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Synthesis and Characterization of the Spore Proteins of *Bacillus subtilis* YdhD, YkuD, and YkvP, Which Carry a Motif Conserved among Cell Wall Binding Proteins[1]

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We have previously reported that YaaH and YrbA are spore proteins of *Bacillus subtilis* that are required for spore resistance and/or germination and that they have a motif conserved among so-called cell wall binding proteins [Kodama *et al.* (1999) *J. Bacteriol.* 181, 4584–4591, Takamatsu *et al.* (1999) *J. Bacteriol.* 181, 4986–4994]. In this study, we analyzed the expression of *ydhD*, *ykuD*, and *ykvP* genes, which encode putative proteins containing the same motif. Transcription of *ydhD* was dependent on SigE, and the mRNA was detectable from 2 h after the cessation of logarithmic growth (T_2 of sporulation). *ykuD* was transcribed by SigK RNA polymerase from T_4 of sporulation. Both SigK and GerE were essential for *ykvP* expression, and this gene was transcribed from T_5 of sporulation. Inactivation of these genes by insertion of an erythromycin resistance gene did not affect vegetative growth, spore resistance to heat, chloroform, and lysozyme, or spore germination in the presence of L-alanine or in a mixture of L-asparagine, D-glucose, D-fructose, and potassium chloride. The His tag fusions of YdhD, YkuD, and YkvP downstream of their natural promoter regions were introduced into a multicopy plasmid. These fusion proteins were produced during sporulation in *B. subtilis* transformants and were detected in mature spores, indicating that YdhD, YkuD, and YkvP are all proteins intrinsic to spores. Excessive YkuD and YkvP in the sporulating cells did not affect spore resistance or germination. The cells producing excessive YdhD also did not show impaired spore resistance, but their germination properties were changed: the spores revealed reduced response to L-alanine and some of them germinated even without germinants. *Escherichia coli* β-lactamase, whose signal sequence had been genetically replaced by the cell wall binding motif of YaaH, was produced in sporulating cells, and Western blot analysis indicated that the fused protein was assembled into spores. We speculate that the conserved motif functions as a kind of signal sequence involved in assembly of these proteins on forespores.

Key words: *B. subtilis*, cell wall binding motif, spore protein.

Bacterial sporulation is a relatively simple model for cell differentiation, and its progress is marked by sequential and drastic changes in the physiological state of the cell. When nutrients are exhausted, the gram-positive soil microorganism *Bacillus subtilis* initiates sporulation by dividing asymmetrically. After asymmetric septation, the resultant larger and smaller cells are the mother cell and the forespore, respectively. As development proceeds, the mother cell engulfs the forespore and eventually lyses, releasing the mature spore. Mature spores are resistant to long periods of starvation, heat, toxic chemicals, lytic enzymes, and other factors that could damage a cell (*1*). Spores germinate and start growing when surrounding nutrients become available (*2*). Genes involved in this developmental system have been identified, and their biological functions have been analyzed (*3–6*). These genes are mostly transcribed during sporulation by RNA polymerase containing developmentally specific sigma factors; these sigma factors, including SigF, SigE, SigG, and SigK, are temporally and spatially activated and regulate gene expression in a compartment-specific fashion (*3*, *4*, *6–8*). The *B. subtilis* genome sequencing project revealed about 4,100 protein-encoding genes, of which half have unknown functions (*9*). The identification of these genes will contribute useful information to the study of sporulation, germination, and spore dormancy of *Bacillus* at the gene level.

We have previously reported that *B. subtilis* YaaH and YrbA are spore proteins involved in spore resistance and/or germination and that these proteins have the motif conserved among so-called cell wall binding protein (*10*, *11*) (Fig. 7). This motif has been identified from some bacterial and phage proteins which are involved in cell lysis (*12–16*).

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We found that at least 10 proteins of *B. subtilis*, including XkdP, XlyA, XlyB, YocH, YojL, YdhD, YhdD, YkuD, YkvP, and YqbP, also have the same motif (*10*). The properties and functions of these proteins or the genes encoding them are reported as follows: transcription of *yhdD* gene is regulated by SigD RNA polymerase, and its product, YhdD (CwlE/LytF), is a cell wall binding protein involved in cell wall hydrolysis (*17*). YdhD shows significant similarity to LytE and YojL (*9*, *17*, *18*). XlyA is an *N*-acetylmuramoyl-L-alanine amidase involved in defective prophage PBSX-mediated lysis and shows about 50% identity to XlyB in its primary sequence (*19*, *20*). These facts suggest that proteins having the cell wall binding motif play important roles in morphological events during the cell cycle in *B. subtilis*. We identified additional sporulation-specific genes, *ydhD*, *ykuD*, and *ykvP*, and their expression and function are described herein.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and General Techniques—The *B. subtilis* and *Escherichia coli* strains used in this study are listed in Table I. ASK202, ASK203, ASK204, and ASK205 are derivatives of *B. subtilis* strain 168 (wild type) transformed with DNA from *spoIIAC*, *spoIIGAB*, *spoIIIG*, and *spoIVCB* mutants, respectively, obtained from P. Stragier. Oligonucleotide primers YDHDF (5′-AAGAAGCTTATCCATATCGTCGGGC-3′) and YDHDR (5′-GGAGGATCCTCTAATGGCACAAACGC-3′) were used to amplify a 206 bp segment internal to *ydhD* from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Bam*HI and *Hin*dIII sites introduced by the primers and inserted into *Bam*HI- and *Hin*dIII-restricted pMutinT3 to create plasmid pMU1211. Oligonucleotide primers YKUDF (5′-AAGAAGCTTGAAGCAAGGTGATACAC-3′) and YKUDR (5′-GGAGGATCCGATTGAGACTGCGAT-3′) were used to amplify a 173 bp segment internal to *ykuD* from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Bam*HI and *Hin*dIII sites introduced by the primers and inserted into *Bam*HI- and *Hin*dIII-restricted pMutinT3 to create plasmid pMU1212. Oligonucleotide primers YKVPF (5′-AAGAAGCTTGAGAGTCATCCCATGTG-3′) and YKVPR (5′-GGAGGATCCCTTGAATGCTCTGGTG-3′) were used to amplify a 167 bp segment internal to *ykvP* from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Bam*HI and *Hin*dIII sites introduced by the primers and inserted into *Bam*HI- and *Hin*dIII-restricted pMutinT3 to create plasmid pMU1213. pMutinT3 was pMutin1 into which the t1t2 terminator from the *rrnB* operon of *E. coli* had been introduced between the erythromycin resistance gene and the *spac* promoter (*21*, *22*). pMU1211, pMU1212, and pMU1213 were introduced into strain 168 by transformation, a single crossover with selection for erythromycin resistance (0.5 μg/ml) yielding the strains ASK211, ASK212, and ASK213 respectively. Oligonucleotide primers YDHDM624 (5′-GCTCGAGAAAGATGGTTTGTTAAAAGC-3′) and YDHD1317R (5′-TAGATCTTTCTGCTGCTCTGGACTTAATC-3′) were used to amplify a 1,941-bp segment including the *ydhD* gene and its 5′ upstream region from the *B. subtilis* 168 chromosome (Fig. 2a). The PCR product was restricted at the *Xho*I and *Bgl*II sites introduced by the primers and inserted into *Xho*I- and *Bgl*II-restricted pTUBE1200H6 to create plasmid pYDHD8. Oligonucleotide primers YKUDM822 (5′-TCTCGAGCCGCATATGATCGA-3′) and YKUD491R (5′-AAGATCTCCGGTTAATCGTGACTCTCG-3′) were used to amplify a 1,313-bp segment including the *ykuD* gene and its 5′ upstream region from the *B. subtilis* 168 chromosome (Fig. 2b). The PCR product was restricted at the *Xho*I and *Bgl*II sites introduced by the primers and inserted into *Xho*I- and *Bgl*II-restricted pTUBE1200H6 to create plasmid pYKUD8. Oligonucleotide primers YKVPM1074 (5′-TCTCGAGAACGTAACCGGTGTTCAAGG-3′) and YKVP1196R (5′-TAGATCTGAATAATTTGTTATTAATATTTCTAAAC-3′) were used to amplify a 2,270-bp segment including the *ykvP* gene and its 5′ upstream region from the *B. subtilis* 168 chromosome (Fig. 2c). The PCR product was restricted at the *Xho*I and *Bgl*II sites introduced by the primers and inserted into *Xho*I and *Bgl*II restricted pTUBE1200H6 to create plasmid pYKVP8. pTUBE1200H6 is a multicopy

TABLE I. **Bacterial strains and plasmids.**

Strains and plasmids	Genotype or description	Source and/or reference(s)
B. subtilis		
168	*trpC2*	BGSC*
ASK211	*trpC2, ydhD*Ω(pMU1211 (*erm*))	This work
ASK212	*trpC2, ykuD*Ω(pMU1212 (*erm*))	This work
ASK213	*trpC2, ykvP*Ω(pMU1213 (*erm*))	This work
ASK202	*trpC2, pheA1, spoIIAC::kan*	Kodama *et al.* (*10*)
ASK203	*trpC2, pheA1, spoIIGAB::kan*	Kodama *et al.* (*10*)
ASK204	*trpC2, pheA1, spoIIIG::kan*	Kodama *et al.* (*10*)
ASK205	*trpC2, pheA1, spoIVCB::erm*	Kodama *et al.* (*10*)
4751	*leu-2, gerE36*	BGSC*
Plasmids		
pMutinT3	*bla, erm,* $t_1t_2t_0$ *Pspac, lacZ, lacI*	Moriya *et al.* (*21*)
pMU1211	*bla, erm,* t_0 *ydhD-lacZ, lacI, Pspac-'ydhD*	This work
pMU1212	*bla, erm,* t_0 *ykuD-lacZ, lacI, Pspac-'ykuD*	This work
pMU1213	*bla, erm,* t_0 *ykvP-lacZ, lacI, Pspac-'ykvP*	This work
pTUBE1200H6	*tet*	Takamatsu *et al.* (*23*)
pBLA8	*tet, bla(40-285 a.a.)-His*	This work
pYAAH2B	*tet, yaaH(1-2 a.a.)-bla(40-285 a.a.)-His*	This work
pYAAH97B	*tet, yaaH(1-97 a.a.)-bla(40-285 a.a.)-His*	This work
pYDHD8	*tet, ydhC', ydhD-His*	This work
pYKUD8	*tet, ykuE', ykuD-His*	This work
pYKVP8	*tet, ykvO', ykvP-His*	This work

*BGSC, *Bacillus* Genetic Stock Center.

vector having a tetracycline resistance gene, a multicloning site, and a replication origin, pAMα1, which is active in *B. subtilis* cells (*23*). Using pUC18 as the template and two oligonucleotides, BLA116 (5′-TGAAGATCACTCGAGTGCACGA-3′) and BLA855R (5′-GGTCTGACAGAGATCTATGCTTAAT-3′), a DNA fragment (740-bp) encoding the mature region of β-lactamase (amino acid position from 40 to 285) was amplified by PCR, digested by *Xho*I and *Bgl*II, and inserted into the *Xho*I–*Bgl*II site of pTUBE1200H6 containing a DNA region for a His tag to construct plasmid pBLA8. Oligonucleotide primers YAAHM558 (5′-GATCTAGAGGAAACCCTCGCTAAA-3′) and YAAH5R (5′-TCTCGAGACCACATAAATTTGAATGAAAAACG-3′) were used to amplify a 563-bp segment including the initiation codon of *yaaH* gene and its promoter region from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Xba*I and *Xho*I sites introduced by the primers and inserted into *Xba*I- and *Xho*I-restricted pBLA8 to create plasmid pYAAH2B. Oligonucleotide primers YAAHM558 and YAAH290R (5′-TCTCGAGACCACATAAATTTGAATGAAAAACG-3′) were used to amplify a 848-bp segment including the region encoding N-terminal 97 amino acid residues of YaaH protein and *yaaH* promoter from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Xba*I and *Xho*I sites introduced by the primers and inserted into *Xba*I- and *Xho*I-restricted pBLA8 to create plasmid pYAAH97B. pYDHD8, pYKUD8, pYKVP8, pYAAH2B, and pYAAH97B were introduced into strain 168 with selection for tetracycline resistance (20 μg/ml) to produce the transformants pYDHD8/168, pYKUD8/168, pYKVP8/168, pYAAH2B/168, and pYAAH97B/168, respectively. *B. subtilis* strains were grown in Luria-Bertani (LB) and DS media (*24*). *E. coli* was grown in LB medium. The conditions for sporulation of *B. subtilis* have been described previously (*25*). Recombinant DNA methods were carried out as described previously (*26*). Methods for preparing competent cells, for transformation, and for the preparation of chromosomal DNA of *B. subtilis* have been described previously (*27*).

Northern Analysis—The cells were grown in DS medium at 37°C, and an aliquot was harvested by centrifugation. Total RNA was extracted from the cells as described previously (*28*). Aliquots containing 5 μg of total RNA were electrophoresed and blotted on a positively charged nylon membrane (Hybond-N+, Amersham). Hybridizations were performed with digoxigenin-labeled RNA probes (10 ng) according to the manufacturer's recommended procedure (Boehringer Mannheim Biochemicals). Hybridizations specific for *ydhD, ykuD,* and *ykvP* mRNAs were conducted with digoxigenin-labeled RNA probes synthesized *in vitro* with T7 RNA polymerase using as templates PCR products amplified from pMU1211, pMU1212, and pMU1213, respectively. The primers used to introduce a promoter for T7 RNA polymerase for these amplifications were YDHDF, YKUDF, YKVPF, and T7R (5′-TAATACGACTCACTATAGGGCGAAGTGTATCAACAAGCTGG-3′).

Preparation of Spores—*B. subtilis* spores were purified as described previously (*29*). Mature spores were prepared by culturing the bacteria in DS medium for 48 h at 37°C. The spores were harvested by centrifugation and purified by two washes in cold deionized water, lysozyme treatment (0.1 mg lysozyme/ml) at 37°C for 10 min, and sonication (NISSEI US-300 Ultrasonic Generator) six times at 4°C for 15 s each time. The resultant cells were washed with cold deionized water by repeated centrifugation until all cell debris and vegetative cells had been removed.

Spore Resistance—Cells were grown in DS medium at 37°C for 18 h after the end of exponential growth, and spore resistance was assayed as follows. The cultures were heated at 80°C for 30 min, treated with lysozyme (final concentration, 0.25 mg/ml) at 37°C for 10 min, or treated with 10% (v/v) chloroform at room temperature for 10 min as described previously (*30*), diluted in distilled water, plated on LB agar, and incubated overnight at 37°C. The numbers of survivors were determined by counting colonies.

Spore Germination—The purified spores were heat-activated at 65°C for 15 min, then suspended in 50 mM Tris-HCl (pH 7.5) buffer to an optical density at 660 nm of 0.5. Either L-alanine (10 mM) or AGFK (3.3 mM L-asparagine, 5.6 mM D-glucose, 5.6 mM D-fructose, and 10 mM potassium chloride) was added. Germination was monitored by measurement of the decrease in the optical density at 660 nm of the spore suspension at 37°C for up to 90 min.

Solubilization of Proteins from Sporulating Cells and Mature Spores—For preparation of protein-containing extracts from sporulating cells, cultures (5 ml) were harvested every hour throughout sporulation and washed with 10 mM sodium phosphate buffer (pH 7.2). The pellets were suspended in 0.1 ml of lysozyme buffer (10 mM sodium phosphate [pH 7.2], 1% [w/v] lysozyme), kept on ice for 5 min, solubilized in 0.1 ml of loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue), and boiled for 5 min. For preparation of proteins from mature spores, spores were harvested at 18 h after the cessation of logarithmic growth (T_{18}) and washed with 10 mM sodium phosphate buffer (pH 7.2). The pellets were suspended in 0.1 ml of lysozyme buffer, incubated at room temperature for 10 min, then washed with wash buffer (10 mM sodium phosphate [pH 7.2], 0.5 M NaCl). Spore proteins were solubilized in 0.1 ml of loading buffer and boiled for 5 min. The resulting samples were analyzed by SDS-PAGE (15% [w/v] acrylamide) as described previously (*29*).

Immunoblotting—Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; 0.45-μm pore size; Millipore) and detected by use of rabbit immunoglobulin G against the His tag (Qiagen) and β-lactamase (5′-3′ Inc. Co.) as the first antibody and donkey anti–rabbit IgG–horseradish peroxidase conjugate as the second antibody (Amersham). The antisera were diluted to 1:1,000 or 1:5,000 with 20 mM Tris-HCl (pH 7.6) buffer containing 0.8% (w/v) NaCl and 0.5% (v/v) Tween 80.

RESULTS

Identification of Genes Transcribed Only at Sporulation—We have previously reported that *Bacillus subtilis xkdP, xlyA, xlyB, yaaH, ydhD, yhdD, ykuD, ykvP, yocH, yojL, yrbA,* and *yqbP* are deduced to encode polypeptides that have the motif conserved among so-called cell wall binding proteins (*10*). Here we analyzed transcription of *yhdD, ykuD,* and *ykvP,* because the others had already been characterized and/or their functions could be deduced from the primary sequence (*9, 10, 11, 17, 19*). Transcription of *yqbP* was also analyzed, but no mRNA hybridizing to a probe specific to this gene was found (data not shown). To

confirm gene expression pattern and transcription unit, total RNA was isolated from *B. subtilis* 168 (wild type) and analyzed by Northern hybridization. The data in Fig. 1 show that mRNA species of *ydhD, ykuD*, and *ykvP* that hybridized with each specific probe were first detected from T_2, T_4, and T_5 of sporulation, respectively. The gene organization surrounding *ydhD, ykuD*, and *ykvP*, and the sizes of the ORFs are shown in Fig. 2. The majority of genes induced during sporulation are transcribed by RNA polymerase containing sporulation-specific sigma factors (*3*, *4*, *6–8*). To determine which sigma factor was concerned with the transcription of these genes, we performed Northern analysis with RNA prepared from sigma factor–deficient mutants. A probe specific for *ydhD* hybridized to a 1.3 kb mRNA in samples prepared from wild-type cells. This mRNA molecule was not detectable in *spoIIAC* and *spoIIGAB* mutants, which were deficient in SigF and SigE, respectively. On the other hand, the signal was still detectable in *spoIIIG* and *spoIVCB* mutants, which were deficient in SigG and SigK, respectively (Fig. 1a). According to the sigma cascade, the sporulation-specific sigma factors SigF, SigE, SigG, and SigK become active in order in sporulating cells of *B. subtilis* (*3*, *4*, *6*). The results shown in Fig. 1 suggest that expression of *ydhD* is dependent on SigE. A probe specific for *ykuD* hybridized to a 0.9 kb mRNA in samples prepared from wild-type cells. This mRNA molecule was not detectable in *spoIIAC, spoIIGAB, spoIIIG*, and *spoIVCB* mutants. On the other hand, the signal was still detectable in a *gerE* mutant (Fig. 1b). This result suggests that expression of *ykuD* is regulated by SigK. The band density of mRNA for *ykuD* was increased in the sample prepared from *gerE36* cells (lane 12), suggesting that GerE was a negative regulator for *ykuD*. A probe specific for *ykvP* hybridized to a 2.5 kb mRNA in samples prepared from wild-type cells. This mRNA molecule was not detectable in *spoIIAC, spoIIGAB, spoIIIG, spoIVCB*, and *gerE* mutants (Fig. 1c). The smaller bands in the wild-type cell (168) in Fig. 1c were probably generated by degradation of the 2.5 kb mRNA. This result suggested that expression of *ykvP* was regulated by SigK and dependent on GerE.

Detection of the His Tag Fusion Proteins in Sporulating Cells and Mature Spores—As described in our previous paper, use of a particular gene product with His tag fusion provides useful information about the assembly of the particular protein (*10*). pTUBE1200H6, a multicopy vector

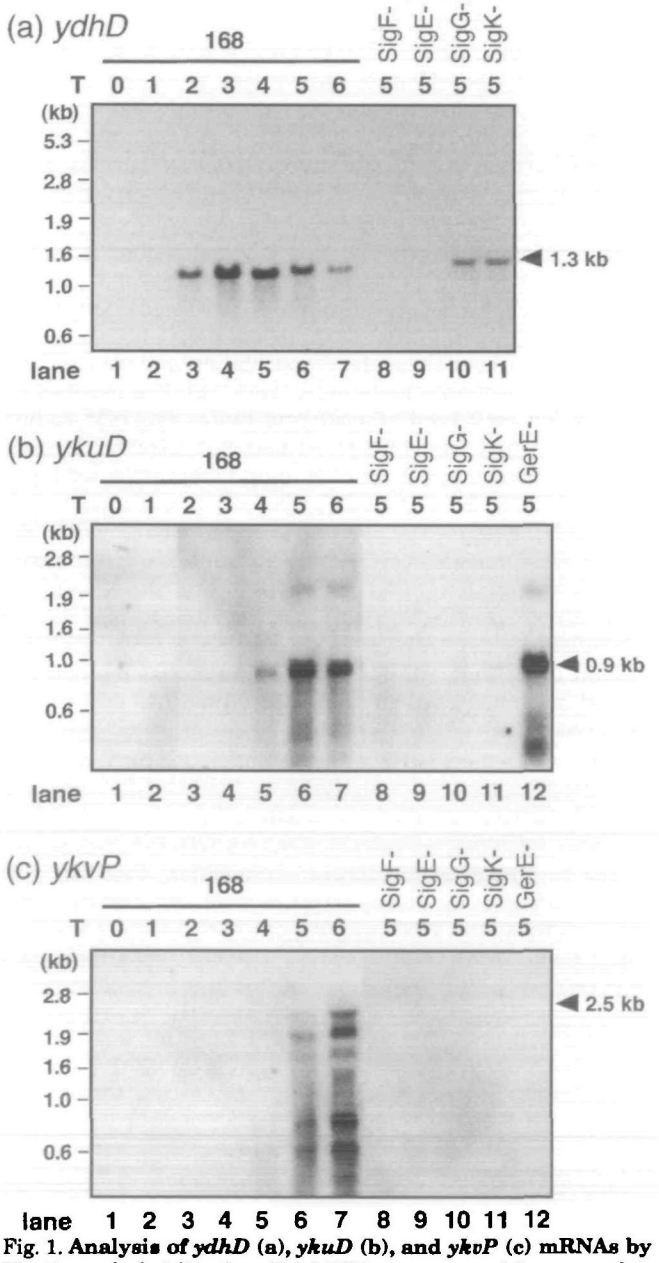

Fig. 1. Analysis of *ydhD* (a), *ykuD* (b), and *ykvP* (c) mRNAs by Northern hybridization. Total RNA was prepared from sporulating cells, and each mRNA was detected by Northern blotting. The arrowheads indicate the position of each mRNA hybridizing with the digoxigenin-labeled RNA probe. Lanes 1 through 7, total RNA isolated from strain 168. T, harvesting times of cells, *i.e.*, hours after the end of the exponential phase of growth. Transcription in *spoIIAC* ($SigF^-$) (lane 8), *spoIIGAB* ($SigE^-$) (lane 9), *spoIIIG* ($SigG^-$) (lane 10), *spoIVCB* ($SigK^-$) (lane 11), and *gerE36* ($GerE^-$) (lane 12) mutants at T_5 were also analyzed by Northern hybridization.

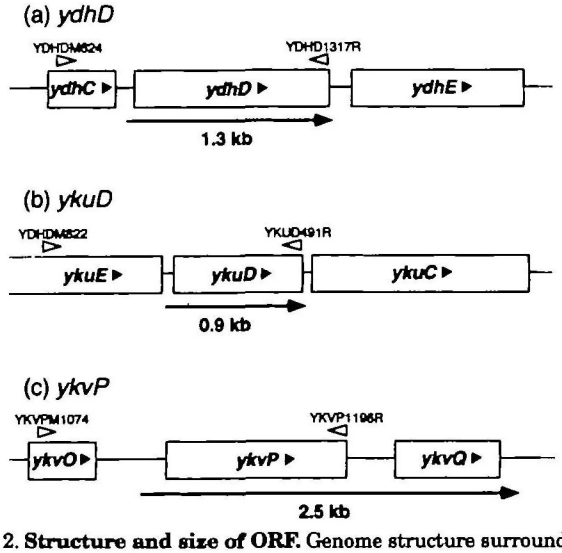

Fig. 2. Structure and size of ORF. Genome structure surrounding *ydhD* (a), *ykuD* (b), and *ykvP* (c), and the sizes of the ORFs are shown, respectively. The filled arrowheads indicate the direction of transcription, and the open arrowheads indicate the positions and directions of PCR primers used for amplification of each gene as described in "MATERIALS AND METHODS." The arrows indicate the direction and length of transcription.

available in both *E. coli* and *B. subtilis* (*23*), was used as an expression vector, and His tag fusion proteins and His tag-specific antiserum were used to detect YdhD, YkuD, and YkvP in this study. pTUBE1200H6 has a tetracycline resistance gene and a multicloning site followed by a sequence encoding six consecutive histidine residues. To analyze the synthesis and location of YdhD, YkuD, and YkvP in sporulating cells, plasmids pYDHD8, pYKUD8, and pYKVP8 containing each gene and promoter region were constructed. The cloned ORFs are fused in frame to a sequence encoding six consecutive histidine residues (His tag) at its 3′ end, and the product is detectable with antiserum specific for the tag. These multicopy plasmids, which potentially produce more fusion proteins than a single copy gene on the chromosome, were introduced into *B. subtilis* 168 (wild type) to generate transformants pYDHD8/168, pYKUD8/168, and pYKVP8/168, respectively. YaaH-His was produced from T_2 of sporulation in pYAAH8/168 cells as previously described (*10*). YdhD-His (45 kDa) was produced from T_2 of sporulation in the transformant cells (Fig. 3a). YkuD-His (19 kDa) and YkvP-His (45 kDa) were produced from T_4 and T_5 of sporulation in the respective transformants (Fig. 3, b and c). No protein band reacts with anti-His tag serum in the sporulating cells carrying the control vector (*10*). The mature spores were collected at T_{18} of sporulating cells for purification, and protein preparations solubilized from the purified spores in the presence of SDS and mercaptoethanol were analyzed using anti–His tag antiserum. YdhD-His, YkuD-His, and YkvP-His were still detectable in the protein extracts of spores (Fig. 3, lanes 8). It is unlikely that the His tag fusion proteins were sticking to the surface of spores due to their overproduction, because the spores used here were washed in the presence of 0.5 M sodium chloride. These results suggest that YdhD, YkuD, and YkvP are proteins intrinsic to spores.

Properties of Mutant Spores—We characterized mutant cells of *ydhD, ykuD,* and *ykvP*; the vegetative growth of mutant cells in DS medium was the same as that of wild-type strain (data not shown). Mature spores of these mutants prepared from the medium after 24 h of cultivation showed resistance to heat, chloroform, and lysozyme, and germinated in the presence of L-alanine or AGFK as did

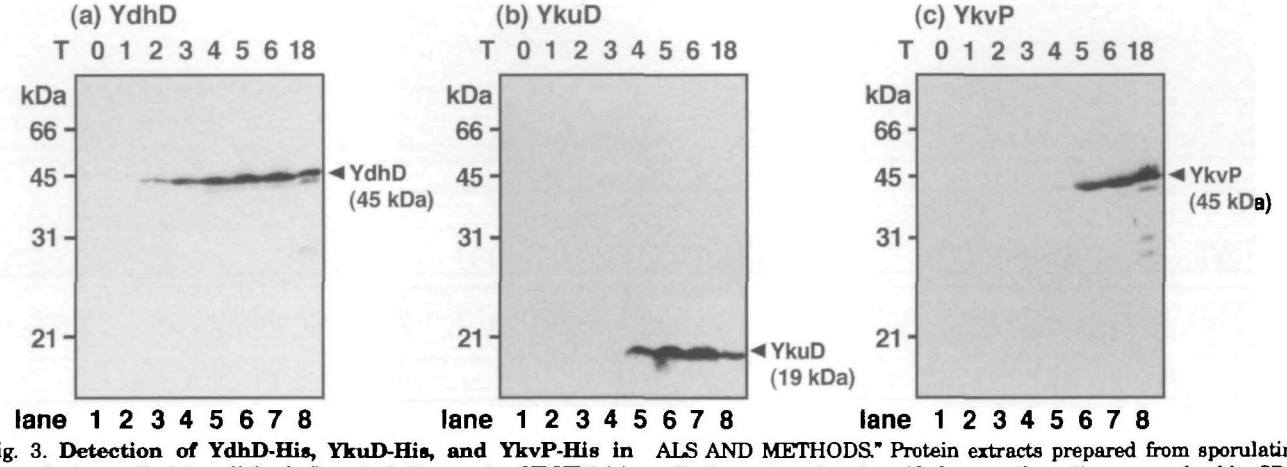

Fig. 3. **Detection of YdhD-His, YkuD-His, and YkvP-His in sporulating cells.** The *ydhD*, *ykuD*, and *ykvP* genes in pYDHD8 (a), pYKUD8 (b), and pYKVP8 (c) are regulated by a promoter located upstream of the gene and fused to a sequence encoding six consecutive histidine residues (His tag), respectively. *B. subtilis* wild-type cells (168) were transformed with these vectors as described in "MATERIALS AND METHODS." Protein extracts prepared from sporulating cells (lanes 1 to 7) and purified spores (lane 8) were resolved by SDS-PAGE (15% acrylamide gel) and visualized by immunoblotting with antiserum against the His tag. T, harvesting times of cells, *i.e.*, hours after the end of the exponential phase of growth. Arrowheads indicate the positions of proteins.

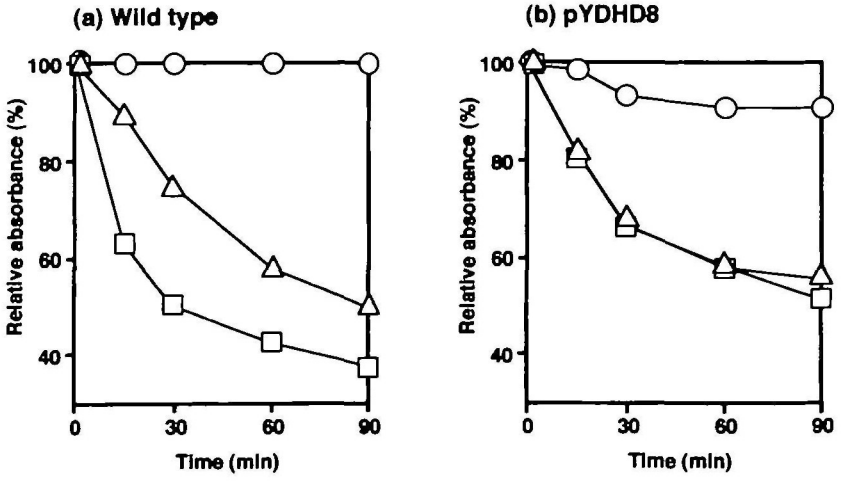

Fig. 4. **Spore germination of *B. subtilis* 168 (wild type, a) and transformants carrying pYDHD8** (b). The germination of *B. subtilis* spores was monitored by measuring the optical density at 660 nm at the indicated times after the addition of L-alanine (squares), AGFK (triangles), or control buffer (circles). The efficiency of germination is expressed as relative absorbance.

wild-type spores (data not shown). Then we characterized the transformants carrying multicopy plasmids pYDHD8, pYKUD8, and pYKVP8, respectively. These cells developed resistant spores as wild-type cells did (data not shown). The spores containing excess YdhD-His showed an altered germination profile, though they were also resistant to heat, chloroform, and lysozyme. Purified spores of pYDHD8/168 responded to L-alanine less effectively than the wild type, and even in the absence of L-alanine or AGFK part of the spores lost their refractivity as observed by the reduction of optical density and their phase-gray appearance on phase contrast microscopy (Figs. 4b and 5b). Spore germination of the transformants pYKUD8/168 and pYKVP8/168 were the same as that of the wild-type spores (data not shown). These results suggested that overexpression of YdhD-His resulted in spontaneous germination in a certain proportion of spores, and in slow germination response to L-alanine, but not to AGFK.

Detection of the β-Lactamase Fusion Proteins in Sporulating Cells and Mature Spores—To examine the biological function of putative cell wall binding motif in YaaH, we constructed multicopy plasmids pYAAH2B and pYAAH97B encoding fusion proteins of *E. coli* β-lactamase and *B. subtilis* YaaH. The original signal sequence of β-lactamase was genetically removed or substituted for the cell wall binding motif of YaaH. YaaHN2-Bla (28 kDa) is composed of the N-terminal 2 amino acid residues of YaaH and the mature region of β-lactamase (amino acid position 40 to 285). YaaHN97-Bla (39 kDa) is composed of the N-terminal 97 amino acid residues of YaaH, including two repeats of the cell wall binding motif, and the mature region of β-lactamase. These fusion proteins were produced from T_2 of

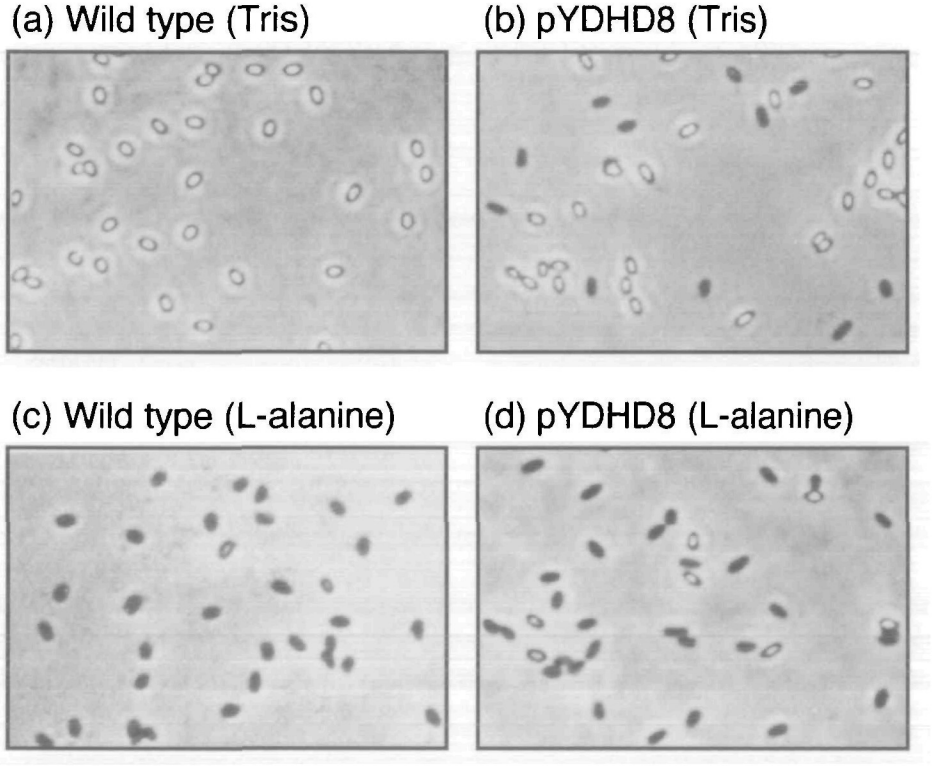

Fig. 5. **Phase contrast microscopy of wild-type and YdhD overproducing spores.** Wild-type spores (a, c) and YdhD overproducing spores (b, d) were incubated 10 mM Tris-HCl (pH 7.5) (a, b) or 10 mM L-alanine (c, d) at 37°C for 90 min.

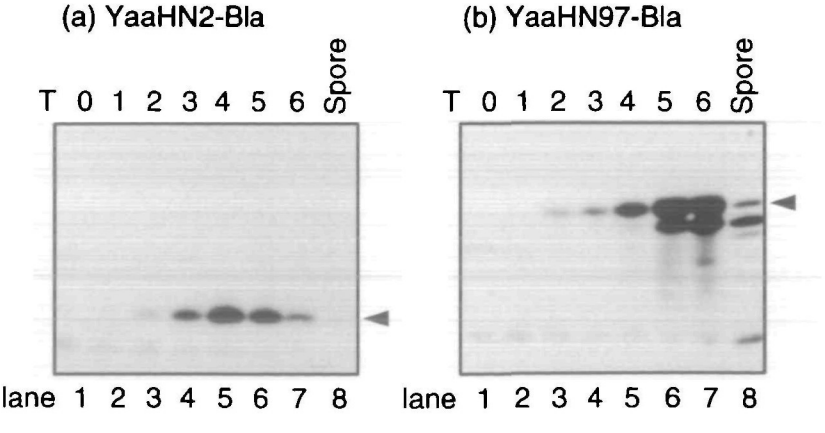

Fig. 6. **Detection of YaaHN2-Bla (a) and YaaHN97-Bla (b) in sporulating cells.** Multicopy plasmids containing fusion genes of *B. subtilis yaaH* and *E. coli bla* (β-lactamase) were introduced into *B. subtilis* 168 (wild type), and the YaaH-β-lactamase fusion proteins were produced under the regulation of a promoter located upstream of *yaaH* gene. YaaHN2-Bla and YaaHN97-Bla are composed of the N-terminal 2 or 97 amino acid residues of YaaH and the mature region of β-lactamase (amino acid position 40 to 285), respectively. *B. subtilis* wild-type cells (168) were transformed with these vectors as described in "MATE-RIALS AND METHODS." Protein extractions prepared from sporulating cells (lanes 1 to 7) and purified spores (lane 8) were resolved by SDS-PAGE (15% acrylamide gel) and visualized by immunoblotting with antiserum against β-lactamase. T, harvesting times of cells, *i.e.*, hours after the end of the exponential phase of growth. Arrowheads indicate the positions of each protein.

sporulation under the control of *yaaH* promoter in the cells carrying pYAAH2B and pYAAH97B, respectively (Fig. 6). YaaHN2-Bla was gradually reduced from T_5 of sporulation and hardly detectable in the protein preparation of purified spores (Fig. 6a). In contrast to this, YaaHN97-Bla was detectable in the protein extract of purified spores (Fig. 6b). The band slightly smaller than 39-kDa protein might be generated by proteolysis of YaaHN97-Bla. These results suggest that the cell wall binding motif in YaaH protein functions as a localization signal to forespores.

DISCUSSION

The motif conserved among so-called cell wall binding proteins is ubiquitous in bacteria and phages (*12–16*), and we have previously reported that YaaH and YrbA, spore proteins of *B. subtilis*, also have the conserved motif (*10*, *11*). In addition, YdhD, YkuD, and YkvP of *B. subtilis* proteins had the conserved motif (Fig. 3). YdhD, YkuD, and YkvP were probably synthesized in the mother cell compartment, because their transcription was dependent on the mother cell–specific transcription factors, SigE and SigK (Fig. 1). The transcripts of *ykuD* and *ykvP* were increased or reduced respectively in *gerE36* cells (Fig. 1), and DNA sequences similar to the GerE-binding consensus existed upstream of these genes (data not shown). GerE is a DNA-binding protein involved in regulation of some sporulation-specific genes controlled by SigK (*31–33*). These results suggest that GerE regulates the expression of *ykuD* and *ykvP* genes negatively or positively. The lengths of *ydhD, ykuD*, and *ykvP* coding sequences are 1,320, 494, and 1,199 bp, respectively (*9*). We assume that the transcription of *ydhD* and *ykuD* was independent from the following genes because the size of mRNA shown in Fig. 1 was insufficient to encode *ydhC* and *ykuE*, respectively (*9*). *ykvP* was possibly transcribed with *ykvQ* because the length of mRNA hybridized with a probe specific to *ykvP* was sufficient to encode both *ykvP* and *ykvQ* (Figs. 1c and 2c). The 2.5 kb mRNA was also hybridized to a probe specific to *ykvQ* but not to that of *ykvO* (data not shown).

As shown in Figs. 1 and 3, the spores of *ydhD, ykuD*, and *ykvP* mutant cells and YkuD or YkvP-overproduced spores showed almost the same resistance and response to germinant as wild-type spores. On the other hand, excessive YdhD altered the germination profile though it did not impair spore resistance (Figs. 4 and 5). Morphogenesis of the spore coat is considered to be involved in the development of the strict response to germinants as well as resistant to harmful environments, and correct assembly of coat protein components will be involved in this process (*34–36*). Some spores became dark even without germinants, and the efficiency of L-alanine–stimulated germination was reduced by the overproduction of YdhD (Fig. 5). YaaH is involved in L-alanine stimulated germination of *B. subtilis* spores, and its primary sequence shows about 30% identity to that of YdhD (*10*). The *sleL* gene product of *B. cereus* encodes spore-specific *N*-acetylglucosaminidase, which is involved in cortex hydrolysis during germination, and SleL has the cell wall binding motif in its N-terminal portion and shows similarity to both YaaH and YdhD (*37*). These facts suggest that the regulatory system for spore germination may have been disturbed by excessive levels of YdhD.

Since the functions of YkuD and YkvP were not clear, we searched for their homologues in the BLAST database. YkuD showed similarity to many bacterial proteins. Among them, *E. coli* CaiA and YnhG also have the motif conserved among cell wall binding proteins and are similar to YkuD over their entire length (30% identity) (*38*, *39*). *B. subtilis* YqjB is a paralogue of YkuD, though it does not have the conserved motif (*9*). The function of these YkuD homologues is also unknown. We could not find any protein sequences similar to that of YkvP except the conserved motif. YocH, YojL, XkdP, and YqbP of *B. subtilis* proteins have cell wall binding motif, and their functions have not yet been analyzed. The primary sequence of YojL is similar to those of cell wall hydrolase, LytE and YdhD (CwlE/LytF) (*9*, *17*, *18*). The C-terminal region of YocH composed of 120 a.a. shows 60% identity to YabE of *B. subtilis* (*9*). The primary sequence of XkdP, a phage-like element PBSX protein, has over 80% identity to that of YqbP (*9*). They possibly have a phage-related function, though no characterized protein is similar to them.

The existence of five proteins, YaaH, YdhD, YkuD, YkvP, and YrbA, having the conserved cell wall binding motif in the spores of *B. subtilis* suggests that the motif is required for protein assembly to forespores during sporulation. These proteins probably exist on the surface of outer spore membrane and/or inner spore coat, because they did not have any signal sequence available for membrane translocation (*9*). YrbA forms a complex with SpoVID and is localized to the cortex-coat interface in mature spores (*40*), and SpoVID is a morphogenetic protein required for assembly of the *B. subtilis* spore coat (*41*, *42*). We searched for spore proteins having the conserved cell wall binding motif in the BSORFDB database (http://bacillus.tokyo-center.genome.ad.jp) and found that SpoVID also has the motif (Fig. 7). While over 20 coat proteins and some essential factors for coat assembly have been identified from *B. subtilis* spores (*28*, *33*, *43–51*), almost no apparent consensus was found in the primary sequences of coat proteins, and any internal information involved in coat assembly has not yet been shown. On the other hand, proteins involved in cortex synthesis, DacB and SpoVD, have a typical signal sequence required for their translocation across the outer forespore

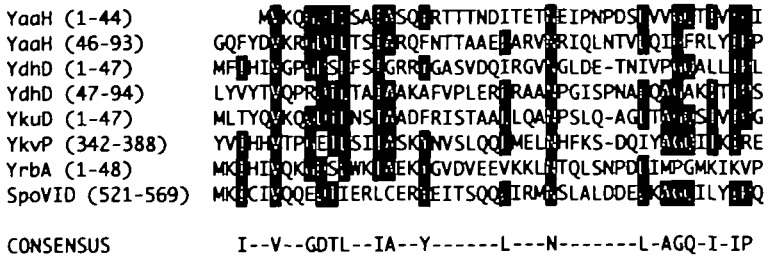

Fig. 7. **Comparison of spore proteins with amino acid sequences similar to the motif conserved among so-called cell wall binding proteins.** YaaH has two repeats of the consensus motif conserved among the bacterial lytic enzymes (*10*). A database analysis showed that *B. subtilis* spore proteins SpoVID, YdhD, YkuD, YkvP, and YrbA also have the motif. Identical amino acid residues are indicated by black boxes.

membrane (*52*, *53*). To assess the function of the cell wall binding motif in sporulating cells, we used a fusion protein of *E. coli* β-lactamase, which has the N-terminal region, including cell wall binding motif of YaaH in its N-terminal instead of the original signal sequence, and found that the fusion protein assembled to spores (Fig. 6). We speculate that cell wall binding motif is required for correct localization of these proteins around forespores, because this motif is the only sequence conserved among these proteins.

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