

# A Polysaccharide Deacetylase Gene (*pdaA*) Is Required for Germination and for Production of Muramic $\delta$ -Lactam Residues in the Spore Cortex of *Bacillus subtilis*

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The predicted amino acid sequence of *Bacillus subtilis* *yjfS* (renamed *pdaA*) exhibits high similarity to those of several polysaccharide deacetylases.  $\beta$ -Galactosidase fusion experiments and results of Northern hybridization with sporulation sigma mutants indicated that the *pdaA* gene is transcribed by  $E\sigma^G$  RNA polymerase. *pdaA*-deficient spores were bright by phase-contrast microscopy, and the spores were induced to germination on the addition of L-alanine. Germination-associated spore darkening, a slow and partial decrease in absorbance, and slightly lower dipicolinic acid release compared with that by the wild-type strain were observed. In particular, the release of hexosamine-containing materials was lacking in the *pdaA* mutant. Muropeptide analysis indicated that the *pdaA*-deficient spores completely lacked muramic  $\delta$ -lactam. A *pdaA*-*gfp* fusion protein constructed in strain 168 and *pdaA*-deficient strains indicated that the protein is localized in *B. subtilis* spores. The biosynthetic pathway of muramic  $\delta$ -lactam is discussed.

*Bacillus subtilis* produces spores under various nutrient limitations, and this provides a survival mechanism under adverse environmental conditions (25). On the addition of germinants, spores start to germinate and refractility quickly decreases (17). At the same time dipicolinic acid is released, and then cortex fragments containing hexosamine are released from the spores (17). The cortex is one of the most characteristic structures in spores and is degraded by germination-specific cortex lytic enzymes (GSLEs) (3, 17). SleB and CwlJ are candidate GSLEs (10, 15, 17), and it is believed that these proteins recognize the muramic  $\delta$ -lactam structure, which is unique in spore cortex (7, 17, 24), because *Bacillus cereus* SleB, which is highly homologous to *B. subtilis* SleB and CwlJ, degrades normal cortex but not muramic  $\delta$ -lactam-deficient cortex (13, 17; S. Makino and R. Moriyama, unpublished results).

Previously, two groups suggested that an *N*-acetylmuramoyl-L-alanine amidase homologue, CwlD, is associated with the biosynthesis of muramic  $\delta$ -lactam (4, 18) because a *cwlD*-deficient mutant completely lacked muramic  $\delta$ -lactam (4, 18) and expressed a germination-negative phenotype (21). The cortex of the mutant exhibited higher cross-linkage than the wild-type spores (4, 18). Under the *B. subtilis* functional analysis project, we analyzed the germination of spores from more than 500 disrupted strains (22). One of the mutations, *yjfS*, exhibited almost no germination (as measured by recovery of colonies from spores). The amino acid sequence similarity of YjfS with sequences in protein databases suggested it is a polysaccharide deacetylase homologue (BSORF database). In legumes, this

enzyme is known as nodulation enzyme B (8), and two homologous gene products in yeast are chitin deacetylases (14).

In this paper we describe the germination profiles of the *yjfS* mutant and the *yjfS* gene, which is associated with muramic  $\delta$ -lactam biosynthesis in the spore cortex.

## 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 (19) at 37°C and then inoculated into LB medium. *B. subtilis* was grown on nutrient agar medium (8 g of Bacto nutrient broth/liter, 0.12 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O/liter, 1 g of KCl/liter, and 15 g of agar/liter; pH 7.0 to 7.2) at 30°C, inoculated into DSM (Schaeffer) medium (20), and then shaken at 37°C. If necessary, cells on the agar medium were precultured in NB medium (nutrient agar medium without agar) before cultivating in DSM medium. For muropeptide analysis, *B. subtilis* spores were produced in CCY medium (4, 26). If necessary, erythromycin, tetracycline, and ampicillin were added to the medium to final concentrations of 0.2, 5 (unless otherwise noted), and 50  $\mu$ g/ml, respectively.

**Plasmids used for construction of a *pdaA* (*yjfS*) mutant.** To construct a *pdaA* disruption plasmid, an internal fragment of the *pdaA* gene was amplified by PCR using two primers, forward primer JS-HF (5'-GCCGAGCTTGATGTGTTCA ATATGCTGC; the *Hind*III site is underlined) and reverse primer JS-BR (5'-GCGCGATCCTCATATCCGTTATCAAACGT; the *Bam*HI site is underlined), with *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Hind*III and *Bam*HI and then ligated to *Hind*III- and *Bam*HI-digested pMUTIN2 followed by transformation of *E. coli* JM109. The resulting plasmid, pM2fjS, was used to construct a *pdaA*-deficient mutant. The digested PCR fragment was also ligated to *Hind*III- and *Bam*HI-digested pGEM3Zf(+) to construct pGEMfjS. pGEMfjS was used to synthesize an RNA probe, and pM2fjS was also used for the transformation of *E. coli* C600 to produce catemeric DNAs (6).

**Construction of *pdaA* complementation plasmids.** pUCfjS was constructed by PCR with PQEJS (5'-GCCGAGCTCATGAAGTGGATGTGTTCA; the *Sac*I site is underlined) and JS-D (5'-GCGCGGATCCTTTACAAAGACGGCAG CC; the *Bam*HI site is underlined) as primers and 168 DNA as a template. The amplified fragment containing the entire *pdaA* gene was digested with *Sac*I and *Bam*HI, followed by ligation to the corresponding sites of pUC119. The resultant pUCfjS was further digested with *Pst*I and *Eco*RI, and the fragment containing the *pdaA* gene was ligated to the corresponding sites of pBluescript II SK(+),

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TABLE 1. Bacterial strains used in this study

Strain or plasmid	Genotype	Source or reference
<b>B. subtilis strains</b>		
168	<i>trpC2</i>	D. Ehrlich
168HR	<i>trpC2</i>	Laboratory stock (S. J. Foster)
YFJSd	<i>trpC2 yjfS::pM2fjS</i>	pM2fjS→168 <sup>a</sup>
HR(YFJS)	<i>trpC2 yjfS::pM2fjS</i>	pM2fjS→168HR
HR(YFJS CWLD)	<i>trpC2 yjfS::pM2fjS cwld::cat</i>	AA107 (4)→HR(YFJS)
1S86	<i>trpC2 spoIIA1</i>	BGSC <sup>b</sup>
1S60	<i>leuB8 tal-1 spoIIIG41</i>	BGSC
SpoIIIGΔ1	<i>trpC2 spoIIIGΔ1</i>	BGSC
1S38	<i>trpC2 spoIIIC94</i>	BGSC
<b>E. coli strains</b>		
JM109	<i>recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44 [F' traD36 proAB<sup>+</sup> lacI<sup>a</sup> lacZ ΔM15]</i>	Takara
C600	<i>supE44 hsdR17 thi-1 thr-1 lueB6 lacY1 tonA21</i>	Laboratory stock
<b>Plasmids</b>		
pMUTIN2	<i>lacZ lacI bla erm</i>	D. Ehrlich
pM2fjS	pMUTIN2::Δ <i>yjfS</i> ( <i>lacZ lacI bla erm ΔyjfS</i> )	This study
pGEM3Zf(+)	<i>lacZ bla</i>	Promega
pGEMfjS	pGEM3Zf(+>::Δ <i>yjfS</i> ( <i>lacZ bla ΔyjfS</i> )	This study
pUC119	<i>lacZ bla</i>	Takara
pBluescriptII-SK(+)	<i>lacZ bla</i>	Stratagene
pHY300PLK	<i>bla tet</i>	Takara
pHYCM1	pHY300PLK with a 292-bp <i>citM</i> promoter region (carrying a CG-to-AT dinucleotide change at +47 and +48 relative to the <i>citM</i> start point)	H. Yamamoto and M. Serizawa
pUCfjS	<i>bla yjfS</i>	This study
pBlfjS	<i>bla yjfS</i>	This study
pUCΔ <i>fjS</i>	<i>bla ΔyjfS</i> (lacking putative signal sequence)	This study
pBlΔ <i>fjS</i>	<i>bla ΔyjfS</i> (lacking putative signal sequence)	This study
pHYCMfjS	<i>bla tet yjfS</i>	This study
pHYCMΔ <i>fjS</i>	<i>bla tet ΔyjfS</i> (lacking putative signal sequence)	This study
pQBI63	<i>bla gfp</i>	Takara
pHYGFP	<i>bla tet gfp</i>	This study
pHY300GFP14	<i>bla tet gfp</i>	This study
pB1PRbaNG	<i>bla ybaN-gfp</i>	This study
pB1PRfjSG	<i>bla yjfS-gfp</i>	This study
pHYPRbaNG	<i>bla tet ybaN-gfp</i>	This study
pHYPRfjSG	<i>bla tet yjfS-gfp</i>	This study

<sup>a</sup> Sources shown before and after the arrows indicate donor DNA and recipient cells on transformation, respectively.

<sup>b</sup> BGSC, *Bacillus* Genetic Stock Center.

resulting in pBlfjS. The *SalI* fragment containing the *yjfS* gene from pBlfjS was ligated to the *SalI* site of pHYCM1 (described below), resulting in pHYCMfjS. pHYCM1 is a pHY300PLK derivative with a 292-bp *citM* promoter region (carrying a CG-to-AT dinucleotide change [mutated *cre*] at +47 and +48 relative to the *citM* start point) (30). Therefore, the *citST*-controlled gene in pHYCM1 is only expressed in the presence of citrate plus Mg<sup>2+</sup> and is not repressed by glucose.

Plasmids containing a putative signal sequence-deleted *pdaA* gene were constructed basically as described above. A fragment containing the putative signal sequence-deleted *pdaA* gene (the start codon was also changed from GTG to ATG) was constructed using two primers, PQEspjS (5'-GCCGGAGCTCATG CCGAATGAGCCGATT; the *SacI* site is underlined) and JS-D. The amplified fragment was ligated to pUC119, resulting in pUCΔ*fjS*. Then, pBlΔ*fjS* and pHYCMΔ*fjS* were constructed as described for the construction of pBlfjS and pHYCMfjS.

**Construction of GFP fusion plasmids.** A basic green fluorescent protein (GFP) fusion plasmid, pHYGFP, was constructed by insertion of the *HindIII*-*BglII* fragment from pQBI63 (rsGFP vector with red-shifted excitation wavelength; Takara) into the corresponding site of pHY300PLK. Then, a *gfp*-containing fragment was obtained by PCR amplification with two primers, GFP-HF (5'-GCCGAGCTTTGTTTAAAGAAGGAG; the *HindIII* site is underlined) and GFP-BgR (5'-GCGCGCAGCCAGATCTTCAGTTG; the *BglII* site is underlined), and pHYGFP as a template. After digestion with *HindIII* and *BglII*, the fragment was ligated to the corresponding site of pHY300PLK, resulting in pHY300GFP14. To construct an intermediate plasmid, pHYPRbaNG, a fragment containing the *ybaN* promoter, Shine-Dalgarno (SD) sequence, and the entire *ybaN* gene was obtained by PCR amplification with two primers, ybaN-

SDB-U (5'-GCCGGGATCCGTAAGAGACATTTTATCGG; the *BamHI* site is underlined) and ybaN-GFP2 (5'-GCGCTCTAGAACCCTCCACCTCCGCTA GCCTTACCTCTGCGGATTTG; the *XbaI* and *NheI* sites are underlined), and strain 168 chromosome DNA as a template. The amplified fragment was digested with *BamHI* and *XbaI*, followed by ligation to the corresponding site of pBluescript II SK(+), resulting in pBIPRbaNG. Then, a *HindIII*-*XbaI* fragment from pBIPRbaNG, containing the *ybaN* promoter, SD, and the *ybaN* gene, was ligated to *HindIII*- and *NheI*-digested pHY300GFP14, resulting in pHYPRbaNG. To finally construct a *pdaA*-GFP plasmid, a fragment containing the *pdaA* region was amplified by PCR using two primers, forward primer jT-HF (5'-GC CGAAGCTTGGGGAACGCCGTAACAA; the *HindIII* site is underlined) and reverse primer jS-DG (5'-GCGCGAATTCGCTAGCCAAAGACGGCAG CCTCA; the *EcoRI* and *NheI* sites are underlined), with 168 DNA as a template. The PCR fragment was digested with *HindIII* and *EcoRI* and then ligated to the corresponding site of pBluescript II SK(+), followed by transformation of *E. coli* JM109. The resulting plasmid, pBIPRfjSG, was further digested with *HindIII* and *NheI*, and the digested fragment was ligated to the corresponding site of pHYPRbaNG, resulting in pHYPRfjSG. Therefore, pHYPRfjSG contains a region between *yjfT* and *pdaA* and the *pdaA* gene translationally fused with the *gfp* gene.

**Mutant construction.** A *pdaA*-deficient mutant, YFJSd, was constructed by transformation of *B. subtilis* 168 with pM2fjS. Disruption of the *pdaA* gene by means of Campbell-type recombination was confirmed by PCR. Thus, the *pdaA* mutant was a *pdaA-lacZ* transcriptional fusion strain.

**Transformation of *E. coli* and *B. subtilis*.** *E. coli* transformation was performed as described by Sambrook et al. (19), and *B. subtilis* transformation was performed by the competent cell method (1).

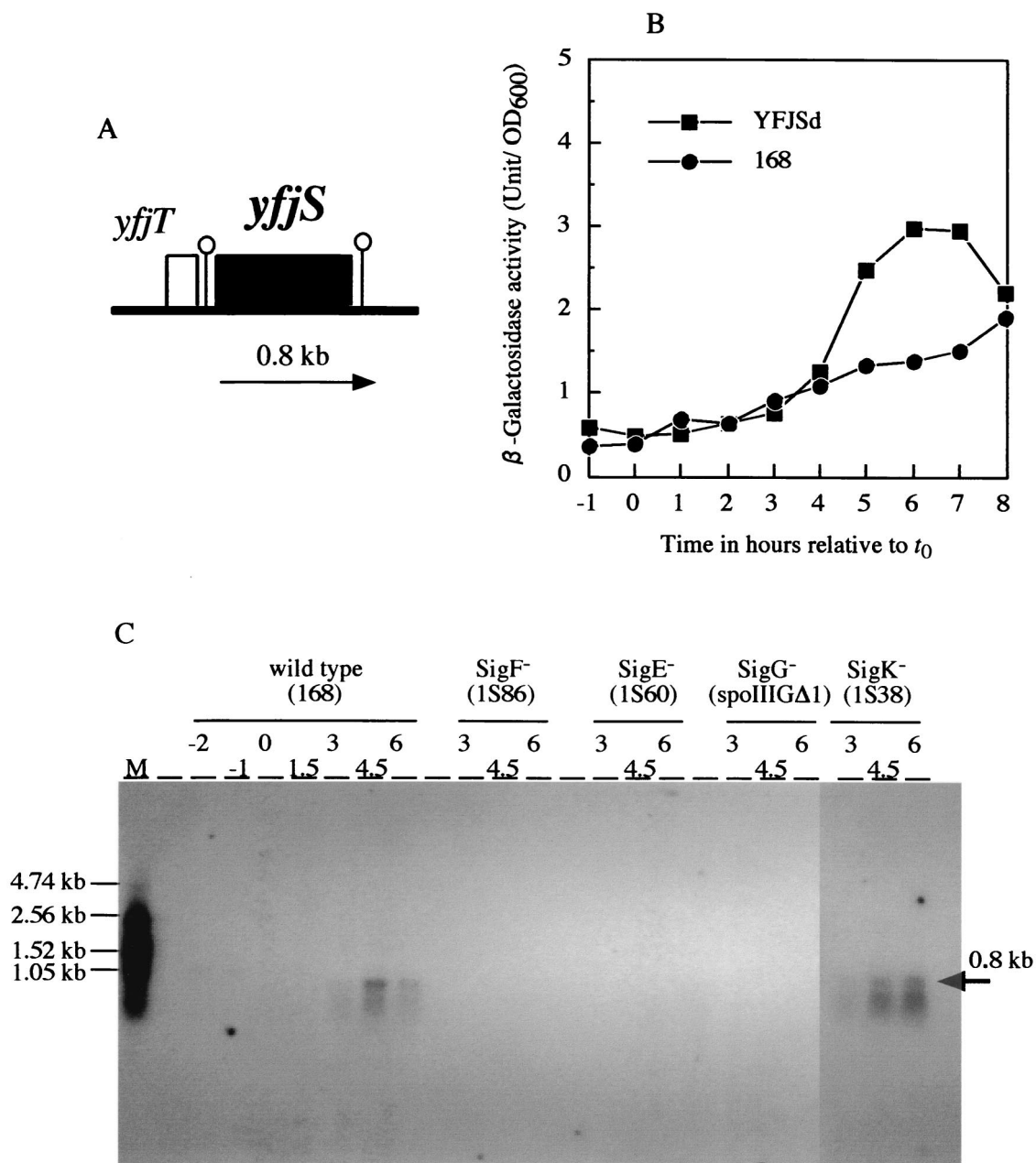


FIG. 1. (A) Physical map of the *yfjT* and *yfjS* (*pdaA*) genes of *B. subtilis*. Stem-loop structures indicate the putative terminators. The arrow indicates the position of *yfjS* mRNA. (B)  $\beta$ -Galactosidase activity of the *pdaA-lacZ* transcriptional fusion strain. Circles, wild-type strain 168; squares, *pdaA-lacZ* fusion strain YFJSd. (C) Northern blotting analysis with a *pdaA* RNA probe. Numbers indicate times after the onset of sporulation. Each RNA was isolated from strain 168, 1S86 (*spoIIA*), 1S60 (*spoIIG*), *spoIIIG* $\Delta$ 1 (*spoIIG*), or 1S38 (*spoIIIC*). Twenty micrograms of each RNA was separated on a 1% formaldehyde-agarose gel. The calculated size of the detected *pdaA* mRNA is indicated by an arrow. M, marker (RNA molecular weight marker 1, digoxigenin labeling [Boehringer Mannheim]).

**Spore germination.** *B. subtilis* 168 and YFJSd were cultured in DSM at 37°C until  $t_{48}$  (48 h after the onset of sporulation). Spores were washed until all cell debris and vegetative cells had been removed (10, 22). The spores were heat activated at 80°C for 20 min, unless otherwise noted, and then diluted with 10 mM Tris-HCl buffer (pH 8.4). Germination was initiated by the addition of L-alanine to 10 mM or AGFK (L-asparagine, D-glucose, D-fructose, and KCl) to final concentrations of 10, 1, 1, and 10 mM, respectively. At appropriate times, the  $A_{580}$  of the mixture was measured. If necessary, the amounts of dipicolinic acid and/or hexosamine released from spores during germination were determined (22). The supernatant (1 ml) was used for measurement of the released dipicolinic acid as described by Nicholson and Setlow (16). The rest of the

supernatant was dried with a concentrator (model CC-180; TOMY), followed by measurement of the released reducing groups by the method of Elson and Morgan, with *N*-acetylglucosamine as a standard (16).

**$\beta$ -Galactosidase assay.** The  $\beta$ -galactosidase assay was performed basically as described by Shimotsu and Henner (23). One unit of  $\beta$ -galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from 2-nitrophenyl- $\beta$ -D-galactopyranoside in 1 min.

**Northern blot analysis.** *B. subtilis* cells (optical density at 600 nm of 15 to 20) cultured in DSM medium were harvested and then suspended in 1 ml of chilled killing buffer (20 mM Tris-HCl [pH 7.5] containing 5 mM MgCl<sub>2</sub>). After centrifugation at 11,000  $\times$  g for 2 min, the pellet was suspended in 1 ml of SET buffer

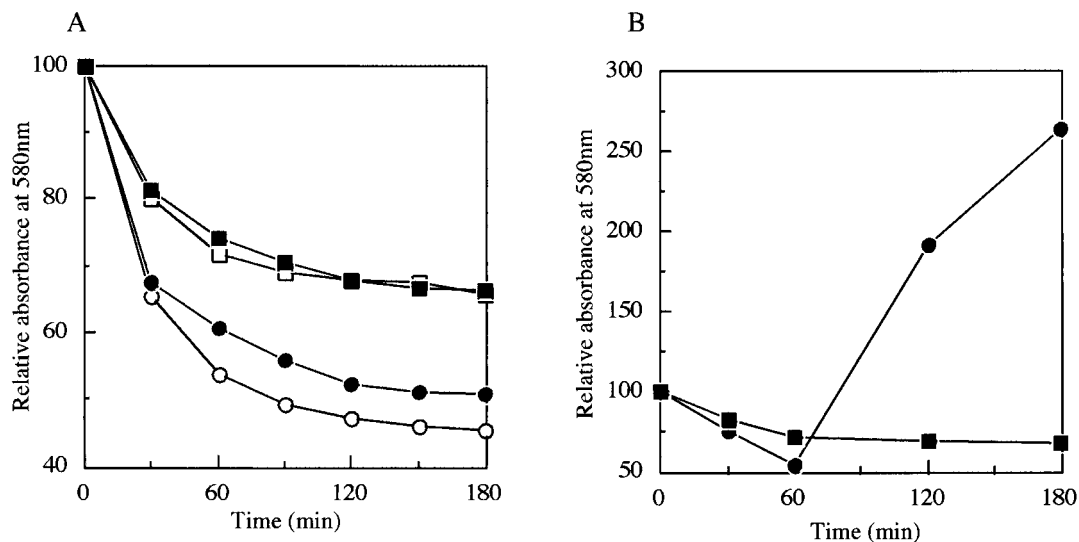


FIG. 2. Spore germination and outgrowth of *B. subtilis* YFJSd and 168. (A) Spore germination was monitored at 580 nm after the addition of L-alanine (closed symbols) or AGFK (open symbols) as a germinant. (B) Outgrowth of *B. subtilis* YFJSd and strain 168 spores in LB medium at 37°C was monitored at 580 nm. Circles, strain 168; squares, YFJSd.

(20% [wt/vol] sucrose, 50 mM EDTA, 20 mM Tris-HCl [pH 7.6]) containing lysozyme (final concentration, 6 mg/ml) (29). After incubation for 9 to 12 min at 0°C, the suspension was centrifuged at  $11,000 \times g$  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. (19). The transfer of RNAs onto a nylon membrane (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 with pGEM-fjS DNA, containing the internal region of *pdaA*, as a template. The amplified fragment was digested with *Hind*III, and the resulting fragments were 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.

**RP-HPLC analysis of spore peptidoglycan.** Spores of *B. subtilis* strains 168HR (a laboratory strain of 168), HR(YFJS), and HR(YFJS CWLD) were produced

in CCY medium, and cortex extraction from dormant spores, muropeptide separation by reverse-phase high-performance liquid chromatography (RP-HPLC), and amino acid and mass spectrometry (MS) analyses were performed as previously described (4, 5).

**Phase and fluorescence microscopy.** Cells expressing green fluorescence were prepared as follows. After inoculation and incubation of YFJSd harboring pHYPRfjSG on LB agar plates containing erythromycin and tetracycline at 37°C for 10 h, each colony was suspended into 4 ml of NB medium containing tetracycline and then precultured at 37 or 30°C for 4 h. Then, the preculture was suspended in 50 ml of DSM medium containing tetracycline (final concentration, 10 µg/ml) to obtain a cell density ( $A_{600}$ ) of 0.01, followed by culturing at 37 or 30°C until  $t_{20}$ . After centrifugation of the culture, cells were washed and stained with 4,6-diamidino-2-phenylindole (DAPI; final concentration, 1.0 µg/ml; Wako). For fluorescence microscopy, an Olympus BX61 microscope was used with a BX-UCB control unit and an UPPlan Apo Fluorite phase-contrast objective (magnification,  $\times 100$ ; numerical aperture, 1.3). The dichroic mirror cube units for GFP and DAPI contained a wide-band-pass (330 to 385 nm) excitation

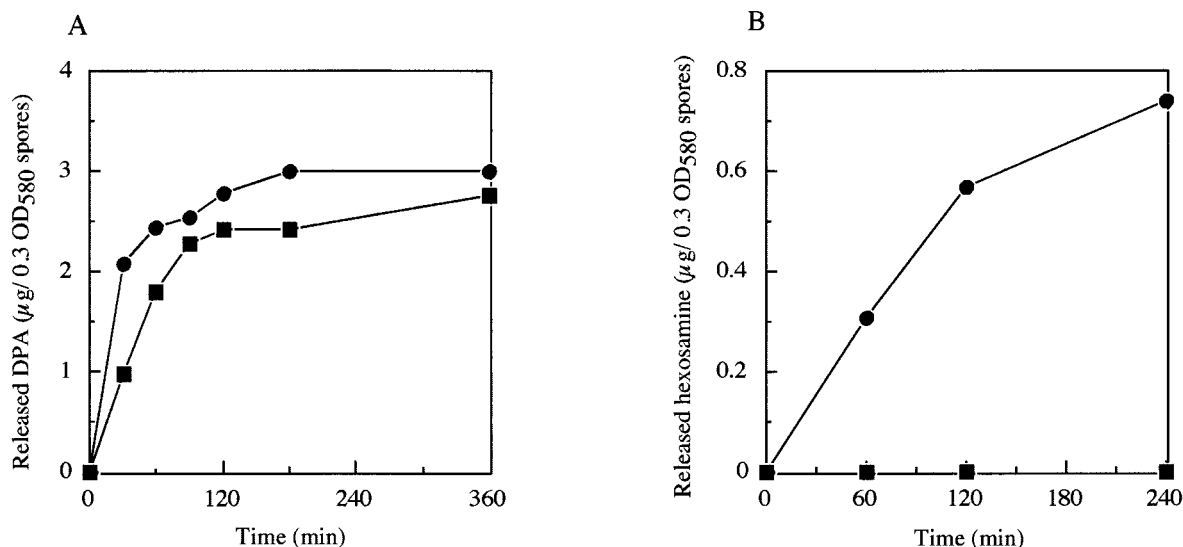


FIG. 3. Dipicolinic acid (DPA) (A) and hexosamine (B) released from *B. subtilis* YFJSd and 168 spores during germination. Released DPA and hexosamine were measured after the addition of L-alanine as a germinant. Circles, strain 168; squares, YFJSd.

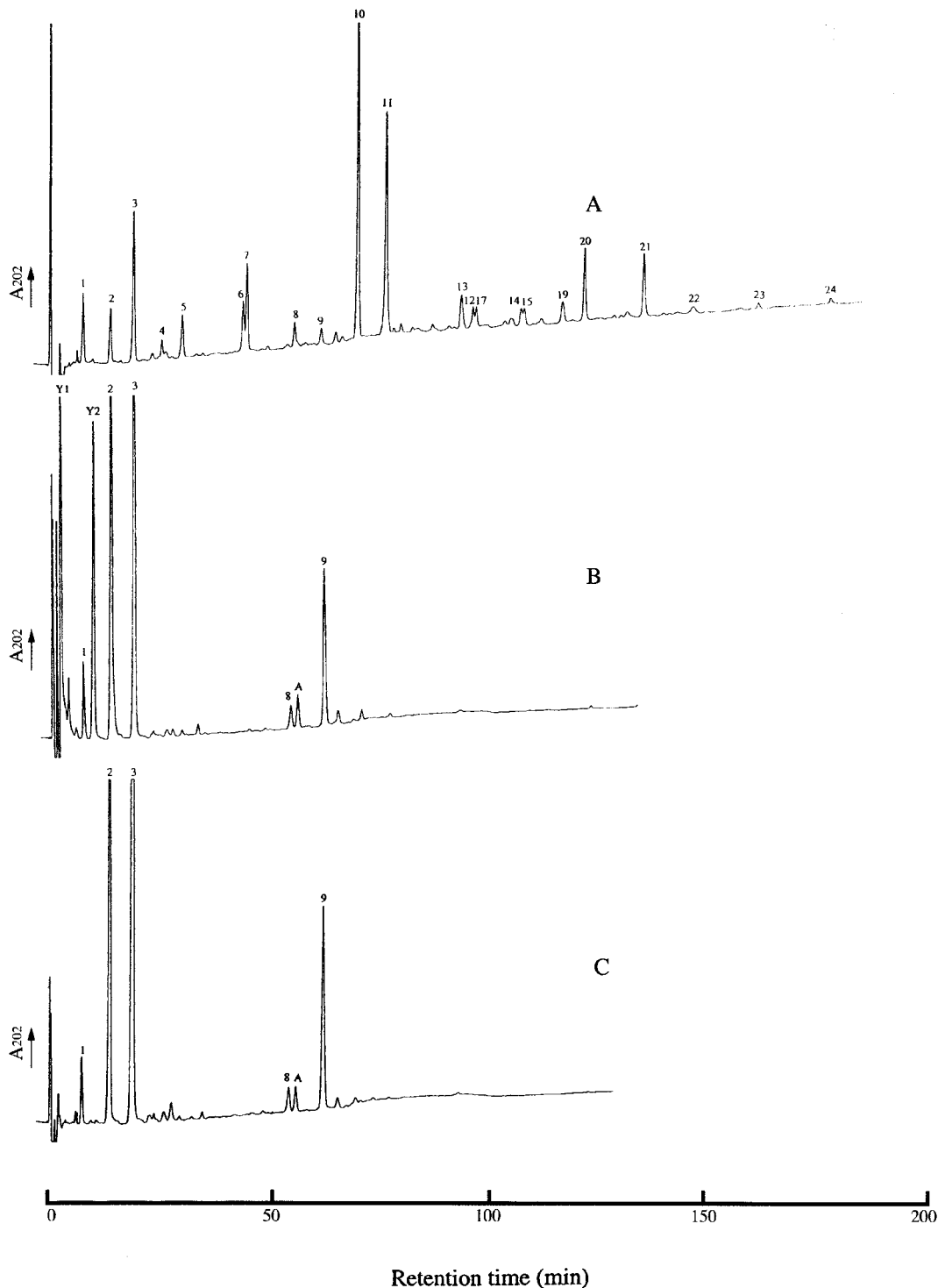


FIG. 4. Analysis of the peptidoglycan composition by RP-HPLC. Samples of peptidoglycan from endospores of wild-type *B. subtilis* strain 168HR (A), a *pdaA* mutant HR(YFJS) (B), and a *pdaA cwlD* mutant HR(YFJS CWLD) (C) were digested with Cellosyl, and the resulting muropeptides were separated by RP-HPLC. The numbered muropeptides were previously identified (4).

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). The exposure times for phase-contrast, DAPI, and GFP detection were 0.1, 0.003, and 1 s, respectively. Cells were photographed by both fluorescence and phase-contrast microscopy by using a charge-coupled device 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.

**Complementation analysis.** Cultures of strains 168(pHYCM1), YFJSd(pHYCMfjS), YFJSd(pHYCMΔSfjS), and YFJSd(pHYCM1) were treated by heating (80°C, 20 min) and then plated on LB agar plates. After the LB plates had been kept at 37°C for 10 h, the colonies were counted. Each sporulation ratio was

TABLE 2. Complementation of *pdaA* with a plasmid containing the entire *pdaA* gene or the putative signal peptide-deficient *pdaA* gene<sup>a</sup>

Strain	Plate assay		Microscopic observation		Germination ratio (%)
	No. of outgrowing spores	Spores	Vegetative cells	Sporulation ratio (%)	
YFJSd (pHYCMfjS)	$5.57 \times 10^7$	$2.85 \times 10^8$	$10.2 \times 10^7$	73.6	19.5
YFJSd (pHYCMΔSfjS)	$9.8 \times 10^4$	$5.15 \times 10^8$	$6.72 \times 10^7$	88.5	0.019
YFJSd (pHYCM1)	$2.0 \times 10^3$	$2.53 \times 10^8$	$11.5 \times 10^7$	68.8	0.0008
168 (pHYCM1)	$7.15 \times 10^7$	$2.94 \times 10^8$	$6.40 \times 10^7$	82.1	24.3

<sup>a</sup> YFJSd is a *pdaA* mutant. pHYCMfjS and pHYCMΔSfjS contained the entire *pdaA* gene and the putative signal peptide-deficient *pdaA* gene, respectively, downstream of the *citM* promoter region. pHYCM1 is a control plasmid (lacking the *pdaA* gene). Expression of the genes was carried out by the addition of 2 mM Mg-citrate at  $t_{0.5}$ , and spores were harvested at  $t_{48}$ . The plate assay was performed as described in Materials and Methods. The representative results for two independent clones from each strain are shown.

calculated on the basis of the number of spores and vegetative cells counted under the microscope. Each germination ratio was calculated on the basis of the number of colonies on an LB plate and the number of spores counted under a microscope.

## RESULTS

The *B. subtilis* genome contains five polysaccharide deacetylase homologues, one of which is the *yfjS* gene located at 74.2° on the *B. subtilis* chromosome (12, 31). YfjS (renamed PdaA) is a 263-amino-acid polypeptide with a predicted molecular mass of 30,069 Da. A consensus SD sequence ( $A_{-15}AGGAG_{-10}$ ; the numbering is with respect to the translational start codon) is present. The N-terminal 21-amino-acid sequence seems to be a signal sequence, because a positively charged amino acid, lysine, at position 2, a hydrophobic core comprising positions 7 to 18, and a typical signal sequence cleavage site,  $A_{19}XA_{21} \downarrow$  (the arrow indicates the cleavage point), are present. The upstream gene is *yffT*, but a good candidate for the rho-independent terminator ( $\Delta G = -20.8$  kcal/mol) is located between *yffT* and *yfjS*. Another typical rho-independent terminator ( $\Delta G = -18.0$  kcal/mol) is present downstream of *yfjS*. Therefore, *yfjS* seems to be transcribed as a monocistronic mRNA (Fig. 1A).

**Expression of the *pdaA* gene.** A *pdaA-lacZ* gene fusion strain, YFJSd, was constructed as described in Materials and Methods and cultured in DSM, and then  $\beta$ -galactosidase activity was determined. The activity was low but significant from  $t_4$  and was maximal at around  $t_6$  (Fig. 1B), which indicates that the *pdaA* gene is regulated by a sporulation-specific sigma factor. RNAs from four sigma factor-deficient (null) strains, 1S86 (SigF<sup>-</sup>), 1S60 (SigE<sup>-</sup>), spoIIIGΔ1 (SigG<sup>-</sup>), and 1S38 (SigK<sup>-</sup>), were analyzed by Northern blotting using an RNA probe containing the internal region of the *pdaA* gene. A weak hybridizing band was detected around 0.8 kb for the 168 strain and SigK mutant, but no such band was observed for the SigF<sup>-</sup>, SigE<sup>-</sup>, and SigG<sup>-</sup> mutants (Fig. 1C). Since the *pdaA* gene consists of 789 nucleotide residues (excluding the stop codon) and the distance between the two rho-independent terminators is approximately 980 nucleotide residues, the size of the transcript (0.8 kb) reasonably matched the estimated size of monocistronic mRNA. These results indicate that *pdaA* was transcribed by  $\sigma^G$  RNA polymerase. Since PdaA seems to contain a signal sequence, it may be expected that the enzyme might be transported into the cortex layer and modify it during sporulation.

**Characterization of a *pdaA* disruptant.** *pdaA*-deficient mutant YFJSd showed normal growth, cell separation, and motility and produced bright refractile spores. After heat activa-

tion at 80°C for 20 min, germination was measured by monitoring the decrease in the  $A_{580}$  of a spore suspension upon the addition of L-alanine or AGFK at 37°C (Fig. 2A). Spores of YFJSd responded to the germinants, and the  $A_{580}$  values of the spore suspensions slowly decreased by about 30%, in contrast with the decrease by about 50% for the wild-type strain (Fig. 2A). During the incubation, bright spores became phase-gray, and dipicolinic acid was released into the suspension. The amount released from YFJSd spores was not greatly different from that from the wild-type spores (Fig. 3A). However, there was a great difference in the release of hexosamine between the YFJSd and wild-type spores (Fig. 3B). A complete lack of hexosamine release has been reported for a mutant of *cwlD*, which is one of the homologous genes for cell wall-lytic *N*-acetylmuramoyl-L-alanine amidase. YFJSd did not show any outgrowth in the LB medium, like the *cwlD* mutant (ADD1) (Fig. 2B) (21).

**Muropeptide analysis of *pdaA*-disrupted spores.** Since the *pdaA*- and *cwlD*-deficient spores have similar phenotypes and the *cwlD*-deficient spores completely lack muramic acid  $\delta$ -lactam (4, 18), RP-HPLC of the spore cortex muropeptides from the *B. subtilis* wild-type strain, 168HR, and its congenic *yfjS* mutant, HR(YFJS), was performed (Fig. 4). There was a remarkable difference in peptidoglycan structure between the mutant and the wild-type strain. The HR(YFJS) peptidoglycan lacked all of the muropeptides containing  $\delta$ -lactam (muropeptides 4 to 7 and 10 to 24), which represent about 70% of the total components in the wild-type strain (Fig. 4) (4). There are increases in muropeptides 2 and 3, which are, respectively, disaccharide alanine and disaccharide tetrapeptide (4). Some novel muropeptides (Y1, Y2, and A) were also detected in HR(YFJS). Amino acid analysis of muropeptides Y1 and Y2 revealed that they are peptidoglycan derived and contain only *N*-acetylglucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), the two amino sugars of glycan strands. MS analysis of muropeptide Y1 in the positive and negative modes gave, respectively, fragment ions at  $m/z$  521.2 and 497.2. These values correspond, respectively, to  $[M+Na]^+$  and  $[M-H]^-$  molecular ions. The Y2 MS value is nearly double that of Y1, and the fragment ions of positive  $[M+Na]^+$  and negative  $[M-H]^-$  were observed, respectively, at  $m/z$  999.4 and 975.2. Based on the amino acid composition and MS results, muropeptides Y1 and Y2 are, respectively, a disaccharide (GlcNAc-MurNAc) and a tetrasaccharide (GlcNAc-MurNAc-GlcNAc-MurNAc). The MurNAc residues at the reducing ends of the disaccharide (Y1) and the tetrasaccharide (Y2) and position 2 of the tetrasaccharide are unsubstituted. Digestion of

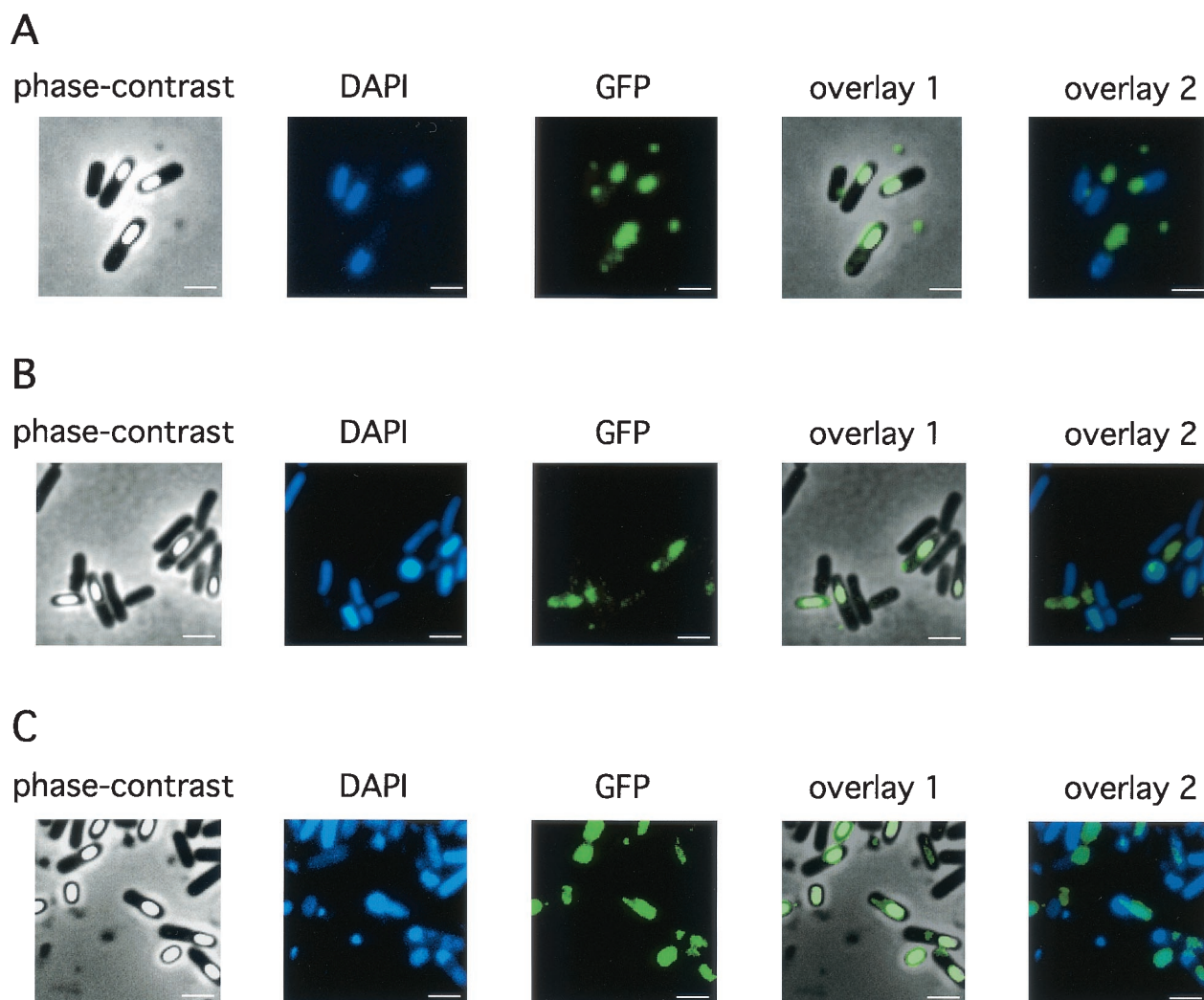


FIG. 5. Localization of the PdaA-GFP fusion protein in the wild-type strain (A) or *pdaA*-deficient mutant YFJSd (B and C). The DAPI or GFP fluorescence of cells at  $t_{20}$  was detected as described in Materials and Methods. The *pdaA-gfp*-containing plasmid (pHYPRfjSG) was used for transformation of *B. subtilis* 168 or YFJSd. Spores and sporangia were collected at  $t_{20}$  at 37 and 30°C in DSM medium (B and C, respectively). The culture at 30°C exhibited stronger fluorescence in spores than that at 37°C. Overlay 1 is a phase-contrast image overlaid on a GFP image, and overlay 2 is a DAPI-stained fluorescence image overlaid on a GFP image. Bars, 2  $\mu$ m.

the YFJSd cortex with a high concentration of lysozyme (2 mg/ml) resulted in a substantial decrease in muropeptide Y2 and an increase in muropeptide Y1 (data not shown). This demonstrates that the MurNAc-GlcNAc bond in muropeptide Y2 is susceptible to digestion, but only with a high concentration of hydrolase. Muropeptide A in Fig. 4 was coeluted with muropeptide A, previously detected for a *cwlD* mutant (4). Amino acid analysis and MS confirmed that this muropeptide is a disaccharide tripeptide disaccharide tetrapeptide and that it is identical to that found in the peptidoglycan of the *cwlD* mutant (4). This muropeptide is possibly amidated at the free carboxylic group of diaminopimelic acid.

The RP-HPLC spore cortex muropeptide profile of the *pdaA cwlD* mutant, HR(YFJS CWLD), is shown in Fig. 4C. The peaks of Y1 and Y2 are absent from the muropeptide profile of HR(YFJS CWLD), and the profile is identical to that of single mutant *cwlD* (4). *CwlD* was previously suggested to be

the amidase that cleaves off muropeptide side chains and is involved in  $\delta$ -lactam biosynthesis (2, 4, 18, 21).

**Complementation with *pdaA*-containing plasmids.** pHYCMfjS and pHYCM $\Delta$ SfjS, containing the entire *pdaA* gene and the signal sequence-deleted *pdaA* gene, respectively, were used to transform the *pdaA*-deficient mutant YFJSd. In the case of sporulation, the frequencies of YFJSd harboring pHYCMfjS and pHYCM $\Delta$ SfjS were not significantly different from those of YFJSd and strain 168 harboring a control plasmid, pHYCM1 (Table 2). But in the case of germination, YFJSd-(pHYCM1) almost completely lost the ability to germinate (germination ratio of 0.0008% on LB agar plates), whereas introduction of pHYCMfjS into the YFJSd strain complemented the germination-negative phenotype at nearly the wild-type level (Table 2). However, YFJSd(pHYCM $\Delta$ SfjS) only partially complemented the germination-negative phenotype (0.019% germination). In this system, the *pdaA* gene was ex-



FIG. 6. Alignment of PdaA and homologous gene products. The polysaccharide deacetylase family members, including PdaA, are aligned with a protein families database of alignments and hidden Markov models (Pfam, Sanger Institute). A seed sequence is shown as the first line. The name and accession number in the SWISS-PROT 40 database are shown at the left and right, respectively, of the alignment. The positions of the first and last amino acid residues of the sequence with respect to the N-terminal amino acid residue are shown after the name. Shading indicates amino acid residues identical to the highly conserved amino acid residues (capital letters) of the seed sequence. NodB, nodulation protein B (chitooligosaccharide deacetylase); NODB\_RHIME, NodB of *Rhizobium meliloti*; NODB\_BRAEL, *Bradyrhizobium elkanii*; NODB\_RHISN, *Rhizobium* sp. strain NGR234; NODB\_RHITR, *Rhizobium tropici*; NODB\_RHILV, *Rhizobium leguminosarum* bv. *viciae*; NODB\_RHILT, *R. leguminosarum* bv. *trifolii*; NODB\_RHIGA, *Rhizobium galegae*; NODB\_RHILP, *R. leguminosarum* bv. *phaseoli*; NODB\_AZOCA, *Azorhizobium caulinodans*; YFU2\_BACST, *B. stearothermophilus* YFU2; YFJS\_BACSU, *B. subtilis* PdaA; CDA1\_YEAST, *Saccharomyces cerevisiae* chitin deacetylase 1 (CDA1); CDA2\_YEAST, *S. cerevisiae* chitin deacetylase 2 (CDA2); CHDE\_MUCRO, *Mucor rouxii* chitin deacetylase (CHDE); XYND\_CELFI, *Cellulomonas fimi* xylanase D (XYND); AXEA\_STRLI, *Streptomyces lividans* acetylxyloxyesterase (AXEA); Q59300\_CELMI, *Cellvibrio mixtus* endo- $\beta$ -1,4-xylanase; Q59674\_PSUFL, *Pseudomonas fluorescens* endo- $\beta$ -1,4-xylanase; O53444\_MYCTU, *Mycobacterium tuberculosis* hypothetical protein; YJEA\_BACSU, *B. subtilis* YjeA; XYNU\_CLOTH, *Clostridium thermocellum* xylanase U (XynU); XYNC\_CELFI, *C. fimi* endoxylanase (XynC); YHEN\_BACSU, *B. subtilis* YheN; XYNA\_RUMAL, *Ruminococcus albus* xylanase A (XynA). Two of the PdaA paralogs (YlxY and YbaN) are not aligned because of a lower sequence similarity.

pressed with Mg-citrate probably both in the forespore and the mother cell. The results indicate that the signal sequence of *pdaA* is important for phenotype expression and they may suggest that the enzymatic reaction site is the cortex surrounded by two membranes.

**Localization of PdaA-GFP.** A GFP-encoding gene was fused to the 3' terminal of *pdaA*, and the resultant plasmid (pHYPRfjSG) was introduced into the wild-type 168 strain. Then, 168(pHYPRfjSG) was cultured in DSM at 37°C and samples were taken at  $t_{20}$ . 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. Strong fluorescence of GFP was found in sporangia at  $t_7$  (data not shown) and spores at  $t_{20}$  (Fig. 5A), but not in vegetative cells or mother cells (Fig. 5A; data not shown). pHYPRfjSG introduced into YFJSd also complemented the *yfjS*-deficient phenotype (plate assay was used; data not shown). Localization of GFP in YFJSd(pHYPRfjSG) was also found in sporangia and spores (Fig. 5B and C). These results indicate that the PdaA protein was localized in spores.

## DISCUSSION

The amino acid sequence of PdaA exhibits extensive similarity with those of polysaccharide deacetylases, including NodB chitooligosaccharide deacetylases, chitin deacetylases, and peptidoglycan *N*-acetylglucosamine deacetylase (8, 11, 14,

28). As to cellular function, these proteins act as bacterial nodulation signals in symbiosis (8), and they are involved in mature spore formation in yeast (14) and modification of peptidoglycan causing resistance to the hydrolytic action of lysozyme (28). Figure 6 shows the alignment of *pdaA* homologous genes. In *Bacillus stearothermophilus*, *yfu2* is highly homologous with *pdaA* (60% identity over 256 amino acid residues). In *B. subtilis*, five paralogs (*pdaA* [*yfjS*], *ybaN*, *yheN*, *yjeA*, and *ylxY*) are present, three of which are highly homologous and also aligned in Fig. 6. In the genera *Bacillus* and *Clostridium*, none of the paralogs except *pdaA* dealt with in this paper were functionally characterized. The *pdaA*-deficient mutant showed the almost complete loss of germination, whereas the spores were fully refractive. This and other profiles, including dipicolinic acid release and the complete loss of hexosamine release from spores, led to the idea that the mutation may be associated with cortex maturation, especially muramic  $\delta$ -lactam formation, as the previously characterized *cwld* mutant completely lacks muramic  $\delta$ -lactam (4, 18, 21).

A remarkable feature of the HR(YFJS) mucopeptide profile is the total lack of products containing  $\delta$ -lactam (Fig. 4). This peptidoglycan composition is similar to that of *cwld*; however, two extra products are present in HR(YFJS). The new mucopeptides have unsubstituted muramic acid residues that suggest that an amidase was cleaved off the side chains. YfjS is homologous to peptidoglycan de-*N*-acetylase, and thus it is likely to cleave the *N*-acetyl groups of muramic acid as an intermediate step of  $\delta$ -lactam biosynthesis. Two mechanisms



have been previously proposed for  $\delta$ -lactam biosynthesis: an amidase action followed by transacetylation, and de-*N*-acetylation followed by transpeptidation (27). In light of the present results, the first step of  $\delta$ -lactam formation appears to be the cleavage of mucopeptide side chains, most likely that of tetrapeptides by CwID, followed by de-*N*-acetylation of muramic acid residues by YfjS. The last step in  $\delta$ -lactam formation would be via transpeptidase activity that gives the final structure. The fact that double mutant *cwID pdaA* exhibits a mucopeptide profile identical to that of the single mutant *cwID* confirms that the first step of  $\delta$ -lactam formation is the cleavage of mucopeptide side chains by CwID. The  $\delta$ -lactam was recently shown to serve as a substrate recognition signal for the different GSLEs involved in cortex hydrolysis during germination (2, 5, 18). Spores lacking this cortex-specific moiety neither hydrolyze peptidoglycan nor outgrow (2, 5, 18). This correlates with the phenotype of the *pdaA* mutant spores.

The *pdaA* gene is transcribed by  $\sigma^G$  RNA polymerase as a monocistronic mRNA (Fig. 1). Upstream of the *pdaA* gene are the sequences G<sub>-70</sub>CATA<sub>-66</sub> and C<sub>-49</sub>AAAGTC<sub>-43</sub> (the numbering is with respect to the translational start codon), with a spacing of 16, similar to the consensus sequences of -35 and -10 (GNATR and CATNNTA, respectively; R stands for A or G, and N stands for nonspecific nucleotide) of  $\sigma^G$  with a spacing of 15 (9). The SD sequence (AAGGAG) is located 14 to 9 bp upstream of the translational start codon. Coupled with the GFP fusion data, this shows that *pdaA* is a forespore-expressed gene. The product is exported into the developing cortex, where it acts with CwID to produce muramic  $\delta$ -lactam residues. How the final structure is attained is unknown and may require the action of an independent transpeptidase.

This is the first report that a polysaccharide deacetylase homologue is associated with *Bacillus* sporulation and germination. The other polysaccharide deacetylase homologues are now being investigated as to whether they are associated with differentiation or vegetative growth.

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