptidase domain. Class B high-molecular-weight PBPs are known to have only transpeptidase activity and are, in some cases, required for cell septation and maintenance of cell shape (30, 49, 50, 56). Low-molecular-weight PBPs generally have D,D-carboxypeptidase activity and, in some cases, are involved in regulating the number of cross-links between the glycan strands (36, 39, 46).

Redundancy in the functions of multiple class A PBPs has been previously demonstrated in vegetative cells of *Bacillus subtilis* (44), *Escherichia coli* (13, 52, 57), and *Streptococcus pneumoniae* (19, 34). Sequence analysis of the *B. subtilis* genome (24) revealed genes encoding four class A PBPs (35, 44). Loss of three (PBP1, PBP2c, and PBP4) of the four slows the vegetative-growth rate, mostly due to the loss of PBP1, and decreases the production of spores 10-fold (44). Recent studies indicated that *YwhE*, the fourth class A PBP, has no effect on vegetative PG synthesis and demonstrated that *ywhE* is expressed only in the forespore under the control of  $\sigma^F$  and, to a lesser degree,  $\sigma^G$  (35). Another class A PBP, PBP2c, is expressed vegetatively but is also induced in the forespore under the control of  $\sigma^G$  (42), suggesting potential roles for both

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TABLE 1. *B. subtilis* strains used

Strain	Genotype <sup>a</sup>	Transformation		Source or reference
		Donor(s)	Recipient	
IA626	<i>hisA82::Tn917</i>			Bacillus Genetic Stock Center
AD51	<i>gerE-lacZ</i>			11
AD52	<i>cotD-lacZ</i>			58
AD799	<i>sspB-lacZ</i>			26
DPVB29	Double $\Delta$ <i>pbpD</i> ::Cm	pDPC271	PS832	This work
DPVB30	$\Delta$ <i>pbpD</i> (Spo <sup>-</sup> )			This work
DPVB40	$\Delta$ <i>pbpD hisA82::Tn917</i> (Spo <sup>+</sup> )	1A626	DPVB30	This work
DPVB42	$\Delta$ <i>pbpD</i> (Spo <sup>+</sup> )	DPVB30	DPVB40	This work
DPVB45	$\Delta$ <i>pbpG</i> ::Kn	pDPV35	PS832	This work
DPVB46	$\Delta$ <i>pbpD \Delta</i> <i>pbpF</i> ::Erm <sup>r</sup>	PS1869	DPVB42	This work
DPVB49	$\Delta$ <i>pbpD \Delta</i> <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	DPVB45	DPVB46	This work
DPVB56	$\Delta$ <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	DPVB45	PS1869	This work
DPVB57	$\Delta$ <i>pbpD \Delta</i> <i>pbpG</i> ::Kn	DPVB45	DPVB42	This work
DPVB61	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpG</i> ::Kn	PS2062	DPVB45	This work
DPVB62	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpD \Delta</i> <i>pbpG</i> ::Kn	PS2062	DPVB57	This work
DPVB63	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	PS2062	DPVB56	This work
DPVB68	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpD</i>	PS2062	DPVB42	This work
DPVB69	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpD \Delta</i> <i>pbpF</i> ::Erm <sup>r</sup>	PS2062	DPVB46	This work
DPVB84	<i>sspB-lacZ</i>	AD799	PS832	This work
DPVB85	<i>sspB-lacZ \Delta</i> <i>pbpF</i> ::Erm <sup>r</sup>	AD799	PS1869	This work
DPVB86	<i>sspB-lacZ \Delta</i> <i>pbpG</i> ::Kn	AD799	DPVB45	This work
DPVB89	<i>sspB-lacZ \Delta</i> <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	DPVB84	DPVB56	This work
DPVB90	<i>gerE-lacZ</i>	AD51	PS832	This work
DPVB91	<i>gerE-lacZ \Delta</i> <i>pbpF</i> ::Erm <sup>r</sup>	AD51	PS1869	This work
DPVB92	<i>gerE-lacZ \Delta</i> <i>pbpG</i> ::Kn	AD51	DPVB45	This work
DPVB93	<i>gerE-lacZ \Delta</i> <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	AD51	DPVB56	This work
DPVB94	<i>cotD-lacZ</i>	AD52	PS832	This work
DPVB95	<i>cotD-lacZ \Delta</i> <i>pbpF</i> ::Erm <sup>r</sup>	AD52	PS1869	This work
DPVB96	<i>cotD-lacZ \Delta</i> <i>pbpG</i> ::Kn	AD52	DPVB45	This work
DPVB97	<i>cotD-lacZ \Delta</i> <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	AD52	DPVB56	This work
DPVB141	$\Delta$ <i>pbpG</i> ::Kn <i>cwlD</i> ::Cm	PS2307	DPVB45	This work
DPVB142	$\Delta$ <i>pbpF</i> ::Erm <sup>r</sup> <i>cwlD</i> ::Cm	PS2307	PS1869	This work
DPVB143	$\Delta$ <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup> <i>cwlD</i> ::Cm	PS2307	DPVB56	This work
DPVB157	$\Delta$ <i>pbpF</i> ::Erm <sup>r</sup> <i>sleB</i> ::Sp <i>cwlJ</i> ::Tet <sup>r</sup>	FB111, FB112	PS1869	This work
DPVB158	$\Delta$ <i>pbpG</i> ::Kn <i>sleB</i> ::Sp <i>cwlJ</i> ::Tet <sup>r</sup>	FB111, FB112	DPVB45	This work
DPVB159	$\Delta$ <i>pbpG</i> ::Kn $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup> <i>sleB</i> ::Sp <i>cwlJ</i> ::Tet <sup>r</sup>	FB111, FB112	DPVB56	This work
FB111	<i>cwlJ</i> ::Tet <sup>r</sup>			33
FB112	<i>sleB</i> ::Sp			33
FB113	<i>cwlJ</i> ::Tet <sup>r</sup> <i>sleB</i> ::Sp			33
PS832	Prototrophic revertant of strain 168			Laboratory stock
PS1869	$\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	pDPC89	PS832	42
PS2062	$\Delta$ <i>ponA</i> ::Sp	pDPC197	PS832	41
PS2251	$\Delta$ <i>ponA</i> ::Sp $\Delta$ <i>pbpF</i> ::Erm <sup>r</sup>	PS2062	PS1869	44
PS2307	<i>cwlD</i> ::Cm	ADD1	PS832	45

<sup>a</sup> Abbreviations: Erm<sup>r</sup> resistance to erythromycin and lincomycin; Sp, resistance to spectinomycin; Cm, resistance to chloramphenicol; Kn, resistance to kanamycin; Tet<sup>r</sup>, resistance to tetracycline.

PBP2c and YwhE in spore PG synthesis or spore germination. In this communication we present studies examining the phenotypes of double and triple class A PBP mutants lacking *ywhE*. We demonstrate that loss of both PBP2c and YwhE has no effect on vegetative growth but that this double mutant is unable to complete sporulation.

#### MATERIALS AND METHODS

**Bacterial growth, transformation, and sporulation.** All strains of *B. subtilis* listed in Table 1 were derivatives of strain 168. Transformation was performed as previously described (1). Transformants were selected and maintained using appropriate antibiotics: chloramphenicol (3  $\mu$ g/ml), spectinomycin (100  $\mu$ g/ml), kanamycin (10  $\mu$ g/ml), tetracycline (10  $\mu$ g/ml), and erythromycin (0.5  $\mu$ g/ml) plus lincomycin (12.5  $\mu$ g/ml; macrolide-lincosamide-streptogramin B resistance). Antibiotics were omitted in cultures grown for determination of growth rates, sporulation efficiencies, and spore PG structure.

Growth rates were determined in 2 $\times$  SG medium (25) at 37°C with shaking. Cultures were allowed to sporulate following nutrient exhaustion for 24 h, at

which time spore heat resistance and chloroform resistance were measured as previously described (31).  $\beta$ -Galactosidase activity, glucose dehydrogenase activity, and dipicolinic acid contents of sporulating cultures were assayed as previously described (31). Sporulating cells were prepared for electron microscopy as previously described, except that grids were stained with uranyl acetate as well as Reynolds lead (9). The amount and structure of spore PG produced within cultures were analyzed as previously described (28).

**Class A PBP mutant construction.** Plasmid pDPC145 (43) was digested with *EcoRI* and *EcoRV* to produce an 800-bp fragment containing the first 147 bp of *pbpD* plus upstream sequences. This fragment was ligated into *EcoRI*- and *PvuII*-digested pDPC179 (43), which contains the last 243 bp of *pbpD*, to create an in-frame deletion of codons 50 to 543 (out of 624 codons). The plasmid with the deletion, pDPC271, was used to transform PS832 with selection for chloramphenicol resistance. Insertion of pDPC271 into the chromosome via a single crossover results in copies of *pbpD* on both sides of the vector sequence. Transformants were screened by PCR to identify a strain in which a recombination event caused both copies of *pbpD* to have the in-frame deletion ( $\Delta$ *pbpD*; strain DPVB29) (data not shown). This strain was grown for 50 generations in nonselective liquid media, allowing for recombination of the plasmid out of the chro-

mosome to leave a single  $\Delta pbpD$ . The culture was plated for single colonies on nonselective media and replica plated to identify a chloramphenicol-sensitive isolate (DPVB30). The single  $\Delta pbpD$  in this strain was verified using PCR and Southern blot analysis (data not shown). DPVB30 was found to have a  $Spo^-$  phenotype that was not present in DPVB29 and that was believed to result from a spontaneous mutation in an unrelated locus. DPVB30 was transformed with chromosomal DNA of strain 1A626 with selection for macrolide-lincosamide-streptogramin B resistance. The resulting colonies were screened for cotransformation to a  $Spo^+$  phenotype, and one  $Spo^+$  isolate was saved as DPVB40. This strain was then transformed with limiting chromosomal DNA from DPVB30 with selection for  $His^+$ . Most transformants retained the  $Spo^+$  phenotype, and one (DPVB42) was verified using PCR to contain  $\Delta pbpD$ .

A PCR product containing a sequence from 417 bp upstream of the *ywhE* start codon to 212 bp downstream of the *ywhE* stop codon (24, 35) was ligated into the pGEM-T vector (Stratagene) to produce pDPV24. This plasmid was digested with *PvuII* and *SalI* to obtain a 524-bp fragment containing the first 90 bp of *ywhE* and with *PvuII* and *SphI* to obtain a 485-bp fragment containing the final 251 bp of *ywhE*. These two fragments were ligated with *SphI*- and *SalI*-digested pJH101 to obtain pDPV33, in which bases 91 to 1691 of *ywhE* are deleted. Plasmid pDG780 (18) was digested with *SmaI* and *HincII* to obtain a kanamycin resistance cassette that was inserted into pDPV33 at the *PvuII* site at the point of the *ywhE* deletion to create pDPV35. pDPV35 was linearized with *ScaI* and used to transform PS832, with selection for kanamycin resistance, to create DPVB45 ( $\Delta ywhE::Kn$ ). Strains containing multiple mutations were made by transformation using limiting chromosomal DNA and selection with the appropriate antibiotics.

**PBP detection.** Penicillin X was synthesized and labeled with  $^{125}I$  as previously described (21, 27). Membranes were prepared from *B. subtilis* cells at the fourth hour of sporulation in  $2\times$  SG medium as previously described (41). Membrane samples containing 40  $\mu$ g of protein were incubated for 30 min at 30°C with 3  $\mu$ Ci of labeled penicillin X in a total volume of 20  $\mu$ l of 50 mM Tris-HCl, pH 8.0–1 mM  $\beta$ -mercaptoethanol–0.1 mM phenylmethylsulfonyl fluoride. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% polyacrylamide gel. PBPs were detected and signal intensities were integrated using a STORM 860 phosphorimager and ImageQuant software (Molecular Dynamics).

## RESULTS

**Construction of class A PBP mutant strains.** Previous genetic analysis of the class A PBPs in *B. subtilis* utilized some Campbell-type plasmid insertion mutations that have the ability to revert. The appearance of revertants produced significant problems in strains with reduced growth rates due to the loss of multiple class A PBPs. To avoid this problem, we utilized non-revertible mutations, such as deletions or deletions with antibiotic resistance insertions, in each of the four genes. Each of the mutations removed  $\geq 79\%$  of the genes' coding sequences. Deletion mutations in *ponA* (41), *pbpF* (42), and *ywhE* terminated the coding sequences at or before codon 30 (out of  $\geq 647$  codons) so that any resulting protein product would be missing all nine highly conserved motifs found within class A PBPs (16). The in-frame deletion in *pbpD* removed 79% of the coding sequence, including conserved motifs 1 to 8 (16).

PCR was used to verify the presence of the expected alleles in each mutant strain (data not shown). Southern blot analysis (48) was then used to verify that all of the expected mutations were present as well as the fact that none of the genes were present in a form undetectable by our PCR assay (undetectable due to some undefined nonhomologous recombination event). Two probes were used for each gene. One probe contained a region of the gene outside the deletion to verify the existence of either the wild-type or mutant allele in each strain, and a second probe contained a region interior to the deleted region to verify the complete absence of this region in each mutant. The expected wild-type and mutant genes are present in each of the triple mutants (data not shown).

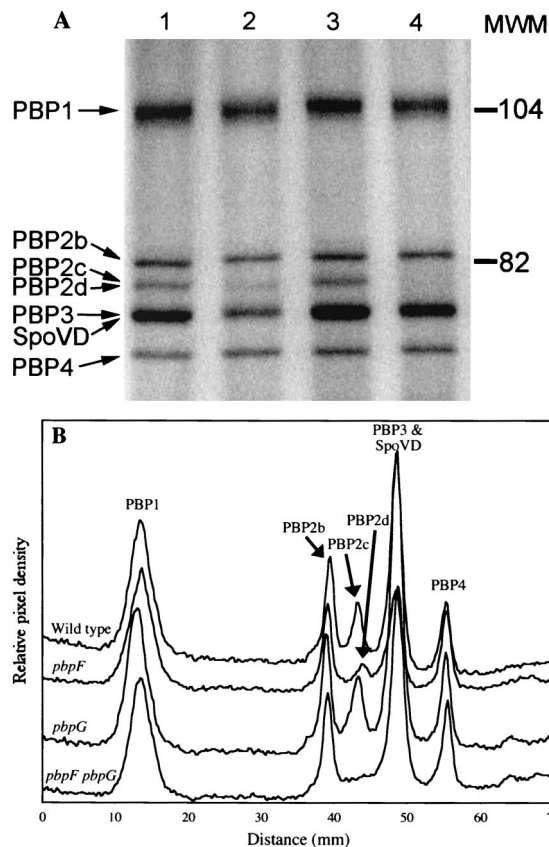


FIG. 1. PBP profiles of wild-type, *pbpF* mutant, and *pbpG* mutant strains. Membranes were purified from cultures at the fourth hour of sporulation. (A) Membranes were incubated with  $^{125}I$ -labeled penicillin X, proteins were separated on an SDS–7.5% PAGE gel, and PBPs were detected using a phosphorimager. Lane 1, wild type; lane 2, *pbpF*; lane 3, *pbpG*; lane 4, *pbpF pbpG*. Calibrated molecular mass standards (MWM; in kilodaltons) were Bio-Rad low-range-prestained SDS-PAGE standards. PBP2a decreases dramatically during sporulation (7) and is not visible on this gel. (B) Histogram of PBP band intensities produced by integrating signal strength within columns that covered 90% of each lane's width. PBPs are numbered as previously described (5, 23).

We used radioactively labeled penicillin to visualize the PBPs present in our wild-type and mutant strains. We identified a PBP that appeared to be the product of *ywhE* in membranes prepared from cells in the fourth hour of sporulation. This PBP migrated on an SDS-PAGE gel in nearly the same place as PBP2c and could only be clearly seen in a *pbpF* mutant (Fig. 1). This result is consistent with the predicted molecular masses of PBP2c (42) and the *ywhE* product (35), 79 and 77 kDa, respectively. We refer to *ywhE* as *pbpG* and to the gene product as PBP2d from this point on.

**Growth and sporulation of class A PBP mutants.** The growth rates and sporulation efficiencies of *ponA*, *pbpD*, and *pbpF* single- and multiple-mutant strains were consistent with those previously reported (Table 2) (44). Deletion of *pbpG*, alone and in multiple mutants, had no effect on growth rate (Table 2) (35). The sporulation efficiency of each strain was determined by measuring the number of heat-resistant CFU and comparing this number to the number of viable CFU after 24 h of sporulation. Small decreases in production of heat- and

TABLE 2. Growth and sporulation of class A PBP mutant strains<sup>a</sup>

Strain	PBP phenotype	Doubling time <sup>b</sup> (min)	Cell counts <sup>c</sup> (CFU/ml)		
			Viable	Heat-resistant spores	Chloroform-resistant spores
PS832	Wild type	20	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$
PS1869	PBP2c <sup>-</sup>	20	$1 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$
DPVB45	PBP2d <sup>-</sup>	20	$4 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$
DPVB42	PBP4 <sup>-</sup>	20	$3 \times 10^9$	$2 \times 10^9$	$1 \times 10^9$
PS2062	PBP1 <sup>-</sup>	25	$2 \times 10^9$	$3 \times 10^9$	$2 \times 10^9$
DPVB57	PBP4 <sup>-</sup> PBP2d <sup>-</sup>	20	$1 \times 10^9$	$7 \times 10^8$	$9 \times 10^8$
DPVB56	PBP2c <sup>-</sup> PBP2d <sup>-</sup>	20	$2 \times 10^8$	$4 \times 10^4$	$2 \times 10^4$
DPVB46	PBP2c <sup>-</sup> PBP4 <sup>-</sup>	20	$2 \times 10^9$	$4 \times 10^8$	$7 \times 10^8$
DPVB61	PBP1 <sup>-</sup> PBP2d <sup>-</sup>	26	$2 \times 10^9$	$2 \times 10^9$	$2 \times 10^9$
PS2251	PBP1 <sup>-</sup> PBP2c <sup>-</sup>	26	$7 \times 10^8$	$1 \times 10^9$	$7 \times 10^8$
DPVB68	PBP1 <sup>-</sup> PBP4 <sup>-</sup>	31	$5 \times 10^8$	$3 \times 10^7$	$4 \times 10^7$
DPVB49	PBP2c <sup>-</sup> PBP4 <sup>-</sup> PBP2d <sup>-</sup>	21	$8 \times 10^8$	$7 \times 10^3$	$3 \times 10^2$
DPVB63	PBP1 <sup>-</sup> PBP2c <sup>-</sup> PBP2d <sup>-</sup>	28	$4 \times 10^8$	$8 \times 10^3$	$5 \times 10^3$
DPVB69	PBP1 <sup>-</sup> PBP2c <sup>-</sup> PBP4 <sup>-</sup>	28	$4 \times 10^8$	$9 \times 10^6$	$5 \times 10^7$
DPVB62	PBP1 <sup>-</sup> PBP4 <sup>-</sup> PBP2d <sup>-</sup>	31	$4 \times 10^8$	$2 \times 10^7$	$1 \times 10^7$

<sup>a</sup> Doubling times and cell counts are averages from at least three separate experiments.

<sup>b</sup> Growth was in liquid 2 × SG medium at 37°C.

<sup>c</sup> Cell counts determined 24 h after initiation of sporulation.

chloroform-resistant spores were observed in strains lacking PBP1 and PBP4 (Table 2), as observed previously (44). These decreases were attributed to poor initiation of sporulation as a result of decreased growth rate rather than to a specific block in the sporulation process (44). In contrast, the *pbpF pbpG* strain exhibited a growth rate equal to that of the wild type but a >10,000-fold decrease in spore production (Table 2, strain DPVB56). A similar sporulation block was observed in triple mutants that lacked *pbpF* and *pbpG* (Table 2, strains DPVB49 and DPVB63).

**Regulation of sporulation gene expression in the *pbpF pbpG* double mutant.** Following engulfment, activation of  $\sigma^G$  leads to the expression of a set of sporulation genes in the forespore, some of which, in turn, are required for activation of  $\sigma^K$  in the mother cell (reviewed in reference 51). It has been theorized that spore PG synthesis could be one component of the signal needed for the activation of  $\sigma^K$  (58). We theorized that loss of expression of two PBPs within the forespore might disrupt spore PG synthesis, in turn blocking  $\sigma^K$  activation and completion of sporulation. Studies were performed with wild-type, *pbpF*, *pbpG*, and *pbpF pbpG* strains to determine if gene expression during late sporulation was altered.  $\sigma^G$ -dependent gene *sspB* (Fig. 2A) and  $\sigma^K$ -dependent genes *cotD* (Fig. 2B) and *gerE* (Fig. 2C) were expressed at or above wild-type levels in all three mutant strains, indicating that there was no block in the signal cascade between the forespore and mother cell.

**Spore PG synthesis in *pbpF* and *pbpG* mutants.** The amount and structure of spore PG synthesized in mutant strains were determined. Culture samples were collected every 30 min for 8 h following the initiation of sporulation. The muramic acid contents (28) of the wild-type and *pbpF* and *pbpG* single- and double-mutant cultures increased similarly throughout sporulation (data not shown). This muramic acid was present in PG strands because similar amounts of spore PG could be purified in a muramidase-sensitive form from all the sporulating cultures. PG was purified from developing forespores (28) collected throughout sporulation of each culture. Structural analysis of this forespore PG using reverse-phase high-pressure liquid chromatography (28) demonstrated that, throughout

sporulation, the *pbpF* and *pbpG* strains produced spore PG with structural parameters similar to those found in the wild type (Table 3) (28). The *pbpF pbpG* strain produced spore PG with altered structural parameters including a twofold reduction in the percentage of muramic acid side chains that were cleaved to form muramic  $\delta$ -lactam and a threefold reduction in the number of side chains cleaved to single L-alanine residues (Table 3). Increases in the numbers of both tripeptide and tetrapeptide side chains were also observed. The amount of muramic acid involved in cross-linking was slightly higher throughout the spore PG in the double mutant than in the single mutants. Although normal amounts of spore PG could be recovered from the double mutant until at least the eighth hour of sporulation, all spore PG was apparently degraded by 24 h after sporulation initiation.

**Microscopic examination of mutant cells.** Examination of the *pbpF pbpG* cells under phase-contrast microscopy 6 h into sporulation revealed that >80% of the cells contained visible phase-dark forespores (data not shown). Twenty four hours following the initiation of sporulation, very few phase-bright endospores were visible. To characterize the status of the spore PG in more detail, we performed thin-section electron-microscopic analysis of mutant cells. In cultures of *pbpG* cells approximately 7 h after the initiation of sporulation, we observed two morphologically distinct populations. The majority of cells resembled those of a wild-type population (Fig. 3A). In particular, the cortex was clearly visible. In a subset (approximately 35%) of the cells that had clearly completed engulfment, we observed a severe and novel defect in development (Fig. 3B and C). These cells possess what appears to be a highly disorganized forespore. The central regions of these cells resemble a forespore cytoplasm in electron density and granularity. However, instead of being surrounded by a lightly staining region clearly corresponding to the cortex, one or two lightly staining masses were adjacent to the apparent forespore cytoplasm, generally at opposite ends of the forespore. The regions of the section containing these masses frequently sustained tears during electron microscopy, suggesting that the embedding resin they tended to infiltrate poorly into the sample.

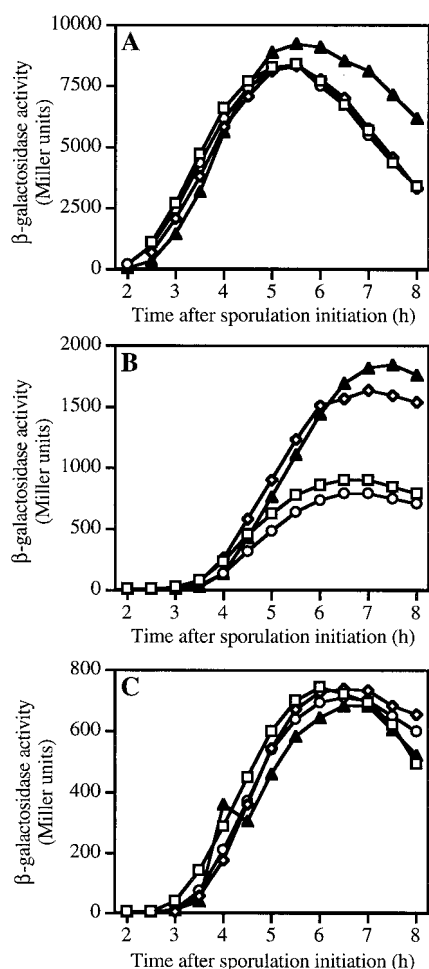


FIG. 2. Expression of late-sporulation genes in *pbpF* and *pbpG* mutant strains. Cultures of wild-type ( $\square$ ), *pbpF* ( $\diamond$ ), and *pbpG* ( $\circ$ ) single-mutant, and *pbpG pbpF* double-mutant ( $\blacktriangle$ ) strains carrying fusions of *lacZ* to late sporulation genes were sampled following initiation of sporulation in  $2\times$  SG medium at  $37^\circ\text{C}$ .  $\beta$ -Galactosidase expression from *sspB-lacZ* under regulatory control of  $\sigma^G$  (A) or from *cotD-lacZ* (B) and *gerE-lacZ* (C) under regulatory control of  $\sigma^K$  was assayed using *o*-nitrophenyl- $\beta$ -D-galactopyranoside as previously described (31). The cause of *cotD* overexpression in the *pbpF* mutants is unknown.

Spore coat material surrounded these regions. These coats possessed inner and outer layers but tended not to form a contiguous shell and to be thinner than wild-type spore coats. Those cells lacking a normal spore PG layer are not expected to achieve normal heat resistance. We believe that the proportion of defective spores in *pbpG* cultures is low enough (and possibly highly variable) that it was not detectable in our assays of heat-resistant spore production (Table 2).

*pbpF pbpG* cells harvested at hour 7 differed from *pbpG* cells in that the defect was present in all the cells (Fig. 3D). At hour 24 the majority of spore structures within double-mutant sporangia no longer contained the masses (Fig. 3E) and mother cells that had lysed released what appeared to be simply shells of spore coat without any interior PG or cytoplasm (Fig. 3F). One interpretation of these data is that in the absence of *pbpG* a significant percentage of cells form a defective cortex, result-

ing in the accumulation of incorrectly assembled PG in pockets near the forespore. This defect is magnified with the addition of a mutation in *pbpF*, such that none of the cells form functional cortices. As a consequence of the resulting failure in dehydration, when the mother cell lyses, the spore interior lyses as well, producing a spore that is a fragment of the coat without a core. Several lines of evidence suggest that the masses seen in the *pbpF pbpG* developing spores consist of disorganized spore PG. The masses are positioned between the inner forespore membrane and the spore coats, as for normal spore PG. The masses disappear by hour 24 of sporulation, and we were unable to recover any spore PG from culture samples at that time (Table 3). Two types of mutations result in stabilization of spore PG in *B. subtilis*: mutations in *cwlJ* and *sleB*, which encode lytic enzymes used in germination (6, 20, 29), and a mutation in *cwlD* (45), which is required for the production of muramic  $\delta$ -lactam in the spore PG (3, 38), a recognition determinant for lytic enzymes used in germination. When *cwlJ* and *sleB* mutations (33) or a *cwlD* mutation (45) was intro-

TABLE 3. Structural parameters of forespore PG produced by *pbpF* and *pbpG* mutant strains<sup>a</sup>

Geno- type	Time in sporula- tion (h)	% Spore PG made <sup>b</sup>	% Muramic acid with:				
			Side chain of <sup>c</sup> :				Cross-linked side chain
			Lactam	L-Ala	TriP	TP	
<i>pbpF</i>	3.5	2	4.6	7.9	72.1	15.4	14.8
	4	4.5	30.3	33.3	27.5	8.9	6.3
	4.5	13.5	41.4	44.2	9.5	4.9	2.7
	5	22.5	43.5	38.6	7.7	10.2	2.6
	5.5	33	44.5	30.9	5.2	19.3	2.9
	6	46.5	45.6	29.3	3.7	21.3	3.0
	6.5	59.5	45.5	26.5	3.3	24.7	3.2
	7	75	46.1	26.6	2.7	24.6	3.0
	7.5	89	46.4	24.8	2.4	26.4	3.1
	8	100	46.5	22.8	2.3	28.4	3.4
	24	100	48.5	21.6	1.5	28.4	3.4
	<i>pbpG</i>	3.5	9.5	13.2	17.1	56.8	12.9
4		18.5	32.4	34.3	26.2	7.2	5.5
4.5		27.5	37.3	35.6	17.7	9.4	4.1
5		37.5	39.8	29.7	12.0	18.5	4.0
5.5		52	41.0	27.4	9.5	22.0	3.8
6		71	42.7	26.0	7.0	24.2	3.9
6.5		78.5	43.4	24.7	6.1	25.7	3.8
7		86	44.4	24.6	5.4	25.6	3.7
7.5		93.5	45.1	23.6	4.9	26.4	3.9
8		100	46.1	23.7	4.2	26.0	3.6
24		100	47.7	18.1	3.2	31.0	4.1
<i>pbpF pbpG</i>		3.5	5	0.0	0.0	80.9	19.1
	4	11	10.9	11.5	53.4	24.2	12.1
	4.5	17.5	18.8	14.9	29.6	27.7	9.7
	5	36	16.7	7.3	21.0	55.0	7.2
	5.5	57	14.3	5.4	11.1	69.3	6.5
	6	70	15.6	5.6	8.2	70.6	6.4
	6.5	81	16.4	5.5	7.2	70.9	6.5
	7	89.5	17.7	5.7	6.6	70.0	6.3
	7.5	95	18.7	6.1	6.1	69.2	6.1
	8	100	19.3	6.0	5.8	68.9	6.0
	24	0					

<sup>a</sup> Forespore PG was purified from culture samples taken every 15 min between the fourth and seventh hours of sporulation, but only data for samples taken every 30 min are shown.

<sup>b</sup> These values are derived from the interpolation of culture muramic acid contents as determined by amino acid analyses (28).

<sup>c</sup> Lactam, muramic  $\delta$ -lactam; L-Ala, single alanine; TriP, tripeptide; TP, tetrapeptide.

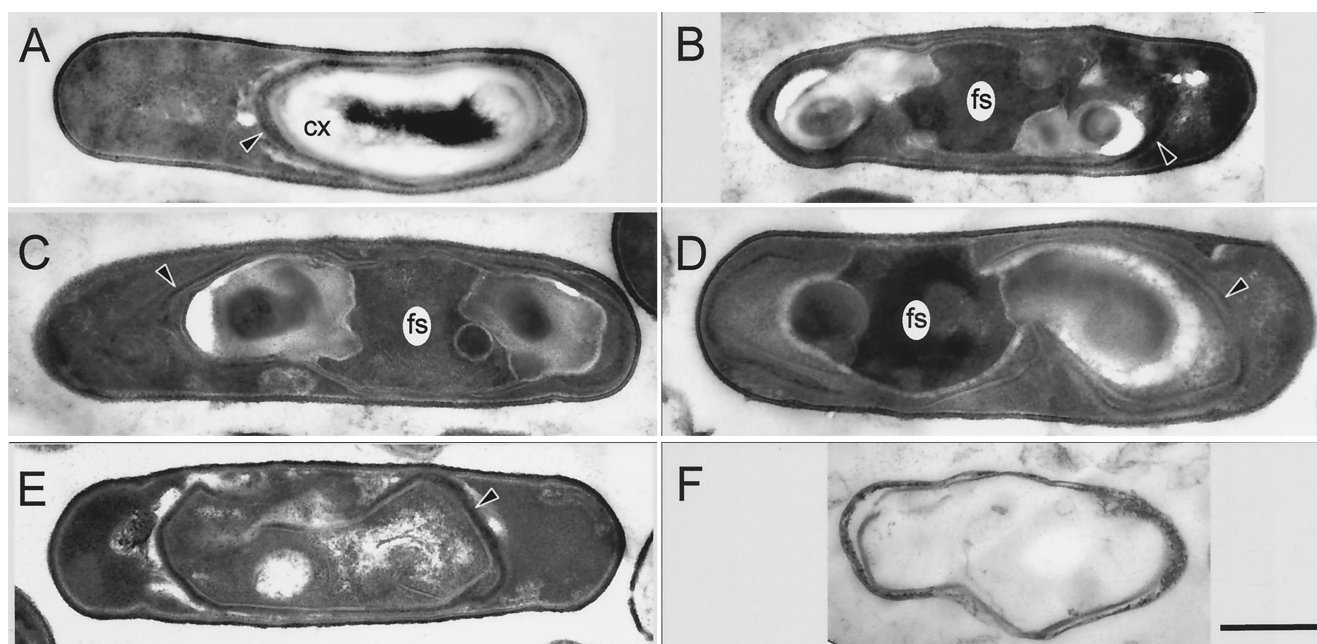


FIG. 3. Thin-section electron microscopy of sporulating mutant cells. Cells of *pbpG* (A to C) or *pbpG pbpF* (D to F) strains were sporulated, harvested at hour 7 (A to D) or 24 (E and F), and prepared for electron microscopy. The majority of *pbpG* cells had an appearance (A) similar to that of the wild type. In a minority of the *pbpG* cells, masses, presumed to be spore PG, do not completely surround the forespore (B and C). The majority of *pbpF pbpG* cells had this appearance (D). (E) A *pbpF pbpG* double mutant cell at the 24th hour of sporulation, in which the masses on either side of the forespore have disappeared. (F) Empty spore coat structure released by lysis of a *pbpF pbpG* double-mutant cell at the 24th hour of sporulation. CX, cortex; fs, forespore cytoplasm. Arrowheads, spore coat structures. Bar (F), 500 nm (all panels have the same magnification).

duced into the *pbpF pbpG* strain, the masses contained within the spore coats were still clearly visible under phase-contrast microscopy at the 24th hour of sporulation (data not shown) and the spore PG that was produced remained stable. Spore PG was isolated from the *cwlJ sleB*, *cwlJ pbpF sleB*, *cwlJ pbpG sleB*, and *cwlJ pbpF pbpG sleB* strains at both the 8th and 24th hours of sporulation. The presence of the *cwlJ* and *sleB* mutations had no effect on the spore PG structure produced by these strains (data not shown). However, the presence of these mutations allowed us to isolate spore PG from the *cwlJ pbpF pbpG sleB* strain at the 24th hour of sporulation, and we found it to have a structure similar to that produced by the *pbpF pbpG* strain at the 8th hour of sporulation (Table 3 and data not shown). Similarly, the introduction of a *cwlD* mutation into each *pbp* mutant strain resulted only in the loss of all muramic  $\delta$ -lactam from the spore PG, but spore PG could still be recovered from the *cwlD pbpF pbpG* strain at the 24th hour of sporulation.

## DISCUSSION

Previous studies performed on three class A PBPs of *B. subtilis* indicated that they have redundant functions in vegetative-PG polymerization but revealed no clear role in spore PG synthesis (44). Pedersen et al. (35) found that *pbpG*, which encodes a fourth class A PBP, is expressed only during sporulation and that a *pbpG* mutation had no effect on vegetative growth. We have now shown that loss of *pbpG* in multiple mutants lacking other class A PBPs reveals no redundant role for PBP2d in vegetative growth. This suggests that in a mutant strain lacking PBPs 1 and 4, which has a greatly reduced

growth rate, *pbpG* is not being induced to take on a significant role in vegetative-wall synthesis.

A *pbpF pbpG* double-mutant strain has a severe sporulation defect in the absence of any vegetative-growth deficiency. Electron microscopy and biochemical assays of glucose dehydrogenase, dipicolinic acid (data not shown), and spore PG production revealed that this double mutant initiated and progressed through stage four of sporulation at a rate equivalent to those of the wild type and both single mutants. Consistent with a block at this stage, mutant cells never achieved full resistance properties, and the spore was degraded during the next 24 h. The fact that expression of both *pbpF* and *pbpG* is induced specifically within the forespore compartment (35, 42) suggests that these proteins might be involved in synthesis of spore PG from the surface of the inner forespore membrane. This is the site of the germ cell wall in the dormant spore, and this structure appears to be synthesized first, prior to synthesis of the cortex PG (28). However, the initial 10 to 20% of the spore PG produced by the double mutant appeared normal, having the structure expected in the germ cell wall (4, 28). The structure of the cortex PG was greatly altered in the double mutant. One major change was a twofold decrease in the amount of muramic  $\delta$ -lactam, a structural marker used to differentiate cortex from germ cell wall (3, 4, 28, 37, 38). This was surprising since several lines of evidence indicate that the cortex PG is synthesized from the mother cell side. The spore PG defects revealed by electron microscopy in a minority of *pbpG* mutant sporangia must not reflect a major alteration of spore PG structural parameters or must have been present in too small a percentage of the cells to produce a large change in the spore PG structural parameters determined for the population.

The particular spore PG structural alterations present in the *pbpF pbpG* strain would not be expected to result in failure to achieve dormancy. Previous studies have shown that mutant strains that produce spore PG containing either no muramic  $\delta$ -lactam (*cwlD* strain) (3, 38), high cross-linking (*dacB* strain) (37, 39), or both (*cwlD dacB* strain) (40) are able to achieve normal spore dehydration and dormancy. The spore PG produced by a *dacB* strain also has a threefold decrease in the amount of single L-alanine side chains, similar to that seen in the *pbpF pbpG* double mutant, but normal spore dormancy. Failure of the *pbpF pbpG* spores to achieve dormancy is almost certainly due to the large change in the three-dimensional PG architecture we observed in electron micrographs.

We consider several possibilities for the mechanism by which loss of *pbpF* and *pbpG* results in altered synthesis of spore cortex PG. One possibility is that cortex PG is not actually produced from the mother cell side and that PBP2c and PBP2d are required on the inner forespore membrane to synthesize this structure. While there is no direct evidence for cortex synthesis from the mother cell side, there are a variety of lines of evidence that suggest this, including the production of spore PG-specific precursors in the mother cell (53), mother cell-specific synthesis of two PBPs that have significant effects on cortex PG synthesis (8, 12, 47), and the fact that the cortex PG appears to be synthesized after the germ cell wall PG (28). A second possibility is that altered synthesis of the first layers of spore PG (alterations of a type undetectable with our current methods of analysis) could disrupt the cell-cell communication carried out by the forespore and mother cell. This communication is necessary for activation of  $\sigma^K$  in the mother cell, and  $\sigma^K$  activity is required for completion of spore PG synthesis (10). It is possible that spore PG synthesis could be one component of a signal transduced from the forespore to the mother cell (58). Although previous studies indicated that expression of *spoIVB* is the only function of forespore-specific transcription factor  $\sigma^G$  required for activation of  $\sigma^K$  (17), and that  $\sigma^G$  was required for initiation of spore PG synthesis (22, 32), we felt that previous electron-microscopic examinations could have missed production of a very small amount of spore PG in a *sigG* mutant. However, expression of genes dependent on both  $\sigma^G$  and  $\sigma^K$  was normal in the *pbpF pbpG* strain. It is interesting that the failure of *pbpF pbpG* double-mutant spores to reach dormancy is similar to the phenotype produced by certain *spoIVB* point mutants which allow  $\sigma^K$  activation but which are deficient in an undefined second role required for spore maturation (32). We plan to examine if similar spore PG defects are present in this type of *spoIVB* mutant, which might suggest that this second role of *spoIVB* is exerted through the spore PG synthesis machinery.

A third explanation for defective cortex synthesis in the *pbpF pbpG* strain is that the *pbpF* and *pbpG* products are actually required on the outer forespore membrane. These particular class A PBPs may be required for the coordination of other activities required for production of muramic  $\delta$ -lactam and L-Ala side chains. Such a model would require both of these gene products to be present and functional on the outer forespore membrane in order to result in the functional redundancy seen in our genetic analysis. The *pbpF* gene is expressed at relatively low levels during vegetative growth, and during the process of engulfment its product, PBP2c, could be distributed

to both the inner and outer forespore membranes. Previous studies of *pbpG* expression identified only forespore-specific transcription (35). We would have to theorize that either (i) extremely low-level mother cell expression of *pbpG*, below the detection limit of previous assays, was sufficient to satisfy a requirement for cortex synthesis on the surface of the outer forespore membrane or (ii) PBP2d is produced within the forespore and crosses the inner forespore membrane but, unlike other class A PBPs (16, 44), does not remain associated with this membrane and is free to move to the surface of the outer forespore membrane. Our detection of PBP2d in membrane preparations of sporulating cells argues against this idea.

Finally, the model we prefer is that alteration of the germ cell wall PG structure presents an improper "template" for synthesis of the cortex PG by proteins on the outer forespore membrane. We propose that either PBP2c or PBP2d can carry out synthesis of germ cell wall PG in a uniform shell surrounding the entire forespore. In the absence of both of these PBPs an incomplete germ cell wall is produced (potentially by class A PBPs 1 and/or 4). Cortex PG polymerization is carried out by PBPs associated with the outer forespore membrane, potentially using the germ cell wall PG as a template. We suggest that, in the absence of a proper template, the cortex is synthesized in disorganized masses, often on either side of the forespore. If this synthesis of cortex PG is specifically targeted to the forespore poles, it could possibly be due to remnants of septum PG synthesis machinery. The last known sites of PG synthesis on the membranes surrounding the forespore were at the centers of a vegetative-division septum and the asymmetric sporulation septum. An alternative explanation is that the cortex PG masses are not actually synthesized at the forespore poles but that a major elongation of the spore in one direction, due to the odd PG synthesis at any single site on the forespore surface, causes the forespore to turn within the cell so that the PG extension appears to be at a pole. Finally, PG synthesis at the apparent poles of the forespore may simply be due to the fact that this is where there is available space within the sporangium. The fact that a fraction of *pbpG* cells produce disorganized cortex PG may be because PBP2d expression in the forespore, directed by  $\sigma^F$ , takes place before forespore expression of PBP2c, directed by  $\sigma^G$ . In some *pbpG* cells, cortex synthesis may advance too far in an altered way before PBP2c is produced in large enough amounts to produce a normal germ cell wall. Investigation of the requirements for *pbpF* and *pbpG* expression in the mother cell and forespore compartments in order to complete spore formation is a step toward eliminating some of these alternate theories.

#### ACKNOWLEDGMENTS

This work was supported by grants GM56695 (D.L.P.) and GM53989 (A.D.) from the National Institutes of Health.

We thank Madan Paidhungat, Peter and Barbara Setlow, and Patrick Stragier for providing strains, David Nelson and Kevin Young for advice on the use of  $^{125}$ I-penicillin X, Jennifer Meador-Parton and Amy Weaver for technical assistance, and Marita Seppanen Popham for editing the manuscript.

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