Characterization of two *Bacillus subtilis* penicillin-binding protein-coding genes, *pbpH* (*ykuA*) and *pbpI* (*yrrR*)

Yuping Wei

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Committee members:

Chairman: David Popham

Charles Rutherford

Timothy Larson

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Characterization of two *Bacillus subtilis* penicillin-binding protein-coding genes, *pbpH* (*ykuA*) and *pbpI* (*yrrR*)

Yuping Wei

David L. Popham, Chairman

Department of Biology, Virginia Polytechnic Institute and State University

ABSTRACT

Penicillin-binding proteins (PBPs) are required in the synthesis of the cell wall of bacteria. In *Bacillus subtilis*, PBPs play important roles in the life cycle, including both vegetative growth and sporulation, and contribute to the formation of the different structures of vegetative cell wall and spore cortex. The *B. subtilis* genome sequencing project revealed there were two uncharacterized genes, *ykuA* and *yrrR*, with extensive sequence similarity to class B PBPs. These two genes are renamed and referred to henceforth as *pbpH* and *pbpI*, respectively.

A sequence alignment of the predicted product of *pbpH* against the microbial protein database demonstrated that the most similar protein in *B. subtilis* is PBP2A and in *E. coli* is PBP2. This suggested that PbpH belongs to a group of the genes required for maintaining the rod shape of the cell. Study of a *pbpH-lacZ* fusion showed that *pbpH* was expressed weakly during vegetative growth and the expression reached the highest level at the transition from exponential phase to stationary phase. The combination of a *pbpA* deletion and the *pbpH* deletion was lethal and double mutant strains lacking *pbpH* and *pbpC* or *pbpI* (also named *yrrR*) were viable. The viable mutants were indistinguishable from the wild-type except that the vegetative PG of the *pbpC pbpH* strain had a slightly

slightly lower amount of disaccharide tetrapeptide with 1 amidation and higher amount of disaccharide tripeptide tetrapeptide with 2 amidations when compared to others strains. This suggests that PbpC (PBP3) is involved in vegetative PG synthesis but only affects the PG structure with a very low efficiency.

A *pbpA pbpH* double mutant containing a xylose-regulated *pbpH* gene inserted into the chromosome at the *amyE* locus was constructed. Depletion of PbpH resulted in an arrest in cell growth and a dramatic morphological change in both vegetative cells and outgrowing spores. Vegetative cells lacking *pbpA* and *pbpH* expression swelled and cell elongation was arrested, leading to the formation of pleiomorphic spherical cells and eventual lysis. In these cells, cell septations were randomly localized, cell walls and septa were thicker than those seen in wild type cells, and the average cell width and volume were larger than those of cells expressing *pbpA* or *pbpH*. The vegetative PG had an increased abundance of one unidentified muropeptide. Spores produced by the *pbpA pbpH* double mutant were able to initiate germination but the transition of the oval-shaped spores to rod-shape cells was blocked. The outgrowing cells were spherical, gradually enlarged, and eventually lysed. Outgrowth of these spores in the presence of xylose led to the formation of helical cells. Thus, PbpH is apparently required for maintenance of cell shape, specifically for cell elongation. PbpH and PBP2a play a redundant role homologous to that of PBP2 in *E. coli*.

A sequence alignment of the predicted product of *pbpI* against the microbial protein database demonstrated that the most similar protein in *B. subtilis* is SpoVD and in *E. coli* is PBP3. This suggested that PbpI belongs to the group of the genes required for synthesis of the spore or septum PG. PbpI was identified using radio-labeled penicillin

and found to run underneath PBP4 on SDS-PAGE. PbpI is therefore renamed PBP4b. Study of a *pbpI-lacZ* fusion showed that *pbpI* was expressed predominantly during early sporulation. A putative sigma F recognition site is present in the region upstream of *pbpI* and studies using mutant strains lacking sporulation-specific sigma factors demonstrated that the expression of *pbpI* is mainly dependent on sigma factor F. A *pbpI* single mutant, a *pbpI pbpG* double mutant, and a *pbpI pbpF* double mutant were indistinguishable from the wild-type. The sporulation defect of a *pbpI pbpF pbpG* triple mutant was indistinguishable from that of a *pbpF pbpG* double mutant. Structure parameters of the forespore PG in a *pbpI spoVD* strain are similar to that of a *spoVD* strain. These results indicate that PBP4b plays an unknown redundant role.

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CHAPTER ONE

REVIEW OF THE LITERATURE

Introduction

Most bacteria have a rigid cell wall, whose indispensable function is providing support for the maintenance of bacterial morphology, protection of the integrity of the cell membrane, and resistance to the turgor pressure (27, 33). The main component of the bacteria cell wall is peptidoglycan (PG), which is a net-like macromolecule of glycan strands cross-linked by peptides. The glycan strands are made up of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues. The carboxyl groups of NAM residues are linked to short peptides. The formation of this PG involves two steps (Reviewed in 19). The first step is the synthesis of the PG subunit in the cytoplasm, the disaccharide pentapeptide. In many organisms, including *Bacillus subtilis* and *Escherichia coli*, the pentapeptide is L-Ala¾ D-Glu¾ L-*meso*-diaminopimelic acid¾ D-Ala¾ D-Ala. The transport of the subunit through the membrane is mediated by attachment to 55-carbon isoprenoid lipid. The second step is the polymerization, which includes the elongation of the glycan strands and the cross-linking of the peptide side chains. This step happens on the outer surface of the membrane and requires two enzyme activities, glycosyl transferase and transpeptidase. Glycosyl transferases connect the disaccharide units together by the β-1,4-glycosidic linkage to form glycan strands. Transpeptidases carry out the function of cross-linking by removing the terminal D-Ala from the side chain of the donor glycan strand and linking the penultimate D-Ala to the free amino group of the diaminopimelic acid in the side chain of the acceptor glycan strand or to a bridge peptide which links to the peptide side chain of the acceptor glycan

strand (3). The maturation of the structure and some regulation of the level of cross-linking are carried out by D, D-carboxypeptidases (49) which remove the terminal D-Ala from the pentapeptide side chains and inhibit the cross-linking.

A family of enzymes known as the penicillin-binding proteins (PBPs) is required in the assembly and metabolism of bacterial cell wall PG. Most of these proteins are membrane-bound with their active sites available on the outer surface of the membrane. Based on their structure, function, and similarities in peptide sequence, they are classified as multimodular class A or B high molecular-weight (MW) PBPs or monofunctional low molecular-weight PBPs (49). Most of the low MW PBPs (Fig. 1) (MW ≤ 55 KDa) have D, D-carboxypeptidase activity, whereas most high MW PBPs (generally ≥ 60kDa) (46) are transpeptidases (70); activities that are carried out by the C-terminal penicillin-binding domains. Class A PBPs are bifunctional proteins. Besides the transpeptidase activity in the C-terminal penicillin-binding domain, they possess an N-terminal glycosyl transferase domain. The N-terminal domain of the class B PBPs is believed to be a morphogenetic determinant (20), but the specific activity is unknown. A common feature of both high MW PBPs and low MW PBPs is they all have a penicillin-binding domain that interacts with β-lactam antibiotics, including penicillin, by covalent binding of these antibiotics to their active-site serine residues. The modified enzymes are inactive and deprived of their essential functions. All bacteria except those without PG synthesis possess multiple PBPs. Multiple PBPs of the same class within a species often exhibit redundant function (53, 54).

B. subtilis is the most well-studied Gram positive bacterium that undergoes sporulation, which is the process of producing an endospore, in a nutrient deprived

environment. When the environmental conditions become favorable the spore can resume vegetative growth by germination. The PG structure of the spore cell wall (spore cortex) is different from that of the vegetative cell wall on the level of cross-linking and the peptide side chains of NAM (reviewed in (19). The PBPs of *B. subtilis* are involved in the synthesis of both the vegetative cell wall and the spore cortex.

Research about PBPs of *B. subtilis* dates back to the 1970s (22). In the 1990s a great deal of progress was made. This progress was stimulated further after the entire genome sequence of this organism was published in 1997 (30). Analysis of the *B. subtilis* genome reveals 16 PBP-encoding genes recognizable from sequence similarities. These include 4 class A PBP-encoding genes: *ponA* (9, 50), *pbpF* (29, 51), *pbpD* (9, 53) and *pbpG* (also called *ywhE*) (30, 36, 45), 6 class B PBP-encoding genes: *pbpA* (29, 41), *pbpB* (29, 41, 70), *pbpC* (8, 9, 42), *spoVD* (13, 14), *ykuA* (30) and *yrrR* (30), and 6 low MW PBP-encoding genes: *pbpX* (30), *pbpE* (51, 58, 64), *dacA* (8, 9, 65), *dacB* (11, 12, 49, 58, 64) *dacC* (30, 44), *dacF* (46, 69). By cloning, mutagenesis, and construction of *lacZ* fusions, thirteen of these genes have been studied extensively whereas genes *pbpX*, *ykuA* and *yrrR* have not. The putative gene product of *pbpX* displays high sequence homology to low MW PBPs. The putative gene products of *ykuA* and *yrrR* are similar to high MW class B PBPs. The studies presented here concern *ykuA*, *yrrR*, and their gene products.

B. subtilis vegetative growth and cell wall structure

B. subtilis vegetative cells are rod-shaped and they grow by elongation in their long axis (17). Cell length is dependent on the growth rate. In general faster growing cells are longer than the slow growers. Cell division occurs at the midpoint of the rod,

generally after a doubling in length. Right after cell division the new wall material between the nascent sister cells holds the cells together. Later, hydrolysis of the septal PG separates the two cells and the new poles of the cells complete their maturation. The hydrolysis is influenced by the growth medium and growth phase. Under exponential growth conditions in a rich medium, long chains of unseparated cells can accumulate. In the vegetative cell wall, the peptide side chains of the PG are mainly tripeptides and the cross-linking level is between 29-33% of muramic acid residues (4, 19)

Sporulation, sporulation related sigma factors and spore cell wall (cortex) structure

As an adaptive response to starvation, some gram-positive bacteria, including *B. subtilis*, will undergo a complex survival mechanism called sporulation. The sporulation process of *B. subtilis* takes about 8 hours and has been described as a series of stages (Fig 2) (Reviewed in (16)). Vegetative cells are defined as being at stage 0. Under starvation, vegetative cells initiate sporulation which is characterized by the formation of an axial filament of DNA at the centers of cells. This is defined as stage I. Next, a septum is formed at an extreme polar position and lead to the partitioning of the cell into large and small compartments known as the mother cell and the prespore. Completion of the septation is defined as stage II. The prespore is then engulfed by the mother cell. When engulfment is completed (stage III), the forespore is surrounded by two membranes, the inner forespore membrane and the outer forespore membrane. Between these two membranes, two layers of PG are being synthesized (stage IV). The thinner inner layer, the germ cell wall or primordial cell wall, is adjacent to the inner membrane. The thicker outer layer, the cortex, is a modified form of PG. The spore coat proteins are deposited on the outside surface of the forespore (stage V). As the spore matures (stageVI), the

resistance, dormancy, and germination ability appear in sequence. Lysis of mother cell and the release of the mature spore is defined as stage VII.

The process of sporulation involves over 125 genes (61), the transcription of which is temporally and spatially directed by DNA-binding proteins and five RNA polymerase sigma factors (sigmas H, F, E, G, and K), which are activated sequentially. First, in response to starvation, sigma A (the major sigma factor of growing cells), sigma H, and other transcription factors regulate the transcription of the genes that direct the formation of the asymmetric septum. After the septation, sigma F becomes active in the forespore and then sigma E is activated in the mother cell. These two sigma factors control the genes required for engulfment. After engulfment, sigma G is activated in the forespore and shortly thereafter, sigma K becomes active in the mother cell. Both sigma G and K are required for the synthesis of the spore cortex and coat proteins and the regulation of spore maturation.

There are two layers of spore PG that have different structures. The germ cell wall has the same basic structure as vegetative cell wall PG (37). During germination, this layer will be developed into the vegetative cell wall. On the contrary, fifty percent of the muramic acid residues of cortex PG do not have side chains and are converted into muramic δ-lactam, which occurs at every alternate disaccharide (5, 47, 68). The muramic δ-lactam residues are not required for the spore dormancy but do play an important role as part of the substrate recognized by germination-specific lytic enzymes (48). About 23% of the NAM residues contain single L-ala side chains. The remaining 27% of the NAM residues contain peptide side chains, some of which are involved in cross-linking. The cross-linking index (the percentage of muramic acid residues with cross-linked

peptides) is only 2.9% (5, 47), a dramatic difference from the 29% found in the vegetative cell PG (4).

PBPs and maintenance of the rod-shaped cell

Class A PBPs have bi-functional activities as glycosyl transferase and transpeptidase, which are located in the N-terminal domain and C-terminal domain, respectively. The four *B. subtilis* class A PBP-encoding genes, *ponA*, *pbpD*, *pbpF,* and *pbpG* appear to play partially redundant, yet distinct, roles in the life cycle. Deletion of *ponA*, which codes for PBP1 (50), causes decreases in growth rate and sporulation efficiencies and the production of cells that are longer, thinner, and slightly bent. Two other genes, *pbpD* and *pbpF*, play some redundant function of *ponA*, the further loss of these genes from a *ponA* mutant exacerbates the phenotypic changes (54). The deletion of both *pbpF* and *pbpG* leads to the severe sporulation defect (36), which indicates these two genes play a redundant role in sporulation.

Class B PBPs are monofunctional transpeptidases. Some are essential for septation and regulation of cell shape. PBP2b, the gene product of *pbpB*, is essential for septation (70), and its sequence is very similar to that of a protein with the analogous function in *E. coli*, PBP3 (gene product of *pbpB*). PBP2a, the gene product of *pbpA*, is involved in the formation of the rod-shaped cell from oval spores during the spore germination and out-growth (41), and is most similar to the *E. coli* PBP2, which is required for the maintenance of the rod shape of the cell (59). The deletion of *B. subtilis pbpA* is not lethal and has no effect on vegetative growth and sporulation, which suggests there is another gene in the same class carrying out the redundant function of *pbpA*. The other gene with known function is *spoVD*, which codes for SpoVD and is required for the

spore PG synthesis. PBP3, encoded by *pbpC*, was found to be dispensable under all growth, sporulation, and germination conditions tested (42). The functions of the two remaining class B PBP-encoding genes, *ykuA* and *yrrR*, are undefined.

Among the six low-molecular-weight class PBPs, PBP5 (encoded by *dacA*) and PBP5* (encoded by *dacB*) are D,D- carboxypeptidase, and DacC and DacF are predicted to have the same activity based on sequence similarity (reviewed in (19). The two remaining gene products (those encoded by *pbpE* (51) and *pbpX* (30)) are predicted to be D, D-endopeptidases according to their sequence similarity to the D, D-endopeptidase, PBP4, of *E.coli* (19).

The cell envelope is important in determining the cell shape in bacteria. In *E. coli* and *B. subtilis*, most of the spherical morphology mutants have mutations in genes associated with cell envelope synthesis, mostly PG synthesis. A hypothesis is that during specific periods of the cell cycle, the cell shape is associated with the cognate PBPs to control the synthesis of PG. To maintain their shape, it has been proposed that rod-shaped bacteria have at least two systems, one for cell elongation and the other for the formation of the new poles by septation. The final shape is determined by the balance of these two systems (26). Each of the systems probably contains PG synthesis enzymes (PBPs), autolysins, and regulation enzymes.

In *E. coli*, two proteins, RodA (*mrdB*, an integral membrane protein, encoded by *rodA*) (35, 63) and class B high MW PBP2 (encoded by *pbpA*, also called *mrdA*)(59), are involved in maintenance of the rod shape of cells during cell elongation. Mutations in *rodA* or *pbpA*, or the inactivation of PBP2, block elongation and cause the production of spherical cells. These two genes are in an operon (35), and their gene products are

expected to interact with each other (34). Two other proteins, FtsW (encoded by *ftsW* and with sequence similarity to RodA) (10, 28) and class B high MW PBP3 (encoded by *pbpB*), are required in formation of the division septum.

In *B. subtilis*, there are two proteins, RodA (encoded by *rodA*, (24)) and PBP2a (41), identified as the homologues of RodA and PBP2 in *E. coli*. RodA has an essential role in elongation. The loss of RodA causes the formation of non-viable spherical cells (24). PBP2a is required for the normal elongation of germinating cells but is not essential for viability, suggesting there is another gene having similar function (41). PBP2b has been identified as a homologue to PBP3 and partial loss-of-function mutation affecting PBP2b results in filamentous growth (70). A candidate for the *B. subtilis* homologue of *E. coli* FtsW is the product of an uncharacterized ORF designated as *ylaO* (30).

Through studies of the two uncharacterized putative class B HMW PBPs of *B. subtilis* we hope to gain further insight into the mechanisms of cell elongation and cell shape maintenance.

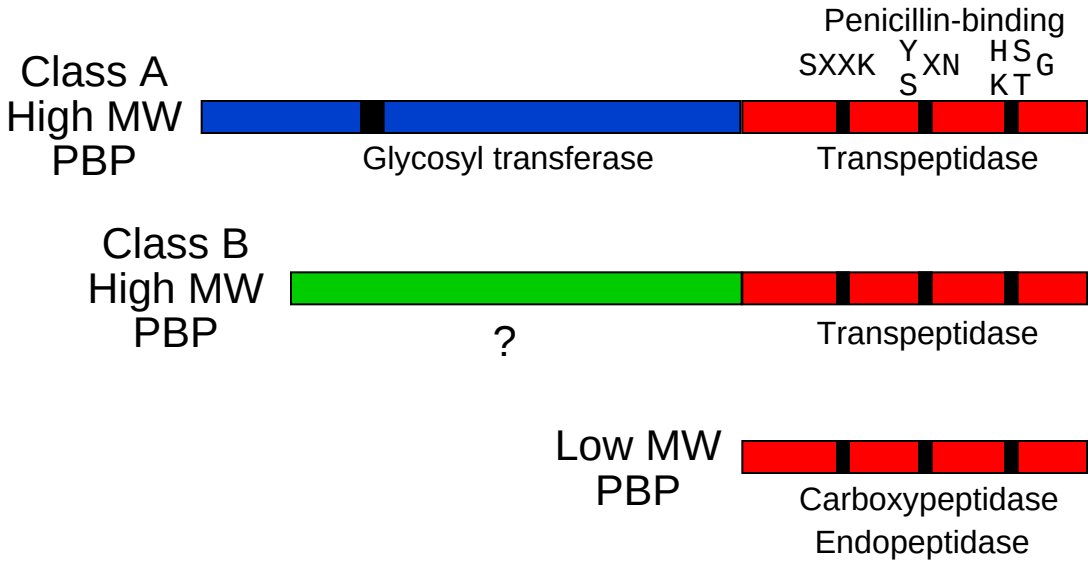

Fig. 1. The domain structures of the class A, class B high MW PBPs, and low MW PBPs. Class A and class B high MW PBPs have two function domains. Class A PBPs have glycosyl transferase and transpeptidase activities located in the N-terminal domain and C-terminal domain, respectively. Class B PBPs have transpeptidase activity in the C-terminal domain while the function of N-terminal domain is unknown. Most of the low MW PBPs have D, D-carboxypeptidase activity, and some of them are endopeptidases. A common feature of all three class PBPs is the penicillin-binding domain that interacts with β-lactam antibiotics, including penicillin, by covalent binding of these antibiotics to their active-site serine residues. The most highly conserved sequences within these domains are shown as black boxes.

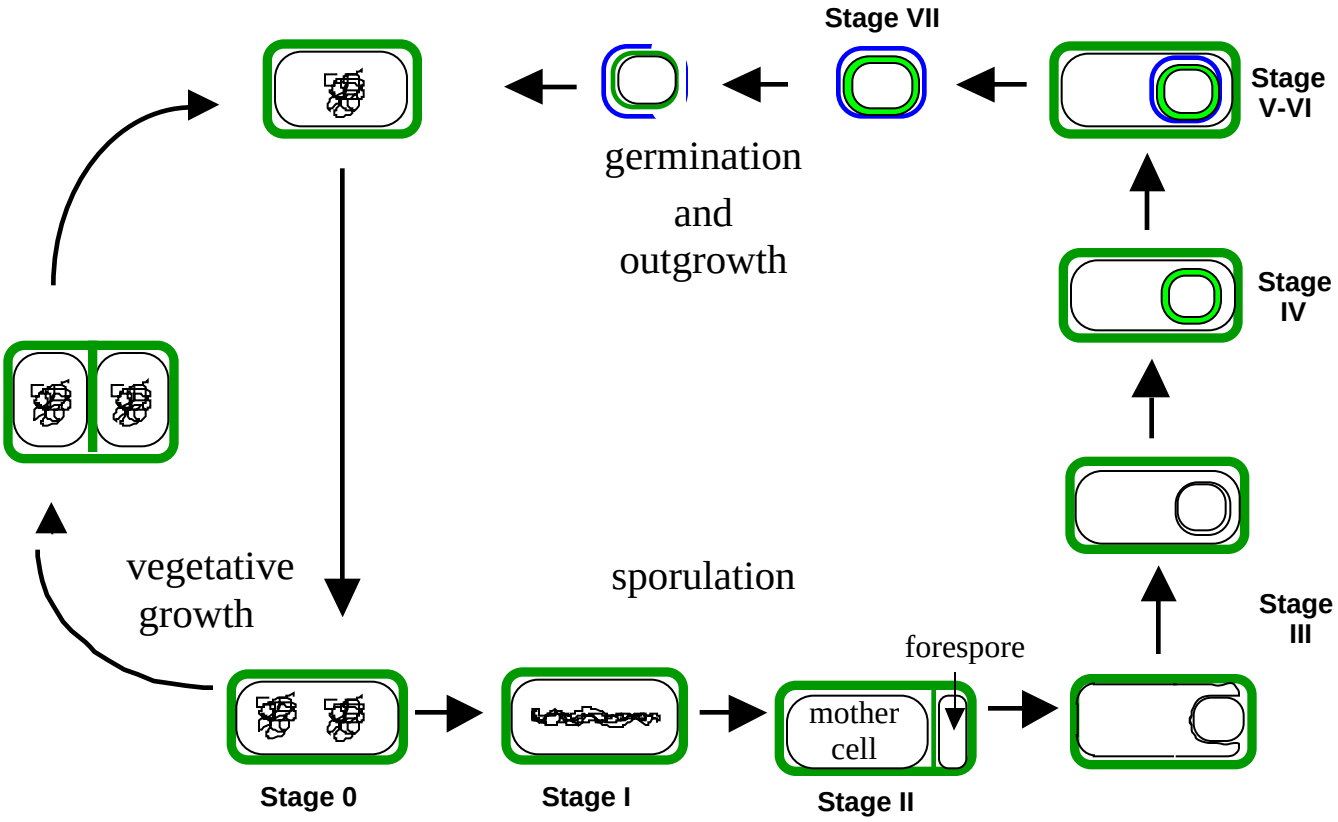

Fig. 2. Stages of the *B. subtilis* life cycle. The vegetative cell wall is shown in dark green. The developing spore (stage IV- VI) is surrounded by the cortex PG (light green). The coat protein layers are shown as a blue line. Membranes are represented as thin black lines. The filamentous structures within the cytoplasm represent the chromosomal DNA.

CHAPTER TWO

CHARACTERIZATION OF THE PBP-CODING GENE *pbpH* (*ykuA*)

Extensive sequence similarity indicated that *ykuA* encodes a class B PBP. This gene was renamed and is now referred to as *pbpH*. The first goal was the identification of the gene product of *pbpH* by comparing the PBP profiles of a *pbpH* over-expressing strain and a wild type strain using radio-labeled penicillin. The second goal was examination of the expression of *pbpH*. The final goal was identification of the functions of *pbpH*.

Materials and Methods

1. Plasmids, bacterial strains, and growth conditions.

All plasmids and *B. subtilis* strains used in this study are listed in Table 1. *E. coli* strain JM109 was used for cloning. All *B. subtilis* strains used were derived from strain 168. Transformation with plasmid DNA or chromosomal DNA was performed as described previously (2). Transformants were selected on 2xSG (32) plates containing appropriate antibiotics: chloramphenicol (3 μg/ml), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), tetracycline (10 μg/ml), and erythromycin (0.5 μg/ml) plus lincomycin (12.5 μg/ml) (macrolide-lincosamide-streptogramin B resistance). Vegetative growth for determining growth rate and sporulation for obtaining spores was routinely done in 2xSG at 37°C. Growth rate was measured by plotting the optical density at 600 nm (OD) of the growing culture versus time on a semi-log plot and reading off the generation time. Spores were purified by water washing (43). Spore germination and outgrowth was analyzed in 2xYT medium (48) containing 4 mM L-ala after a 30 min

heat shock in water at 65°C (43). The OD of all vegetative, germinating, and outgrowing cultures were determined using a Genesys 5 spectrophotometer. Spore heat resistance and chloroform resistance was measured after sporulation for 24 hours as previously described (43).

For induction of the xylose-regulated promoter, strains were grown at 37°C in 2xSG lacking glucose with the concentration of xylose at 0.4% (for the study of *pbpH* function) or 2% (for over-expression). To resuspend an exponentially growing culture into a new medium, the culture was centrifuged at 5,000xg for 5 min at 24°C and resuspended into prewarmed (37°C) fresh medium.

2. Construction of plasmids and strains.

Primers pbpH1 and pbpH2 (Table 2) were used to amplify a 2628 bp fragment containing 272 bp of upstream sequence, the coding sequence, and 302 bp of sequence downstream of *pbpH* from the chromosomal DNA of PS832. This fragment was cloned into pGEM-T (Promega), generating plasmid pDPV103, in which the *pbpH* sequence is in the same orientation as *lacZ*. The insert of this plasmid was sequenced using SP6 and T7 primers to confirm that the PCR product had the correct sequence. To construct a deletion mutation in *pbpH*, pDPV103 was digested with *Hin*dIII and *Bam*HI to remove 40.3% of the coding sequence, including the penicillin-binding active site. The deleted region was replaced with a spectinomycin resistance gene cassette obtained from pJL73 (31) by digested with *Hin*dIII and *Bam*HI. In this way plasmid pDPV113 (Δ*pbpH*::Sp) was generated. The plasmid was linearized by restriction digestion at an *Xmn*I site within the pGEM-T vector sequence. The linearized DNA was transformed into the wild type *B. subtilis* strain, PS832, with selection for the spectinomycin resistant marker to allow the

mutated gene to integrate into the chromosome via double crossover. Thus the *pbpH* deletion mutant (DPVB133) was obtained.

Primer pbpHa was designed to contain an added *Eco*RI site to assist in cloning a fragment for constructing *lacZ*-fusion. Primer pbpHa and pbpH2 were used to PCR amplified the 2628 bp insert in pDPV103. The 2637 bp PCR product was cut with *Eco*RI and *Hin*dIII to get a 682 bp fragment that contained the upstream region and the first 402 bp of the *pbpH* coding sequence. The fragment was cloned into *Eco*RI- and *Hin*dIII-digested pDPC87 (52) to generate pDPV125, in which the 682 bp fragment was placed in front of a promoterless *lacZ* gene. The insert of the plasmid was sequenced and confirmed to be identical to the sequence from the genome. The plasmid was used in transforming *B. subtilis* PS832 to allow the *lacZ* fusion to recombine into the chromosome via a single crossover. Transformants were selected on 2xSG plates containing chloramphenicol. In this way strain DPVB168 was generated, containing the transcriptional *lacZ* fusion to the *pbpH* promoter.

DPVB203 was constructed by transforming PS2465 (*pbpA*::Cm) using plasmid pCM::Erm^r (60) to switch the antibiotic resistance that is inserted in the *pbpA* locus from Cm to Erm^r.

Primers pbpH5 and pbpH3 (Table 2) were designed with added *Pac*I site and *Bgl*II sites, respectively. They were used to PCR amplify a 2162 bp fragment containing 80 bp upstream, the coding sequence, and 25 bp downstream of *pbpH* from chromosomal DNA of PS832. The resulting 2176 bp *Pac*I-*Bgl*II fragment was cloned into *Pac*I- and *Bgl*II-digested pSWEET-*bgaB* (7) which contains a xylose-regulated expression system. The *pbpH* gene in the resulting plasmid, pDPV138, was sequenced and found to be

identical to the sequence from the genome. The plasmid was linearized at the *Pst*I site in the vector and used to transform DPVB133 to generate a *xylAp-pbpH* fusion at the *amyE* locus. The resulting Amy$^-$, Cm, Sp strain DPVB202 (Δ*pbpH*::Sp *amyE*::*xylAp-pbpH*::Cm) was obtained. It was transformed with chromosomal DNA from DPVB203 with the selection for Erm^r and Sp in the presence of 0.2% xylose. The resulting transformant, DPVB 207, can only survive in medium containing xylose. As a control, DPVB213 was generated by transforming PS832 using the plasmid pSWEET-*bgaB*.

3. Enzyme assays, membrane preparation, and gel identification of PBPs.

β-galactosidase assays of vegetative cells, sporulating cells, and germinating spores were done using the substrate *o*-nitrophenyl-β-D-galactopyranoside (43, 52) and the activity was expressed in Miller units (38). Glucose dehydrogenase activity was assayed as previously described (43).

For over-expression of PbpH, strains were grown at 37°C to an OD of 0.1. Xylose was added to the culture to make the final concentration of 2%, and incubation was continued until the OD reached 1.0. Membranes were prepared as previously described (50). PBPs were detected using ^{125}I-labeled penicillin X as previously described (36).

4. Phase contrast and electron microscopy.

For phase contrast light microscopy, 0.5ml samples of germinating and outgrowing spores or vegetative cells of PS832 and DPVB207 were centrifuged, resuspended in 0.5 ml of fixing solution (2.4% paraformaldehyde and 0.01% glutaraldehyde in 30 mM sodium phosphate buffer pH 7.0) for 15 min at room temperature and 30 min on ice, washed twice with 0.5 ml of phosphate-buffered saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4), and

resuspended in 50-150 µl PBS. Fixed cell suspensions were placed on slides with polylysine-coated cover slips (One drop of 0.1% polylysine was air-dried on each cover slip.) and were photographed on an Olympus Provis AX70 microscope equipped with an Olypus UPlanF1 100X/1.30 Oil Ph3 objective. Images were processed for publishing using Adobe Photoshop software on a Macintosh computer.

For electron microscopy, 10 ml samples of vegetative cells of PS832 and DPVB207 were centrifuged; resuspended gently in the fixing solution (840 µl of 0.5 M sodium phosphate buffer, pH7.0 plus 50 µl of 25% EM grade glutaraldehyde); placed at 4°C overnight; washed 4 times with 1 ml cold 0.1 M sodium phosphate buffer, pH 6.7; resuspended in 1% osmium in cold 0.1 M sodium phosphate buffer, pH 6.7; and stored at 4°C overnight. Fixed cells were than washed in 0.5 M NH_4Cl, suspended in 2% warm agar, and immediately spun at 10,000xg for 3-5 min while cooling to pellet the cells in agar. A standard dehydration was performed at 30%, 50%, 70%, 95% and 100% EtOH, then 1: 1 with EtOH and Spurr's resin overnight, and finally suspended in 100% Spurr's resin overnight. Consequently the samples were embedded in Spurr's resin and cut with a diamond knife. The sections were placed on 200 mesh copper grids and stained with 1% uranyl acetate for 12 min and Reynold's lead for 5 min. Samples were viewed and photographed using a JEOL 100 CX-II transmission electron microscope at an accelerating voltage of 80kV. Cell length and width were measured from electron micrographs. The negatives of photographs were scanned to produce digital images and processed for publication using Adobe Photoshop software.

5. Vegetative peptidoglycan analysis.

Cultures (100 ml) of PS832 and DPVB207 were grown to OD of 1.0-1.7. cultures of DPVB133 and DPVB171 were grown to an OD of 0.5. The cultures were then chilled in ice water with swirling for 5 min, and centrifuged at 15,000xg for 10 min at 4°C. Pellets were resuspended in 2 ml of 4°C water, added dropwise to 50 ml of boiling 4% SDS, and boiled for 30 min. The cooled suspensions were centrifuged at 12,000xg for 10 min at room temperature and washed with water until free of SDS. Pellets were resuspended in 1 ml 100 mM Tris-HCl, pH7.5 and digested with α-amylase for 2 hr, then with DNase I (10 μg) and RNase A (50 μg) for 2 hr in the presence of 20 mM $MgSO_4$, and finally with trypsin (100 μg) overnight after the addition of $CaCl_2$ to a final concentration of 10 mM. SDS was added to a final concentration of 1% and the mixture was boiled for 15 min, diluted into 7 ml warm H_2O, and then centrifuged at 12,000xg for 10 min at 20°C. The pellets were washed twice in 8 ml H_2O, once in 8 M LiCl, twice in H_2O, and resuspended in a small amount of H_2O. The suspensions were transferred to 2 ml screw-cap microcentrifuge tubes and centrifuged at 13,000xg for 5 min. The pellets were lyophilized for 1 hr, resuspended in 2 ml 49% HF, and then placed on a rocker at 4°C for 48 hr to cleave off teichoic acids. The suspensions were centrifuged at 13,000xg for 5 min. The pellets were washed three times in 1 ml of H_2O and resuspended in fresh 100 mM NH_4HCO_3. The suspensions were transferred to microcentrifuge tubes, digested with 5 units of alkaline phosphatase (Boehringer Mannheim, calf intestinal) at 37°C overnight, boiled for 5 min, and centrifuged at 13,000xg for 15 min. The pellets were washed three times in H_2O and digested with 125 U of a muramidase (Mutanolysin; Sigma) in a total volume of 250 μl of 12.5 mM sodium phosphate (pH 5.5) for 16 hr at

37°C. The later steps of the lyophilization and the reduction (15 min) of the muropeptides were performed as previously described (47). The separation of the muropeptides by reverse-phase HPLC was carried out as described previously (4). Analysis of the chromatograms was done using Powerchrom V2.2.2 software on a Macintosh OS 9.1 computer.

Table 1. *Bacillus subtilis* strains and plasmids

B. subtilis strain	Genotype[a]	construction	Source or reference
DPVB133	Δ*pbpH*::Sp	pDPV113→ PS832	This work
DPVB168	*pbpH*-lacZ	pDPV125→PS832	This work
DPVB171	Δ*pbpH*::Sp *pbpC*::Cm	DPVB133→PS2352	This work
DPVB172	Δ*pbpH*::Sp *spoVD*::Kn	DPVB133→DPVB64	This work
DPVB173	Δ*pbpH*::Sp Δ*pbpI*::Erm^r	DPVB133→DPVB160	This work
DPVB175	Δ*pbpI*::Erm^r *pbpC*::Cm	DPVB160→PS2352	This work
DPVB187	Δ*pbpH*::Sp *pbpC*::Cm *spoVD*::Kn	DPVB64→DPVB171	This work
DPVB190	Δ*pbpH*::Sp Δ*pbpI*::Erm^r *pbpC*::Cm	DPVB133→DPVB175	This work
DPVB202	Δ*pbpH*::Sp xylP-*pbpH* at amyE	pDPV138→DPVB133	This work
DPVB203	*pbpA*::Erm^r	pCm::Erm^r→PS2465	This work
DPVB207	Δ*pbpH*::Sp *pbpA*::Erm^r xylP-*pbpH* at amyE	DPVB203→DPVB202	This work
DPVB213	xylP-bgaB at amyE	pSWEET-bagB→PS832	This work
PS832	Wild type, trp$^+$ revertant of 168		Laboratory stock
PS2352	*pbpC*::Cm		(42)
PS2465	*pbpA*::pTMA4 Cm		(41)

Plasmid	Construction or description of use	Source or reference
pCM::erm	Cm::Erm^r, for switching the antibiotic resistance from Cm to Erm^r	(60)
pDPC87	*B. subtilis* integrating *lacZ* transcriptional fusion vector	(52)
pDPV103	Upstream, downstream, and coding region of *pbpH* in pGEM-T	This work
pDPV113	Deletion of *pbpH* and the replacement of Sp cassette, pDPV103 digested with *Hind*III and *Bam*HI, ligated with a 1200 bp *Hind*III-*Bam*HI Sp cassette from pJL73	This work
pDPV125	*PpbpH-lacZ*, pDPC87 carrying 682 bp *Eco*RI-*Hind*III fragment of *pbpH* from pDPV103	This work
pDPV138	*xylAP- pbpH*, pSWEET carrying 2170 bp *Pac*I-*Bgl*II PCR fragment of *pbpH*	This work

pJF751	*B. subtilis* integrating *lacZ* fusion vector	(18) (45)
pJL73	Carrying the Sp cassette	(31)
pSWEET-bgaB	*xylAP- bgaB*, pSWEET carrying *bgaB*	(7)

[a] Abbreviations for antibiotic resistance: Cm, chloramphenicol; Erm^r, lincomycin and erythromycin; Kn, kanamycin; Sp, spectinomycin.

Table 2. Primer sequences

Name	Primer sequence	Restriction enzyme site added	Uses	Position in chromosome
pbpH1	5' CGT TGA ATC ACT AAG AAT AAG G		For constructing pDPV103	-272 upstream of *pbpH*
pbpH 2	5' TCA TCT CTC CTT GGA GAT AGC C		For constructing pDPV103	302 downstream of *pbpH*
pbpH a	5' CGG AAT TCC* GTT GAA TCA CTA AGA ATA AGG	*Eco*RI	*LacZ* fusion	-272 upstream of *pbpH*
pbpH 5	5' GCG CTT AAT TAA* TGA TTT GAG AGG GGT A	*Pac*I	pSWEET-ykuA	-80 upstream of *pbpH*
pbpH 3	5' GAA G*AT CTT TTA GTT GTG CAC CCT G	*Bgl*II	pSWEET-ykuA	28 downstream of *pbpH*
* The added restriction enzyme site.				

Results

Identification of the *pbpH* gene

A sequence alignment of the predicted gene product of *pbpH* against the microbial protein database was done using the BLAST software (1). The most similar protein in *B. subtilis* is PBP2a (42% identical and 60% similar) and in *E. coli* is PBP2 (22% identical and 40% similar). PBP2a is the high molecular weight (MW) class B PBP that is required for the normal outgrowth of spores. Insertional mutagenesis of its coding gene, *pbpA*, had no effect on the phenotype of cells in log phase or sporulation, and *pbpA* spores initiated germination normally, but these spores had difficulty in the transition from a spherical germinated spore to a cylindrical cell (41). PBP2 of *E. coli*, the gene product of *pbpA*, is a HMW class B PBP and is required for cell elongation and maintenance of the rod shape (59). A strain lacking *pbpA* grew as spherical cells and was not viable (66). These data highly suggest that *pbpH* belong to the group of the genes required for maintaining the rod shape of the cell.

Production of an inducible *xylAp-pbpH* construct and attempted identification of the gene product of *pbpH*

Plasmid pSWEET contains a xylose-dependent expression system that can be integrated into the chromosome of *B. subtilis* at the *amyE* locus (7). In order to clone the whole coding sequence of *pbpH* into the plasmid, two primers were designed and the gene was PCR-amplified and ligated into the digested vector. This produced the plasmid pDPV138 (pSWEET-*pbpH*), in which *pbpH* is under the control of the xylose-regulated promoter. Plasmid pDPV138 was linearized and used to transform strain DPVB133. Strain DPVB202 (Δ*pbpH*::Sp, *amyE::xylP-pbpH*) was obtained in which the level of

pbpH expression could be controlled by adjusting the xylose concentration. Plasmid pSWEET-*bgaB* was linearized and used to transform PS832 to produce strain DPVB213 (x*ylAP-bgaB*) as a control. Radioactively labeled penicillin was used to visualize the PBPs present in the xylose-induced DPVB202, DPVB213, and our wild type strain PS832. The predicted gene product of *pbpH* has a molecular mass of 77.0 kD. A PBP that appeared to be the product of *pbpH* is not visualized in membranes prepared from these cells.

Expression of *pbpH*

A PCR-amplified fragment containing 402 bp of the N-terminal coding region and 272 bp upstream of *pbpH* was used to generate a transcriptional *lacZ* fusion. This construct was used to transform *B. subtilis* PS832 to allow the *lacZ* fusion to recombine into the *pbpH* locus via a single crossover, producing strain DPVB168. To determine at which stage *pbpH* was expressed, the activity of β-galactosidase in vegetative cells, sporulating cells, and outgrowing spores was assayed (Fig. 3). Transcription of *pbpH* was very low, even when compared to that of other PBP-encoding genes (50, 52, 53), increased during vegetative growth, and reached the highest level at the transition from exponential phase to stationary phase. Expression was minimal during sporulation and in outgrowing spores. According to the time of its expression and its upstream sequence, it is hard to predict which sigma factor controls *pbpH* transcription. Further work is needed to define the transcription start site.

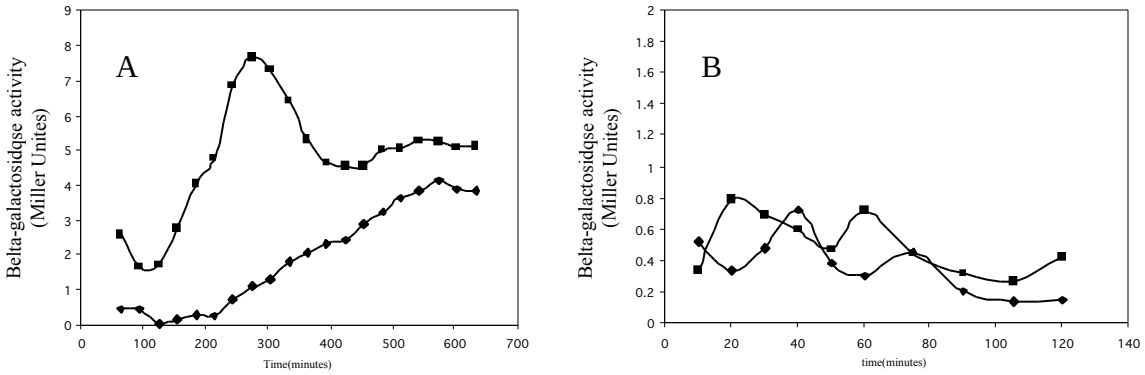

Fig. 3. Expression of a *pbpH-lacZ* fusion. (A) Expression during vegetative growth and sporulation in 2xSG medium at 37°C. The beginning of the sporulation was at 210 min. (B) Expression during spore germination and outgrowth in 2xYT medium with 4 mM L-Ala at 37°C. Symbols:◆, wild type (strain PS832);■, *pbpH-lacZ* (strain DPVB168).

Phenotypic properties of *pbpH* single mutant and multiple mutants

PBPs of the same group often have function redundancy. To identify the function of *pbpH,* we constructed a strain with a *pbpH* deletion (DPVB133), and multiple mutant strains containing the *pbpH* deletion and the deletions of other class B PBP-coding genes. It was found that the combination of a *pbpA* deletion and the *pbpH* deletion was lethal. Double mutant strains lacking *pbpH* and *pbpC* or *pbpI* were viable.

The phenotypic properties of the mutant strains were compared to those of the wild type strain PS832.There were no significant changes in the doubling times of the mutant strains (Table 3). Differences of 1 or 2 min were within experimental error. No distinguishable difference was found between mutant and the wild type cell morphologies when observed under the phase contrast microscope (data not shown). Sporulation efficiency was assayed by determining the numbers of chloroform- and heat-resistant spores per ml of the culture after sporulation for 24 hours. The results showed that all three mutant strains sporulated as efficiently as the wild type (Table 3). Spores were heat activated for 30 minutes at 65°C and germinated in 2xYT medium containing L-Ala. The progression of germination and outgrowth was measured by the change in OD over time (Fig. 4). All mutants' spores were able to initiate spore germination as shown by the decrease in OD of spore suspensions. The germination kinetics of mutants were very close to that of the wild type. The outgrowth kinetics were also indistinguishable from those of the wild type.

Table 3. Growth and sporulation of *B. subtilis* wild type and mutant strains.

Strains	Genotype[a]	Generation time (min)	Cell counts ($x10^9$ cfu/ml)		
			Viable	Chloroform-resistant spores	Heat-resistant spores
PS832	Wild type	18	2	3	3
PS2352	*pbpC*::Cm	19.5	1	1	1
DPVB133	Δ*pbpH*::Sp	20	2	3	2
DPVB160	Δ*pbpI*:: Erm^r	18.5	2	3	2
DPVB171	*pbpC*::Cm Δ*pbpH*::Sp	18.5	2	3	2
DPVB173	Δ*pbpH*::Sp Δ*pbpI*:: Erm^r	20	2	3	2
DPVB175	*pbpC*::Cm Δ*pbpI*:: Erm^r	19	2	3	2
DPVB190	*pbpC*::Cm Δ*pbpH*::Sp Δ*pbpI*:: Erm^r	19	2	3	2

[a] Abbreviations for antibiotic resistance: Cm, chloramphenicol; Erm^r, lincomycin and erythromycin;Sp, spectinomycin.

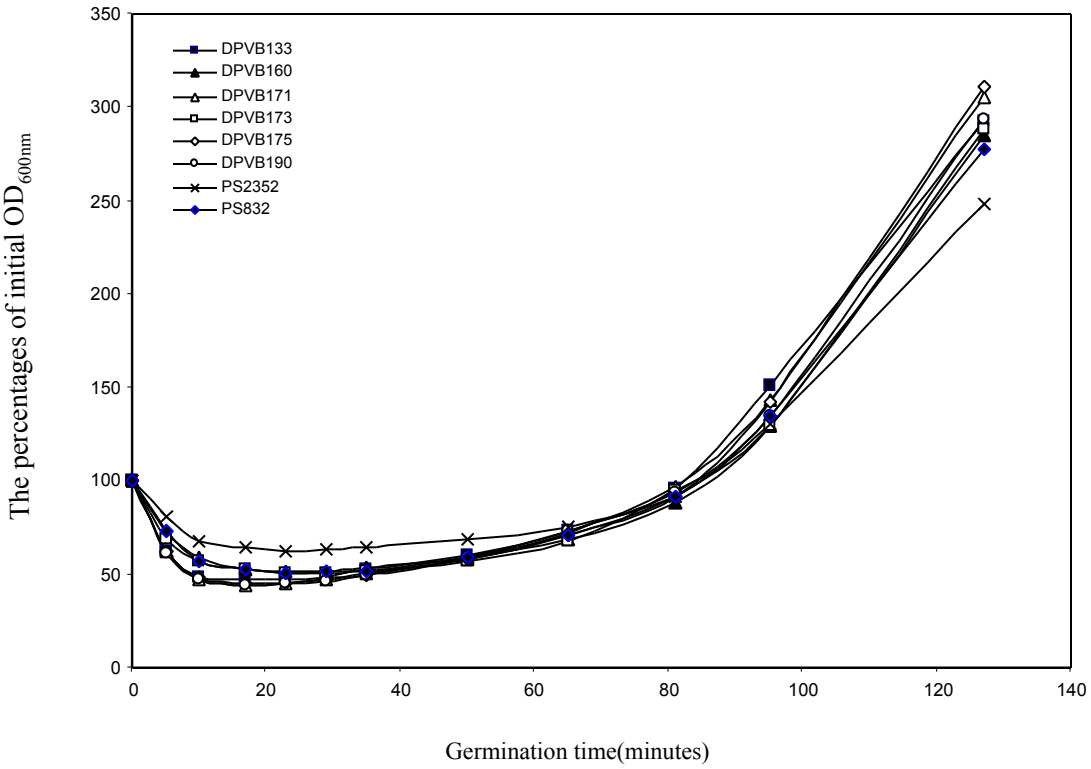

Fig. 4. The germination kinetics of *B. subtilis* wild type and mutant spores. The spores were heat activated and germinated in 2xYT medium with 4 mM L-Ala. There was no distinguishable difference of the germination kinetics among wild-type (PS832), a *pbpH* mutant (DPVB133), a *pbpI* mutant (DPVB160), a *pbpC* single mutant (DPVB2352), a *pbpC pbpH* mutant (DPVB171), a *pbpH pbpI* mutant (DPVB173), a *pbpC pbpI* mutant (DPVB175) and a *pbpC pbpH pbpI* mutant (DPVB190).

Non-viability of a *pbpA pbpH* double mutant strain

Our inability to obtain the double mutant lacking *pbpA* and *pbpH* suggested that such a strain was not viable. To study the reason for the non-viability and the phenotype of this double mutant, we constructed a double mutant containing a xylose-regulated *pbpH* gene inserted into the chromosome at the *amyE* locus. A PCR fragment containing the *pbpH* putative ribosome binding site and coding sequence was cloned into the xylose-regulated promoter vector pSWEET (7), creating pDPV138 (*pSWEET-pbpH*). The plasmid pDPV138 was linearized and used to transform DPVB133. Strain DPVB202 was obtained as a result of the integration of pDPV138 into the chromosome by a double cross-over at the *amyE* locus. Chromosome of DPVB202 was used to transform DPVB203 (*pbpA*::*Erm*), and the transformants were selected on LB plates containing 0.4% xylose and antibiotics. Strain DPVB207 was obtained, which has null mutations in both *pbpA* and *pbpH* and the xylose-inducible copy of *pbpH* inserted at the *amyE* locus. On solid medium, in the absence of xylose, DPVB207 did not grow and only a few suppressor colonies were formed. These most likely resulted from a mutation inactivating the xylose repressor or from recombination events that exchanged the *pbpH* null and wild type alleles between the normal and the xylose-inducible constructs. With the addition of 0.4% xylose to plates, DPVB207 grew and sporulated normally. This xylose concentration was used to grow this strain on both solid and liquid media.

Growth of DPVB207 in the presence and absence of xylose. Cultures of DPVB207 and PS832, inoculated from overnight plates containing xylose, were grown to an OD of 0.5 in 2xSG liquid medium containing xylose and lacking glucose, then 5 ml of the culture was centrifuged and diluted 20-fold during resuspension into fresh medium

with the presence or absence of xylose. The vegetative growth of DPVB207 and wild type strain PS832 was studied (Fig 5). Growth of the wild type was not affected by the presence or absence of xylose. In the presence of xylose, the growth of DPVB207 was almost the same as that of wild type. However, when the culture of DPVB207 was resuspended into medium lacking xylose, the cells resumed their growth for only three generations. These results indicate that *pbpA* and *pbpH* have redundant function and these two genes together have an essential role in vegetative growth.

Spore germination and outgrowth in the presence and absence of xylose. When a culture of DPVB207, at an OD of 0.5 in medium containing xylose, was spun down and diluted two-fold into fresh xylose-containing and xylose-free medium, the cells resumed growth, entered into sporulation, and generated apparently normal spores. DPVB207 and PS832 spores were purified following sporulation in both medium containing and lacking 0.4% xylose. Germination was initiated in both the presence and absence of xylose in 2xYT medium containing 4 mM L-ala (Fig. 6). The germination and outgrowth of wild type spores was the same under all conditions. The initiation of germination (visualized by the decrease in OD) of the xylose-grown (xylose-positive) spores of DPVB207 was delayed whereas that of the spores produced in the absence of xylose (xylose-negative) was indistinguishable from the wild type. The early stage of outgrowth (the initial slow increase in OD) of xylose-negative spores was similar to that of the wild type spores while that of the xylose-positive spores was still delayed. Subsequently, the outgrowth of both xylose-negative and -positive spores were significantly impaired in the medium lacking xylose, whereas all the spores exhibited similar rates of growth in the presence of xylose. This indicates that either PbpH or

PBP2a is required for outgrowth and vegetative growth but not for sporulation or initiation of spore germination. The delayed germination of DPVB207 spores produced in the presence of xylose could be due to a change in the level of cross-linking of the spore cortex due to the activity of PbpH. However, the cortex PG of DPVB207 xylose-positive and -negative spores was indistinguishable from that of wild type (data not shown). Further work is required to answer this question.

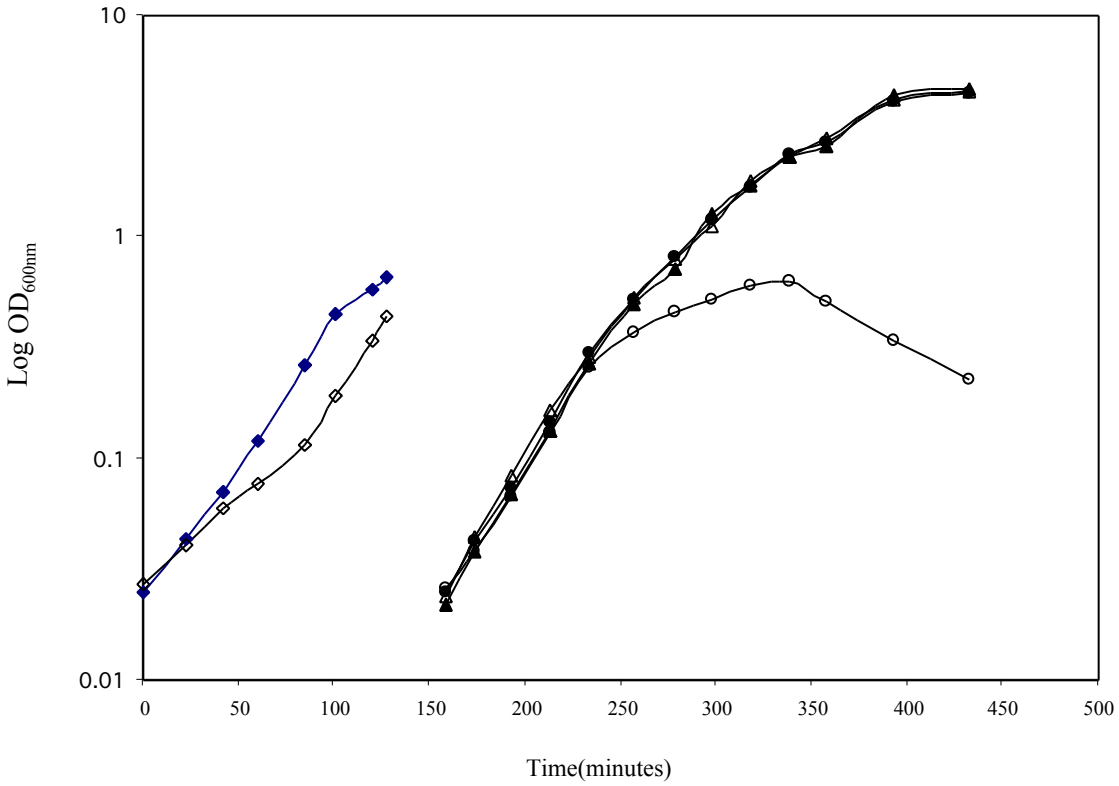

Fig. 5. Growth of DPVB207 and PS832 in the presence and absence of xylose. Filled symbols are for PS832 and open symbols are for DPVB207. Both strains were grown in the presence of xylose (diamonds) to an OD of ~0.7. The cultures were centrifuged and resuspended with a 20-fold dilution in medium containing (triangles) or lacking (circles) xylose and incubation was continued at 37°C.

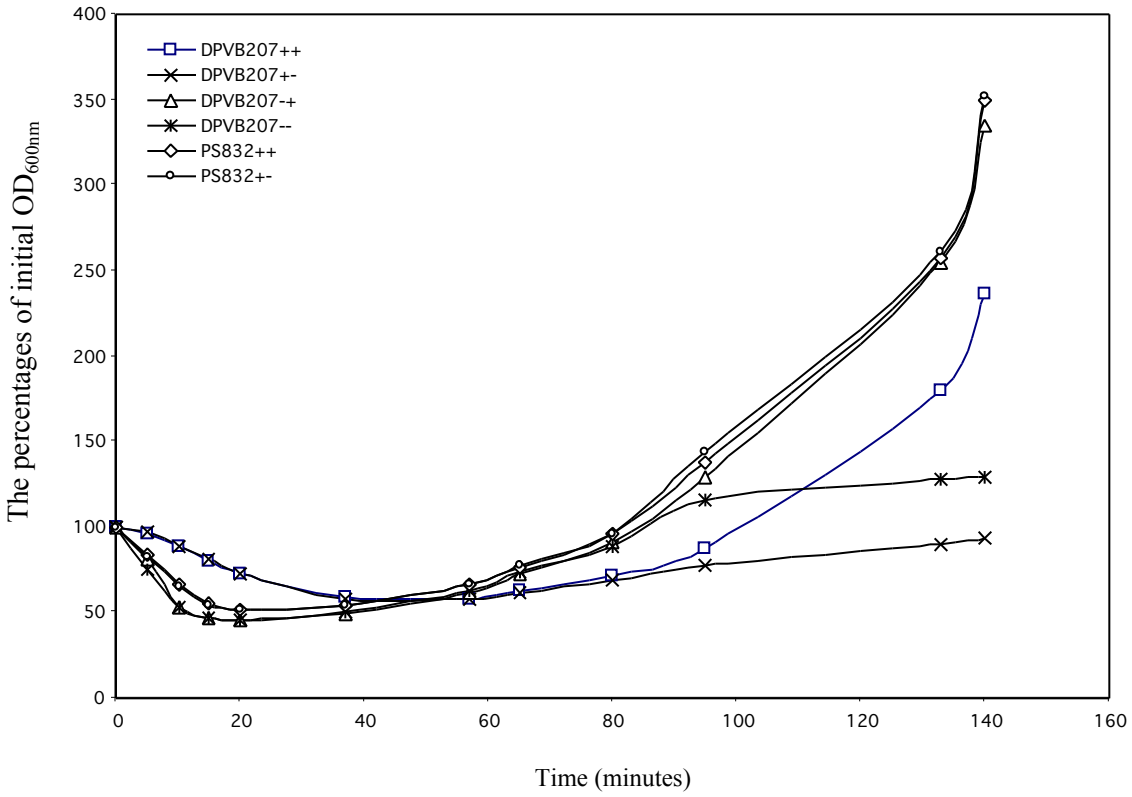

Fig. 6. Germination and outgrowth kinetics of spores produced by DPVB207 and PS832. '++', the spores were produced in the presence of xylose and germinated in the presence of xylose; ' +– ', the spores were produced in the presence of xylose and germinated without xylose; ' –+ ', the spores were produced without xylose and germinated in the presence of xylose.

Cell morphology of the *pbpA pbpH* double mutant. Cell samples from both vegetative growth and spore germination were fixed for examination under phase contrast microscopy. Examination of vegetative growth samples revealed that the wild type cells in the presence and absence of xylose and the *pbpA pbpH* cells in the medium containing xylose looked similar (Fig. 7). However, between 60 and 80 minutes after resuspension in the absence of the xylose (about two to three doubling of mass), cells of strain DPVB207 started to swell. After 2 h a few cells lysed, and many cells swelled dramatically and had a cone shape. Some cell divisions were asymmetric. After 3 h many cells had lysed, and most cells were nearly spherical. After 4 h, most cells had lysed and within the remaining cells, division septa were extremely irregular.

The morphologies of germinating and outgrowing wild type spores were identical in the presence and absence of xylose (Fig. 8 and data not shown). Examination of *pbpA pbpH* outgrowing spores revealed that those spores produced in xylose-containing medium were able to initiate germination either with or without xylose even though the initiation was delayed (Fig. 8). From then on, the outgrowth was different. Cells germinated without xylose were spherical, gradually enlarged, and eventually lysed (Fig. 8). Few cells were able to divide and the septation was asymmetrical. At later stages (240 min), a few rod-shaped cells appeared, apparently due to the acquisition of suppressor mutations. For spores germinated in xylose-containing medium, the outgrowing cells had a greater width than those of the wild type and were highly bent. Some of these cells became helical but all eventually became shorter bent rods and grew in a manner similar to the wild type. Sporulation without xylose apparently led to the production of a significant number of spores with suppressor mutations that allowed them to carry out

outgrowth in a manner similar to the wild type. When this spore suspension was germinated without xylose, the growth of the suppressor strains was highly selected. So in the beginning, the growth of the culture was like a mixture of wild type and DPVB207 xylose-negative spores germinated without xylose, and later the fast-growing mutants predominated. When this spore suspension was germinated with xylose, the cell morphology was similar to the DPVB207 xylose-negative spores germinated with xylose except a few normal rod shaped cells were present. This data suggest that in the *pbpA* strain PbpH is required in the transition of an oval-shaped outgrowing spore to a rod-shaped cell and for the maintenance of the rod-shape. It also indicates that the over-production of PbpH during early outgrowth leads to the distortion of the normal regular rod-shape of cells and the formation of bent or helical cells.

Vegetative cells were examined in greater details by transmission electron microscopy (Fig. 9). The observations were consistent with phase contrast microscopy results. Cells of the *pbpA pbpH* strain incubated in the presence of xylose had the same morphology as those of the wild type. However, these cells had a pleiomorphic spherical or distorted irregular shape when incubated in the absence of xylose. Cell septations were randomly localized. In some cells successive divisions seem to occur in perpendicularly alternating planes. Most cell walls and septa were thicker than those seen in wild type cells. Eventually many of the cells lysed. The average cell width and volume were larger than those of the wild type and *pbpA pbpH* cells grown in xylose-containing medium (Table 4).

Table 4. The cell dimensions of wild type and *pbpA pbpH* cells grown in medium with the presence and absence of xylose

Strains	Presence or absence of xylose	Cell width (μm)	Cell length (μm)
PS832	+xylose [a]	0.57	1.8
	-xylose [b]	0.56	1.5
DPVB207	+xylose [c]	0.59	1.5
(*pbpA pbpH*)	-xylose [d]	1.4	1.4

[a] the values here are the average of 40 cells;

[b] the values here are the average of 21 cells;

[c] the values here are the average of 10 cells;

[d] the values here are the average of 56 cells.

Vegetative PG structure

To determine if PG structural changes could be correlated with the altered morphology of the *pbpA pbpH* double mutant, the muropeptides derived from vegetative PG were separated and quantified using reversed-phase HPLC. The PG of DPVB133 (*pbpH*), DPVB207 grown in the presence and absence of xylose, and wild type were analyzed. In addition, the PG of DPVB171 (*pbpC pbpH*) was also studied.

Analysis of the muropeptide chromatograms (Fig. 10) revealed no difference among the vegetative PG structures of VB133, DPVB207 with xylose, PS832 with xylose, and PS832 without xylose. DPVB207 without xylose and DPVB171 produced slightly different muropeptide profiles than the others. DPVB207 minus xylose had an increased abundance of one unidentified muropeptide. DPVB171 had a slightly lower amount of disaccharide tetrapeptide with 1 amidation (peak 7) and higher amount of disaccharide tripeptide tetrapeptide with 2 amidations (peak 10) when compared to others strains. This suggests PBP3 is involved in vegetative PG synthesis but only affects the PG structure with a very low efficiency.

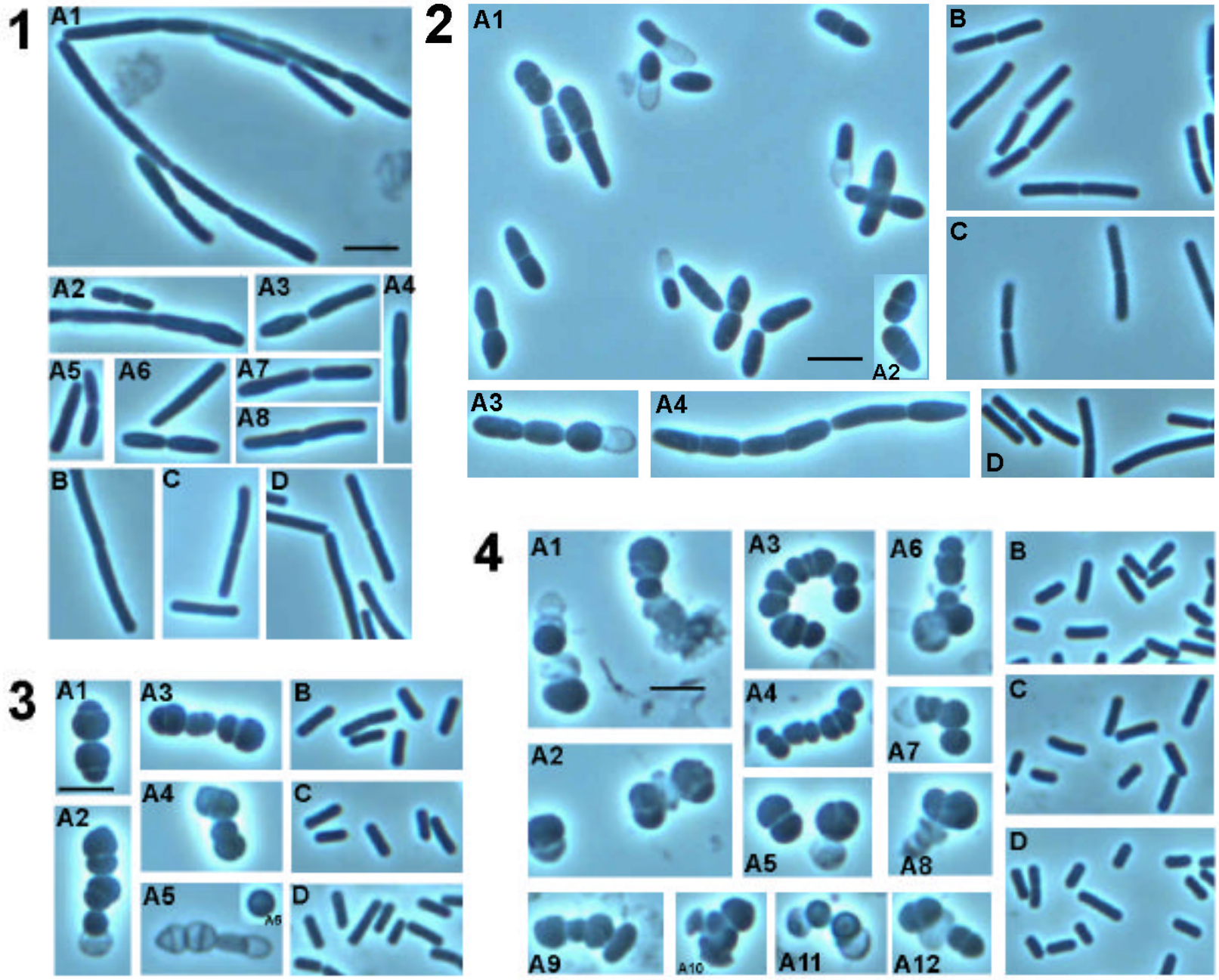
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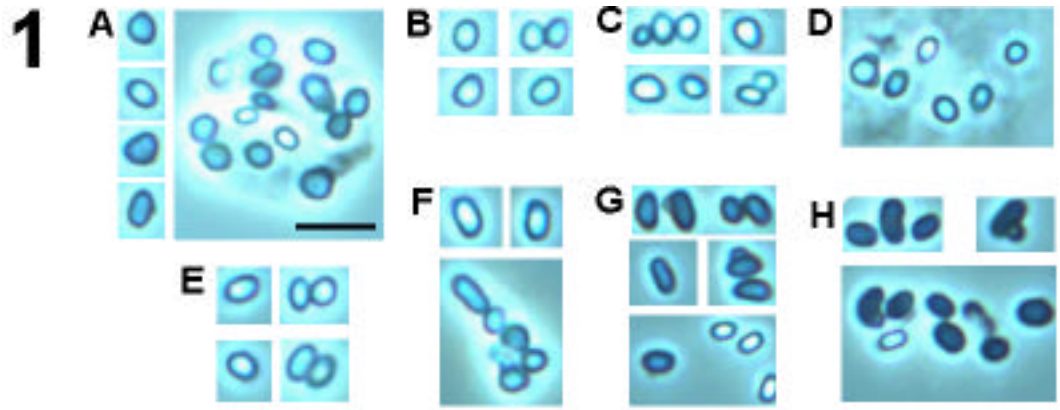
Fig. 7. Phase contrast microscopy of wild type and DPVB207 (the xylose regulated *pbpA pbpH* double mutant) vegetative cells. (A), DPVB207 without xylose; (B), DPVB207 with xylose; (C), PS832 with xylose; (D), PS832 without xylose. (1), (2), (3) and (4) are 80, 120, 180 and 240min, respectively, after the resuspension into the new medium. Scale bars, 4μm.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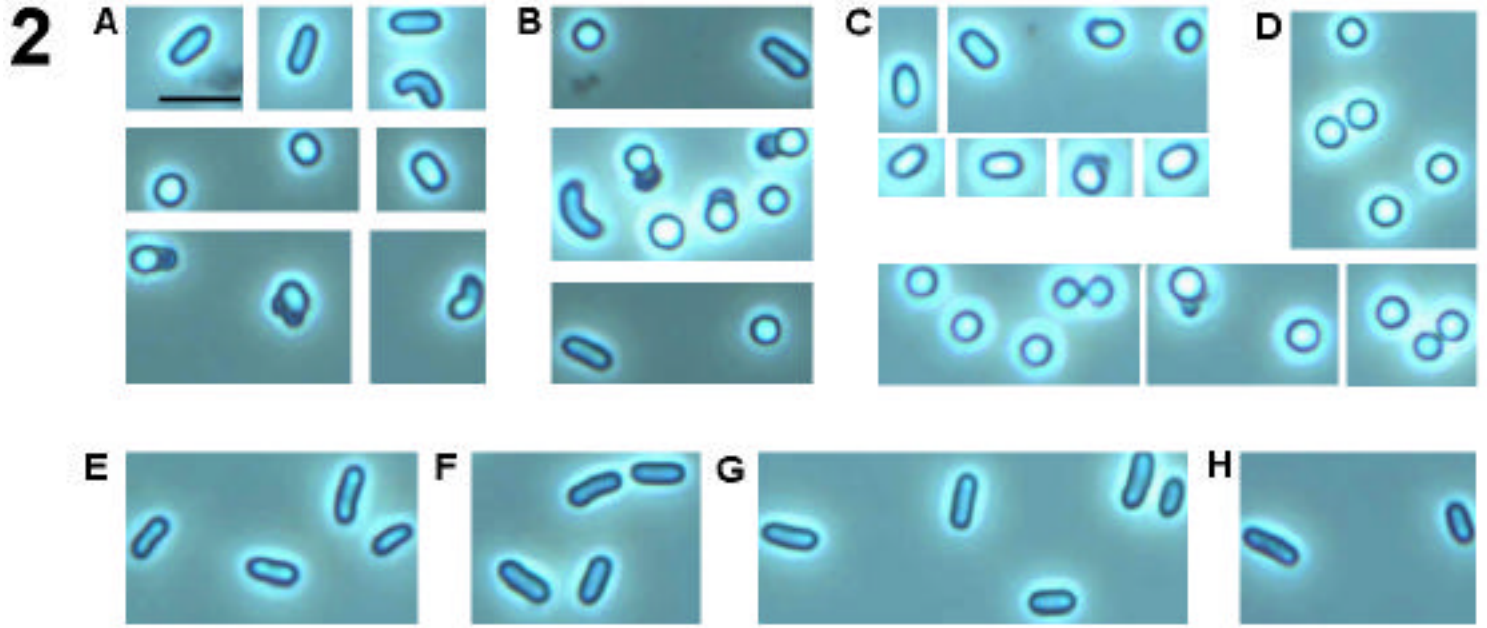
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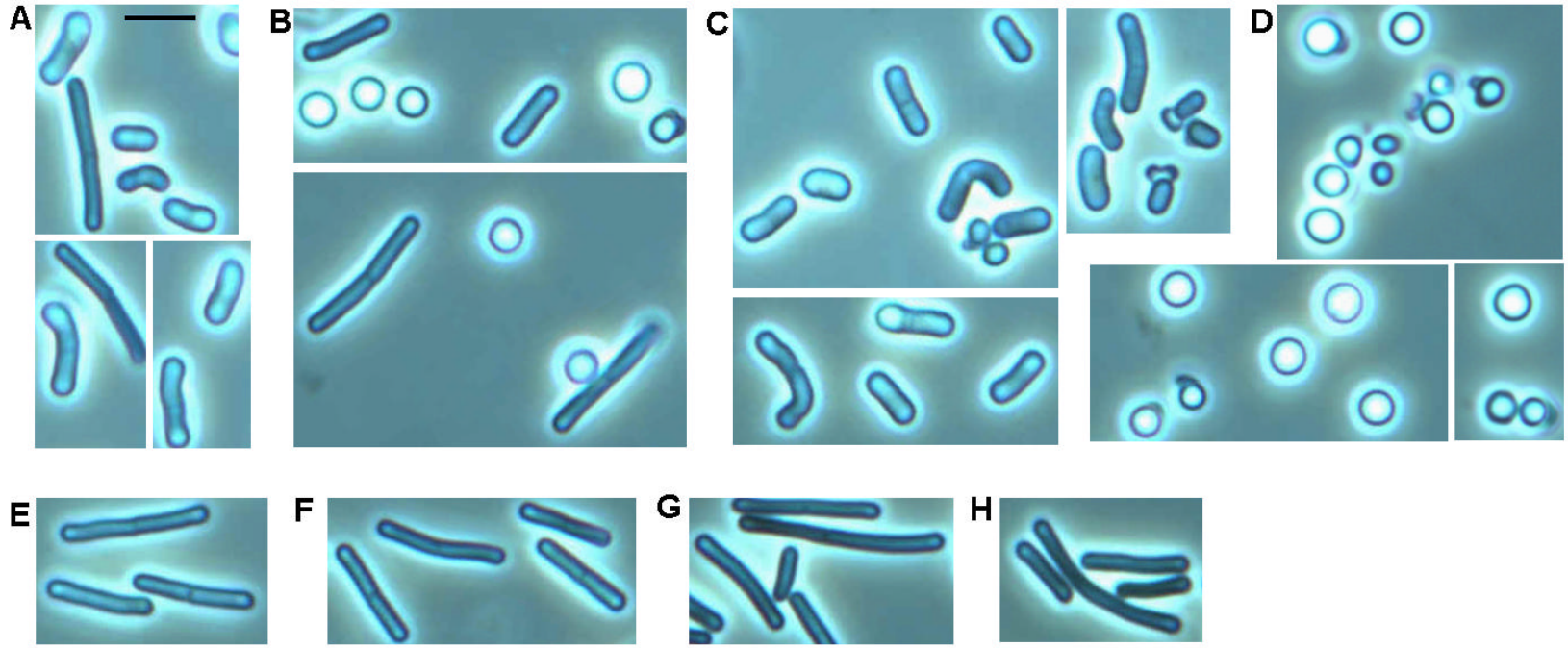

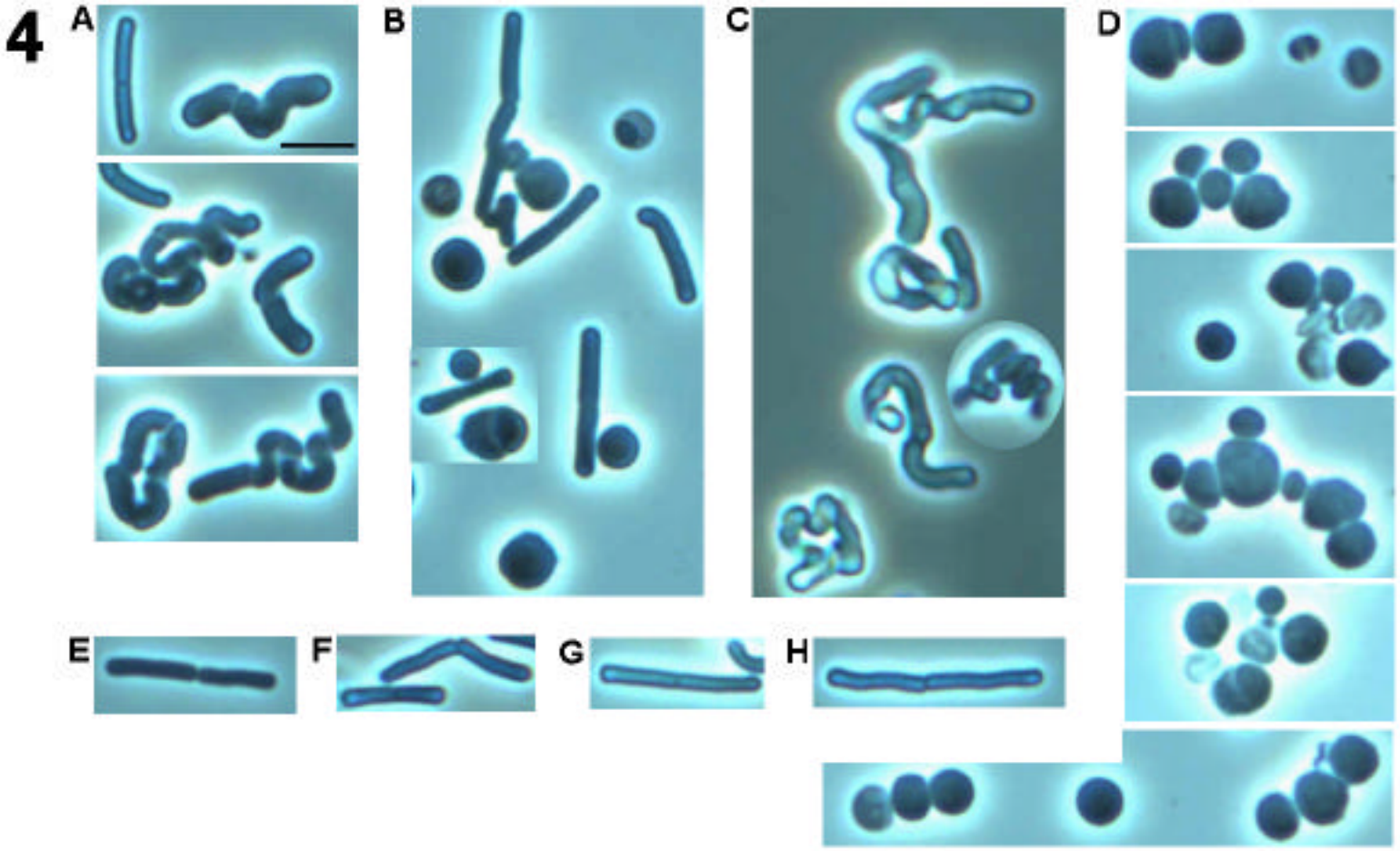
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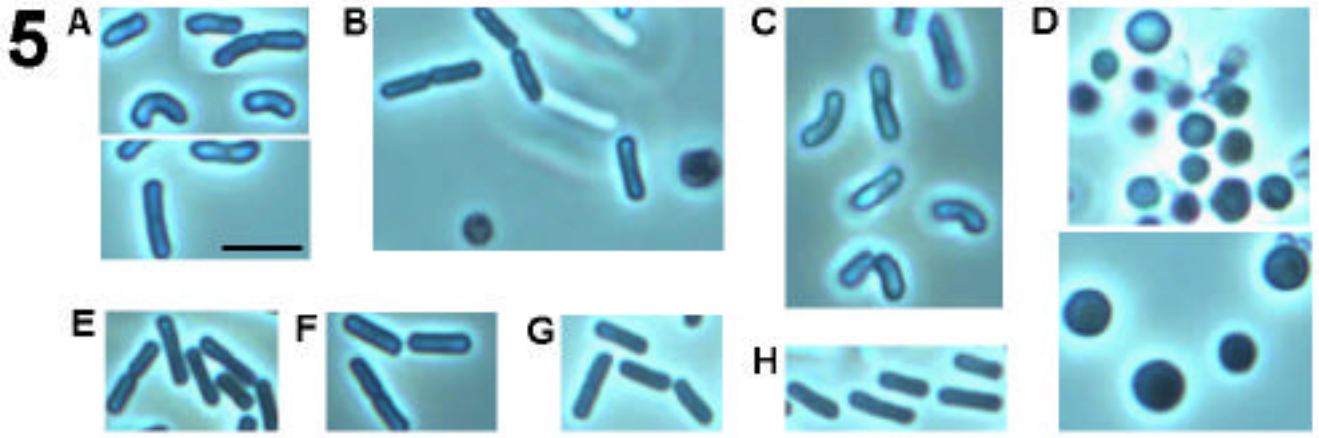
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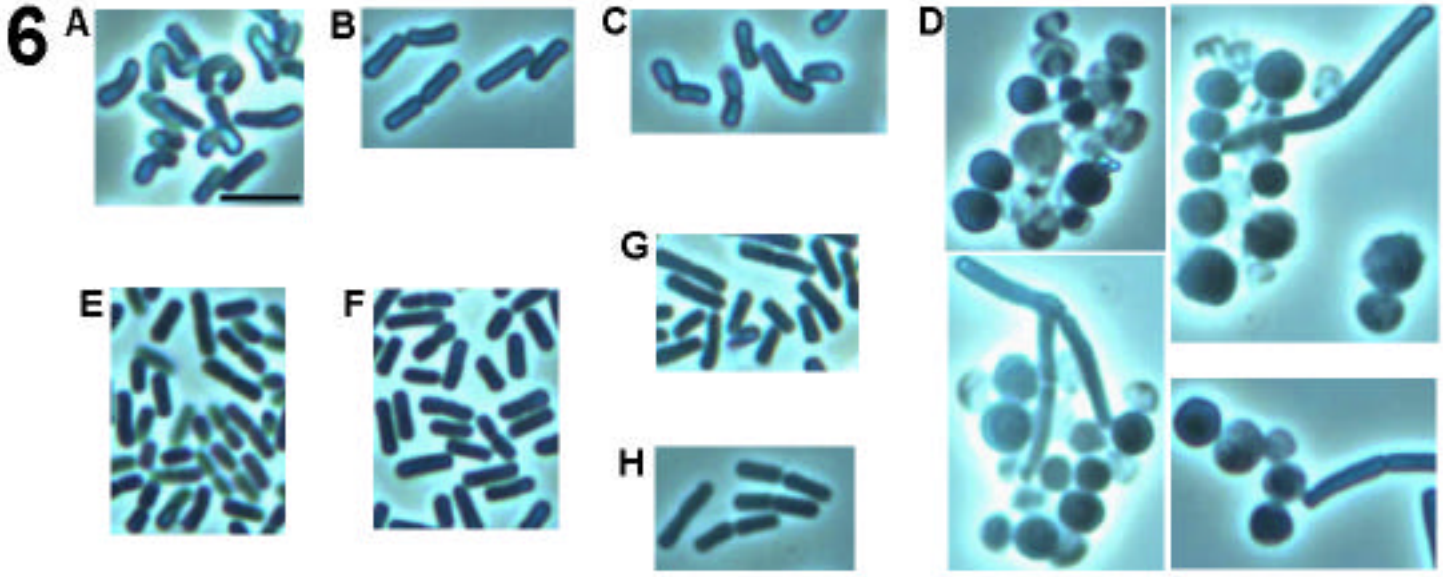
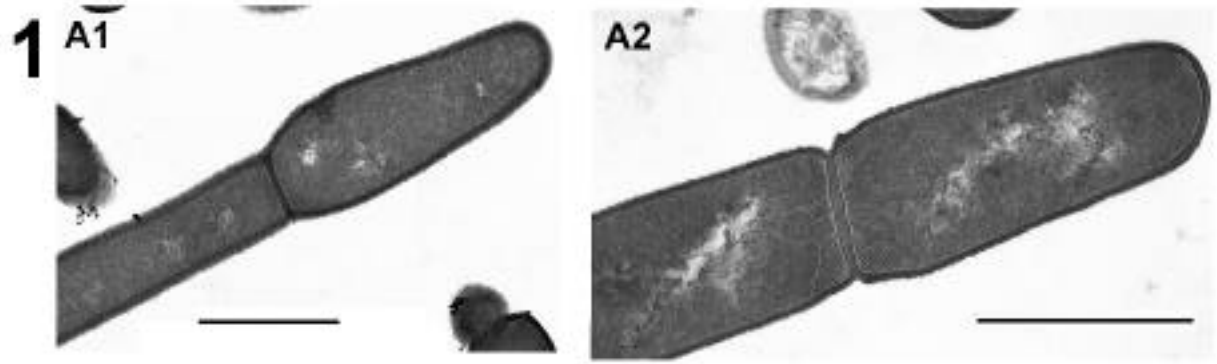

6
A
B
C
D
E
F
G
H

Fig. 8. Phase contrast light microscopy of outgrowing DPVB207 (xylose regulated *pbpA pbpH* double mutant) and wild-type spores. A, the spores of DPVB207 were from the medium without xylose and germinated with the presence of xylose; B, the spores of DPVB207 were from the medium without xylose and germinated without xylose; C, the spores of DPVB207, were obtained in the medium with the presence of xylose and germinated in the presence of xylose; D, the spores of DPVB207, were from the medium with xylose and germinated without xylose; E, the spores of PS832 were from the medium without xylose and germinated with xylose; F, the spores of PS832 were obtained in the medium without xylose and germinated without xylose; G, the spores of PS832, were from the medium with the presence of xylose and germinated with xylose; H, the spores of PS832, were from the medium with xylose and germinated without xylose. 1, 2, 3, 4, 5, and 6, are 45, 60, 90,120, 180, and 240min, respectively, after initiation of germination. Scale bars, 4μm.


1
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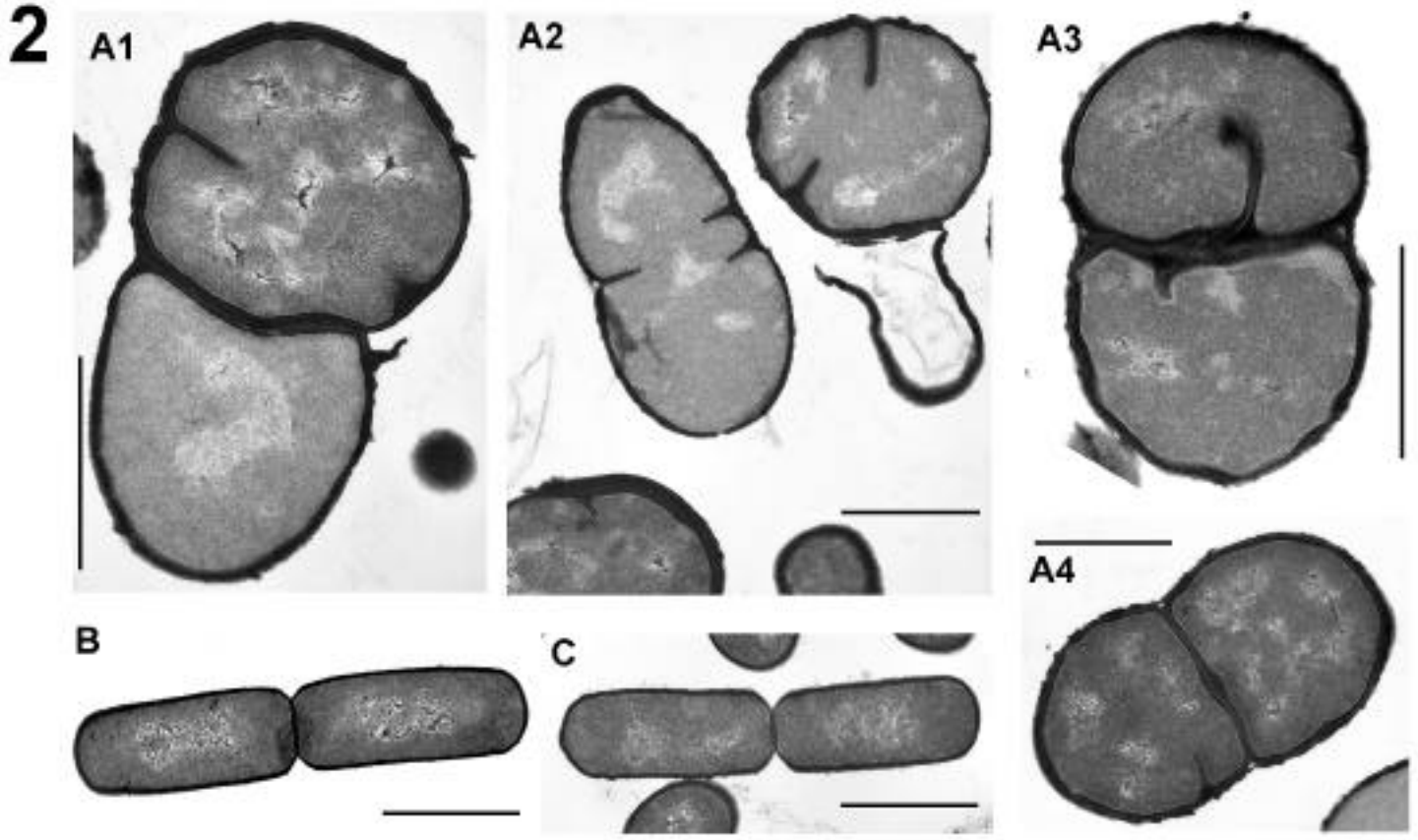
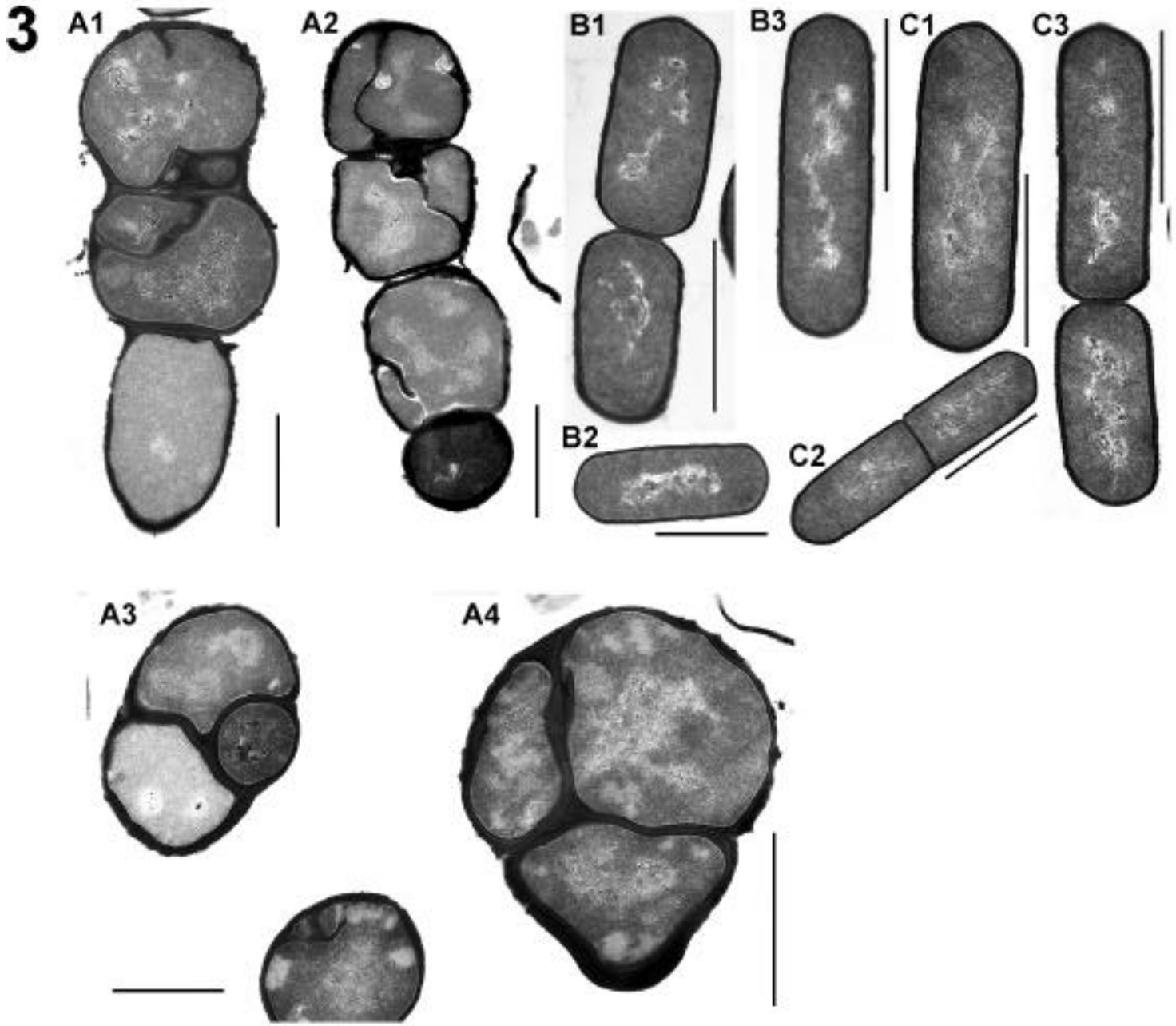
A2


2
A1
A2
A3
A4
B
C


3
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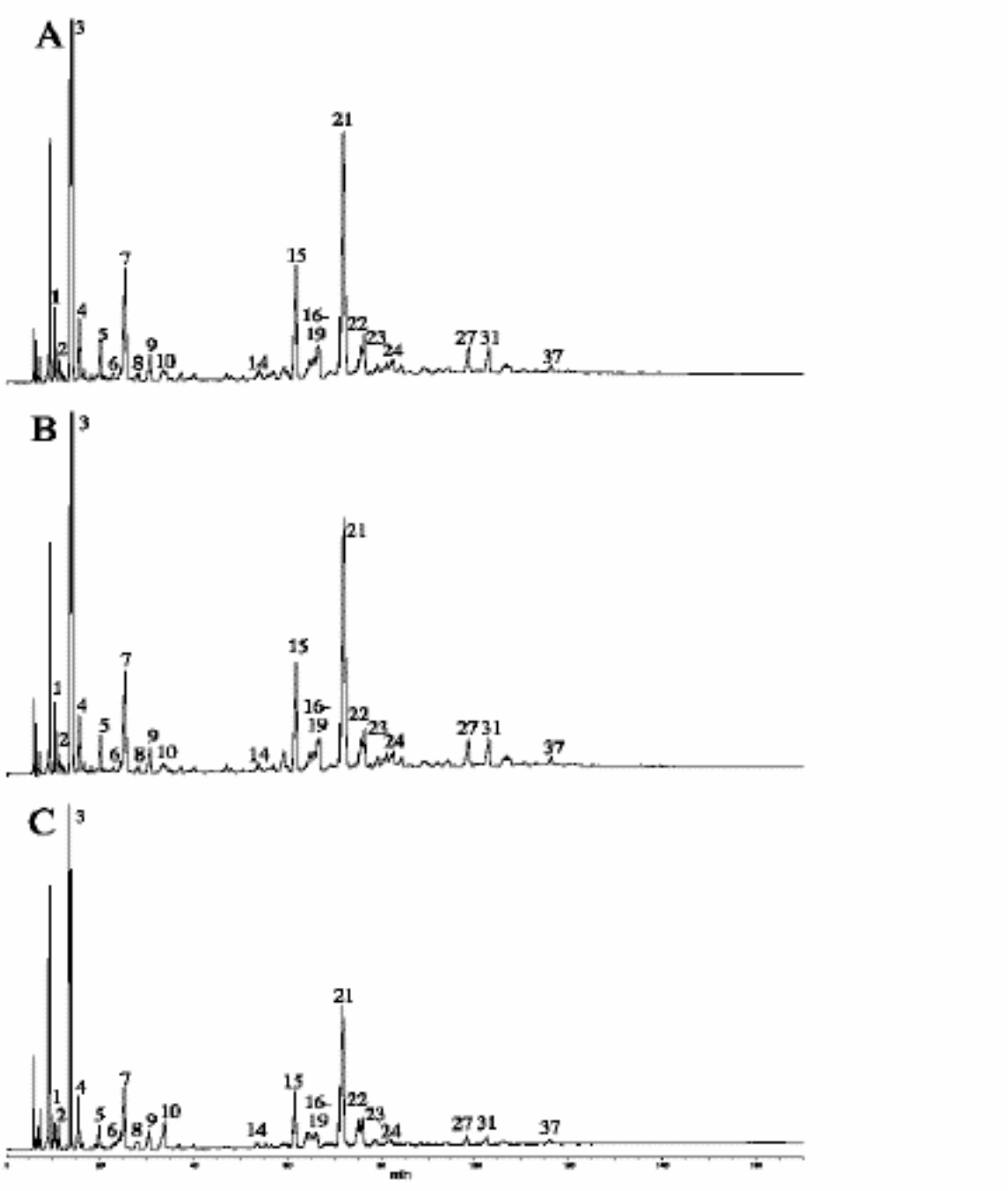
A2
B1
B3
C1
C3
B2
C2
A3
A4

Fig. 9. Electron microscopy of wild type and DPVB207 (xylose regulated *pbpA pbph* double mutant) vegetative cells. (A), DPVB207 without xylose; (B), DPVB207 with xylose; (C), PS832 with xylose. (1), (2) and (3) are 80, 180 and 240min, respectively, after the resuspension into the new medium. Scale bars, 1μm.


A
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9
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C
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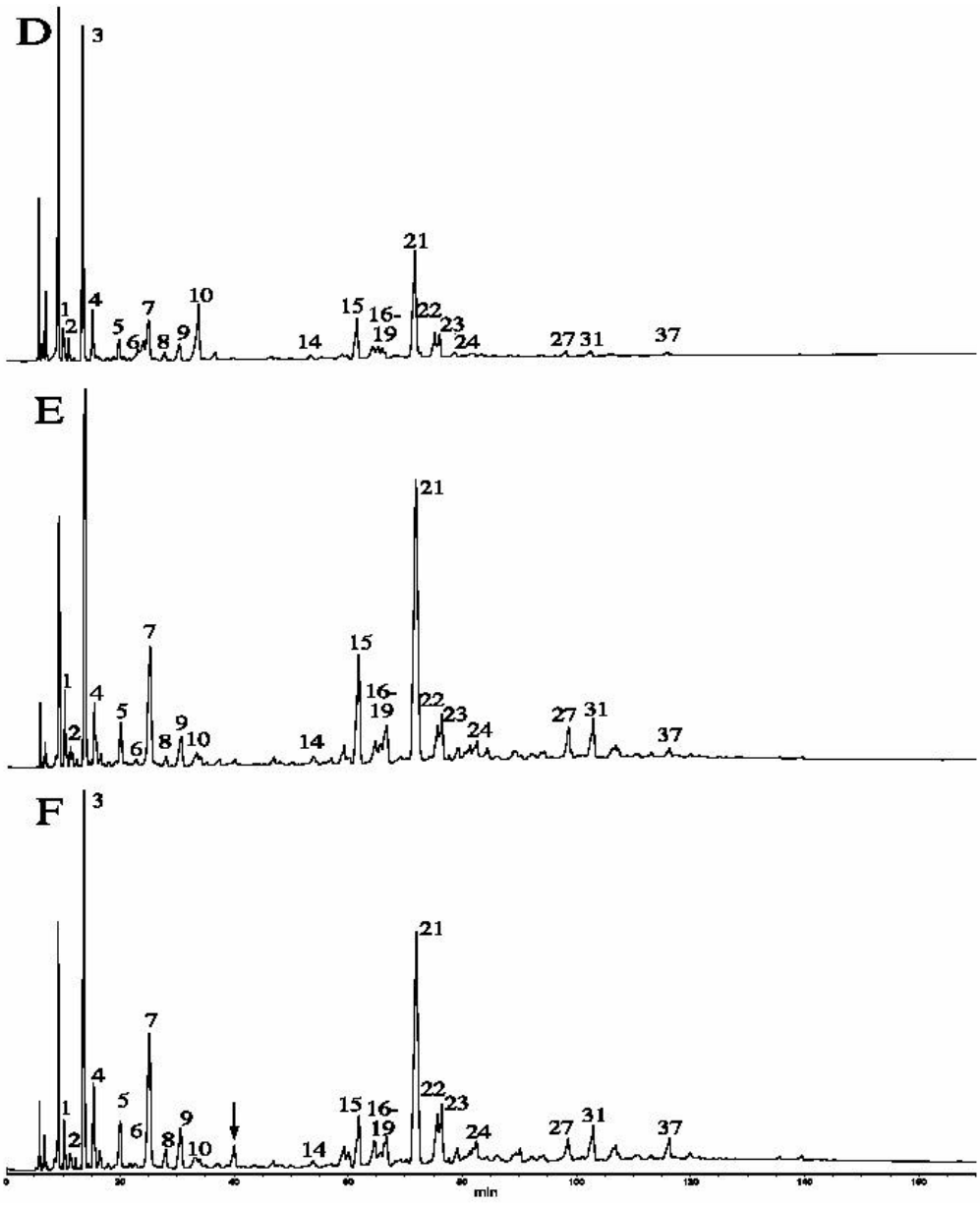
min


D
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16-
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24
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31
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9
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15
16-
19
22
23
24
27
31
37
0
30
60
90
100
120
140
160
min

Fig. 10. RP-HPLC Muropeptide elution patterns of peptidoglycan from vegetative cells of various strains. (A), PS832 (w.t.) with xylose; (B), PS832 without xylose; (C), DPVB133 (*pbpH*) without xylose; (D), DPVB171 (*pbpC pbpH*) without xylose; (E), DPVB207 (*pbpA pbpH*) with xylose; (F), DPVB207 without xylose. The Y-axis is the Absorbance at 206 nm. The arrows point at the new peak or the peaks of different size.

Discussion

The identification and characterization of *pbpH* was carried out through the study of the putative coding sequence and the construction and analysis of a series of *B. subtilis* mutant and altered gene expression strains.

The identification of gene product of *pbpH*. Analysis of the expression of a transcriptional *lacZ* fusion to *pbpH* showed that transcription of *pbpH* is at a very low level. Over-expression of the gene was attempted in order to detect the gene product. In this study, strain DPVB202, which contains an inducible *xylAp-pbpH* fusion, was used to overexpress *pbpH*. Membranes were purified from the induced strain, incubated with labeled penicillin, run on a SDS-PAGE gel, and scanned on a phosphoimager. However, we could not visualize the gene product. The chromosome of DPVB202 was used to transform a *pbpA* mutant to generate a *pbpH pbpA* double mutant in which viability was maintained only upon xylose induction of *pbpH* expression, indicting that *pbpH* was being expressed at some level from the *xylA* promoter construct. In the other part of this project the gene product of *pbpI* was identified using labeled penicillin, indicating that our performance of this method was successful. The inability to detect PbpH may due to a low binding affinity for penicillin, a low level of *pbpH* expression upon xylose induction, or instability of the protein.

The function of *pbpH* gene product. Analysis of the coding sequence of *pbpH* revealed its gene product's sequence similarity to class B high-molecular weight PBPs, especially to PBP2a in *B. subtilis*. The non-viability of a *pbpA pbpH* double mutant indicated that *pbpA* and *pbpH* play a redundant essential function. Our studies of the inducible *pbpA pbpH* double mutant verified that these two genes are determinants of cell

elongation and maintenance of the rod-shape of *B. subtilis*. These two proteins, therefore, appear to carry out a role homologous to that of PBP2 in *E. coli*. *E. coli* cells in which PBP2 is inactivated grow as spherical cells and are not viable (59, 66).

Insertional mutagenesis of *B. subtilis pbpA* had no effect on the phenotype of cells in log phase or sporulation or on the properties of the dormant spores. Spores produced by the *pbpA* strain initiated germination normally, but these spores had difficulty in cell elongation and the determination of the cell diameter during the spore outgrowth (41). However, *pbpA* spores eventually gave rise to vegetative cells that were indistinguishable from those of the wild-type strain. It was suggested that PBP2a's role in vegetative elongation is minimal or that other PBPs are able to compensate for PBP2a's contribution to vegetative cell wall elongation in its absence. Our results confirmed that this other PBP is the product of *pbpH*. PBP2a and PbpH have the same indispensable function of contribution to the elongation of the cylindrical cell wall and maintenance of the normal rod shape of *B. subtilis*. The non-viability of a *pbpH pbpA* strain may be due to an inability to properly segregate the cell contents and to carry out cell division without causing a wall disruption resulting in lysis.

Analysis of the expression of a transcriptional *lacZ* fusion to *pbpH* showed that the expression of *pbpH* was increased during the vegetative growth and reached the highest level during the transition to the stationary phase. Then, the expression of *pbpH* dropped to a very low level during sporulation. It was not expressed during germination. On the contrary, the expression of *pbpA* began to increase 30 to 40 min after the initiation of germination, increased throughout vegetative growth, and then decreased upon entry into stationary phase and sporulation (41). The expression pattern of *pbpH* may explain

the phenotype of the *pbpA* mutant. The initiation of germination doesn't require the activity of any wall synthesis protein, thus both the *pbpA* and *pbpA pbpH* strains can initiate spore germination. At the stage of spore outgrowth in the *pbpA* mutant, *pbpH* is expressed at an extremely low level, thus PbpH cannot fulfill its function in maintenance of rod shape, leading to the delayed outgrowth and defective cell morphology. Eventually, the expression of *pbpH* is increased to the level that *pbpA*'s function is compensated and the cell resumes normal shape and growth. In the *pbpA pbpH* double mutant, after the initiation of spore germination, the cells never change to rod shape due to the loss of the cell elongation function carried out by PbpH and/or PBP2a.

In our study we found that the expression level of *pbpH* is much less than that of *pbpA* (41). It will be interesting to study the expression level of *pbpH* in the *pbpA* mutant and the expression level of *pbpA* in the *pbpH* mutant. It is possible that in the absence of one of these gene products, expression of the other may be increased.

Cell shape maintenance and PBPs. It is believed that cell shape is determined by the balance of cell elongation and cell septation systems (26). Each of these systems is predicted to contain PG synthesis enzymes (PBPs), autolysins, and possibly regulation proteins. During the normal cell cycle, cells appear to switch between these systems, elongating until the cell is prepared to divide and then temporarily directing the majority of the PG synthetic activity to septum production.

In *E. coli*, RodA (an integral membrane protein, encoded by *rodA*) (35, 63) and class B high MW PBP2 (encoded by *pbpA*)(59), are involved in the maintenance of the rod shape during cell elongation. Mutations in *rodA* or *pbpA*, or the inactivation of PBP2 block elongation and cause the production of spherical cells. (These mutant cells look

similar to some of spherical cells of our *B. subtilis pbpA pbpH* double mutant (24, 55).) These two genes are in an operon (35), and their gene products are believed to interact with each other (34). Two other proteins, FtsW (also an integral membrane protein, encoded by *ftsW*) (10, 28) and class B HMW PBP3 (encoded by *pbpB*), are required for cell division. It has been suggested that there is an interaction between RodA and PBP3 (6) and PBP2 is essential for both elongation and division (67). Thus the cell elongation system and cell division system are associated. FtsW and RodA have similar sequences (24). Other proteins belonging to this FtsW/RodA (*mrdB*) family seem to be associated with the function of every class B protein that has been examined in detail (25).

This study has given some insights to *B. subtilis* cell elongation and cell shape maintenance. In *B. subtilis*, there are three proteins, RodA (encoded by *rodA*, (24)), PBP2a (41), and PbpH as the homologous system of RodA and PBP2 in *E. coli*. RodA has an essential role in elongation. The mutation of *rodA* causes the formation of non-viable spherical cells (24). PBP2a and *pbpH* are required in the elongation of cells as specific transpeptidases. In *B. subtilis* cell division, PBP2b (encoded by *pbpB*) is essential for septation and has been identified as a homologue to PBP3 (70). Based on sequence similarities, a candidate for the *B. subtilis* homologue of *E. coli* FtsW is the product of an uncharacterized ORF designated as *ylaO*. It is not known at this point whether these two systems are associated or not.

In the spherical cells produced by a *B. subtilis pbpA pbpH* double mutant there is still septum formation, but the septa are formed extremely irregularly and cells have different sizes and shapes. This may be due to the activity of only the cell septation PG synthetic system. It is not clear why these cells lyse. The septation localization system

appears to be affected by the loss of the rod-shape. There can be several possibilities to explain this phenomenon. One hypothesis is that PbpH or PBP2a is required for localization of the division sites or for completion of septation. Another possibility is the larger-than-normal diameter of the mutant cells impedes the formation of a complete FtsZ ring as required for normal septation (15). Polymerization of the FtsZ at the division site may lead to partial rings or spirals, which are unable to direct a normal invagination of the envelope.

Helical cells of inducable *pbpH pbpA* double mutant when germinated with xylose. During spore germination and outgrowth, the cell shape of the induced xylose-dependent *pbpA pbpH* double mutant was helical or twisted. This helical phenotype has been previously reported in *B. subtilis* in several circumstances, such as wild-type cells grown in a chemostat with a low [Mg^{2+}] in the medium (56), cells treated with penicillin G or chlorpromazine (62), mutants resistant to Triton X-100 (62), strains with conditional mutation in *pbpB* (57), a strain with mutations in *prfA*, *ponA*, *pbpD* and *pbpF* (54), and a strain with mutations in *ponA* and *pbpD* grown in medium with a low [Mg^{2+}] (40). The twist of our cells indicates abnormal cell wall PG synthesis, arrangement or turn-over in the cell.

The thicker cell wall of *pbpH pbpA* double mutant. It was reported that the cell wall of outgrowing spores of *pbpA* mutants possess a thicker cell wall, which is probably due in part to the slower PG turnover early in *pbpA* spore outgrowth (39). The spores of this strain eventually outgrow to vegetative cells indistinguishable from wild-type cells. If the hypothesis is true then any imbalance between PG synthesis and degradation early in the outgrowth will be corrected to give rise to normal cell (39). And this will indicate that

PbpH is associated with the correction of this imbalance. The other hypothesis proposed to explain the increased cell wall thickness of *pbpA* spores early in outgrowth is that newly synthesized PG is inserted into a smaller surface area in the outgrowing spherical *pbpA* spore than in the more cylindrical outgrowing wild-type spore, since a sphere has a smaller surface area than a cylinder and outgrowing wild type and *pbpA* spores have similar volumes (39). The unevenly thicker cell wall of the *pbpA pbpH* double mutant is more likely due to the imbalance of the synthesis and degradation of PG. Another explanation is that PG in the cell poles is more stable than PG in the cylindrical cell wall. If, in the *pbpA* outgrowing spores or vegetative cells, all the PG synthesis is carried out by the cell septation machinery then this PG will more closely resemble cell pole PG and will be degraded more slowly, and thus will be thicker.

Conclusions

A sequence alignment of the predicted product of *pbpH* against the microbial protein database demonstrated that the most similar protein in *B. subtilis* is PBP2A and in *E. coli* is PBP2. This suggested that PbpH belongs to a group of the genes required for maintaining the rod shape of the cell. Study of a *pbpH-lacZ* fusion showed that *pbpH* was expressed weakly during vegetative growth and the expression reached the highest level at the transition from exponential phase to stationary phase. Construction of mutant strain lacking PbpH and mutant strains lacking multiple class B PBPs including PbpH revealed that the combination of a *pbpA* deletion and the *pbpH* deletion was lethal and double mutant strains lacking *pbpH* and *pbpC* or *pbpI* (also named *yrrR*) were viable. The viable mutants were indistinguishable from the wild-type except that the vegetative PG of the *pbpC pbpH* strain had a slightly slightly lower amount of disaccharide tetrapeptide with 1 amidation and higher amount of disaccharide tripeptide tetrapeptide with 2 amidations when compared to others strains. This suggests that PbpC is involved in vegetative PG synthesis but only affects the PG structure with a very low efficiency.

A *pbpA pbpH* double mutant containing a xylose-regulated *pbpH* gene inserted into the chromosome at the *amyE* locus was constructed to study the mechanism of the non-viability and the phenotype of this double mutant. Depletion of PbpH resulted in an arrest in cell growth and a dramatic morphological change in both vegetative cells and outgrowing spores. Vegetative cells lacking *pbpA* and *pbpH* expression swelled and cell elongation was arrested, leading to the formation of pleiomorphic spherical cells and eventual lysis. In these cells, cell septations were randomly localized, cell walls and septa

were thicker than those seen in wild type cells, and the average cell width and volume were larger than those of cells expressing *pbpA* or *pbpH*. The vegetative PG had an increased abundance of one unidentified muropeptide. Spores produced by the *pbpA pbpH* double mutant were able to initiate germination but the transition of the oval-shaped spores to rod-shape cells was blocked. The outgrowing cells were spherical, gradually enlarged, and eventually lysed. Outgrowth of these spores in the presence of xylose led to the formation of helical cells. Thus, PbpH is apparently required for maintenance of cell shape, specifically for cell elongation. PbpH and PBP2a play a redundant role homologous to that of PBP2 in *E. coli*.

CHAPTER THREE

CHARACTERIZATION OF PBP-CODING GENE *pbpI* (*yrrR*)

Extensive sequence similarity indicates that *yrrR* encodes a class B PBP. This gene has been renamed and will now be referred to as *pbpI*. The first goal was the identification of the gene product of *pbpI* by comparing the PBP profile of a PbpI over-expressing strain to that of the wild type strain using radio-labeled penicillin. The second goal was examination of the expression of *pbpI*. The final goal was the identification of the function of *pbpI*.

Materials and Methods

1. Plasmids, bacterial strains, and growth conditions.

All plasmids and strains used in this study are listed in Table 5. *E. coli* strain JM109 was used for cloning. All *B. subtilis* strains used were derived from strain 168. Transformation with either plasmid DNA or chromosomal DNA was performed as described previously (2). Transformants were selected on 2xSG (32) plates containing appropriate antibiotics: chloramphenicol (3 μg/ml), spectinomycin (100 μg/ml), kanamycin (10 μg/ml), tetracycline (10 μg/ml), and erythromycin (0.5 μg/ml) plus lincomycin (12.5 μg/ml; macrolide-lincosamide-streptogramin B resistance). Vegetative growth and sporulation was routinely carried out in 2xSG liquid medium at 37°C. Spores were purified by water washing (43). Spore germination and outgrowth was analyzed in 2xYT medium (48) containing 4 mM L-ala after a 30 min heat shock in water at 65°C (43). The optical densities at 600nm (OD) of all cultures were determined using a

Genesys 5 spectrophotometer. Spore heat- and chloroform-resistance assays were carried out after sporulation for 24 hours as previously described (43).

For induction of the xylose-regulated promoter, the strains were grown at 37°C in 2xSG lacking glucose (16 g nutrient broth, 2 ml 1M $MgSO_4$, 10.7 ml 2M KCl, 100 µl 1M $MnCl_2$, 2.72 µl 0.36M $FeSO_4$, and 20 ml 1.18% $Ca(NO_3)_2$ per liter) with a final xylose concentration of 2%.

2. Construction of plasmids and strains.

Primers pbpI1 and pbpI2 (Table 6) were used to amplify a 2451 bp fragment containing 345 bp of upstream sequence, the coding sequence, and 355 bp of downstream sequence. This fragment was cloned into pGEMT, generating plasmid pDPV107. The insert of this plasmid was sequenced using SP6 and T7 promoter primers and compared to the genome sequence of *B. subtilus*. The cloned *pbpI* gene was 100% identical to that in the genome sequence. To construct a deletion mutation in *pbpI*, pDPV107 was digested with *Eco*RV and *Hin*dIII to remove 89.2% of the coding sequence, including the conserved penicillin-binding active site sequences. The deleted region was replaced with an erythromycin resistance gene cassette obtained from pDG646 (21) by digestion with *Hin*dIII and *Sma*I. In this way we generated plasmid pDPV114 (Δ*pbpI*::*erm*). The plasmid was linearized by restriction digestion at a *Sca*I site within the pGEMT vector sequence. The linearized DNA was transformed into our wild type *B. subtilis* strain, PS832, to allow the mutated gene to integrate into the chromosome via double crossover with selection for the erythromycin resistant marker. Thus, the *pbpI* deletion mutant strain (DPVB160) was obtained.

Primer pbpIa (Table 6) was designed to contain an added *Eco*RI site on pbpI1 to assist in cloning a fragment for constructing a *lacZ*-fusion. Primers pbpIa and pbpI2 were used to PCR amplify the 2451 bp insert in pDPV107. The 2459 bp PCR product was cut with *Eco*RI, *Hin*dIII and *Dra*I to get a 975 bp frament which contains the upstream region and the first 622 bp of the *pbpI* coding sequence. The fragment was cloned into *Eco*RI- and *Hin*dIII-digested pDPC87 to generate pDPV126, in which the 975 bp fragment was placed in front of a promoterless *lacZ* gene. The insert of the plasmid was sequenced and confirmed to be identical to the sequence from the genome. The supercoiled plasmid was used in transforming *B. subtilis* PS832 to allow the *lacZ* fusion to recombine into the chromosomal copy of *pbpI* via a single crossover. Transformants were selected on 2xSG plates containing chloramphenical. In this way we generated strain DPVB169, containing a transcriptional fusion of *lacZ to the pbpI* promoter.

The *pbpI-lacZ* fusion was transformed into a set of strains carrying null mutations in genes encoding sporulation-specific sigma factors: SC1159 (*spoIIAC1*=σF^-), SC137 (*spoIIGB*::Tn917=σE^-::MLS^R) and SC500 (*spoIIIG*Δ*1*=σG^-) using chromosomal DNA of DPVB169. Thus, DPVB184 (*spoIIAC1 pbpI-lacZ*), DPVB185 (*spoIIGB*::Tn917 *pbpI-lacZ*), DPVB186 (*spoIIIG*Δ*1 pbpI-lacZ*) were generated. These sigma factor mutants' isogenic wild type background is strain PY79. As a control, DPVB183 was generated by transforming PY79 using the chromosomal DNA of DPVB169.

Primers pbpI5 and pbpI3 (Table 6) were designed with added *Pac*I site and *Bgl*II sites, respectively. They were used to PCR amplify a 1802 bp fragment containing 25 bp of upstream sequence, the *pbpI* coding sequence, and 26 bp of downsteam sequence. The resulting 1820 bp *Pac*I-*Bgl*II fragment was cloned into *Pac*I- and *Bgl*II-digested

pSWEET-*bgaB* (7) which contains a xylose-regulated expression system. The *pbpI* gene in the resulting plasmid, pDPV146, was sequenced and found to be identical to the sequence from the genome. The plasmid was linearlized at the *Pst*I site in the vector and used to transform PS832 to generate a *xylAp-pbpI* fusion at the *amyE* locus. The resulting AmyE$^-$ and Cm^R strain, DPVB210 (*amyE*::*xylAp-pbpI*), was obtained. As a control, DPVB213 was generated by transforming PS832 using plasmid pSWEET-*bgaB*.

3. Enzyme assays, membrane preparation, and gel identification of PBPs

β-galactosidase assays of vegetative cells, sporulating cells, and germinating spores were done by using the substrate *o*-nitrophenyl-β-D-galactopyranoside (43, 52) and the activity was expressed in Miller units. Glucose dehydrogenase activity was assayed as previously described (43).

For small-scale membrane preparation, strains were grown in 2xSG medium at 37°C to an OD of 0.1. Then xylose was added to the culture to a final concentration of 2%, and incubation was continued until the OD reached 1.0. Membranes were prepared as previously described (49). PBPs were detected using ^{125}I-labeled penicillin X as previously described (36).

4. Immature forespore and spore peptidoglycan structure analysis.

Hexosamines were assayed as described (37). The preparation and analysis of immature forespore PG were performed as described in detail previously (37). Spore PG structure were determined as described (47). The analysis of the chromotagrams was done using Powerchrom V2.2.2 software on a Machintosh OS 9.1 computer.

5. Other phenotypic and biochemical assays.

Dipicolinic acid (DPA) accumulation was assayed as previously described (43). An assay of spore heat resistance was carried out as described previously (49).

Table 5. *Bacillus subtilis* strains and plasmid used

Strains	Genotype[b]	Construction	Source or reference
DPVB45	Δ*pbpG*::Kn		(36)
DPVB56	Δ*pbpG*::Kn Δ*pbpF*::Erm^r		(36)
DPVB64	*spoVD*::Kn		Laboratory stock
DPVB160	Δ*pbpI*::Erm^r	pDPV114→PS832	This work
DPVB169	*pbpI*-lacZ	pDPV126→PS832	This work
DPVB176	Δ*pbpI*::Erm^r *spoVD*::Kn	DPVB160→DPVB64	This work
DPVB183	*pbpI*-lacZ	DPVB169→PY79	This work
DPVB184	*spoIIAC*1 *pbpI-lacZ*	DPVB169→SC1159	This work
DPVB185	*spoIIGB*::Tn917Ωnv325 *pbpI-lacZ*	DPVB169→SC137	This work
DPVB186	*spoIIIG*Δ1 *pbpI-lacZ*	DPVB169→SC500	This work
DPVB198	Δ*pbpI*::Erm^r Δ*pbpG*::Kn	DPVB45→DPVB160	This work
DPVB199	Δ*pbpI*::Erm^r pbpF::Cm	PS1838→DPVB160	This work
DPVB200	Δ*pbpI*::Erm^r Δ*pbpG*::Kn *pbpF*::Cm	PS1838→DPVB199	This work
DPVB210	*xylAP-pbpI* at *amyE*	pDPV146→PS832	This work
DPVB213	*xylAP-bgaB* at *amyE*	pSWEET-bagB→PS832	This work
PS832	Wild type, trp$^+$ revertant of 168		Laboratory stock
PS1838	*pbpF*::Cm		(52)
PY79[a]	wild type		(45, 71)
SC137[a]	*spoIIGB*::Tn917Ωnv325		S. Cutting (45)
SC500[a]	*spoIIIG*Δ1		S. Cutting (45)
SC1159[a]	*spoIIAC*1		S. Cutting (45)

Plasmid	Construction or description of uses	Source or reference
pDG646	Carrying Erm^r cassette	(21)
pDPC87	B. subtilis integrating *lacZ* trascriptional fusion vector	(52)
pDPV107	Upstream, downstream, and coding region of *pbpI* in pGEM-T	This work

pDPV114	Deletion of *pbpI* and the replacement of Erm^r cassette, pDPV107 digested with *Sma*I and *Hind*III, ligated with a 1603 *EcoR*V-*Hind*III bp Erm^r cassette from pDG646	This work
pDPV126	*PpbpI-lacZ*, pDPC87 carrying 975 bp *EcoR*I-*Hind*III fragment of *pbpI* from pDPV107	This work
pDPV146	*xylAP- pbpI*, pSWEET carrying 1819 bp *Pac*I-*Bam*HI PCR fragment of *pbpH*	This work
pJF751	B. subtilid integrating lacZ fusion vector	(18) (45)
pSWEET-bgaB	*xylAP- bgaB*, pSWEET carrying *bgaB*	(7)

[a] Genetic background is PY79. The other strains' genetic background is PS832

[b] Abbreviations for antibiotic resistance: Cm, chloramphenicol; Erm^r, lincomycin and erythromycin; Kn, kanamycin; Sp, spectinomycin..

Table 6. Primer sequences

Name	Primer sequence	Restriction enzyme site added	Uses	Position in chromosome
pbpI1	5' AGA GGG CCG CGT GAC GAC TCT TG		For constructing pDPV103	-345 upstream of *pbpI*
pbpI2	5' ATC AGA GTC AGA AGA CTT CTC AG		For constructing pDPV103	355 downstream of *pbpI*
pbpIa	5' CGG AAT TC*A GAG GGC CGC GTG ACG ACT CTT G	EcoRI	LacZ fusion	-345 upstream of *pbpI*
pbpI5	5' GCG CTT AAT TAA* CAC AAT GTG GGT GAG GTG TTT	PacI	PSWEET-yrrR	-25 upstream of *pbpI*
pbpI3	5' CGG GAT CC*T TAA CAT GTG CTG AGA AGT TG	BamHI	PSWEET-yrrR	26 downstream of *pbpI*
* The added restriction enzyme site.				

Results

Identification of the *pbpI* gene

A sequence alignment of the predicted product of *pbpI* against a microbial protein database was done using the BLAST software (1). The most similar proteins in *B. subtilis* are the high-molecular-weight class B PBPs, among which SpoVD is the most similar one (27% identical and 42% similar). In *E. coli* the most similar protein is PBP3 (22% identical and 38% similar). SpoVD is transcribed from a sigmaE-dependent promoter in the mother cell of a sporulating sporangium and is required for synthesis of the modified peptidoglycan of the spore cortex (14). PBP3 of *E. coli*, the gene product of *pbpB* (*ftsI*), is a class B PBP and is essential for synthesis of septal peptidoglycan during cell division (59). These data suggest that the product of *pbpI* belongs to the group of genes required for synthesis of the spore or septum PG.

Construction of an inducible *xylAp-pbpI* and identification of the product of *pbpI*

Plasmid pSWEET contains a xylose-dependent expression system that can integrate into the chromosome of *B. subtilis* at the *amyE* locus. In order to clone the whole coding sequence of *pbpI* into the plasmid, two primers were designed and the gene was PCR-amplified and ligated into the digested vector. Thus we constructed the plasmid pDPV146 (pSWEET-*pbpI*) in which *pbpI* is under the control of the xylose-regulated promoter. Then pDPV146 was linearized and used to transform strain PS832 to produce strain DPVB210 (*amyE::xylAp-pbpI*). PbpI was over-expressed upon the addition of xylose. Plasmid pSWEET-*bgaB* was linearized and used to transform PS832 to produce strain DPVB213 (*xylAp-bgaB*) as a control. 2% xylose was added to cultures at an OD of 0.1 and membranes were prepared following growth to an OD of 1.0. Radioactively-

labeled penicillin was used to visualize the PBPs present in xylose-induced DPVB210, DPVB213, and our wild type PS832. In DPVB210 (over-expressing *pbpI*) we identified a new PBP with an apparent mass of 65 kDa which matches the predicted molecular mass of the *pbpI* product (65.3 kDa) (Fig. 11). This PBP appeared to be the product of *pbpI* and we will refer to it as PBP4b since it runs just underneath PBP4. This follows the convention of naming PBPs based upon their position on an SDS-PAGE gel.

Expression of *pbpI*

A PCR amplified fragment containing 622 bp of the N-terminal coding region and 345 bp upstream of *pbpI* was used to generate a transcriptional *lacZ* fusion. This construct was transformed into *B. subtilis* PS832 to allow the *lacZ* fusion to recombine into the *pbpI* locus in the chromosome via a single crossover, producing strain DPVB169. To determine at which stage of the *B. subtilis* life cycle that *pbpI* was expressed, the activity of β-galactosidase in vegetative cells, sporulating cells, and outgrowing spores was assayed (Fig. 12).

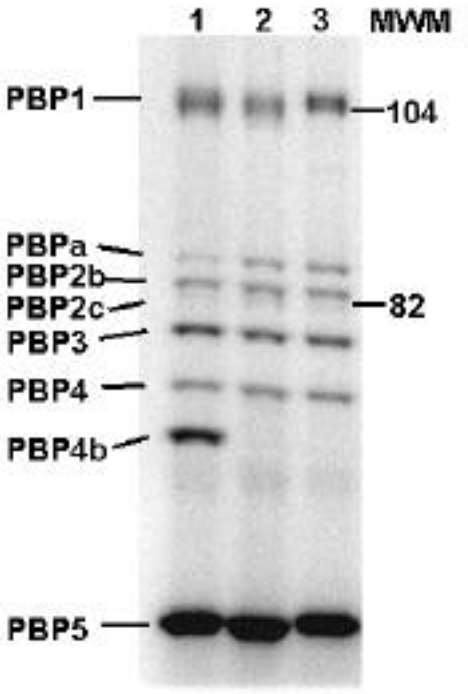

Fig. 11. Identification of PbpI. Membranes were purified from cultures at an OD of 1.0 and were incubated with ^{125}I-labeled penicillin X. Proteins were separated on an SDS-7.5% PAGE gel, and PBPs were detected using a phosphorimager. Lane 1, DPVB210 (over-expressed *pbpI*); lane 2, DPVB213 (over-expressed BgaB); lane 3, PS832 (wild-type). Calibrated molecular mass standards (MWM; in kilodaltons) were Bio-Rad low-range-prestained SDS-PAGE standards.

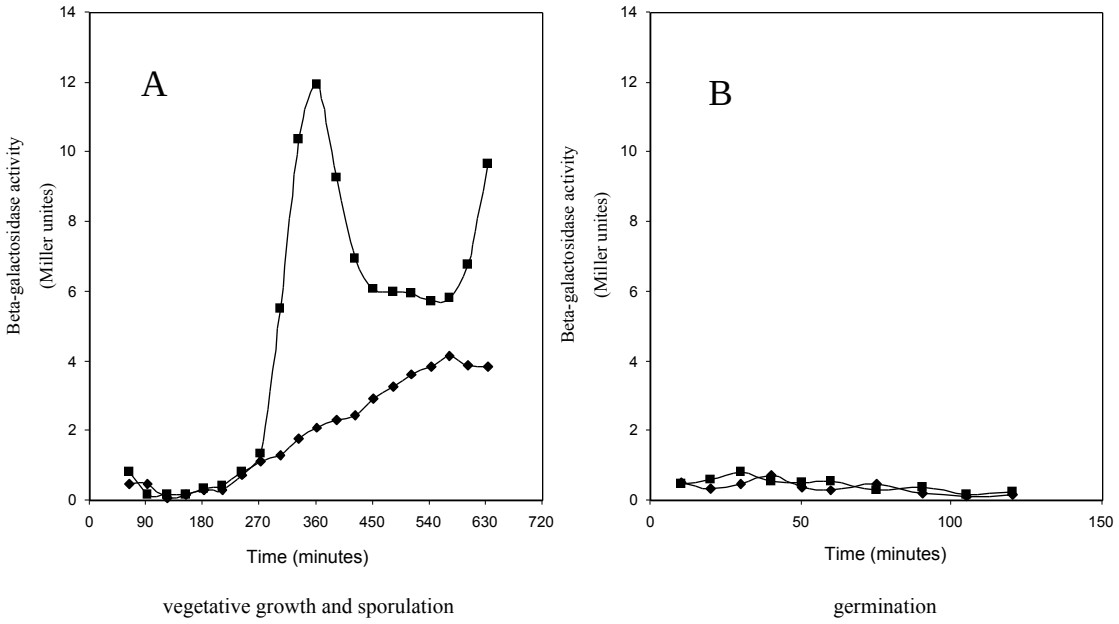

Fig. 12. Expression of a *pbpI-lacZ* fusion. (A) Expression during vegetative growth and sporulation in 2xSG medium at 37°C. The beginning of the sporulation was at 210 min. (B) Expression during spore germination and outgrowth in 2xYT medium with 4 mM L-Ala at 37°C. Symbols:◆, wild type (strain PS832);■, *pbpI-lacZ* (strain DPVB169).

Analysis of β-galactosidase expression demonstrated that the transcription of *pbpI* was very low compared to that of several other PBP-encoding genes (50, 52, 53). Expression of *pbpI* was not observed during vegetative growth and germination, but only during sporulation. After sporulation for about 2 hours, its expression reached the highest level. Sporulation is regulated temporally and spatially by four sigma factors. Sigma factor F is the first one working specifically in sporulation, followed by the sequential activation of sigma factors E, G, and K. Based on its expression time, we predicted that *pbpI* was expressed under the control of sigma factor E or F. Examination of the sequence of the region upstream of *pbpI* suggested that a sigma F recognition site was present (Fig. 13).

Sigma factor dependence of the expression of *pbpI*

To understand which sigma factor controls the expression, the *pbpI-lacZ* fusion was introduced into strains that contained null mutations affecting sigma factors E, F, or G (Fig. 14). In *spoIIGB* (encoding sigma factor E) and *spoIIIG* (encoding sigma factor G) strains, there was no significant difference in the timing and the level of the expression of *pbpI* comparing to that in the wild type strain. In contrast, the mutation in *spoIIAC* (encoding sigma factor F) completely abolished the expression. These results indicate that the expression of *pbpI* is mainly dependent on sigma factor F and the putative sigma F recognition site is probably the promoter of *pbpI*. To confirm this primer extension analysis will be done in the future.

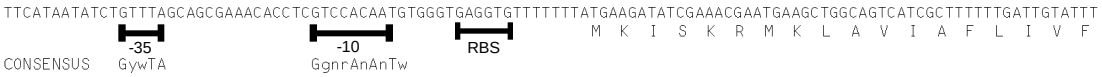

Fig. 13. Nucleotide sequence of the upstream region and the beginning of *pbpI*. Amino acids are placed below the center nucleotides according to their coding sequences. Brackets indicate the predicted "-35" and "-10" sigma F recognition sites and ribosome binding site. The consensus sigma F recognition sequences are from (23).

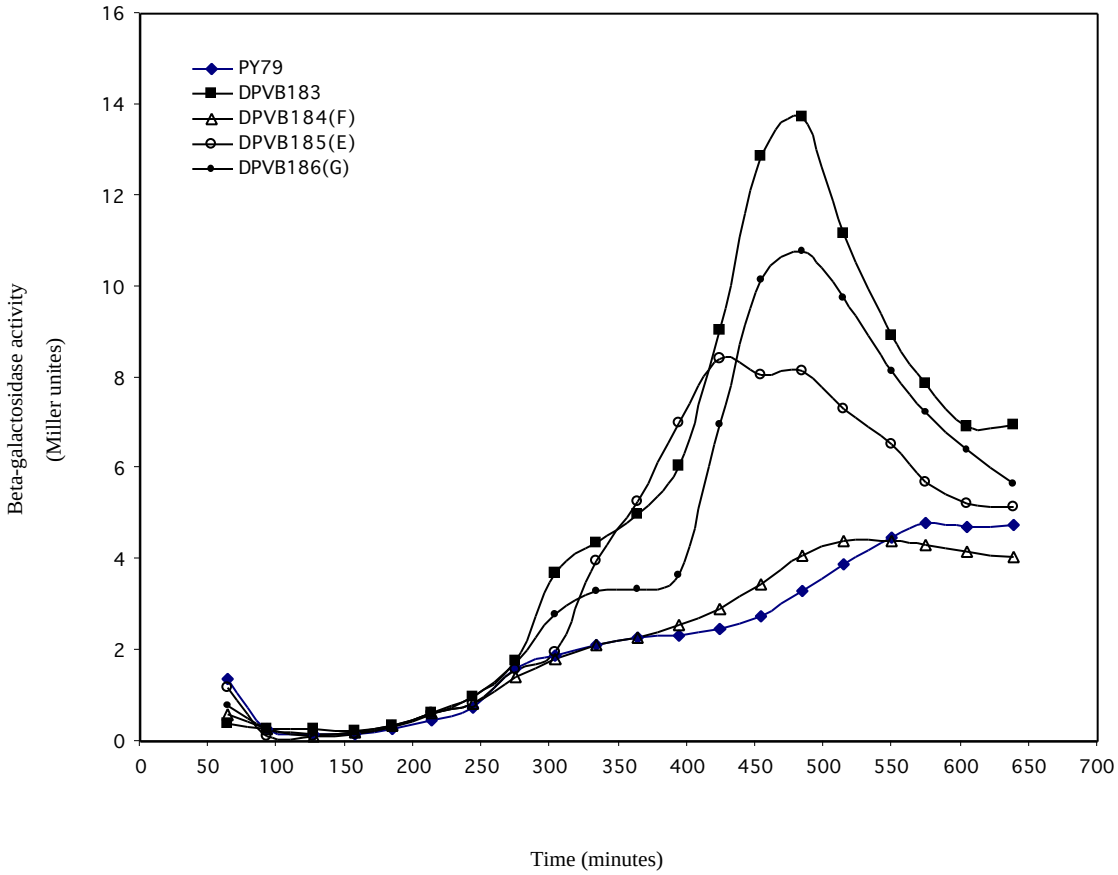

Fig. 14. Sigma factor dependence of *pbpI* expression. The expression of *pbpI-lacZ* in wild type (DPVB183), a sigma F mutant (DPVB184), a sigma E mutant (DPVB185), and a sigma G mutant (DPVB186) was determined. Strain PY79 contained no *lacZ* fusion and revealed the background activity produced by *B. subtilis*. Sporulation initiated at approximately 200 minutes.

Phenotypic properties of *pbpI* single mutant and multiple mutants

To identify the function of *pbpI,* we constructed a mutant strain containing a *pbpI* deletion (DPVB160). PBPs of the same class within a cell frequently exhibit functional redundancy, so we constructed a double mutant strain containing the *pbpI* mutation along with a null mutation in *spoVD*, the only other class B-encoding gene specifically expressed during sporulation (14). *pbpF* and *pbpG* are two genes encoding class A PBPs that are expressed specifically within the forespore. A double mutant lacking *pbpF* and *pbpG* produces defective spore PG. Since *pbpI* is also expressed within the forespore, we were interested to see if a combination of *pbpI* and *pbpF* or *pbpG* mutations would affect spore PG synthesis. Multiple mutants of *pbpI* and these two genes were also studied. 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.

Phenotypic studies of a *pbpI* single mutant, a *pbpI pbpG* double mutant and a *pbpI pbpF* double mutant. There were no significant changes in growth rate of the mutant strains (Table 7) (a difference of 1 or 2 minutes is within the experimental error).

Table 7. Growth and sporulation of *pbpI* mutant strains

Strains	Genotype[a]	Generation Time (min)	Cell counts (x10^9 cfu/ml)		
			Viable	Chloroform-resistant spores	Heat-resistant spores
PS832	Wild type	19.5	2	2	3
PS1838	Δ*pbpF*::Cm	18.75	2	2	3
DPVB45	Δ*pbpG*::Kn	19.5	2	2	2
DPVB160	Δ*pbpI*::erm^r	19.75	2	2	2
DPVB198	Δ*pbpG*::Kn Δ*pbpI*:: erm^r	19.5	2	2	2
DPVB199	Δ*pbpF*::Cm Δ*pbpI*:: erm^r	19	2	2	3

[a] Abbreviations for antibiotic resistance: Cm, chloramphenicol; Erm^r, lincomycin and erythromycin; Kn, kanamycin.

Cell morphology was studied by examining the vegetative cells and the germinating spores under the phase-contrast microscope. No distinguishable difference was found between the mutants and the wild type (data not shown). Sporulation efficiency was assayed by determining the numbers of chloroform- and heat-resistant spores per ml of the culture after sporulation for 24 hours. The results showed that all three mutants sporulated as efficiently as the wild type (Table 7). To determine spore heat resistance more precisely, the purified spores were heated in water at 80°C for various times and the cfu/ml were determined. The cfu/ml was divided by the cfu/ml of the non-heated sample and this value was graphed against time (Fig. 15). D values (the time required to reduce viability 10-fold) were determined from the curves: 24 min, 14 min, 14 min, 26 min, 33 min, and 20 min for PS832, DPVB160, DPVB198, DPVB199, DPVB45 and PS1838, respectively. There was no significant difference among the death rates of these strains.

Spore germination and outgrowth was examined by first heat-activating the spores for 30 minutes at 65°C, and then germinating them in 2xYT medium containing L-Ala while measuring the OD changes over time. The curves showed the kinetics of germination and outgrowth (Fig. 16). All mutants' spores were able to initiate spore germination as demonstrated by the decreases in OD of spore suspensions. The germination kinetics of the mutants were very close to those of the wild type. The outgrowth kinetics were indistinguishable from that of the wild type.

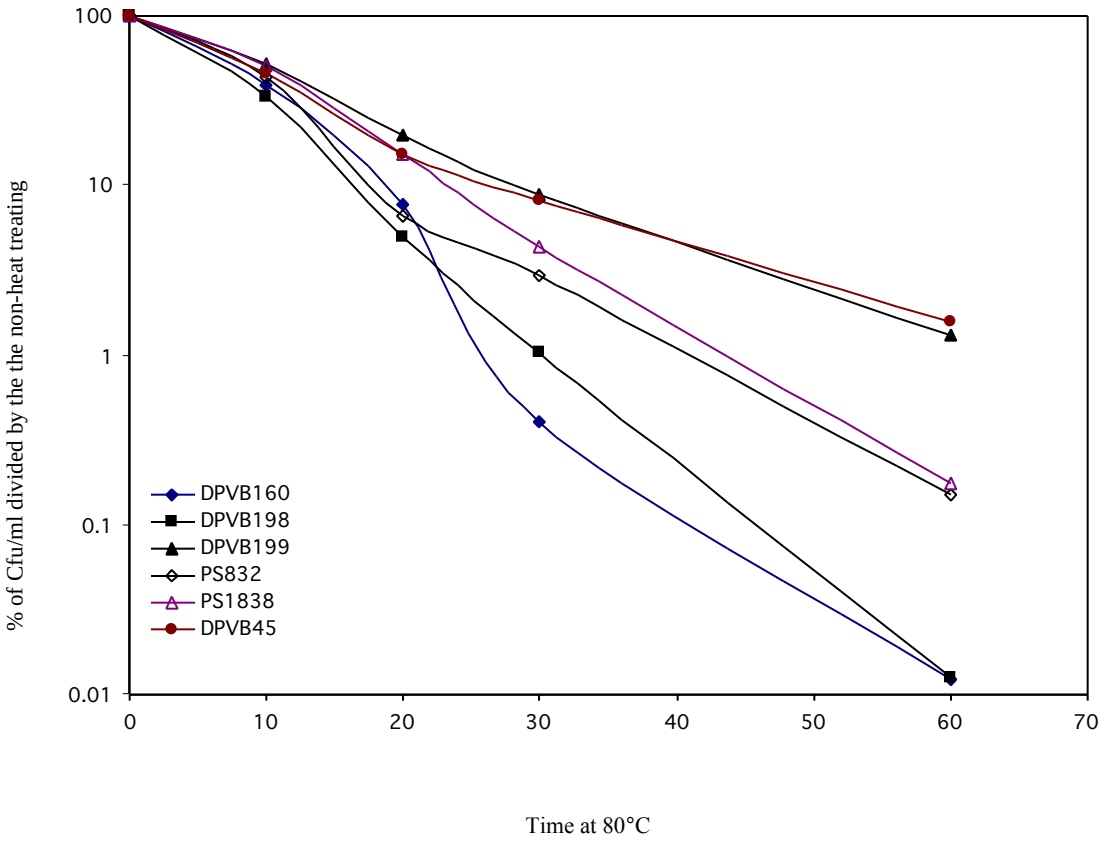

Fig. 15. Heat-killing of wild type and mutant spores. The assay was done by heating the spore suspensions of *pbpI* (DPVB160), *pbpI pbpG* (DPVB198), *pbpI pbpF* (DPVB199) strains and control strains, which were wild-type (PS832), *pbpF* (PS1838) and *pbpG* (DPVB45), at 80°C for various time, plating the heated suspensions on LB plate and calculating the cfu/ml.

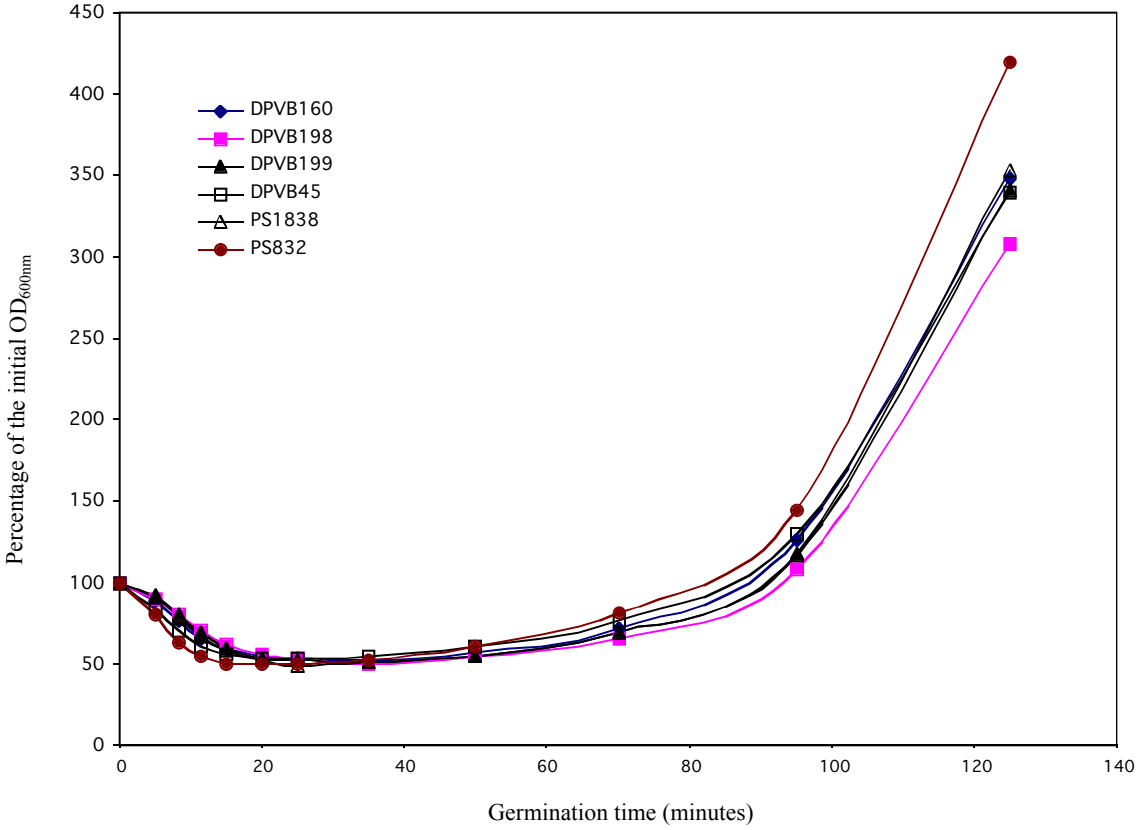

Fig. 16. The germination and outgrowth kinetics of wild type and mutant spores. The spores were heat activated and germinated in 2xYT medium with 4 mM L-Ala. The germination and outgrowth of spore suspensions of mutant strains *pbpI* (DPVB160), *pbpI pbpG* (DPVB198), *pbpI pbpF* (DPVB199) had no difference to those of control strains, wild-type (PS832), *pbpF* (PS1838) and *pbpG* (DPVB45).

To determine if there were any changes in the spore cortex structure of our mutant strains, PG was purified from each of the spore preparations. Muropeptides obtained from the PG were analyzed by reverse-phase HPLC, and the derived structural parameters of the PG are shown in Table 8. The overall structure of the spore PG of the *pbpI*, *pbpI pbpF*, and *pbpI pbpG* mutant strains was almost identical to that of the wild type.

Phenotypic studies of a *pbpI spoVD* double mutant and a *pbpI pbpF pbpG* triple mutant. To help understand the physiological significance of *pbpI* during the sporulation, forespore PG synthesis during the sporulation was analyzed 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 the control wild type (PS832), *spoVD* (DPVB64), and *pbpF pbpG* (DPVB56) strains, respectively. Sporulation biochemical markers were analyzed in the cultures of each strain and its control in order to match their progression through the sporulation process. The DPA contents at each time point were compared between the pairs and they were at similar levels for strains in a pair (data not shown). The amount of spore PG produced during the 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, the *pbpI* strain produced spore PG with structural parameters similar to those found in the wild type (Table 9). The *pbpI spoVD* strain produced spore PG with structural parameters similar to those found in the *spoVD* strain. The *pbpI pbpF pbpG* triple mutant produced spore PG with similar parameters to those found in *pbpF pbpG* strain. These results indicate that PBP4b plays a redundant role.

Table 8. Structural parameters for the spore cortex PG of various strains

Strain	Genotype [a]	% Muramic acid with				
		Side chains of				Cross-link
		lactam	alanine	tripeptide	tetrapeptide	
PS832	Wild type	49.4	20.4	1.4	28.9	3.1
PS1838	Δ*pbpF*::Cm	49.8	19.4	0.8	29.9	3.2
DPVB45	Δ*pbpG*::Kn	49.1	20.3	1.7	28.9	3.3
DPVB160	Δ*pbpI*::erm^r	49.3	16.7	1.3	32.7	3.6
DPVB198	Δ*pbpG*::Kn Δ*pbpI*:: erm^r	48.6	18.1	1.3	32.0	3.4
DPVB199	Δ*pbpF*::Cm Δ*pbpI*:: erm^r	49.5	18.1	1.0	31.4	3.3

[a] Abbreviations for antibiotic resistance: Cm, chloramphenicol; Erm^r, lincomycin and erythromycin; Kn, kanamycin.

Table 9. Structural parameters for the forespore PG of various strains

Strain	Time in sporulation (hours)	% Muramic acid with Side chains of				Cross-link	% tripeptide cross-linked
		Lactam	alanine	tripeptide	tetrapeptide		
PS832 (wild type)	4.75	11.9	44.1	38.6	5.5	1.4	2.5
	5	15.3	48.2	31.1	5.4	1.2	2.0
	5.25	19.6	45.4	29.6	5.4	2.0	4.4
	5.5	26.4	44.4	23.7	5.5	2.3	5.8
	5.75	32.3	45.2	17.4	5.1	1.9	5.1
	6	39.3	44.5	10.5	5.8	2.1	10.7
	6.5	43.2	32.3	7.1	17.4	2.2	9.8
	7	44.3	30.2	5.1	20.5	2.5	12.7
	7.5	44.9	27.4	4.4	23.3	2.8	17.1
	8	45.4	27.5	4.0	23.1	2.8	20.2
	24	47.7	19.1	2.7	30.5	3.7	40.0
DPVB160 (*pbpI*)	4.75	9.2	43.2	44.1	3.6	1.2	2.0
	5	15.2	44.0	36.3	4.5	1.9	3.7
	5.25	18.6	44.8	31.5	5.1	2.6	6.3
	5.5	25.6	43.2	24.7	6.5	3.6	11.3
	5.75	29.6	42.7	20.2	7.5	3.9	15.2
	6	38.4	40.6	12.2	8.8	4.3	20.9
	6.5	42.5	32.0	7.3	18.2	2.2	7.3
	7	43.8	30.6	5.4	20.2	2.5	11.7
	7.5	44.5	28.3	4.7	22.5	2.8	18.2
	8	44.7	29.7	4.4	21.1	2.7	19.7
	24	47.2	21.1	2.8	28.9	3.9	41.5
DPVB176 (*pbpI spoVD*)	4.75	0.0	17.4	59.4	23.2	0.8	0.7
	5	0.2	18.0	55.8	26.0	1.2	0.8
	5.25	0.0	18.8	55.5	25.7	1.1	0.7
	5.5	0.9	17.1	52.0	30.0	1.6	1.2
	5.75	1.3	17.8	51.6	29.4	1.8	1.5
	6	0.8	17.6	53.2	28.3	1.7	1.4
	6.5	1.8	16.0	51.6	30.6	1.9	1.7
	7	3.3	24.3	46.7	25.7	1.6	0.9
	7.5	7.0	20.4	56.7	15.9	2.6	2.5
	8	9.1	24.0	43.3	23.6	2.2	2.8
DPVB64 (*spoVD*)	4.75	1.0	6.4	65.0	27.6	1.4	1.3
	5	0.6	6.4	68.7	24.3	1.2	1.1
	5.25	1.0	7.5	69.2	22.2	1.5	1.4
	5.5	0.6	17.8	55.2	26.4	1.6	1.5
	5.75	1.5	18.2	58.9	21.5	1.7	1.7
	6	1.4	15.2	52.2	31.1	1.9	1.7
	6.5	3.6	18.8	55.5	22.1	2.1	2.0
	7	6.4	20.7	55.3	17.6	2.2	2.3
	7.5	7.3	19.7	53.2	19.8	2.4	2.5
	8	8.6	20.8	50.1	20.5	2.2	2.7
DPVB200 (*pbpI pbpF pbpG*)	4.75	0.2	1.5	67.8	30.5	5.0	7.2
	5	0.1	1.4	67.6	31.0	4.6	6.5
	5.25	0.7	2.0	63.7	33.6	8.2	11.8
	5.5	1.1	1.7	61.6	35.6	8.3	12.2
	5.75	2.4	2.1	53.0	42.4	10.4	16.6
	6	4.1	3.2	44.6	48.1	8.3	14.7
	6.5	7.0	4.1	24.6	64.4	9.7	24.7
	7	8.3	3.0	16.4	72.3	10.3	34.5
	7.5	10.7	4.1	13.6	71.5	11.1	40.1
	8	12.9	4.6	11.1	71.4	11.5	44.9
DPVB56 (*pbpF pbpG*)	4.75	1.7	3.8	66.6	27.9	7.0	8.4
	5	1.7	4.1	64.4	29.7	8.1	8.9
	5.75	4.3	4.0	50.2	41.5	7.0	11.5
	6	7.7	5.0	32.3	55.1	8.9	19.5
	6.5	9.0	4.8	19.7	66.6	10.2	32.3
	7	10.0	4.5	14.7	70.8	10.5	40.8
	7.5	12.2	5.1	12.8	70.0	10.7	44.2
	8	14.4	5.4	12.1	68.1	11.0	45.8

Discussion

The identification and characterization of *pbpI* was carried out through the study of the putative coding sequence of the gene and the construction and analysis of a series of *B. subtilis* mutant strains and strains with altered gene expression.

Studies of a transcriptional *pbpI-lacZ* fusion demonstrated that *pbpI* is expressed under the control of sigma factor F. Thus, *pbpI* is expressed in the forespore during sporulation. The difficulty in assessing the function of *pbpI* is due to the fact that the PBPs within a species often possess at least partially redundant enzyme activities (46, 54, 72). Since *pbpI* is controlled by sigma F and expressed in sporulation, we constructed multiple mutant strains containing a mutation in *pbpI* and other sporulation-specific PBP-encoding genes and studied their phenotypes. No reproducible differences could be found between the spore PG produced by *pbpF pbpG* and *pbpF pbpG pbpI* strains. The *pbpF* and *pbpG* products are class A PBPs that are expressed only in the forespore (45, 52). In a *pbpF pbpG* strain the germ cell wall is apparently abnormal, though this is not obvious from analyses of immature forespore PG, and this results in abnormal synthesis of the cortex PG (36). If we accept a model suggesting that class B PBPs, such as PBP4b, function in complex with the class A PBPs and simply cross-link the glycan strands produced by those enzymes, then it would be no surprise that in their absence PBP4b would have no effect. If PBP4b cannot bind to a class A PBP and arrive at the site of polymerization of nascent PG strands then it cannot effect their cross-linking.

No changes were detected in the cross-linking of the spore PG produced by a *pbpI spoVD* strain relative to that of the *spoVD* mutant. In the absence of SpoVD essentially no spore cortex PG is synthesized ((14) and D.L. Popham, unpublished). The PG that is

recovered from developing forespores of this strain is therefore indicative of the germ cell wall structure. In the absence of the cortex PG, it is simpler to observe changes in the germ cell wall structure. The same level of cross-linking upon loss of *pbpI* in this background suggests that PBP4b is not involved or plays a redundant function in cross-linking the germ cell wall PG that is polymerized by the *pbpF* and/or *pbpG* products. The fact that the germ cell wall PG is still cross-linked suggests that the transpeptidase domains of the *pbpF* or *pbpG* products are carrying out some cross-linking, or that another class B PBP, remaining from expression in the vegetative cell and enclosed in the forespore during the sporulation septation event, is capable of participating in germ cell wall PG cross-linking.

Conclusions

A sequence alignment of the predicted product of *pbpI* against the microbial protein database demonstrated that the most similar protein in *B. subtilis* is SpoVD and in *E. coli* is PBP3. This suggested that PbpI belongs to the group of the genes required for synthesis of the spore or septum PG. PbpI was identified using radio-labeled penicillin and found to run underneath PBP4 on SDS-PAGE. PbpI is therefore renamed PBP4b. Study of a *pbpI-lacZ* fusion showed that *pbpI* was expressed predominantly during early sporulation. A putative sigma F recognition site is present in the region upstream of *pbpI* and studies using mutant strains lacking sporulation-specific sigma factors demonstrated that the expression of *pbpI* is mainly dependent on sigma factor F. A *pbpI* single mutant, a *pbpI pbpG* double mutant, and a *pbpI pbpF* double mutant were indistinguishable from the wild-type. The sporulation defect of a *pbpI pbpF pbpG* triple mutant was indistinguishable from that of a *pbpF pbpG* double mutant. Structure parameters of the forespore PG in a *pbpI spoVD* strain are similar to that of a *spoVD* strain. These results indicate that PBP4b plays an unknown or redundant role.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.
2. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**74-76.
3. **Archibald, A. R., I. C. Hancock, and C. R. Harwood.** 1993. Cell Wall Structure, Synthesis, and Turnover, p. 381-410. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and Other Gram-Positive Bacteria. American Society for Microbiology, Washington, D.C.
4. **Atrih, A., G. Bacher, G. Allmaier, M. P. Williamson, and S. J. Foster.** 1999. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. J. Bacteriol. **181:**3956-3966.
5. **Atrih, A., P. Zollner, G. Allmaier, and S. J. Foster.** 1996. Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. J. Bacteriol. **178:**6173-6183.
6. **Begg, K. J., B. G. Spratt, and W. D. Donachie.** 1986. Interaction between membrane proteins PBP3 and RodA is required for normal cell shape and division in *Escherichia coli*. J Bacteriol. **167:**1004-8.
7. **Bhavsar, A. P., X. Zhao, and E. D. Brown.** 2001. Development and characterization of a xylose-dependent system for expression of cloned genes in *Bacillus subtilis*: conditional complementation of a teichoic acid mutant. Appl Environ Microbiol. **67:**403-10.

8. **Blumberg, P. M., and J. L. Strominger.** 1972. Five penicillin-binding components occur in *Bacillus subtilis* membranes. J. Biol. Chem. **247:**8107-8113.

9. **Blumberg, P. M., and J. L. Strominger.** 1972. Isolation by covalent affinity chromatography of the penicillin-binding components from membranes of Bacillus subtilis. Proc Natl Acad Sci U S A. **69:**3751-5.

10. **Boyle, D. S., M. M. Khattar, S. G. Addinall, J. Lutkenhaus, and W. D. Donachie.** 1997. *ftsW* is an essential cell-division gene in *Escherichia coli*. Mol Microbiol. **24:**1263-73.

11. **Buchanan, C. E., and A. Gustafson.** 1992. Mutagenesis and mapping of the gene for a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J. Bacteriol. **174:**5430-5435.

12. **Buchanan, C. E., and M.-L. Ling.** 1992. Isolation and sequence analysis of *dacB*, which encodes a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. J. Bacteriol. **174:**1717-1725.

13. **Coote, J. G.** 1972. Sporulation in *Bacillus subtlis*. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. J. Gen. Microbiol. **71:**1-15.

14. **Daniel, R. A., S. Drake, C. E. Buchanan, R. Scholle, and J. Errington.** 1994. The *Bacillus subtilis spoVD* gene encodes a mother-cell-specific penicillin-binding protein required for spore morphogenesis. J. Mol. Biol. **235:**209-220.

15. **de Pedro, M. A., W. D. Donachie, J. V. Holtje, and H. Schwarz.** 2001. Constitutive septal murein synthesis in *Escherichia coli* with impaired activity of the

morphogenetic proteins RodA and penicillin-binding protein 2. J Bacteriol. **183:**4115-26.

16. **Errington, J.** 1993. *Bacillus subtilis* sporulation: Regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1-33.
17. **Errington, J., and R. A. Daniel.** 2001. Cell division during growth and sporulation, p. 97-109. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and Its Close Relatives: From Genes to Cells. American Society for Microbiology, Washington, D.C.
18. **Ferrari, E., S. M. Howard, and J. A. Hoch.** 1985. Effect of sporulation mutations on subtilisin expression, assayed using a subtilisin-β-galactosidase gene fusion, p. 180-184. *In* J. A. Hoch and P. Setlow (ed.), Molecular biology of microbial differentiation. American Society for Microbiology, Washington, D.C.
19. **Foster, S. J., and D. L. Popham.** 2001. Structure and synthesis of cell wall, spore cortex, teichoic acids, S-layers, and capsules, p. 21-41. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and Its Close Relatives: From Genes to Cells. American Society for Microbiology, Washington, D.C.
20. **Goffin, C., and J. M. Ghuysen.** 1998. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol. Mol. Biol. Rev. **62:**1079-1093.
21. **Guérout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene. **167:**335-337.
22. **Hamilton, T. E., and P. J. Lawrence.** 1975. The formation of functional penicillin-binding proteins. J Biol Chem. **250:**6578-85.

23. **Helmann, J. D., and C. P. Moran, Jr.** 2001. RNA polymerase and sigma factors, p. 289-312. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and Its Close Relatives: From Genes to Cells. American Society for Microbiology, Washington, D.C.

24. **Henriques, A. O., P. Glaser, P. J. Piggot, and C. P. Moran, Jr.** 1998. Control of cell shape and elongation by the *rodA* gene in *Bacillus subtilis*. Mol Microbiol. **28:**235-47.

25. **Henriques, A. O., H. D. Lencastre, and P. J. Piggot.** 1992. A *Bacillus subtilis* morphogene cluster that includes *spoVE* is homologous to the *mra* region of *Escherichia coli*. Biochimie. **74:**735-748.

26. **Holtje, J. V.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol Mol Biol Rev. **62:**181-203.

27. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. Embo J. **13:**4684-94.

28. **Khattar, M. M., K. J. Begg, and W. D. Donachie.** 1994. Identification of FtsW and characterization of a new *ftsW* division mutant of *Escherichia coli*. J Bacteriol. **176:**7140-7.

29. **Kleppe, G., and J. L. Strominger.** 1979. Studies of the high molecular weight penicillin-binding proteins of *Bacillus subtilis*. J. Biol. Chem. **254:**4856-4862.

30. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell,**

V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, and et al. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature. **390:**249-256.

31. **LeDeaux, J. R., and A. D. Grossman.** 1995. Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. J. Bacteriol. **177:**166-175.

32. **Leighton, T. J., and R. H. Doi.** 1971. The stability of messenger ribonucleic acid during sporulation in *Bacillus subtilis*. J. Biol. Chem. **254:**3189-3195.

33. **Massova, I., and S. Mobashery.** 1998. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. Antimicrob Agents Chemother. **42:**1-17.

34. **Matsuhashi, M., M. Wachi, and F. Ishino.** 1990. Machinery for cell growth and division: penicillin-binding proteins and other proteins. Res Microbiol. **141:**89-103.

35. **Matsuzawa, H., S. Asoh, K. Kunai, K. Muraiso, A. Takasuga, and T. Ohta.** 1989. Nucleotide sequence of the *rodA* gene, responsible for the rod shape of *Escherichia coli*: *rodA* and the *pbpA* gene, encoding penicillin-binding protein 2, constitute the *rodA* operon. J Bacteriol. **171:**558-60.

36. **McPherson, D. C., A. Driks, and D. L. Popham.** 2001. Two class A high-molecular-weight penicillin-binding proteins of *Bacillus subtilis* play redundant roles in sporulation. J Bacteriol. **183:**6046-53.

37. **Meador-Parton, J., and D. L. Popham.** 2000. Structural analysis of *Bacillus subtilis* spore peptidoglycan during sporulation. J. Bacteriol. **182:**4491-4499.

38. **Miller, J.** 1972. Assay of β-galactosidase, p. 352-355, Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

39. **Murray, T., D. L. Popham, C. B. Pearson, A. R. Hand, and P. Setlow.** 1998. Analysis of outgrowth of *Bacillus subtilis* spores lacking penicillin- binding protein 2a. J. Bacteriol. **180:**6493-6502.

40. **Murray, T., D. L. Popham, and P. Setlow.** 1998. *Bacillus subtilis* cells lacking penicillin-binding protein 1 require increased levels of divalent cations for growth. J. Bacteriol. **180:**4555-4563.

41. **Murray, T., D. L. Popham, and P. Setlow.** 1997. Identification and characterization of *pbpA* encoding *Bacillus subtilis* penicillin-binding protein 2A. J. Bacteriol. **179:**3021-3029.

42. **Murray, T., D. L. Popham, and P. Setlow.** 1996. Identification and characterization of *pbpC*, the gene encoding *Bacillus subtilis* penicillin-binding protein 3. J. Bacteriol. **178:**6001-6005.

43. **Nicholson, W. L., and P. Setlow.** 1990. Sporulation, germination, and outgrowth., p. 391-450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley & Sons Ltd., Chichester, England.

44. **Pedersen, L. B., T. Murray, D. L. Popham, and P. Setlow.** 1998. Characterization of *dacC*, which encodes a new low-molecular-weight penicillin-binding protein in *Bacillus subtilis*. J. Bacteriol. **180:**4967-4973.

45. **Pedersen, L. B., K. Ragkousi, T. J. Cammett, E. Melly, A. Sekowska, E. Schopick, T. Murray, and P. Setlow.** 2000. Characterization of *ywhE*, which encodes

a putative high-molecular-weight class A penicillin-binding protein in *Bacillus subtilis*. Gene. **246:**187-196.

46. **Popham, D. L., M. E. Gilmore, and P. Setlow.** 1999. Roles of low-molecular-weight penicillin-binding proteins in *Bacillus subtilis* spore peptidoglycan synthesis and spore properties. J. Bacteriol. **181:**126-132.

47. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. J. Bacteriol. **178:**6451-6458.

48. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc. Natl. Acad. Sci. U S A. **93:**15405-15410.

49. **Popham, D. L., B. Illades-Aguiar, and P. Setlow.** 1995. The *Bacillus subtilis dacB* gene, encoding penicillin-binding protein 5*, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration. J. Bacteriol. **177:**4721-4729.

50. **Popham, D. L., and P. Setlow.** 1995. Cloning, nucleotide sequence, and mutagenesis of the *Bacillus subtilis ponA* operon, which codes for penicillin-binding protein (PBP) 1 and a PBP-related factor. J. Bacteriol. **177:**326-335.

51. **Popham, D. L., and P. Setlow.** 1993. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis pbpE* operon, which codes for penicillin-binding protein 4* and an apparent amino acid racemase. J. Bacteriol. **175:**2917-2925.

52. **Popham, D. L., and P. Setlow.** 1993. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis pbpF* gene, which codes for a putative class A high-molecular-weight penicillin-binding protein. J. Bacteriol. **175:**4870-4876.

53. **Popham, D. L., and P. Setlow.** 1994. Cloning, nucleotide sequence, mutagenesis, and mapping of the *Bacillus subtilis pbpD* gene, which codes for penicillin-binding protein 4. J. Bacteriol. **176:**7197-7205.

54. **Popham, D. L., and P. Setlow.** 1996. Phenotypes of *Bacillus subtilis* mutants lacking multiple class A high-molecular-weight penicillin-binding proteins. J Bacteriol. **178:**2079-2085.

55. **Rodriguez, M. C., and M. A. de Pedro.** 1990. Initiation of growth in *pbpAts* and *rodAts* mutants of *Escherichia coli*. FEMS Microbiol Lett. **60:**235-9.

56. **Sevinc, M. S., B. W. Bainbridge, and M. J. Bazin.** 1990. Genetic transformation and cell morphology of *Bacillus subtilis* grown in Mg+(+)-limited chemostat culture. Microbios. **62:**29-35.

57. **Shohayeb, M., and I. Chopra.** 1987. Mutations affecting penicillin-binding proteins 2a, 2b and 3 in *Bacillus subtilis* alter cell shape and peptidoglycan metabolism. J. Gen. Microbiol. **133:**1733-1742.

58. **Sowell, M. O., and C. E. Buchanan.** 1983. Changes in penicillin-binding proteins during sporulation of *Bacillus subtilis*. J. Bacteriol. **153:**1331-1337.

59. **Spratt, B. G.** 1975. Distinct penicillin-binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA. **72:**2999-3003.

60. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through in vivo recombination. Gene. **142:**79-83.

61. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu Rev Genet. **30:**297-341.

62. **Svarachorn, A., A. Shinmyo, T. Tsuchido, and M. Takano.** 1988. Autolysis of *Bacillus subtilis* induced by monovalent cations. Appl. Microbiol. Biotechnol. **30:**299-304.

63. **Tamaki, S., H. Matsuzawa, and M. Matsuhashi.** 1980. Cluster of *mrdA* and *mrdB* genes responsible for the rod shape and mecillinam sensitivity of *Escherichia coli*. J Bacteriol. **141:**52-7.

64. **Todd, J. A., E. J. Bone, P. J. Piggot, and D. J. Ellar.** 1983. Differential expression of penicillin-binding protein structural genes during *Bacillus subtilis* sporulation. FEMS Microbiol. Lett. **18:**197-202.

65. **Todd, J. A., A. N. Roberts, K. Johnstone, P. J. Piggot, G. Winter, and D. J. Ellar.** 1986. Reduced heat resistance of mutant spores after cloning and mutagenesis of the *Bacillus subtilis* gene encoding penicillin-binding protein 5. J. Bacteriol. **167:**257-264.

66. **Vinella, D., R. D'Ari, A. Jaffe, and P. Bouloc.** 1992. Penicillin binding protein 2 is dispensable in *Escherichia coli* when ppGpp synthesis is induced. Embo J. **11:**1493-501.

67. **Vinella, D., D. Joseleau-Petit, D. Thevenet, P. Bouloc, and R. D'Ari.** 1993. Penicillin-binding protein 2 inactivation in *Escherichia coli* results in cell division inhibition, which is relieved by FtsZ overexpression. J Bacteriol. **175:**6704-10.

68. **Warth, A. D., and J. L. Strominger.** 1972. Structure of the peptidoglycan from spores of *Bacillus subtilis*. Biochemistry. **11:**1389-1396.

69. **Wu, J.-J., R. Schuch, and P. J. Piggot.** 1992. Characterization of a *Bacillus subtilis* operon that includes genes for an RNA polymerase σ factor and for a putative DD-carboxypeptidase. J. Bacteriol. **174:**4885-4892.

70. **Yanouri, A., R. A. Daniel, J. Errington, and C. E. Buchanan.** 1993. Cloning and sequencing of the cell division gene *pbpB*, which encodes penicillin-binding protein 2B in *Bacillus subtilis*. J. Bacteriol. **175:**7604-7616.

71. **Youngman, P., J. B. Perkins, and K. Sandman (ed.).** 1984. New genetic methods, molecular cloning strategies, and gene fusion techniques for *Bacillus subtilis* which take advantage of Tn*917* insertional mutagenesis. Academic Press, NY.

72. **Yousif, S. Y., J. K. Broome-Smith, and B. G. Spratt.** 1985. Lysis of *Escherichia coli* by beta-lactam antibiotics: deletion analysis of the role of penicillin-binding proteins 1A and 1B. J Gen Microbiol. **131:**2839-45.

Curriculum Vita

1. Personal statement
   Name: Yuping Wei
   Citizenship: Peoples Republic of China

2. Education:

Aug. 2000 – Aug. 2002, Biology department of Virginia Polytechnic and State University, Master candidate, in the lab of David L. Popham

Oct. 1996 – July 1999: Master of Biochemistry/Molecular Biology, in the lab of Songgang Wu and Qiaoqin Shi, Biology department of Fujian Teacher's University, Fuzhou, China

Oct. 1992 – July 1996: Bachelor of Biology education, Biology department of Fujian Teacher's University, Fuzhou, China

3. Scholarship:
   In 1993, the second prize scholarship of Fujian Teacher's University
   In 1995, the second prize scholarship of Fujian Teacher's University
   In 1997, the first prize of graduate student scholarship of Fujian Teacher's University

4. Teaching Experience:
   In Sept., 1995, biology in Shouning First Middle School
   In Apr., 1998, microbiology and microbiology lab in Fujian Teacher's University

5. Research

2000-2002, the identification characterization of penicillin-binding protein coding genes, *pbpX*, *ykuA* and *yrrR* in *Bacillus subtilis*. Biology Department, Virginia Polytechnic Institute and State University, USA.

- Constructed mutants containing deletion mutations.
- Studied the phenotypic properties of the mutants.
- Examined the peptidoglycan structure using reversed HPLC.
- Constructed transcriptional *lacZ* fusions of each gene and studied the expression phase.
- To study the functional redundancy of these genes.
- To examine the sigma factors that control the expression and the transcriptional start site.
- To identify the gene products.

1997-1999, the research of improving the productivity of alkline lipase of *Penicillium expansum* S-14 and determine the characters of the mutants. Biology Department, Fujian Teachers University, China.

- Mutated *Penicillium expansum* by UV, Co-γ rays, and Chemical muatagen NTG.
- Rationally selected Strains that improved the alkaline lipase productivity dramatically.
- Succeeded in obtaining high productivity mutants that met the industrial requirement after mutation and selection for 7 generations.
- Purified the lipase.

- Studied the phenotypes of the mutants by comparing to the original organism.
- Studied the esterase isozyme and amylase isozyme of both the mutants and the original organism.
- Determine the contents of ergosteral of the mutants.

1996, the study of the cultivation conditions for the unsaturated fatty acids (UFAs) producing strain of *Fusarium lini* and improving the productivity of the content of the UFAs of the strain by mutation. Biology Department, Fujian Teachers University

- Selected the optimal media for *Fusarium lini* JZ5 .
- Mutated *Fusarium lini* JZ5 by UV and Co-γ rays for 3 generations and regulated the media ingredients for the mutants.
- Obtained a mutant with productivity increased by 33% at optimal media.

6. Presentation and Poster

Microbiology and Immunology seminar: 'The thermophilic life of some bacteria', Nov. 14th, 2001. Virginia Polytechnic Institute and State University.

Conference on Prokaryotic Development, American Society for Microbiology, Poster: "Rod-shape determination by the *Bacillus subtilis* class B penicillin-binding proteins encoded by *pbpA* and *ykuA*." Yuping Wei and David L. Popham*, July 10-14, 2002, Quebec City, Quebec, Canada.

Thesis defense seminar: 'Characterization of two *Bacillus subtilis* penicillin-binding protein-coding genes, *pbpH* (*ykuA*) and *pbpI* (*yrrR*)'. Aug. 1st, 2002. Virginia Polytechnic Institute and State University.

7. Publication

(a) Rod-shape determination by the *Bacillus subtilis* class B penicillin-binding proteins encoded by *pbpA* and *pbpH*. Yuping Wei and David L. Popham, in preparation.

(b) Characterization of the penicillin-binding protein coding gene, *yrrR,* in *Bacillus subtilis*. Yuping Wei, Derrell C. McPherson, and David L. Popham, in preparation.

(b) The mutation and rational screening of alkline lipase producing strain *Penicillium expansum*. Wei Yuping, Shi Qiaoqin and Wu Songgang. Biotechnology (China). Vol(9).4. 45-48.1999.

8. Attending meeting

The New England (International) Spore Conference, Harvard University, April, 2001

9. Technical Skills

Electrophoresis.
FT-IR.
Electron-Microscopy(SEM).
Mutation and selection of industrial fungi.
Microbiological breeding.
Operation of HPLC and FPLC.
UV-visible spectroscopy
Molecular biology technique for bacteria.
Pathogenic microorganism lab research.

10. Computer Skills

Proficiency in Windows95, 98, 2000, NT, Macintosh operating systems.
Fluency in various software packages including Microsoft Office and Painting.

11. Significant Coursework

Microbiological Genetics	Molecular Biology and Technology
Advanced Biochemistry	Microbiological Engineering
Gene Regulation in Prokaryotes	Industrial Microorganism Breeding
Microbiological Physiology	Fermentation Equipment
Pathogenic Microbiology	

12. Research Interests:

Biochemistry and Molecular Biology of microorganism, food microorganism, industrial Microbiology, extremophiles, microbial genetics and gene regulation.