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Characterization of a Polysaccharide Deacetylase Gene Homologue (*pdaB*) on Sporulation of *Bacillus subtilis*

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The predicted amino acid sequence of *Bacillus subtilis ybaN* (renamed *pdaB*) exhibits high similarity to those of several polysaccharide deacetylases. Northern hybridization analysis with sporulation sigma mutants indicated that the pdaB gene is transcribed by EσE RNA polymerase and negatively regulated by SpoIIID. The *pdaB* mutant was deficient in spore formation. Phase- and electron microscopic observation showed morphological changes of spores in late sporulation periods. The *pdaB* spores that had lost their viability were empty. Moreover, GFP driven by the promoter of the *sspE* gene was localized in the forespore compartment for the wild type, but was localized in both the mother cell and forespore compartments for phase-gray/dark forespores of the *pdaB* mutant. This indicates that GFP expressed in the forespores of the mutant leaks into the mother cells. Therefore, PdaB is necessary to maintain spores after the late stage of sporulation.

Key words: *Bacillus subtilis*, *pdaB* gene, polysaccharide deacetylase, sporulation mutation, *ybaN* gene.

When nutrients are abundant, a symmetrically positioned septum partitions the *B. subtilis* vegetative cell and gene expression is mainly controlled by σ^A. However, when nutrients are limited, *B. subtilis* cells begin to produce spores. After the initiation of sporulation, each cell divides into a developing cell (mother cell) and a sporangium (forespore), and then gene expression in the mother cell and forespore is controlled by σ^E and σ^F, respectively. When the forespore has become engulfed by the mother cell, σ^E and σ^F are replaced by σ^K and σ^G. Following this event, spore peptidoglycans (cortex and germ cell wall) are synthesized between the two spore membranes and the spore coat begins to be deposited on the spore surface. As the final event, the forespore becomes mature, and then the mature spore is released through lysis of the mother cell (*1*, *2*). The released spore is resistant to heat, toxic chemicals and lytic enzymes. During sporulation, sigma factors are activated in the order of σ^F, σ^E, σ^G and σ^K (sigma cascade) (*2*). For example, σ^E activation requires the SpoIIR protein, which is a secreted signaling protein and transcribed by Eσ^F RNA polymerase. Thus, σ^E is activated by σ^F, and σ^E cannot be activated if σ^F is inactive. Dipicolinic acid (DPA) begins to be accumulated at the period of cortex synthesis (*3*), and then DPA is present in mature spore core at a high level (*4*, *5*). It has been reported that DPA contributes to spore dormancy (*6*).

On systematic genome functional analysis of *B. subtilis*, we found that a *pdaB*-deficient mutant exhibited a defect in sporulation. Recently, Eichenberger *et al.* also reported that this mutant exhibits a sporulation-negative phenotype (*7*). The PdaB (YbaN) protein shows high sequence similarity to several polysaccharide deacetylases (BlastP in NCBI, Fig. 1). Polysaccharide deacetylases including NodB chitooligosaccharide, chitin deacetylases and peptidoglycan *N*-acetylglucosamine deacetylase have various functions: as bacterial nodulation signals in symbiosis, and in maturation of spores in yeast and modification of peptidoglycans causing resistance to the hydrolytic action of lysozyme (*8–10*). Recently, we reported that disruption of the *Bacillus subtilis pdaA* gene leads to a complete lack of spore germination and that the mutant spores are deficient in muramic-δ-lactam, which is one of the major components of the spore cortex (*11*). *pdaA* is a polysaccharide deacetylase homologue and the *B. subtilis* genome has six paralogs (*pdaA, pdaB* [*ybaN*]*, yheN, yjeA, yxkH* and *ylxY*). Since we were curious to know the functions of these paralogs, the *ybaN* (renamed *pdaB*) gene was further investigated.

In this communication, we describe the expression and characterization of the *pdaB* gene, and the function of the *pdaB* product during sporulation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—The strains of *B. subtilis* and *Escherichia coli* used in this study are listed in Table 1. *E. coli* was grown on Luria-Bertani (LB) agar medium (*16*) at 37°C, and then inoculated into LB medium. *B. subtilis* was grown on nutrient agar medium (8 g/liter Bacto Nutrient Broth, 0.12 g/liter $MgSO_4 \cdot 7H_2O$, 1 g/liter KCl and 15 g/liter agar, pH 7.0–7.2) at 30°C, inoculated into DSM (Schaeffer) medium, which is a nutrient agar medium without agar containing 1 mM $Ca(NO_3)_4$, 10 μM $MnCl_2$ and 1 μM $FeSO_4$ (*17*), and

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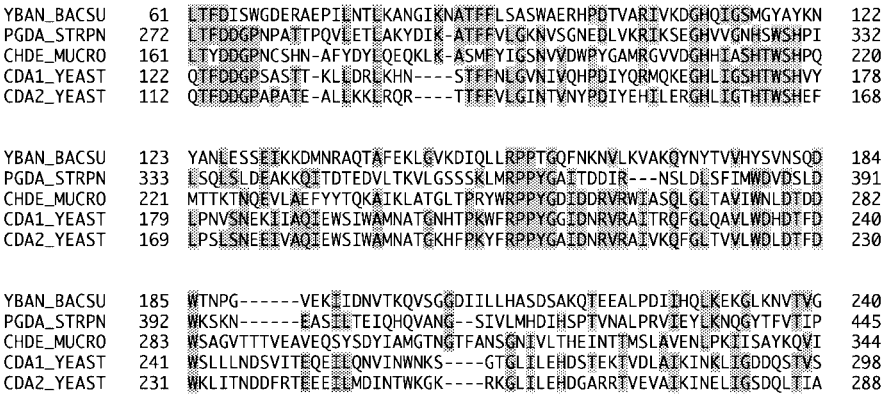

Fig. 1. **Alignment of amino acid sequences of PdaB (YbaN) and several polysaccharide deacetylases.** Numbers are with respect to the N-terminal amino acid of each protein. Shading indicates amino acid residues identical to the highly conserved amino acid residues. YBAN_BACSU, *Bacillus subtilis* YbaN (PdaB); PGDA_STRPN, *Streptococcus pneumoniae* peptidoglycan *N*-acetylglucosamine deacetylase (PgdA); CHDE_MUCRO, *Mucor rouxii* chitin deacetylase (CHDE); CDA1_YEAST, *Saccharomyces cerevisiae* chitin deacetylase 1 (CDA1); CDA2_YEAST, *S. cerevisiae* chitin deacetylase 2 (CDA2).

then shaken at 37°C. If necessary, ampicillin, tetracycline and spectinomycin were added to *E. coli* cultures to final concentrations of 50 μg/ml, 20 μg/ml and 50 μg/ml, respectively, and tetracycline, erythromycin and spectinomycin were added to *B. subtilis* cultures to final concentrations of 10 μg/ml, 0.3 μg/ml and 50 μg/ml, respectively.

Construction of Plasmids to Produce a pdaB (ybaN)-Deficient Mutant—To construct a *pdaB*-disruption plasmid, an internal fragment of the *pdaB* gene was amplified by PCR using two primers, forward primer ybaN-F and reverse primer ybaN-R, with *B. subtilis* 168 (wild-type strain) DNA as the template. The primers used in this study are listed in Table 2. The PCR fragment was digested with *Hin*dIII and *Bam*HI, and then ligated to the corresponding sites of pMUTINT3, followed by transformation of *E. coli* JM109. The resulting plasmid, pM3ybaN, was used to construct a *pdaB*-deficient mutant. To construct a double-crossover mutant, BANSdd, pBlPRBANG was first prepared as follows. A fragment containing the entire *pdaB* gene, and its promoter and SD

Table 1. **Bacterial strains and plasmids used in this study.**

Strain or plasmid	Genotype	Source, reference or construction[a]
B. subtilis strains		
168	*trpC2*	D. Ehrlich
1S86	*trpC2 spoIIA1*	BGSC[b]
1S60	*leuB8 tal-1 spoIIG41*	BGSC
SpoIIIGΔ1	*trpC2 spoIIIGΔ1*	P. Setlow
1S38	*trpC2 spoIIIC94*	BGSC
1G12	*gerE36 leu-2*	BGSC
497.1	*trpC2 spoIIID83*	(*12*)
YBANd	*trpC2 ybaN*::pM3ybaN	pM3ybaN→168
BANSdd	*trpC2 ybaN*::*spc*	Linearized pHYBANSP→168
SSPEdg	*trpC2 sspE*::pM4sspE-gfp	pM4sspE-gfp→168
BANSSPEdg	*trpC2 sspE*::pM4sspE-gfp *ybaN*::*spc*	pM4sspE-gfp→BANSdd
E. coli strains		
JM109	*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15]	Takara
C600	*supE44 hsdR thi-1 leuB6 lacY1 tonA21*	Laboratory stock
Plasmids		
pMUTINT3	*lacZ lacI bla erm*	(*13*)
pDG1726	*bla spc*	BGSC
pBluescriptII-SK(+)	*lacZ bla*	Stratagene
pHY300PLK	*bla tet*	Takara
pGEM3Zf(+)	*lacZ bla*	Promega
pM3ybaN	pMUTINT3:: Δ*ybaN* (*lacZ lacI bla erm* Δ*ybaN*)	This study
pBlPRBANG	pBluescriptII (SK+)::*ybaN* (*bla ybaN*)	This study
pHYBANΔAMP	pHY300PLK::*ybaN* (*tet ybaN*)	This study
pHYBANSP	pHYBANΔAMP::*spc* (*tet spc ybaN*)	This study
pGMSDBAN	pGEM3Zf (+)::*ybaN* (*bla ybaN*)	This study
pHYSDBAN	pHY300PLK::*ybaN* (*bla tet ybaN*)	This study
pGMΔBANU	pGEM3Zf(+)::Δ*ybaN* (*bla* Δ*ybaN*)	This study
pGSSPE	pGEM3Zf(+)::Δ*sspE* (*bla* Δ*ybaN*)	(*14*)
pM4GFP	*gfp lacI bla erm*	(*15*)
pM4sspE-gfp	pM4GFP:: Δ*sspE* (Δ*sspE-gfp lacI bla erm*)	This study

[a]An arrow indicates transformation of the recipient strain by donor DNA. [b]BGSC, *Bacillus* Genetic Stock Center.

Table 2. **Primers used in this study.**

Primer	Sequence (5′→3′) or source	Restriction enzyme
ybaN-F	ggaagcttATATCCAAAGAGCTGTCC$_{106}$	*Hin*dIII
YbaN-R	ttggatccGGTAGGGGGTCTTAATAG$_{456}$	*Bam*HI
ybaN-SDB-U	gccgggatccGTGAAGAGACATTTTATCGG$_{-70}$	*Bam*HI
ybaN-GFP-2	gcgctctagaacctccacctccgctagcC$_{762}$TTTACCTCTGCGGATTTG	*Xba*I, *Nhe*I
ybaN-SDU	gccgggatccTTGACCT-25GGACAAGATAAGGAGGC	*Bam*HI
ybaN-SDD	gcgcggatccaagcttGTAATTACTTTACCTCTGCG$_{750}$	*Bam*HI, *Hin*dIII
ybaN-proU2-H	gccgaagcttAATCACTTCTATGTGTGGC$_{22}$	*Hin*dIII
ybaN-proD2-B	gcgcggatccCTGCTCTCTCATCACCC$_{201}$	*Bam*HI
ybaN-PE	GGCAGCTGCAAATGCG$_{63}$	
-21M13	Universal primer (Takara)	
M13RV	Universal primer (Takara)	

Additional sequences and restriction sites that do not correspond to the sequences near the *pdaB* (*ybaN*) locus are shown by small letters and underlines, respectively. The numbering is with respect to the first G of the translational start codon of *pdaB* (*ybaN*).

sequence was amplified by PCR using forward primer ybaN-SDB-U and reverse primer ybaN-GFP2, with *B. subtilis* 168 DNA as the template. The PCR fragment was digested with *Bam*HI and *Xba*I, and then ligated to *Bam*HI and *Xba*I-digested pBluescript II SK(+), followed by transformation of *E. coli* JM109. The resulting pBl-PRBANG plasmid was digested with *Hin*cII and *Sac*II, and then the digested fragment containing the entire *pdaB* gene, and its promoter and SD sequence was ligated to the *Fsp*I and *Sac*II sites of pHY300PLK, followed by dephosphorylation. After transformation of *E. coli* JM109 with the ligation mixture, pHYBANΔAMP was selected by tetracycline resistance. pHYBANΔAMP was digested with *Fsp*I, followed by dephosphorylation, and then ligated to *Eco*RV and *Hin*cII-digested pDG1736 (Spc^r). After transformation of *E. coli* JM109 with the ligation mixture, pHYBANSP was selected by spectinomycin resistance. pHYBANSP was then linearized with *Bgl*II, followed by transformation of *B. subtilis* 168. The resulting strain, BANSdd, was a double-crossover recombinant as to *pdaB* deficiency.

Construction of pdaB Complementation Plasmids—pGMSDBAN was constructed by PCR with primers ybaN-SDU and ybaN-SDD, and *B. subtilis* 168 DNA as the template. The amplified fragment containing the entire *pdaB* gene with its SD sequence was digested with *Hin*dIII and *Bam*HI, followed by ligation to the corresponding sites of pGEM3Zf(+). After transformation of *E. coli* JM109, the resultant pGMSDBAN was digested with *Hin*dIII and *Bam*HI, and then the fragment containing the *pdaB* gene with its SD sequence was ligated to the corresponding sites of pHY300PLK, resulting in pHYSDBAN. pHYSDBAN was used for the transformation of *E. coli* C600 to prepare concatemeric DNA. Then the DNA was used for the transformation of *B. subtilis* YBANd.

Construction of a Plasmid for Northern Blot Analysis—The internal fragment of *pdaB* was amplified by PCR with primers ybaN-proU2-H and ybaN-proD2-B, and *B. subtilis* 168 DNA as the template. The amplified fragment was digested with *Hin*dIII and *Bam*HI, followed by ligation to the corresponding sites of pGEM3Zf(+). After transformation of *E. coli* JM109, pGMΔBANU was constructed.

Construction of a Strain Containing Green Fluorescent Protein (GFP)—The *sspE* gene is transcribed in the forespore compartment by Eσ^G RNA polymerase (*14*). A plasmid containing *sspE* (pGSSPE, *14*) was digested with *Hin*dIII and *Eco*RI, followed by ligation to the corresponding sites of pM4GFP (*15*). After transformation of *E. coli* JM109, the resultant plasmid, pM4sspE-gfp, was used for the transformation of BANSdd and 168 (wild-type) to construct the BANSSPEdg and SSPEdg strains, respectively. These strains are transcriptional GFP fusion strains. Thus the truncated *sspE* gene and the *gfp* gene are both transcribed by Eσ^E RNA polymerase.

Transformation of E. coli and B. subtilis—*E. coli* transformation was performed as described by Sambrook *et al.* (*16*), and *B. subtilis* transformation was performed by the competent cell method (*18*).

Northern Blot and Primer Extension Analyses—*B. subtilis* cells (15–20 OD_{600} units) cultured in DSM medium were harvested and then suspended in 1 ml of chilled killing buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$). After centrifugation at 11,000 x *g* for 2 min, the pellet was suspended in 1 ml of SET buffer (20% (w/v) sucrose, 50 mM EDTA, 20 mM Tris-HCl [pH 7.6]) containing lysozyme (final concentration, 6 mg/ml) (*14*). After incubation at 9–12 min at 0°C, the suspension was centrifuged at 11,000 x *g* for 2 min. The pellet was used for RNA preparation with Isogen (Nippon Gene) according to the manufacturer's instructions. Agarose-formaldehyde gel electrophoresis was performed as described by Sambrook *et al.* (*16*). The transfer of RNAs onto nylon membranes (Magnagraph; Micron Separations) was performed with a vacuum blotter (model BE-600; BIOCRAFT). The DNA fragment used for preparing an RNA probe was amplified by PCR with M13 (–21) and M13RV as primers, and pGMΔBANU DNA, containing the internal region of *pdaB*, as the template. The amplified fragment was digested with *Hin*dIII, the resulting fragments being purified by phenol and chloroform treatment, and precipitated with ethanol. The RNA probe was prepared with a DIG (digoxigenin) RNA labeling kit (Roche), and Northern (RNA) hybridization was performed according to the manufacturer's instructions. Primer extension analysis was carried out with ybaN-PE as a primer as described previously (*19*).

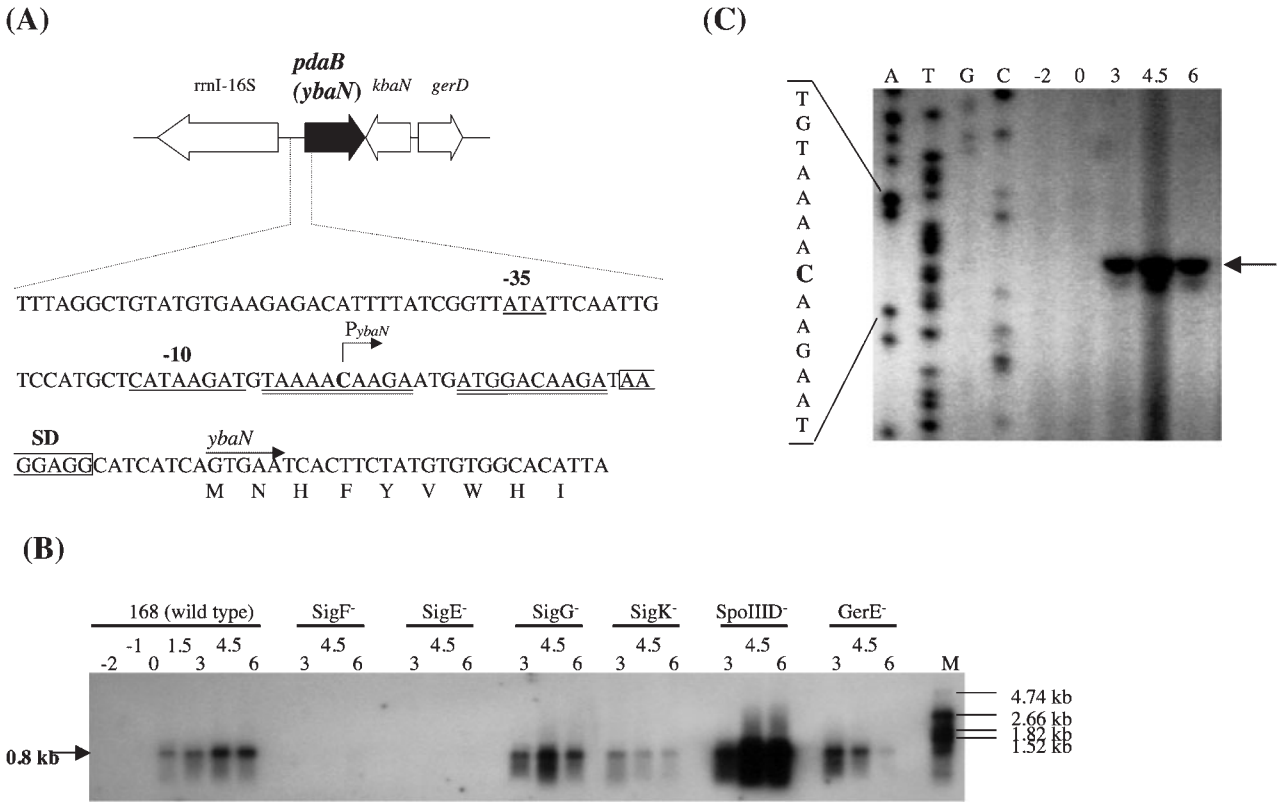

Fig. 2. **A map of *pdaB* (*ybaN*) and the neighboring genes of *B. subtilis* (A), and Northern blotting analysis (B) and primer extension analysis (C) of *pdaB*.** (A) Single and double underlines indicate the consensus sequences of the σ^E promoter and the deduced binding sites of SpoIIID, respectively. SD, Shine-Dalgarno sequence. (B) Northern blotting analysis with a *pdaB* RNA probe. Numbers indicate the times after the onset of sporulation. RNA was isolated from each of 168, SigF$^-$ (1S86, *spoIIA*), SigE$^-$ (1S60, *spoIIG*), SigG$^-$ (spoIIIGΔ1, *spoIIIG*), SigK$^-$ (1S38, *spoIIIC*), SpoIIID$^-$ (497.1, *spoIIID*), and GerE$^-$ (1G12, *gerE*). Twenty micrograms of each RNA was separated on a 1% formaldehyde-agarose gel. The calculated size of the detected *pdaB* mRNA is indicated by an arrow. M, markers (RNA molecular weight marker 1, digoxigenin-labeling; Roche). (C) RNAs from wild-type cells cultured in DSM to t_{-2} (–2), t_0 (0), t_3 (3), $t_{4.5}$ (4.5), and t_6 (6) were used as RNA samples. Signals were detected with ^{32}P-labeled primer ybaN-PE. A dideoxy DNA sequencing reaction mixture with the same primer was electrophoresed in parallel (lanes A, T, G, and C). The nucleotide sequence of the sense strand is given beside the sequence ladder, and arrow and the boldface letter indicate the position and nucleotide at the transcriptional start site, respectively.

Density Gradient Centrifugation—Phase-bright and dark spores, and mother cells were separated by urografin density gradient centrifugation as described below, i.e. basically as described by Nicholson and Setlow (*20*). Five different concentrations (w/v) of urografin (2.1 ml of 70%, 10.5 ml of 60%, 4.2 ml of 50%, 4.2 ml of 40%, and 4.2 ml of 30% urografin) were added stepwise to a 50-ml centrifuge tube to obtain a density gradient. Then a spore suspension (4.2 ml of 20% urografin-containing spores) was added to the top of the gradient, followed by centrifugation at 40,000 × *g* for 60 min at 4°C. Mother cells were pelleted to the bottom of the tube, and phase-bright spores at the middle and phase-gray/dark spores at the top. Each layer was separated and then the spores were washed with water.

Phase and Fluorescent Microscopy—Cells exhibiting green fluorescence were prepared as follows. A transcriptionally fused *sspE-gfp* strain (BANSSPEdg) was cultured in DSM medium at 37°C for various periods. After centrifugation of a culture, the cells were washed and stained with 4,6-diamidino-2-phenylindole (DAPI; final, 1.0 μg/ml; Wako). For fluorescence microscopy, an Olympus BX61 microscope was used with a BX-UCB control unit and a UPPlan Apo Fluorite phase-contrast objective (magnification, X100; numerical aperture, 1.3). The dichroic mirror cube units for DAPI and GFP contained a wide-band-pass (330 to 385 nm) excitation filter with a long-pass (420 nm) barrier filter (U-MWU2; Olympus), and a band pass (470 to 490 nm) excitation filter with a narrow-band-pass (510 to 550 nm) barrier filter (U-MNIBA2; Olympus), respectively. The exposure times for phase-contrast, DAPI, and GFP detection were 0.1 s, 0.003 s, and 0.1 s, respectively. Cells were photographed on both fluorescence- and phase-contrast microscopy, using a CCD camera (CoolSNAP HQ; Nippon Roper), and photo images were analyzed with MetaMorph software (Nippon Roper). Image overlays and micrograph figures were prepared with Adobe Photoshop software.

Viability of Mutant Spores—Spores were treated by heating (80°C, 10 min) and then plated onto LB agar plates. After incubation at 37°C for 13 h, colonies were counted. The sporulation ratio was calculated on the basis of the number of spores and vegetative cells determined under a microscope. The viability of spores was calculated on the basis of the numbers of colonies on LB plates and spores counted under a microscope.

Table 3. **Complementation of *pdaB* (*ybaN*) with a plasmid containing the entire *pdaB* gene.**

Strain	Bright spores	Gray and dark spores
YBANd (pHYSDBAN)	99% (106[a])	0.9% (1)
YBANd (pHY300PLK)	18% (19)	82% (89)
168 (wild type)	99% (117)	0.8% (1)
YBANd	11% (12)	89% (95)

[a]In parentheses are the numbers of spores.

Determination of Dipicolinic Acid (DPA) Contents of Spores—Extraction of DPA from spores was performed basically as described by Nicholson and Setlow (*20*). Determination of DPA was performed by the high sensitive method (Scott and Ellar's method) described by Nicholson and Setlow (*20*).

Transmission Electron Microscopy of Wild and Mutant Spores—Spores were fixed with 2% glutaraldehyde and 1% osmium tetraoxide, and then embedded in an epoxy resin (Spurr; Sigma). Samples were stained with uranyl acetate.

RESULTS

The *B. subtilis* genome contains six polysaccharide deacetylase gene homologues, and one of the genes, *ybaN* (named *pdaB*), exhibits approximately 30% identity with the germination-associated gene *pdaA* at the amino acid level. The *pdaB* gene encodes a 254-amino acid polypeptide starting from a GTG codon, with a predicted molecular mass of 28,174 Da. A consensus Shine-Dalgarno sequence (A_{-15}AGGAGG$_{-9}$, the numbering being with respect to the translational start codon) is present. PdaB has a potential signal sequence that is cleaved at position 24 ($A_{22}FA\downarrow A_{25}$, the arrow indicating the cleavage point) with respect to the N-terminal amino acid residue (PSORT; Prediction of Protein Sorting Signals and Localization Sites, http://psort.ims.u-tokyo.ac.jp/). The upstream and downstream genes are *rrnI* and *kbaN*, respectively. These genes are transcribed in different orientations (Fig. 2A). Therefore, *pdaB* seems to be transcribed as a monocistronic mRNA (Fig. 2A).

(1) Expression of the PdaB Gene—RNAs were prepared from wild-type cells cultured in DSM (Schaeffer) medium (see Materials and Methods) for various periods, and then Northern blot analysis was performed with an RNA probe containing the internal region of the *pdaB* gene. For the 168 (wild-type) strain, a strong hybridizing band was detected at around 0.8 kb at 4.5 and 6 hr after the onset of sporulation (Fig. 2B). Since the *pdaB* gene consists of 762 nucleotide residues (excluding the stop codon), the size of the transcript reasonably matched the estimated size of the monocistronic mRNA. The RNAs from four sigma factor-deficient (null) strains, 1S86 (SigF$^-$ mutant [phenotype: SigF$^-$, SigE$^-$, SigG$^-$, SigK$^-$]), 1S60 (SigE$^-$ mutant [phenotype: SigF$^+$, SigE$^-$, SigG$^-$, SigK$^-$]), spoIIIGΔ1 (SigG$^-$ mutant [phenotype: SigF$^+$, SigE$^+$, SigG$^-$, SigK$^-$]), and 1S38 (SigK$^-$ mutant [phenotype: SigF$^+$, SigE$^+$, SigG$^+$, SigK$^-$]), and two regulatory mutants, 497.1 (SpoIIID$^-$) and 1G12 (GerE$^-$), were further analyzed by Northern blotting (Fig. 2B). During sporulation, sigma factors are activated in the order of σ^F, σ^E, σ^G and σ^K (sigma cascade) (*2*). A hybridizing band was not observed for the SigF- and SigE-deficient mutants, but was for the SigG- and SigK-deficient mutants. These results were obtained only in the case of SigE$^+$ as a transcriptional sigma. The amount of the *pdaB* transcript in the SigK$^-$ mutant was lower than those in the wild type strain and SigG$^-$ mutant. The reason might be that the stability of its transcript in the SigK$^-$ mutant is slightly lower than those in the SigG$^-$ and wild type strains. SpoIIID and GerE are DNA binding proteins that regulate transcription from σ^E- and σ^K-dependent promoters, respectively. The *spoIIID* mutation greatly affected the expression of *pdaB*, but the *gerE* mutation did not affect it significantly. These results indicate that *pdaB* was transcribed by Eσ^E RNA polymerase and negatively regulated by SpoIIID.

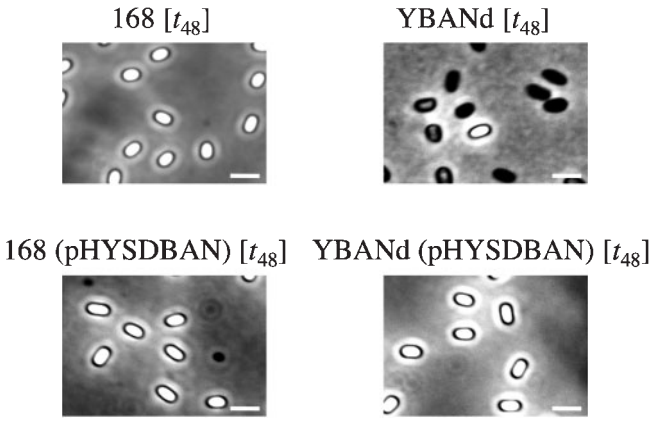

Fig. 3. **Phase-contrast microscopy of dormant spores of wild-type strain 168, *pdaB*-deficient strain YBANd, and strains harboring a *pdaB*-complementation plasmid pHYSDBAN.** Spores were collected from cultures in DSM (with or without 10 μg/ml tetracycline) at t_{48}. The addition of tetracycline induced the expression of *pdaB* in pHYSDBAN. The white bars indicate 2 μm.

Primer extension analysis was performed with RNAs from *B. subtilis* 168 (wild-type strain) and primer ybaN-PE. An extended product was detected from t_3 to t_6, and the product started at C, which was 34 bp upstream of the translational start codon, GTG (Fig. 2A and C). The upstream sequences, ATA and CATAagAT (capital letters, consensus sequence), with spacing of 16 bp, were identical and very similar to the –35 and –10 consensus sequences (ATA and CATACA-T, respectively, with spacing of 16 to 18 bp) of sigma E (*21*). Two sequences (TAAAACAAGa and ATGGACAAGa) found between –10 and the SD sequence were very similar to the consensus sequence of SpoIIID (WWRRACAR-Y; W, A/T; R, A/G; Y, T/C) (Fig. 2A).

(2) Characterization of a PdaB Disruptant—*pdaB*-deficient mutant YBANd showed normal growth, cell separation and motility, but produced spores that appeared gray/dark on phase-contrast microscopy, amounting to 89% of total spores at 48 hr in DSM medium (Table 3 and Fig. 3). On the other hand, 168 (wild-type) strain spores were phase-bright ones (Table 3 and Fig. 3). Since the mutant spores consisted of a mixture of gray/dark and bright ones, it was examined whether or not this is caused by the *pdaB* mutation. A plasmid (pHYSDBAN) containing the entire *pdaB* gene with its SD sequence was introduced into *pdaB*-deficient

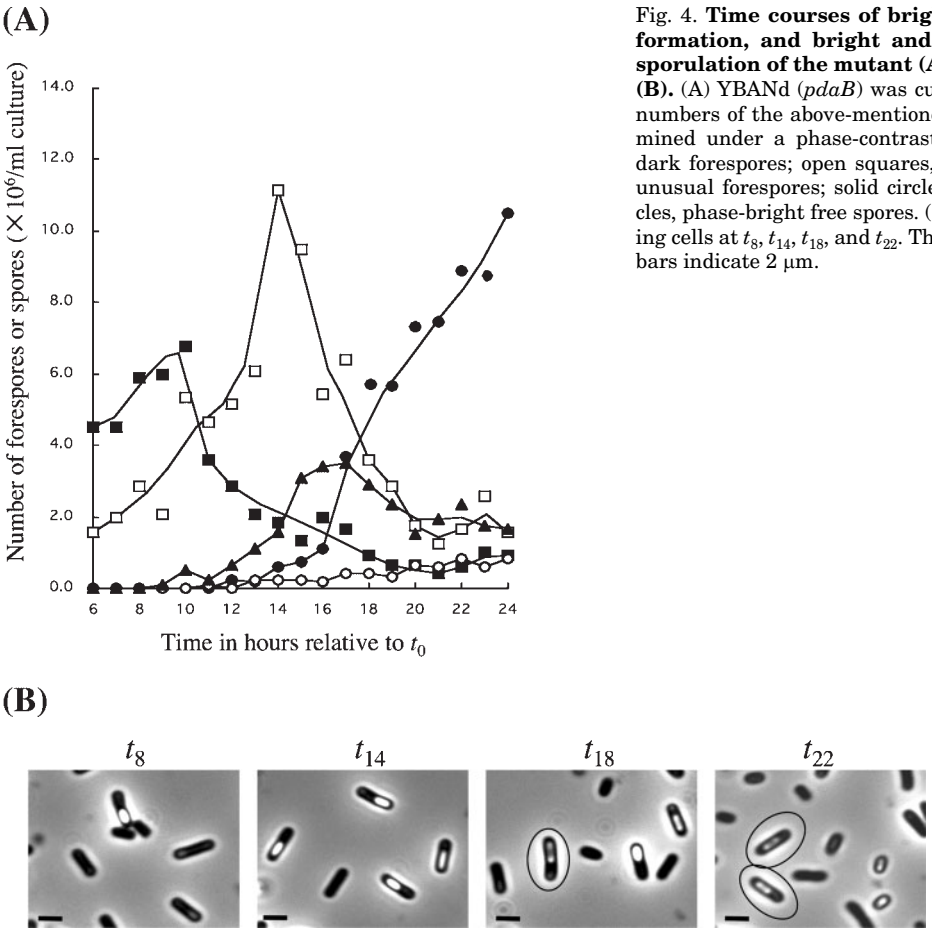

Fig. 4. **Time courses of bright, gray/dark and unusual forespore formation, and bright and gray/dark free spore formation on sporulation of the mutant (A), and their microscopic observation (B).** (A) YBANd (*pdaB*) was cultured in DSM medium at 37°C, and the numbers of the above-mentioned forespores and free spores were determined under a phase-contrast microscope. Solid squares, phase-gray/dark forespores; open squares, phase-bright forespores; solid triangles, unusual forespores; solid circles, phase-gray/dark free spores; open circles, phase-bright free spores. (B) Phase-contrast microscopy of sporulating cells at t_8, t_{14}, t_{18}, and t_{22}. The circled cells are unusual forespores. The bars indicate 2 μm.

mutant YBANd, and then the frequency of gray/dark spores as to total spores was determined. Table 3 indicates that almost all the spores of YBANd harboring pHYSDBAN were phase-bright, similar to in the case of 168 (wild-type), but introduction of a control plasmid, pHY300PLK, into the YBANd strain did not complement the phenotype (Table 3 and Fig. 3). These results indicate that the mixed phenotype is caused by the mutation of *pdaB*.

(3) Time Courses of Forespore and Free Spore Formation on Sporulation of the Mutant—To determine the time when the phenotype of the mutant differs from that of the wild-type, phase bright and gray/dark spore-forming cells were counted and observed under a phase-contrast microscope. PdaB-deficient cells after t_6 (6 h after onset of sporulation) in DSM medium were observed under a phase-contrast microscope, and the numbers of the following were determined: phase-bright forespores, phase-gray/dark forespores, phase-bright free spores, phase-gray/dark free spores and unusual forespores (Fig. 4A). Unusual forespores were defined as ones having phase-dark centers and phase-bright peripheries (Fig. 4b, shown by circles). At intermediate sporulation stages (t_6 to t_{10}), phase-gray/dark forespores were the major population among sporulating cells. But at t_{12} to t_{14}, phase-bright forespores were the major population and phase-gray/dark forespores were decreased in number. This pattern was very similar to that for wild-type sporulating cells (data not shown), because phase-gray/dark forespores at the intermediate stage of sporulation became phase-bright ones at the late stage of sporulation. Unusual forespores appeared from the *pdaB*-deficient cells after t_{12} and the number increased until t_{17}. Free phase-gray/dark spores increased extensively after t_{14}, but phase-bright forespores decreased rapidly after t_{14} (Fig. 4A). These results suggest that the free phase-gray/dark spores were derived from the phase-bright forespores. Figure 4B shows phase-contrast microscopy of spore-forming cells and free spores. Phase-gray/dark forespores were found at t_8, and phase-bright forespores were found at t_8, t_{14} and t_{18}. Unusual forespores were found at t_{18} and t_{22}. Free phase-gray/dark spores became the major population among sporulating cells at t_{22} (Fig. 4B). Therefore, forespores in sporangia showed a change from phase bright to phase gray/dark during the late sporulation phase.

(4) Purification and Characterization of Phase-Bright or Gray/Dark Spores of the pdaB Mutant—To determine the viability and heat sensitivity of mutant spores, we separated bright spores and gray/dark spores by urografin density gradient centrifugation. 1.0×10^8 purified spores were diluted and plated onto DSM agar plates, or plated after heating at 80°C for 10 min. After incubation at 37°C, the numbers of colonies were determined (Table 4). Under the non-heating conditions, 168 (wild-type) spores gave colonies at about 50% frequency, but phase-gray/dark spores from YBANd gave colonies at 0.04% frequency. Phase-bright spores from YBANd gave colonies

Table 4. **Effect of heat treatment on germination of the wild type spores, and phase-gray/dark and phase-bright spores of the *pdaB* (*ybaN*) mutant.**

	Ratio of viable cells per 10^8 spores (%)	
Strain	Non heat	Heat
168 (wild type)	51%	49%
YBANd (mixture)	9.1%	4.5%
YBANd (gray/dark spores)	0.04%	0.0087%
YBANd (bright spores)	23%	19%

Table 5. **DPA contents of the wild type spores, and phase-gray/dark spores and phase-bright spores of the *pdaB* (*ybaN*) mutant.**

Strain	DPA content (μg/10^9 spores)
168 spores (wild type)	57
YBANd spores (mixture)	8.0
YBANd dark spores	0.7
YBANd bright spores	50

at 23% frequency. These results indicated that the phase-gray/dark spores had almost completely lost their viability. Heating did not affect the viability of the wild-type or mutant bright spores.

DPA is known to be a major component of spores (*4*, *5*). Beall and Moran, Jr. reported that the *spoVR* (stage V [Spore coat formation] sporulation protein R involved in spore cortex synthesis) mutant produced phase-bright and dark spores, and could accumulate a little DPA (*3*). The phenotype of phase-bright and dark spore production is similar to that of the *pdaB* mutant. Therefore, the amounts of DPA in the mutant dark and bright spores were determined (Table 5). Phase-bright spores of the mutant contained a similar amount of DPA to in the wild type, but phase-gray/dark spores of the mutant contained only 1.2% of the wild type level. This suggests that the phase gray/dark spores of the *pdaB* mutant can not accumulate DPA.

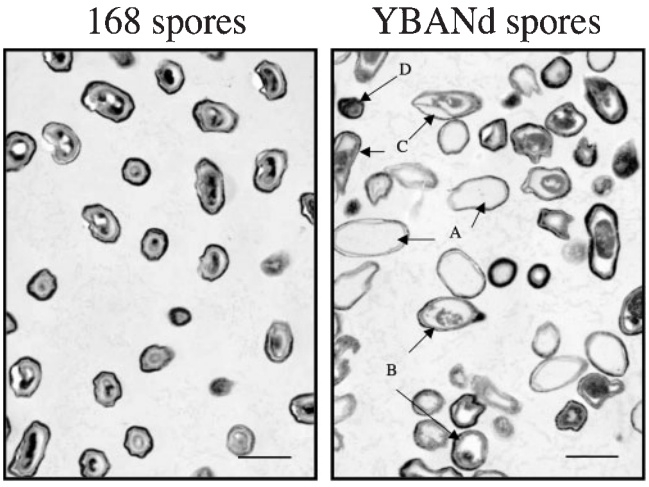

Fig. 5. **Electron micrographs of the wild-type 168 spores and *pdaB*-minus YBANd spores.** Cells cultured in DSM medium for 48 h at 37°C were collected by centrifugation and then spores (a mixture of phase-bright and -gray/dark spores) were separated from vegetative cells by urografin density gradient centrifugation. Empty spores, A; cortex-less spores, B; deformed spores, C; wild-type like spore, D. The bars indicate 2 μm.

(5) Electron Microscopy of the pdaB Mutant Spores— The refractility observed on phase-contrast microscopy appears to be largely due to completion of cortex synthesis and dehydration of spores (*22*). Moreover, since SpoVR is associated with cortex formation (*3*), it is predicted that PdaB is also associated with it. Thus, the structure of the *pdaB* mutant spores was observed under an electron microscope. Electron microscopy (EM) of 168 (wild-type) and *pdaB*-deficient spores from 48-h cultures in DSM is shown in Fig. 5. EM of the wild-type spores revealed bright regions corresponding to the spore core, gray regions corresponding to the spore cortex, and dark peripheral regions corresponding to the spore coat (Fig. 5). EM of the mutant showed a heterogeneous population of spores: empty spores (only the spore coat; A in Fig. 5),

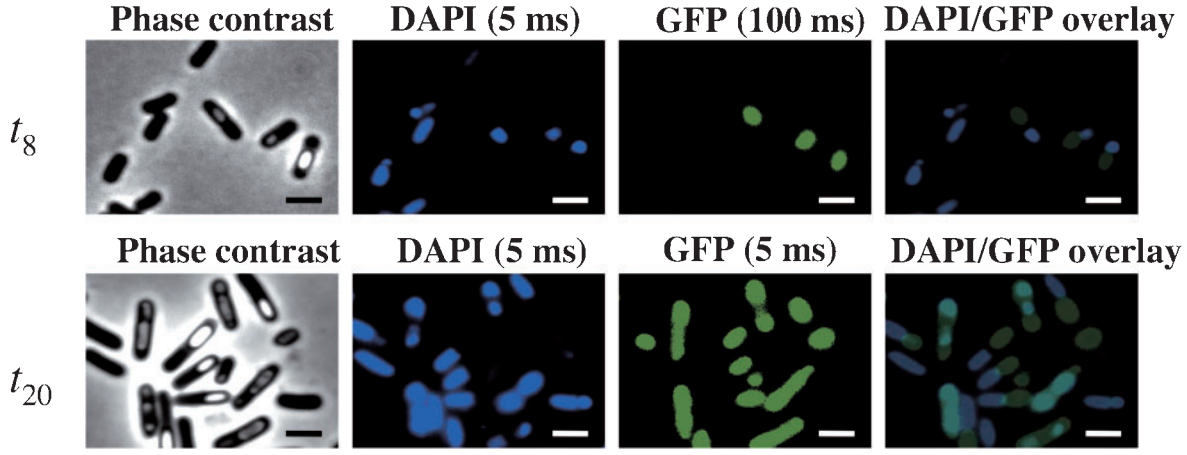

Fig. 6. **Localization of the SspE-GFP transcriptional fusion protein.** The *sspE-gfp* transcriptional fusion strain, BANSSPEdg, was cultured in DSM medium at 37°C until t_8 and t_{20}. After centrifugation of the cultures, the cells were suspended in ultrapure water followed by staining with DAPI. Phase and fluorescence microscopy were carried out as described under Materials and Methods. Overlay indicates a DAPI-staining fluorescence image overlaid on a GFP image. The exposure times for DAPI and GFP detection are given in parentheses. The white bars indicate 2 μm.

cortex-less spores (dark center surrounded by a bright region; B), deformed spores (C), and a few wild-type like spores (D). Therefore, it is suggested that PdaB is involved in cortex formation because the cortex of *pdaB* spores is unusual.

(6) Abnormal Localization of Sigma G-Directed GFP in Spore-Forming Cells of the Mutant—From these results, it is predicted that substrates in the forespore core can not be accumulated in the *pdaB* mutant. Thus, localization of sigma G-directed GFP in spore-forming cells of the mutant was investigated. *SspE-gfp* is a GFP transcriptionally fused to *sspE,* and thus its gene was introduced into BANSdd (*pdaB*) to produce a *pdaB*-deficient *sspE-gfp* strain (BANSSPEdg). This strain was cultured in DSM at 37°C, and samples were taken at t_8 and t_{20}. Cells were stained with a DNA staining fluorescent dye, DAPI, and then the fluorescence of GFP and DAPI was observed under a phase-contrast microscope equipped with a fluorescence apparatus. Since the *sspE* gene is transcribed by σ^G RNA polymerase (*14*), GFP is localized in the forespore compartment and free spores for the wild type *sspE-gfp* strain (data not shown). Similar to the wild-type, GFP of the mutant was localized in the forespore compartment at t_8 (Fig. 6). However, cells containing phase-gray/dark and unusual forespores at t_{20} exhibited abnormal localization of GFP not only in the forespore compartment but also in the mother cell compartment (Fig. 6). Moreover, DNA in mature wild-type forespores (mature spores in cells) at t_{20} was not stained by DAPI. But cells containing unusual forespores at t_{20} exhibited abnormal staining by DAPI not only in the mother cell compartment but also in the forespore compartment. A σ^E-driven *spoIID-lacZ* transcriptional fusion introduced into the *pdaB*-deficient strain did not cause any differences in the activity or time of expression from that introduced into the 168 (wild-type) strain (data not shown). Moreover, significant differences between the *pdaB*-deficient and wild-type strains were not found for the σ^G-driven *sspE-lacZ* and σ^K-driven *cotD-lacZ* transcriptional fusions (data not shown). These data suggest that the transcription of the mutant during sporulation was normal, but that GFP in the forespore compartment at the middle stage of sporulation (t_8) had leaked into the mother cell compartment at the late stage of sporulation (t_{20}). Therefore, PdaB was necessary to maintain spores after the late stage of sporulation.

DISCUSSION

About 90% of the *pdaB*-deficient spores appeared gray/dark on phase-contrast microscopy. The mutant exhibits no defect in spore engulfment (t_8 in Fig. 4B; data not shown). During sporulation stages IV (spore cortex formation) to V (spore coat formation) (*1*), dehydration seems to be normal, because sporulating cells contained phase-bright forespores (t_{14} in Fig. 4B). However, during the maturation stage, phase-bright forespores may become unusual forespores or phase-gray/dark ones ($t_{18, 22}$ in Fig. 4B). The refractility observed on phase-contrast microscopy appears to be largely due to completion of cortex synthesis and dehydration of spores (*22*). Thus, unusual forespores and phase-gray/dark forespores may be unable to retain the dehydrated condition.

Purified phase-gray/dark spores lacked DPA (Table 5). Electron microscopy showed that the *pdaB*-deficient spores were almost empty or cortex-less (Fig. 5). Beall and Moran Jr. reported that the *spoVR* mutant produces phase-dark spores that are almost empty or cortex-less, as judged on EM (*3*). These phenotypes of the *spoVR* mutant are similar to that of the *pdaB* mutant. Moreover, the *spoVR* mutant spores accumulated about three-fold less DPA than the wild-type spores (*3*). This corresponds with the decreased DPA accumulation by the *pdaB* mutant. It is predicted that the lack of DPA in both types of mutant spores is involved in cortex formation. As shown in Fig. 6, GFP expressed in the forespores of the *pdaB* mutant leaked into the mother cells. Therefore, cortex formation is required to prevent leakage of the forespore contents into the mother cell, and PdaB is essential to maintain spores after the late stage of sporulation.

Northern blotting of the *pdaB* gene using spore sigma factor-minus mutants indicated that *pdaB* is transcribed by $E\sigma^E$ RNA polymerase as a monocistronic mRNA (Fig. 2). Primer extension analysis also indicated that the promoter shows high similarity with the σ^E consensus sequence (Fig. 2). SpoVB (stage V [spore coat formation] sporulation protein B, *23*), SpoVD (Stage V sporulation protein D, which is a sporulation-specific penicillin-binding protein, *24*), SpoVE (stage V sporulation protein E, *25*), and SpoVR (*3*) have been shown to play direct roles in cortex formation. The expression of these genes is controlled by σ^E. Moreover, it is predicted that the spore cortex is synthesized from the mother cell side (*1*, *26*). PdaB and these proteins are expressed in mother cells and associated with cortex synthesis. Recently, Eichenberger *et al.* reported that PdaB was localized in the outer forespore membrane (*7*). Therefore, PdaB produced in the mother cell compartment is transported into the forespores, and thus the cortex may be a target of PdaB.

Polysaccharide deacetylases such as a peptidoglycan *N*-acetylglucosamine deacetylase in *Streptococcus pneumoniae* (*10*), and several chitin deacetylases in *Mucor rouxii* (*27*) and *Saccharomyces cerevisiae* (*9*) deacetylate *N*-acetylglucosamine residues. Furthermore, polysaccharide deacetylase homologue PdaA is assumed to be a *N*-acetylmuramic acid deacetylase, because a deficiency of it leads to a complete lack of muramic δ-lactam residues in the spore cortex (*11*). *B. subtilis* and *B. cereus* strains have high proportions of *N*-substituted glucosamine residues in their peptidoglycan components, and they are particularly rich in the deacetylase (*28*). Similar to the peptidoglycans, the spore cortex contains the *N*-acetylglucosamine-*N*-acetylmuramic acid linkage as a polysaccharide layer. Therefore, the glycans of the spore cortex may be candidate PdaB substrates.

Various bacilli and clostridia contain *pdaB* homologous genes exhibiting extraordinary high similarity. They are the genes of *B. anthracis* (encoding 254 aa polypeptide; fasta E-value of gene product, 7.2e-52), *B. cereus* (254 aa; 4.1e-51), *B. halodurans* (253 aa; 1.2e-44), *Oceanobacillus iheyensis* (251 aa; 2.7e-38), *Thermoanaerobacter tengcongensis* (239 aa; 2e-27), *Clostridium tetani* (257 aa, 1.7e-21), and *C. acetobutylicum* (255 aa, 6.5e-18). The most highly homologous gene in *B. subtilis* is *ylxY,* and its product shows an E value of 2.1e-17 and 31.2% identity with PdaB. However, YlxY is a 319-aa polypeptide and

PdaB is a 254-aa one, and disruption of *ylxY* did not cause any sporulation defect (data not shown). Disruption of the other paralogs (*yheN*, *yjeA* and *yxkH*) did not cause any phenotypic difference, including in vegetative cell morphology, sporulation and germination (data not shown). We further constructed double mutants between *pdaB* and the other paralogs (*ylxY, yheN, yjeA* and *yxkH*). They exhibited sporulation defects similar to the *pdaB* single mutant (data not shown).

Since PdaB is associated with sporulation and is highly conserved in spore-forming bacteria, its biochemical properties are interesting but remain to be determined.

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