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Phenotypes of *Bacillus subtilis* Mutants Lacking Multiple Class A High-Molecular-Weight Penicillin-Binding Proteins

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Examination of *Bacillus subtilis* strains containing multiple mutations affecting the class A high-molecular-weight penicillin-binding proteins (PBPs) 1, 2c, and 4 revealed a significant degree of redundancy in the functions of these three proteins. In rich media, loss of PBPs 2c and 4 resulted in no obvious phenotype. The slight growth and cell morphology defects associated with loss of PBP 1 were exacerbated by the additional loss of PBP 4 but not PBP 2c. Loss of all three of these PBPs slowed growth even further. In minimal medium, loss of PBPs 2c and 4 resulted in a slight growth defect. The decrease in growth rate caused by loss of PBP 1 was accentuated slightly by loss of PBP 2c and greatly by loss of PBP 4. Again, a lack of all three of these PBPs resulted in the slowest growth. Loss of PBP 1 resulted in a 22% reduction in the cell radius. Cultures of a strain lacking PBP 1 also contained some cells that were significantly longer than those produced by the wild type, and some of the rod-shaped cells appeared slightly bent. The additional loss of PBP 4 increased the number of longer cells in the culture. Slow growth caused by a mutation in *prfA*, a gene found in an operon with the gene encoding PBP 1, was unaffected by the additional loss of PBPs 2c and 4, whereas loss of both *prfA* and PBP 1 resulted in extremely slow growth and the production of highly bent cells.

The peptidoglycan component of eubacterial cell walls is required to maintain cell shape and resist the turgor pressure produced by high concentrations of cell solutes. Polymerization of the peptidoglycan is carried out on the outer surface of the cytoplasmic membrane by the family of penicillin-binding proteins (PBPs) (7). The PBPs have been divided into three classes based on sequence similarities (7). The low-molecular-weight class of PBPs generally have D,D-carboxypeptidase activity and have not been found to be essential proteins (7). Some of the class B high-molecular-weight PBPs have been clearly shown to possess a transpeptidase activity involved in cross-linking peptidoglycan chains (9, 10, 12) and are required for cell septation and maintenance of cell shape (29, 30, 37). The class A high-molecular-weight PBPs appear to be bifunctional proteins with a transglycosylase N-terminal domain and a transpeptidase C-terminal domain (7, 11, 18, 31). The two class A high-molecular-weight PBPs of *Escherichia coli* have been shown to have partially redundant functions (32, 38); loss of either PBP produces little or no phenotypic effect, whereas loss of both is lethal.

Bacillus subtilis is studied as a model for cell differentiation and as a model gram-positive bacterium because of the availability of well-developed genetic tools. We have been studying the PBPs of *B. subtilis* in order to elucidate their specific roles in growth, division, and sporulation. Among the PBPs found in vegetative *B. subtilis* cells are the class A high-molecular-weight PBPs 1, 2c, and 4, the products of the *ponA*, *pbpF*, and *pbpD* genes, respectively. We have previously described the lack of growth and morphology phenotypes associated with *pbpD* (23) and *pbpF* (22) mutations and the minor growth defect caused by a *ponA* mutation (24), suggesting that there might be redundancy in the function of these genes' products. We report here the construction and characterization of strains lacking multiple class A high-molecular-weight PBPs as well as further characterization of strains carrying a mutation in the *ponA*-linked *prfA* gene.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains were derivatives of strain 168 and are listed in Table 1. *B. subtilis* was transformed as previously described (1). Growth was as 37°C in 2×SG (17), 2×YT (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter), or Spizizen's minimal medium (28) containing trace elements (8). For selection of transformants and strain maintenance, appropriate antibiotics were included in the medium at the concentrations given in parentheses: chloramphenicol (3 μg/ml), spectinomycin (100 μg/ml), and erythromycin (0.5 μg/ml) plus lincomycin (12.5 μg/ml). For determination of growth rate and sporulation efficiency, antibiotics were omitted. Spore chloroform and heat resistance were measured as previously described (20). Spores were purified by water washing (20) and stored in water at 4°C. Spores were heat activated at 70°C for 30 min, and their germination and outgrowth kinetics in 2×YT medium containing 4 mM L-alanine were determined as previously described (20).

Strain constructions. Mutant strains were constructed by transformation with plasmid or chromosomal DNA and selection for the appropriate antibiotic markers. Transformants were purified by streaking for single colonies on fresh plates. Multiple transformants were purified from each transformation, and their growth rates on plates were compared to guard against the selection of a strain carrying a suppressor mutation. Chromosomal DNA was prepared from each strain that was chosen for further analysis, and the presence of the expected mutations was verified by Southern hybridization (27).

The 484-bp *Sau*3aI-*Hin*dIII fragment from pDPC142 (23), containing the terminal portion of *orf2* (downstream of *pbpD*), including a *Pst*I site and some downstream sequences, was inserted into *Bam*HI-*Hin*dIII-digested pUC19 to produce pDPC175. The 2.2-kb *Eco*RI-*Pst*I fragment of pDPC186 (23), containing the initial 49 codons of *pbpD* and the *ermC* gene, was then inserted into *Eco*RI-*Pst*I-digested pDPC175 to produce pDPC272. This plasmid was linearized and transformed into *B. subtilis* with selection for erythromycin and lincomycin resistance to produce strain PS2140, in which most of the *pbpD* and *orf2* coding sequences were deleted and replaced by *ermC*.

PBP detection with FLU-C_6-APA. Fluorescein-hexanoic acid-6-aminopenicillanic acid (FLU-C_6-APA) was prepared essentially as previously described (16); however, the compound that we synthesized carries a six-carbon spacer between the fluorescein and the 6-aminopenicillanic acid rather than a glycine residue (16). To prepare FLU-C_6-APA, 3.7 mg (17 μmol) of 6-aminopenicillanic acid in 77 μl of 4% $NaHCO_3$ (unadjusted pH = 8.3) was mixed with 5 mg (8.5 μmol) of fluorescein-hexanoic acid-succinimide ester (Molecular Probes) in 77 μl of dimethyl formamide and left at 22°C for 4 h. The solution was then diluted with 644 μl of distilled H_2O, and the pH was adjusted to 2 with 2 N HCl. The FLU-C_6-APA was extracted into 800 μl of ethyl acetate and then back-extracted into 800 μl of distilled H_2O, which was maintained at pH 7 with 2 N NaOH during the

TABLE 1. Strains used

B. subtilis strain	Genotype[a]	Transformation donor	Transformation recipient	Source or reference
PS832	Prototrophic revertant of strain 168			Laboratory stock
PS1838	*pbpF*::Cm^r	pDPC70	PS832	21
PS1869	Δ*pbpF*::Erm	pDPC89	PS832	21
PS1958	*pbpD*::Cm^r	pDPC147	PS832	22
PS2007	*pbpD* operon::Cm^r (*pbpD*$^+$ Orf2$^-$)	pDPC179	PS832	22
PS2022	Δ*pbpD*::Erm	pDPC186	PS832	22
PS2035	*pbpD*::Cm^r Δ*pbpF*::Erm	PS1869	PS1958	This work
PS2061	Δ*prfA*::Sp^r	pDPC195	PS832	23
PS2062	Δ*ponA*::Sp^r	pDPC197	PS832	23
PS2067	Δ(*prfA-ponA*)::Sp^r	pDPC196	PS832	23
PS2140	Δ(*pbpD-orf2*)::Erm	pDPC272	PS832	This work
PS2156	*pbpF*::Cm^r Δ*ponA*::Sp^r	PS2062	PS1838	This work
PS2158	Δ(*pbpD-orf2*)::Erm Δ*ponA*::Sp^r	PS2062	PS2140	This work
PS2159	*pbpD*::Cm^r Δ*pbpF*::Erm Δ*prfA*::Sp^r	PS2061	PS2035	This work
PS2160	*pbpD*::Cm^r Δ*pbpF*::Erm Δ*ponA*::Sp^r	PS2062	PS2035	This work
PS2182	Δ*pbpD*::Erm Δ*ponA*::Sp^r	PS2062	PS2022	This work
PS2250	Δ*pbpF*::Erm Δ*prfA*::Sp^r	PS2061	PS1869	This work
PS2251	Δ*pbpF*::Erm Δ*ponA*::Sp^r	PS2062	PS1869	This work
PS2253	*pbpD*::Cm^r Δ*prfA*::Sp^r	PS2061	PS1958	This work
PS2254	*pbpD*::Cm^r Δ*ponA*::Sp^r	PS2062	PS1958	This work
PS2256	*pbpD* operon::Cm^r (*pbpD*$^+$ Orf2$^-$) Δ*ponA*::Sp^r	PS2062	PS2007	This work

[a] Abbreviations: Cm^r, resistance to 3 μg of chloramphenicol per ml; Erm, resistance to 0.5 μg of erythromycin and 12.5 μg of lincomycin per ml; Sp^r, resistance to 100 μg of spectinomycin per ml.

extraction. The FLU-C_6-APA was lyophilized, resuspended in 50 mM $NaPO_4$ (pH 7.0), and stored at −20°C. The purity of the FLU-C_6-APA was verified, and the yield was determined by reverse-phase high-pressure liquid chromatography analysis of the initial reaction mix, the extracted fractions, and the final product essentially as described previously (16). The yield of FLU-C_6-APA was found to be 50% of the theoretical value.

Membranes from vegetative *B. subtilis* cells were prepared as previously described (24). PBPs were labeled with FLU-C_6-APA by incubating 30 μg of total membrane protein with FLU-C_6-APA at 30°C for 30 min in a total volume of 12 μl of 50 mM Tris-HCl (pH 8.0)–1 mM β-mercaptoethanol–0.1 mM phenylmethylsulfonyl fluoride. The reaction was stopped by the addition of 12 μl of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating at 90°C for 5 min. Samples containing 2 μg of protein were separated by SDS-PAGE on a 10% polyacrylamide gel. FLU-C_6-APA-labeled PBPs in the gel were detected and quantified with a Molecular Dynamics Fluorimager 575.

Light microscopy. Samples of 2×SG liquid cultures in mid-exponential growth (optical density at 600 nm of 0.3) or cells removed from 2×SG plates prior to the initiation of sporulation were centrifuged, washed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 5.4 mM Na_2HPO_4, 1.7 mM KH_2PO_4), fixed with 3.3% glutaraldehyde in PBS for 60 min, and washed twice in PBS. For photography, fixed cell suspensions were placed on polylysine-coated cover slips, allowed to bind to the surface for 30 min, rinsed with H_2O, and then air dried. In some cases, cells were stained with 0.01% crystal violet during binding to the coverslip. The coverslips were then placed on slides with a small amount of H_2O and observed with bright-field optics on a Zeiss Axiovert 100TV microscope. Images were recorded with a Photometrics charge-coupled device camera. Contrast was adjusted and images were printed by using Adobe Photoshop software and a Codonics NP1600 printer. For determinations of cell dimensions, fixed cell suspensions were placed on silanized slides and observed under phase-contrast optics with an Olympus BHS microscope. Images were recorded with a Cohu charge-coupled device camera and analyzed by using Optimas software (Bioscan Inc.). Cell radii were calculated from measurements of cell length and area, using the assumption that cells are composed of cylinders capped by hemispherical poles.

RESULTS

Construction of class A high-molecular-weight PBP multiple mutants. To determine if the products of the *pbpD*, *pbpF*, and *ponA* genes have redundant functions, we constructed strains carrying mutations in various combinations of these genes (Table 1). We used two different *pbpF* mutations (Table 1, PS1838 and PS1869) which both interrupt the gene very early in the coding sequence (22). We have used four different mutations in the *pbpD* locus because of uncertainty about a potential operon structure (23). A plasmid insertion in *pbpD* (Table 1, PS1958) terminates the gene after 172 codons (out of 624), and a deletion-antibiotic resistance insertion mutation terminates the gene after 49 codons (Table 1, PS2022). These two mutations probably have a polar effect on expression of the downstream gene, *orf2*. In strain PS2140, most of *pbpD* and *orf2* are deleted and replaced by an antibiotic resistance gene. Strain PS2007 carries a plasmid insertion which leaves *pbpD* intact but which interrupts the apparent operon structure and terminates *orf2* after the second codon (23). A deletion-antibiotic resistance insertion mutation interrupts the *ponA* gene after the third codon (Table 1, PS2062), and a similar deletion/insertion terminates the *ponA*-linked *prfA* gene after 79 codons (out of 206) (24) (Table 1, PS2061). Although *prfA* is upstream of *ponA* in an operon structure, this interruption of *prfA* does not significantly alter the level of *ponA* expression, presumably because of *ponA* transcription originating from the antibiotic resistance gene promoter (24).

We examined the PBP profiles of some representative strains to verify the constructions and to determine if the amounts of other PBPs varied upon loss of the *pbpD*, *pbpF*, and *ponA* products. A recently devised technique (6, 16) for the synthesis and use of a fluorescein-labeled penicillin derivative allowed us to positively identify PBP 2c as the product of *pbpF* (Fig. 1, lane 2). Visual inspection of Fig. 1 and quantitation of the fluorescence suggests that in strains lacking PBP 1 and one or two other PBPs (Fig. 1, lanes 6 to 8), there may be a slight increase in the amount of PBP 2a. Further experiments will be required to verify this minor change. We determined the concentration of FLU-C_6-APA required to achieve 50% saturation of several of the PBPs. PBPs 1, 3, 4, and 5 were 50% saturated at 0.3, 140, 2, and 6 μM FLU-C_6-APA, respectively. Complete saturation of PBP 3 required 800 μM FLU-C_6-APA. Our estimation of the amounts of the PBPs in mutant strains was carried out at a FLU-C_6-APA concentration of 100 μM (Fig. 1). However, we calculate that FLU-C_6-APA was at a greater than 10,000-fold molar excess over PBP molecules in these reactions. Changes in the FLU-C_6-APA/PBP ratios resulting from loss of some PBPs were thus insignificant and

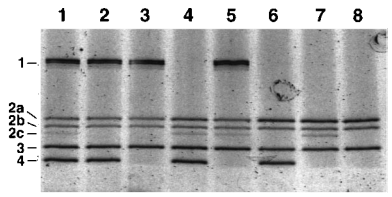

FIG. 1. PBP profiles of *B. subtilis* mutants. Membranes were prepared from vegetative cultures and incubated with 100 μM FLU-C_6-APA as described in Materials and Methods. Two micrograms of total membrane protein was loaded into each lane, and the gel was run until PBP 4 approached the bottom (PBP 5 ran off the bottom of the gel). The fluorescein-labeled PBPs were detected with a laser-scanning fluorescence imager. Strains and PBPs expected to be lacking: lane 1, PS832, wild type; lane 2, PS1869, PBP 2c; lane 3, PS2035, PBPs 2c and 4; lane 4, PS2062, PBP 1; lane 5, PS2140, PBP 4; lane 6, PS2156, PBPs 1 and 2c, lane 7, PS2158, PBPs 1 and 4; and lane 8, PS2160, PBPs 1, 2c, and 4. PBPs are numbered on the left as previously described (2, 13).

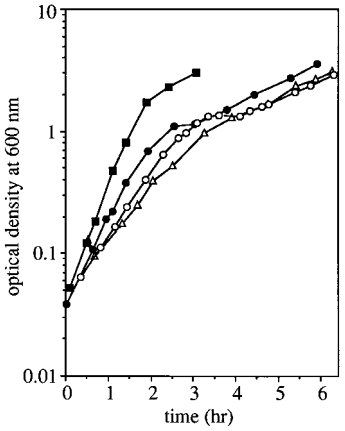

FIG. 2. Growth of *B. subtilis* mutants lacking class A high-molecular weight PBPs. Strains were grown at 37°C in 2×SG medium, and the optical density was monitored. The optical densities of all cultures remained between 3 and 4 until the later stages of sporulation. Strains and the PBPs that they lack: ■, PS832, wild type; ●, PS2062, PBP 1; ○, PS2158, PBPs 1 and 4; △, PS2061, *prfA* mutant.

could not be a cause of the apparent change in the abundance of other PBPs.

Growth of class A high-molecular-weight PBP multiple mutants. We determined the growth rates of our single and multiple mutants in three different media: 2×YT, a rich medium; 2×SG, a rich medium which supports efficient sporulation upon transition from exponential growth into stationary phase; and a minimal medium. The patterns of growth rate reductions produced by different mutations and combinations of mutations were similar in the two rich media, with slightly more pronounced effects in 2×YT (Table 2). As we had previously found (22, 23), mutations eliminating PBP 2c or 4 had no effect on growth in rich or minimal medium (Table 2). Loss of both of these PBPs also produced no obvious phenotype in rich medium but did cause a slight reduction in growth rate in minimal medium (Table 2). As demonstrated earlier (24), loss of PBP 1 produced a significant decrease in growth rate in all media (Table 2). The additional loss of PBP 2c resulted in no change in growth rate in rich medium but did cause a slight reduction in minimal medium (Table 2). In contrast, loss of both PBPs 1 and 4 caused a growth defect greater than that produced by loss of PBP 1 alone in all media tested. We note that the three strains tested in this double-mutant class all probably lacked expression of both *pbpD* and *orf2*, and thus the phenotypic change cannot be definitively attributed to loss of PBP 4. However, interruption of *orf2* in a strain lacking PBP 1 (Table 1, PS2256) did not alter the growth rate in 2×SG medium (data not shown), suggesting that loss of *orf2* expression is not deleterious in these growth conditions. Loss of all three identified class A high-molecular-weight PBPs resulted in a growth defect greater than that caused by loss of PBPs 1 and 4 (Table 2). In rich medium, either 2×SG or 2×YT, a strain lacking PBP 1 deviated significantly from exponential growth at a lower optical density than did the wild type (Fig. 2 and data not shown). After remaining at an optical density of 1 for approximately 30 min, this strain resumed growth and reached a maximum optical density similar to that of the wild-type strain. Varying the amount of glucose in 2×SG medium did not alter this phenomenon (data not shown). All of the multiple mutants lacking PBP 1 retained the interruption of growth at an optical density of 1 (Fig. 2 and data not shown), and all of the multiple PBP mutants retained motility, as judged by microscopic examination.

As we previously observed (24), a mutation affecting *prfA* expression results in a marked decrease in growth rate (Fig. 2;

TABLE 2. Growth of PBP mutants

PBP phenotype	Strain designation(s)[a]	Doubling time[b] (min)			Viable count[c] (CFU/ml)	$CHCl_3$-resistant spores[c] (CFU/ml)	Sporulation efficiency (%)
		Minimal medium	2×YT medium	2×SG medium			
Wild type	PS832	49	20	19	7.2×10^8	1.1×10^9	100
PBP 1^-	PS2062	61	37	27	1.7×10^9	2.3×10^8	14
PBP $2c^-$	PS1838, PS1869	48	20	19	1.2×10^9	7.4×10^8	62
PBP 4^-	PS1958, PS2022, PS2140	49	21	19	9.2×10^8	5.6×10^8	61
PBP $2c^-,4^-$	PS2035	55	21	19	1.5×10^9	9.2×10^8	61
PBP $1^-,2c^-$	PS2156, PS2251	66	36	28	2.4×10^9	3.4×10^8	14
PBP $1^-,4^-$	PS2158, PS2182, PS2254	65	45	37	1.5×10^9	3.0×10^8	20
PBP $1^-,2c^-,4^-$	PS2160	71	60	45	1.0×10^{9d}	1.1×10^{8d}	11
$PrfA^-$	PS2061	66	42	36	2.6×10^8	2.0×10^8	77
$PrfA^-$ PBP $2c^-$	PS2250	ND[e]	41	37	7.4×10^8	4.0×10^8	54
$PrfA^-$ PBP 4^-	PS2253	70	42	38	6.6×10^8	3.8×10^8	58
$PrfA^-$ PBP $2c^-,4^-$	PS2159	70	42	35	3.8×10^8	2.0×10^8	53

[a] Where multiple strains are listed, they were found to be phenotypically indistinguishable and numerical data for only one strain are presented.
[b] Growth was in liquid medium at 37°C.
[c] Viable counts determined 24 h after initiation of sporulation in 2×SG medium.
[d] Viable counts determined 36 h after initiation of sporulation in 2×SG medium.
[e] ND, not determined.

Table 2). The additional loss of PBP 2c, PBP 4, or both did not alter the phenotype of a *prfA* mutant strain (Table 2). We also previously found that a strain lacking both *prfA* and PBP1 exhibited a dramatic reduction in growth rate (24). Only very tiny colonies appeared on the plate used for the strain construction. When these colonies were streaked onto fresh plates, they appeared to contain subpopulations of cells containing suppressor mutations. These suppressor-containing strains exhibited a wide range of phenotypes, suggesting that several different loci might be involved. The growth and sporulation of a strain lacking *prfA* and PBP 1 which we previously reported (24) was apparently due to the presence of such a suppressor mutation. The addition of 0.5 M NaCl to the medium did not appear to increase the growth rate of a strain lacking *prfA* and PBP1. Construction of strains lacking *prfA*, PBP1, and either PBP 2c or PBP 4 or both of the latter two PBPs was hampered by the same problem. These strains appeared to have even greater growth defects and were rapidly overgrown by strains with suppressor mutations.

Sporulation of class A high-molecular-weight PBP mutants. We previously found that mutations affecting PBPs 2c and 4 did not result in a decrease in sporulation efficiency (22, 23) (Table 2). Loss of both of these proteins also had no significant effect (Table 2). The maximum viable counts achieved by all mutant cultures lacking PBP 1 were consistently higher than those of other strains, whereas the number of spores produced was slightly lower, demonstrating a decreased sporulation efficiency (Table 2). The additional loss of PBPs 2c and/or 4 in a strain lacking PBP 1 did not alter the sporulation efficiency (Table 2). Loss of expression of *prfA*, in the presence and absence of PBPs 2c and 4, did not have a significant effect on sporulation efficiency (Table 2). The spores produced by all strains listed in Table 2 exhibited normal heat resistance (>50% survival after 15 min at 85°C) and normal germination kinetics. Following germination, the spores of all of the strains immediately initiated outgrowth, and the rates of outgrowth varied similarly to the vegetative growth rates (data not shown).

Cell morphology changes in class A high-molecular-weight PBP mutants. Cells in exponential growth in 2×SG medium were harvested, fixed, and observed under a light microscope. Wild-type cultures were found to contain predominantly single cells and chains of two cells, and these cells had very straight side walls (Fig. 3A). The length distribution of these cells was tightly regulated between 4 and 10 μm (Fig. 4A). The cells of strains lacking PBP 2c, PBP 4, or both were indistinguishable from those of the wild type (data not shown). We previously reported that loss of PBP 1 resulted in no clear change in cell shape (24), but after more careful examination of this strain, we find that within these cultures there are some cells that are significantly longer than the wild-type cells (Fig. 3B and 4B) and that some of the cells appear slightly bent (Fig. 3B). Cells lacking PBPs 1 and 2c were similar to those lacking only PBP 1 (data not shown), whereas cultures of strains lacking PBPs 1 and 4 contained greater numbers of long cells (Fig. 4C) and many cells with pronounced bends (Fig. 3C). Loss of all three PBPs resulted in a morphological phenotype and cell length distribution similar to that in the strain lacking PBPs 1 and 4 (Fig. 3D and data not shown). The appearance of cell bending in these strains does not seem to simply be an artifact associated with increased cell length, as bending is seen even in the shorter cells in the culture. The radii of cells from vegetative cultures were calculated from direct microscopic measurements of cell length and area. All strains lacking PBP 1 exhibited a reduced radius with respect to strains possessing PBP 1 (Fig. 5). The combined data from these two classes of strains indicated a 22% reduction in cell radius upon loss of PBP 1 (strains PS832, PS2035, PS2061, and PS2159, $n = 595$, mean radius = 0.58 ± 0.07 μm; strains PS2062, PS2182, and PS2160, $n = 434$, mean radius = 0.45 ± 0.05 μm).

We previously reported that a mutation eliminating *prfA* expression produced no effect on cell morphology (24). After closer examination of this strain, we find that while the cells have essentially normal morphology, there is a population of longer cells in the culture, similar to what is found for the PBP 1 mutant but without a change in cell radius (Fig. 5). Preliminary observations suggested that cells lacking *prfA* are often in chains which appear to exhibit an abnormal division pattern (Fig. 3E). Whereas the cells within a wild-type chain are generally all the same length (Fig. 3A), those of a *prfA* mutant vary considerably. Although we were unable to get *prfA* mutants which lacked PBP 1 to grow in liquid culture without the appearance of cells containing suppressor mutations, we did examine the cells from a large number of colonies. Though we examined the cells from these colonies as soon as they were visible on plates, we cannot exclude the possibility that they already contained suppressor mutations. These cells exhibited the apparent odd division frequency of the *prfA* single mutant and were often in long, highly bent filaments (Fig. 3F). Portions of these filaments appeared to be helical, and lysed cells were often seen.

DISCUSSION

Our data indicate that as in *E. coli* (32, 38), the class A high-molecular-weight PBPs of *B. subtilis* have partially redundant functions. PBP 1 appears to be the most important of these proteins, as only loss of this single protein results in a change in growth rate and morphology. In the absence of PBP 1, PBP 4 appears to take on a significant role in maintenance of the growth rate and a straight cell side wall. Attribution of this function to PBP 4 must remain provisional because each of our *pbpD* mutant strains probably also lacks expression of a downstream gene, *orf2*. However, a mutation which probably disrupts only *orf2* expression did not produce the phenotypic changes associated with mutations affecting PBP 4, suggesting that these changes are due to loss of PBP 4 or to the loss of both gene products. Elimination of PBPs 1 and 4 revealed a subordinate role for PBP 2c, and the additional loss of this protein further decreased the growth rate.

Strains lacking PBP 1 have three characteristics which were unaffected by the loss of additional PBPs: (i) a pause in growth at an optical density of 1 in rich medium, (ii) an increase in viable count relative to a wild-type culture at maximum optical density, and (iii) a decrease in sporulation efficiency. It is not clear if these three phenomena are related. The pause in growth could possibly be due to more rapid utilization of one medium component, giving rise to a diauxic growth curve. One candidate for such a compound is glucose, which might be more rapidly incorporated into peptidoglycan as *N*-acetylglucosamine. This could be manifested as either a thicker cell wall or more rapid shedding of cell wall fragments into the medium (21). However, variation in the concentration of glucose added to 2×SG medium did not result in a change in the time or presence of this deviation from exponential growth. The increase in maximum viable count associated with a *ponA* mutation is consistent with a previous observation that a strain containing a mutation eliminating PBP 1 produced cells of reduced diameter (3, 14). Our measurements of photomicrographs indicated that *ponA* mutation resulted in a 22% decrease in cell radius, a value similar to the 16% reduction observed previously (14). The stage of sporulation at which the

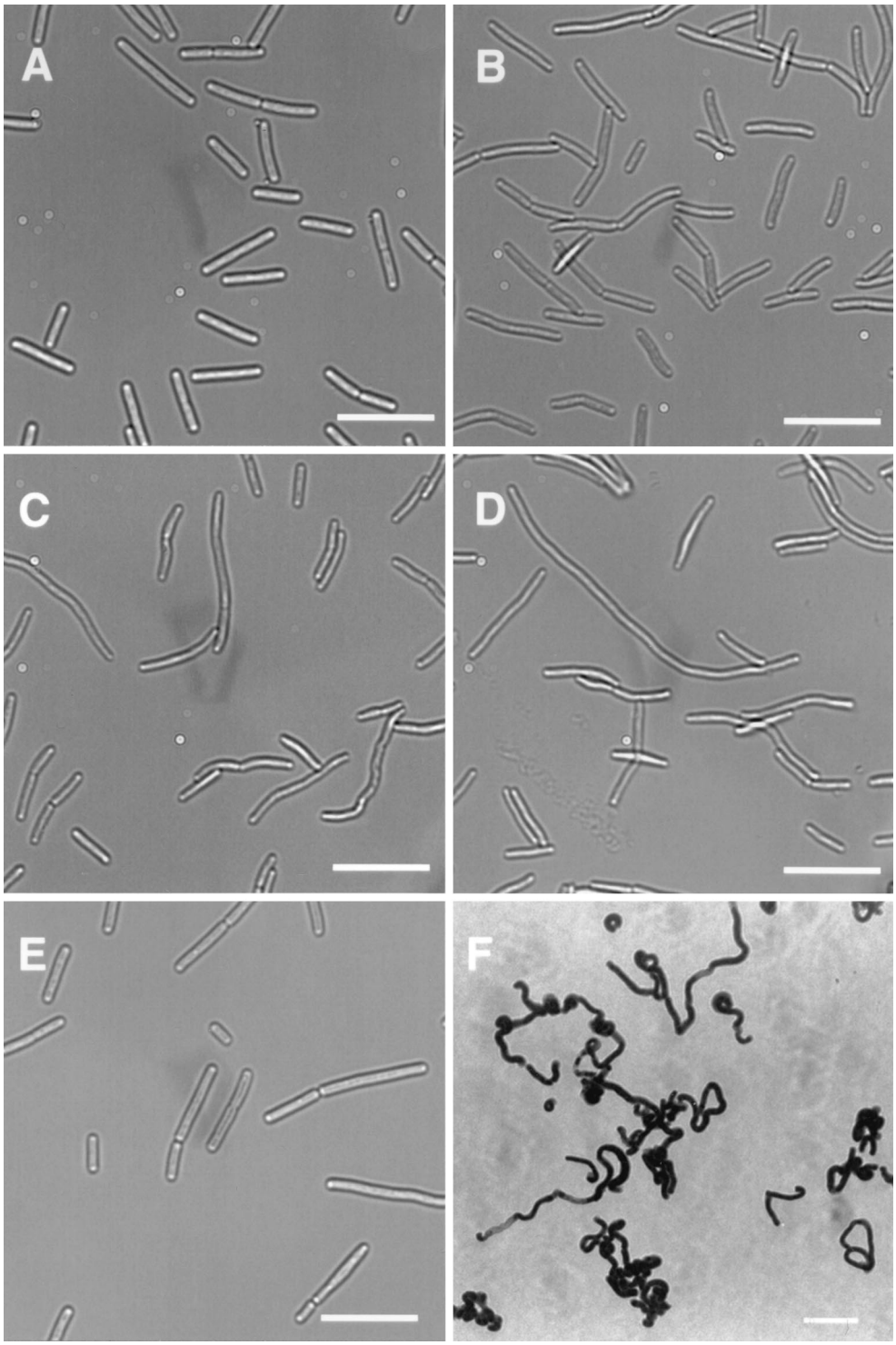

FIG. 3. Cell morphologies of *B. subtilis* mutants lacking class A high-molecular-weight PBPs. Cells were washed, fixed, and observed under bright-field optics as described in Materials and Methods. (A to E) Unstained vegetative cells; (F) cells taken from a plate and stained with crystal violet. Control experiments demonstrated that this staining did not alter cell morphology and that the cell morphologies of the strains shown in panels A to E were similar when harvested from plates. The bar markers represent 10 μm. Strains and the PBPs that they lack: (A) PS832, wild type; (B) PS2062, PBP 1; (C) PS2158, PBPs 1 and 4; (D) PS2160, PBPs 1, 2c, and 4; (E) PS2061, *prfA* mutant; (F) *prfA* mutant lacking PBPs 1, 2c, and 4c. The small round spots in panels A to D were caused by dust in the camera rather than by particles in the cultures.

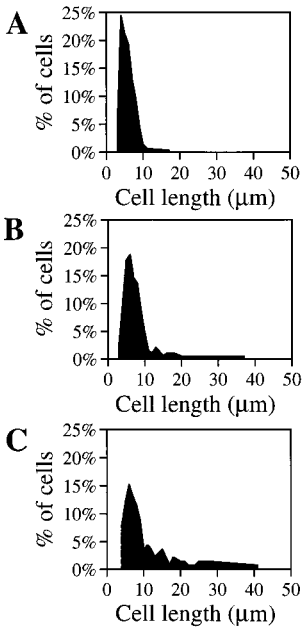

FIG. 4. Vegetative cell length distributions in wild-type and mutant *B. subtilis* strains. Cells were harvested from 2×SG medium during exponential growth, fixed, and observed under phase-contrast optics. Cell lengths were determined as described in Materials and Methods. (A) PS832 (wild type, $n = 295$); (B) PS2062 (PBP 1^-, $n = 192$); (C) PS2182 (PBP 1^-,4^-, $n = 138$).

partial block observed in mutants lacking PBP 1 is effected remains to be determined; however, preliminary microscopic observations suggest that most cells in these cultures fail to initiate sporulation.

The precise roles of the class A high-molecular-weight PBPs in *B. subtilis* cell wall formation remain unclear. A role for these proteins in formation of the cylindrical side wall is suggested by the change in cell diameter and cell bending associated with their loss. However, it is not clear whether these phenotypes are dependent on loss of the transglycosylase activity, the transpeptidase activity, or both activities associated with this class of PBPs. The viability of a strain lacking all three of the identified *B. subtilis* class A high-molecular-weight PBPs indicates that there is at least one more protein that is capable of fulfilling their function. The apparent increase in the amount of PBP 2a in some strains lacking multiple class A high-molecular-weight PBPs makes this protein a candidate for the source of the required enzymatic redundancy. An increase in the amount of PBP 2a was previously seen in a strain lacking PBP 1 (4, 14); however, the presence of multiple mutations in this strain precluded a rigorous demonstration of a cause-and-effect relationship between these two phenomena. Indeed, later studies revealed that the loss of PBP 1 and the increase in PBP 2a were separable phenomena (3). A role for PBP 2a in cylindrical wall formation was previously postulated on the basis of its early expression during spore outgrowth (19). That there might be an additional class A high-molecular-weight PBP is suggested by hybridization data (24). We are currently investigating whether PBPs 2a and 3, for which genes have not been identified, might fall into this class. Alternatively, a class B high-molecular-weight PBP such as PBP 2b (37) may be able to provide the required transpeptidase (9, 10, 12) or transglycosylase (9) activity. One model for PBP action suggests that the class A PBPs polymerize short glycan primers which are elongated by class B PBPs (36). In such a system, a lack of class A PBPs could result in the class B PBPs utilizing previously synthesized long chains or internally cleaved chains as primers. Perhaps the synthesis of abnormally long glycan chains by this mechanism could result in the cell bending that we observed. Bending of *B. subtilis* cells has previously been found to result from a variety of causes, including mutations affecting autolysin activity (5), Triton X-100 resistance (33), or PBPs 2a, 2b, and 3 (26) and treatment of cells with surfactants (35), fatty acid esters (34), local anesthetics (33), penicillin G (33), or electric fields (25). In some cases, this bending has been clearly associated with a decrease in autolytic activity (5, 35), suggesting that it may be due to a perturbation of the balance between peptidoglycan synthetic and degradative processes. Obviously, loss of peptidoglycan synthetic activities associated with class A high-molecular-weight PBPs could also produce such an imbalance. Characterization of mutations which suppress the slow-growth and cell-bending phenotypes of our multiple PBP mutants may identify other factors involved in cell wall synthesis. Some possible mechanisms of suppression might be a decrease in autolytic activity or an increase in the activity of another PBP.

The mechanisms by which *prfA* affects growth rate and cell size are unknown. Variation in cell size within a chain could be due to asymmetric division septum placement or to the production of nongrowing cells. The presence of inviable cells in the culture could also explain the change in growth rate. It is clear that either *prfA* or PBP 1 is required to maintain the wild-type growth rate and cell morphology, suggesting that they participate in redundant processes. Perhaps *prfA* is needed to coordinate peptidoglycan synthetic activities in situations in which PBP 1 is not active, i.e., in the presence of antibiotics that selectively inhibit PBP 1 or in the formation of parts of the cell wall where PBP 1 is not normally involved. Alternatively, *prfA* and PBP 1 may be involved in disparate

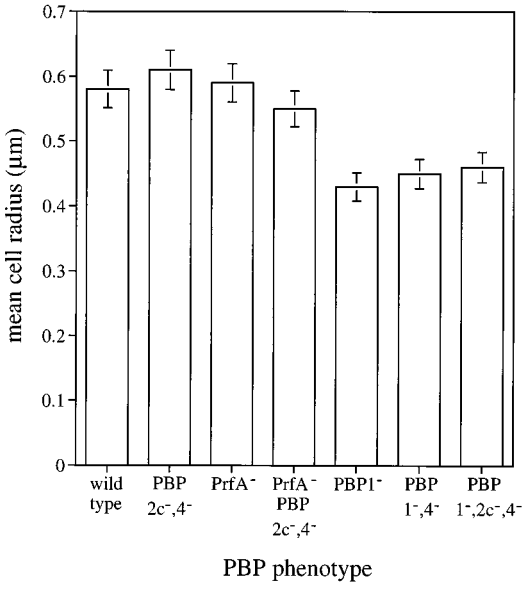

FIG. 5. Mean cell radii in vegetative cultures of *B. subtilis* wild-type and mutant strains. Cells were harvested from 2×SG medium during exponential growth, fixed, and observed under phase-contrast optics. Cell lengths and areas were determined and radii were calculated as described in Materials and Methods. Error bars represent 1 standard deviation. Strains and numbers of cells measured: PS832 (wild type), 221; PS2035 (PBP $2c^-$,4^-), 111; PS2061 (PrfA$^-$), 125; PS2062 (PBP 1^-), 173; PS2159 (PrfA$^-$ PBP $2c^-$,4^-), 138; PS2160 (PBP 1^-,$2c^-$,4^-), 158; PS2182 (PBP 1^-,4^-), 103.

systems, for example, peptidoglycan polymerization and degradation, which can individually be crippled but for which damage to both systems is incompatible. Interesting experiments will involve determination of the localization of the *prfA* product in the cell and identification of other molecules with which it might associate. Characterization of mutations which suppress the slow-growth phenotypes associated with loss of *prfA* or *prfA* and PBP 1 might aid in such analyses.

The use of molecular biological methods and the progression of the *Bacillus* Genome Sequencing Project (15) are rapidly bringing us to the point at which all the PBPs of *B. subtilis* will be identified. Further studies of the type presented here in combination with biochemical studies of peptidoglycan structure may allow construction of a strain possessing a minimal complement of PBPs that lacks the apparent redundancy found in the wild type. Such a strain could be used for a detailed analysis of the roles played by each of the classes of PBPs in synthesis of the cell wall.

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