Expression of *yeeK* during *Bacillus subtilis* Sporulation and Localization of YeeK to the Inner Spore Coat using Fluorescence Microscopy[▽]

Hiromu Takamatsu,* Daisuke Imamura, Ritsuko Kuwana, and Kazuhito Watabe

Faculty of Pharmaceutical Sciences, Setsunan University, Osaka, Japan

The *yeeK* gene of *Bacillus subtilis* is predicted to encode a protein of 145 amino acids composed of 28% glycine, 23% histidine, and 12% tyrosine residues. Previous studies were unable to detect YeeK in wild-type spores; however, the 18-kDa YeeK polypeptide has been identified in *yabG* mutant spores. In this study, we analyze the expression and localization of YeeK to explore the relationship between YeeK and YabG. Northern hybridization analysis of wild-type RNA indicated that transcription of the *yeeK* gene, which was initiated 5 h after the onset of sporulation, was dependent on a SigK-containing RNA polymerase and the GerE protein. Genetic disruption of *yeeK* did not impair vegetative growth, development of resistant spores, or germination. Fluorescent microscopy of in-frame fusions of YeeK with green fluorescent protein (YeeK-GFP) and red fluorescent protein (YeeK-RFP) confirmed that YeeK assembles into the spore integument. CotE, SafA, and SpoVID were required for the proper localization of YeeK-GFP. Comparative analysis of YeeK-RFP and an in-frame GFP fusion of YabG indicated that YeeK colocalized with YabG in the spore coat. This is the first use of fluorescent proteins to show localization to different layers of the spore coat. Immunoblotting with anti-GFP antiserum indicated that YeeK-GFP was primarily synthesized as a 44-kDa molecule, which was then digested into a 29-kDa fragment that corresponded to the molecular size of GFP in wild-type spores. In contrast, a minimal amount of 44-kDa YeeK-GFP was digested in *yabG* mutant spores. Our findings demonstrate that YeeK is guided into the spore coat by CotE, SafA, and SpoVID. We conclude that YabG is directly or indirectly involved in the digestion of YeeK.

Endospore formation by *Bacillus subtilis* involves a series of temporal and spatial changes in cell morphology and gene expression (37, 46). In response to starvation, *B. subtilis* initiates a developmental process by forming an asymmetric septum, which divides the bacterium into two compartments, the mother cell and the forespore. 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 capable of damaging cells (34). Spores germinate and begin growing when surrounding nutrients become available (36). The genes involved in sporulation have been identified, and their biological functions have been analyzed. Specifically, SigF, SigE, SigG, and SigK are temporally and spatially activated and regulate gene expression in a compartment-specific fashion (37).

The outermost portion of *Bacillus* spores consists of a cortex, a spore coat layer, and, in some cases, an exosporium. The cortex, a thick layer of peptidoglycan, is responsible for maintaining the highly dehydrated state of the core, thereby contributing to the extreme dormancy and heat resistance of spores (11, 12, 13, 14, 47). The spore coat is composed of dozens of proteins that are arranged in an electron-dense outer layer (i.e., the outer coat) and a lamellar inner layer (i.e., the inner coat) (11, 12, 13, 14, 47). These layers provide a protective barrier against bactericidal enzymes and chemicals, such as

lysozyme and organic solvents (34). Recent studies have suggested that some coat proteins are involved in the protection of spores from predators, such as nematodes and protozoa (24, 30). Some proteins are required for proper spore coat formation in *B. subtilis* spores (11, 12, 13, 14, 47). In particular, the SpoIVA protein is synthesized 2 h after the cessation of exponential growth (i.e., during the T_2 stage) in the mother cell compartment and plays a central role in the proper formation of both the cortex and the coat. Sporulating *spoIVA* mutants fail to synthesize a cortex, resulting in an erroneously localized coat (40, 45). The SpoIVA protein is assembled into a spherical shell around the outer surface of the forespore and is thought to be required for the formation of a basement layer, where spore coat proteins assemble (7, 38, 40, 45). The SpoVID and SafA (also known as YrbA) proteins are also synthesized during the T_2 stage of sporulation in the mother cell compartment and are required for the assembly of some coat proteins in *B. subtilis* spores (5, 35, 50). One of the coat protein components, CotE, also plays a central role in morphogenesis of the spore coat and is required for the assembly of the outer coat (4, 32, 56). The *cotE* mutant spores are resistant to heat and chemicals but are lysozyme sensitive and germinate more slowly and less efficiently than do wild-type spores (56).

Previous genomic and proteomic studies have identified several spore polypeptides of smaller or larger sizes than expected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (29, 31). Thus, protein modifications appear to be common during sporulation of *B. subtilis*. Several studies have examined the processing of pro-SigE and pro-SigK, and the importance of this process has been well established. The pro-SigE and pro-SigK precursors become active when their

* Corresponding author. Mailing address: Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-0101, Japan. Phone and fax: 81 (72) 866-3114. E-mail: takamatu@pharm.setsunan.ac.jp.

N-terminal prosequences are removed by specific proteases (46). However, little is known about the modification of proteins that are not essential for spore resistance or germination. We previously focused on the *yabG* gene, which encodes a sporulation-specific protease, in an effort to identify the mechanisms and importance of coat protein processing in *B. subtilis* (48, 49). The transcription of *yabG* is regulated by SigK during stage T_4 of sporulation, resulting in the production of a 33-kDa polypeptide in *B. subtilis* (48, 49). The YabG protein is involved in the processing of several coat proteins (48, 49). The protein composition of mature *yabG* mutant spores differs from that of the wild type. Proteins CotF, CotT, SpoIVA, YeeK, SafA, and YxeE are present at increased levels and often exist as precursor proteins in *yabG* mutant spores (48, 49). The CotF, CotT, and YxeE proteins are spore coat proteins whose synthesis depends on SigK (1, 6, 9, 26). The expression patterns and function of the YeeK protein remain unclear. Proteomic analysis, one-dimensional PAGE analysis, and liquid chromatography coupled with tandem mass spectrometry, have revealed that CotF, SpoIVA, and SafA are digested by proteases in the wild-type spores of *B. subtilis* (29). We identified under the same conditions a YxeE protein that corresponded in size to that of the intact YxeE polypeptide in mature *yabG* mutant spores (26). However, proteomic analysis failed to detect CotT or YeeK in wild-type spores (29). Here, we examine the expression profile of the *yeeK* gene and investigate the involvement in coat assembly of the YeeK protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and general techniques. The *B. subtilis* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. All *B. subtilis* strains were derivatives of strain 168, and those constructed in this work were prepared via transformation with plasmid DNA, confirmed by PCR analysis, and sequenced to ensure that no mutations had been introduced. *E. coli* strain JM109 was used for the production of plasmids. The oligonucleotides used for PCR amplification are listed in Table 2.

We used a Campbell-type single-crossover recombination method to construct a series of insertion mutants of *B. subtilis* (17, 29, 51). Recombination of each mutant DNA was confirmed by PCR. Segments of the *sigE* (*spoIIGB*), *sigF* (*spoIIAC*), *sigG* (*spoIIIG*), *sigK* (*spoIVCB*), *and yeeK* genes were amplified via PCR, digested with HindIII and BamHI at the corresponding primer-incorporated restriction sites, and inserted into a HindIII/BamHI-digested pMutin3 vector to obtain plasmids pSIGE5E, pSIGF5E, pSIGG5E, pSIGK5E, and pYEEK5E, respectively (Table 1) (51). Segments of the *yabG* gene were amplified via PCR, digested with HindIII and BamHI at primer-incorporated restriction sites, and inserted into a HindIII/BamHI-digested pCAT5 vector to obtain plasmid pYABG5C (28). *Bacillus subtilis* 168 was transformed with these plasmids, and the corresponding SIGE5E, SIGF5E, SIGG5E, SIGK5E, YEEK5E, and YABG5C strains were identified via selection for erythromycin resistance (i.e., 0.5 μg/ml erythromycin) or chloramphenicol resistance (i.e., 5 μg/ml chloramphenicol) (Table 1). Chromosomal DNA from YEEK5E was introduced into YABG5C to construct a double mutant (YEEK5EYABG) (Table 1).

Oligonucleotide primers CGEAM490, CGEA398R, COTA86, COTA1538R, COTE59, COTE542R, COTT3, COTT246R, YABG236, YBAG869R, YEEK7, YEEK434R, YHCN51, and YHCN566R were used to amplify the *cgeA*, *cotA*, *cotE*, *cotT*, *yabG*, *yeeK*, and *yhcN* genes from the *B. subtilis* 168 chromosome. The PCR products were digested at the BamHI and XhoI sites introduced by the primers and ligated into BamHI-XhoI-digested pGFP7C (27) to create plasmids pCGEA8G, pCOTA8G, pCOTE8G, pCOTT8G, pYABG8G, pYEEK8G, and pYHCN8G (Table 1). The DNA fragments inserted into these plasmids were introduced into the coding regions of *cgeA*, *cotA*, *cotE*, *cotT*, *yabG*, *yeeK*, and *yhcN* of strain 168 via transformation. Single-crossover mutations were identified via selection for chloramphenicol resistance (i.e., 5 μg/ml), yielding the CGEA8G, COTA8G, COTE8G, COTT8G, YABG8G, YEEK8G, and YHCN8G constructs (Table 1). Each wild-type gene was replaced with an in-frame *gfp* fusion.

Oligonucleotide primers SPCM189, SPC813R, PUC1697, and PUC723R were used to amplify the kanamycin resistance gene and the gene fragment of pUC18 including the replication origin of *E. coli*. The PCR products were digested at the EcoRV and ScaI sites and ligated to create plasmid pKMR5 (Table 1). Oligonucleotide primers DRED3 and DRED681R were used to amplify the gene encoding DsRed-M1 from the plasmid pDsRED-Monomer-N1 (Clontech Laboratories, Inc.). The resulting PCR product was digested at the XbaI and XhoI sites and inserted into the XbaI- and XhoI-digested sites of pKMR5 to create plasmid pRFP3K (Table 1). Oligonucleotide primers APRE20 and APRE1090R were used to amplify the gene fragment of *aprE* from the *B. subtilis* 168 chromosome. The resulting PCR product was digested at EcoRI sites and inserted into an EcoRI-digested pRFP3K to create the pRFP3KP plasmid (Table 1). The 1,070-bp fragment of *aprE* was cloned upstream and in the opposite direction of the DsRed-M1 gene of pRFP3KP. To construct a YeeK-RFP fusion, the YEEKM400 and YEEK434R oligonucleotide primers were used to amplify the *yeeK* gene fragment from the *B. subtilis* 168 chromosome. The resulting PCR product was digested at the BamHI and XhoI sites and inserted into BamHI- and XhoI-digested pRFP3KP to create plasmid pYEEK3RP (Table 1). This plasmid was introduced into the *aprE*-coding region of strain 168 by transformation, and single-crossover mutants were identified via selection for kanamycin resistance (i.e., 10 μg/ml), yielding strain YEEK3RP (Table 1).

The *B. subtilis* strains were grown in Luria-Bertani and Difco sporulation (DS) media (43). The conditions for sporulation and the methods for purifying mature spores have previously been described (29). Recombinant DNA methods were performed as described by Sambrook et al. (42). The methods of competent cell preparation, transformation, and preparation of chromosomal DNA were performed as described by Cutting and Vander Horn (10).

RNA preparation and Northern analysis. *B. subtilis* cells were grown in DS medium, and 20-ml samples were harvested every hour throughout the sporulation process. The RNA used in Northern blot analysis was prepared using a modification of the procedure described by Igo and Losick (21). Aliquots (10 μg) of the RNA preparation were analyzed by size fractionation through a 1% (wt/vol) agarose gel containing 2.2 M formaldehyde. The RNA aliquots were then transferred to a positively charged nylon membrane (Roche) stained with 0.04% methylene blue to measure the concentrations of 16S and 23S RNAs (20; data not shown). The RNA on the membrane was then hybridized to *yeeK*-specific probes. The 0.3-kb probe for *yeeK*, which corresponded to nucleotides (nt) 6 to 280 downstream of the putative translation initiation codon of *yeeK*, was prepared via PCR using primers YEEK6 and YEEK280RT7 (Table 2). The RNA probe was prepared using the Roche digoxigenin labeling system, and hybridization was performed using the DIG Northern starter kit (Roche).

Mapping of the 5′ terminus of *yeeK* mRNA. The YEEK5E cells were grown in DS medium, and 20-ml samples were harvested 6 h after the onset of sporulation (i.e., T_6). The *yeeK* promoter region of strain YEEK5E was fused downstream of the BamHI cloning site to the promoterless *lacZ* gene of pMutinT3. The RNA used in our primer extension analysis was prepared using a modification of a previously described procedure (21). The RNA sample was subjected to primer extension assays using a digoxigenin end-labeled primer (PMT353RD), which was specific for the sequences surrounding the BamHI site and the *lacZ* gene of pMutinT3 (Table 2). Therefore, this primer was capable of detecting *yeeK*-specific transcription within the YEEK5E strain. The RNA (20 μg) and oligonucleotide primer were hybridized at 60°C for 1 h. SuperScript II reverse transcriptase (Invitrogen) was then added, and the mixture was incubated at 42°C for 1 h. We created DNA ladders using the same 5′ digoxigenin-labeled primers, via the dideoxy chain termination method (Takara). The products of primer extension were resolved on DNA sequencing gels and detected as recommended by Roche.

Preparation of spores. The *B. subtilis* strains were grown in DS medium at 37°C as previously described. Mature spores were harvested at stage T_{18} and washed once with 10 mM sodium phosphate buffer (pH 7.2) (48). To remove cellular debris and vegetative cells, the pellets were suspended in 0.1 ml lysozyme buffer (10 mM sodium phosphate [pH 7.2], 1% [wt/vol] lysozyme, and complete protease inhibitor cocktail [Roche]) and incubated at room temperature for 10 min. The pellets were then washed repeatedly with buffer (10 mM sodium phosphate, pH 7.2; 0.5 M NaCl) at room temperature (48). More than 99% of the wild-type and *yeeK*-inactivated spores were refractive, and few dark or gray spores were visible under phase-contrast microscopy.

Spore resistance. Cells were grown in DS medium at 37°C for 18 h following exponential growth, and spore resistance was assayed as previously described (50). The cultures were heated at 80°C for 30 min or were treated with lysozyme (final concentration of 250 μg/ml) at 37°C for 10 min. After the cultures were serially diluted 100-fold with distilled water, appropriate volumes of the dilutions

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype/description	Source or reference (construction)
Strains		
B. subtilis		
168	*trpC2*	*Bacillus* Genetic Stock Center 1A1
SIGF5E	*trpC2, sigF (spoIIAC)*::pMutin3	This work (pSIGF5E, 168)
SIGE5E	*trpC2, sigE (spoIIGB)*::pMutin3	This work (pSIGE5E, 168)
SIGG5E	*trpC2, sigG (spoIIIG)*::pMutin3	This work (pSIGG5E, 168)
SIGK5E	*trpC2, sigK (spoIVCB)*::pMutin3	This work (pSIGK5E, 168)
MTB826	*trpC2, gerE*::pMutin3	28
MTB905	*trpC2, cotE*::pMutin3	28
YRBA5E	*trpC2, safA (yrbA)*::pMutin3	27
S6D5E	*trpC2, spoVID*::pMutin3	27
YABG5E	*trpC2, yabG*::pMutin3	27
YABG5C	*trpC2, yabG::cat*	This work (pYABG5C, 168)
YEEK5E	*trpC2, yeeK*::pMutin3	This work (pYEEK5E, 168)
YEEK5EYABG	*trpC2, yeeK*::pMutin3 *yabG::cat*	This work (YEEK5E, YABG5C)
YEEK8G	*trpC2, yeeK'-gfp cat*	This work (pYEEK8G, 168)
YEEK8GCOTE	*trpC2, cotE*::pMutin3, *yeeK'-gfp cat*	This work (YEEK8G, COTE5C)
YEEK8GSAFA	*trpC2, safA (yrbA)*::pMutin3, *yeeK'-gfp cat*	This work (YEEK8G, SAFA5C)
YEEK8GS6D	*trpC2, spoVID*::pMutin3, *yeeK'-gfp cat*	This work (YEEK8G, S6D5C)
YEEK8GYABG	*trpC2, yabG*::pMutin3, *yee'-gfp cat*	This work (YEEK8G, YABG5C)
YEEK3RP	*trpC2, aprE'::yeeK-rfp kmr*	This work (pYEEK3RP, 168)
YEEK3RPYEEK8G	*trpC2, aprE'::yeeK-rfp kmr, yeeK'-gfp cat*	This work (YEEK8G, YEEK3RP)
YEEK3RPCGEA8G	*trpC2, aprE'::yeeK-rfp kmr, cgeA'-gfp cat*	This work (YEEK3RP, pCGEA8G)
YEEK3RPCOTA8G	*trpC2, aprE'::yeeK-rfp kmr, cotA'-gfp cat*	This work (YEEK3RP, pCOTA8G)
YEEK3RPCOTE8G	*trpC2, aprE'::yeeK-rfp kmr, cotE'-gfp cat*	This work (YEEK3RP, pCOTE8G)
YEEK3RPCOTT8G	*trpC2, aprE'::yeeK-rfp kmr, cotT'-gfp cat*	This work (YEEK3RP, pCOTT8G)
YEEK3RPYABG8G	*trpC2, aprE'::yeeK-rfp kmr, yabG'-gfp cat*	This work (YEEK3RP, pYABG8G)
YEEK3RPYHCN8G	*trpC2, aprE'::yeeK-rfp kmr, yhcN'-gfp cat*	This work (YEEK3RP, pYHCN8G)
E. coli		
JM109	*relA, supF44, endA1, hsdR17, gyrA96, mcrA, mcrB⁺, thiD (lac-proAB)*/F' (*traD36, proAB⁺, lacIq, lacZDM15*)	42
Plasmids		
pCAT5	*Cat*	29
pYABG5C	*Cat, yabG'*	This work
pMutin3	*Bla, emr, lacZ, lacI, Pspac*	33
pYEEK5E	*Bla, emr, yeeK'-lacZ, lacI, Pspac-yeeK'*	This work
pGFP7C	*Gfp, cat*	27
pYEEK8G	*yeeK'-gfp, cat*	This work
pCGEA8G	*cgeA'-gfp, cat*	This work
pCOTA9G	*cotA'-gfp, cat*	This work
pCOTE8G	*cotE'-gfp, cat*	This work
pCOTT8G	*cotT'-gfp, cat*	This work
pYABG8G	*yabG'-gfp, cat*	This work
pYHCN8G	*yhcN'-gfp, cat*	This work
pKMR5	*kmr*	This work
pDsRED-Monomer-N1	DsRED-monomer, *kmr, neoR*	Clontech Laboratories, Inc.
pRFP3K	*rfp, kmr*	This work
pRFP3KP	*rfp, kmr, aprE'*	This work
pYEEK3RP	*aprE'::yeeK-rfp, kmr*	This work

were spread on Luria-Bertani agar plates, which were incubated overnight at 37°C. The proportions of survivors were determined by colony counting.

Spore germination. Purified spores were heat activated at 80°C for 15 min, cooled, and suspended in 10 mM Tris-HCl (pH 7.5) buffer to an optical density at 660 nm of 0.5 and then L-alanine (10 mM) or AGFK (10 mM L-asparagine, 10 mM D-glucose, 10 mM D-fructose, and 10 mM potassium chloride) was added. Germination was monitored by measuring the decrease in optical density (at 660 nm) of the spore suspension at 37°C for 90 min (25).

SDS-PAGE and immunoblotting analyses. Spore proteins were solubilized in 0.1 ml loading buffer (62.5 mM Tris-HCl [pH 6.8], 10% [wt/vol] SDS, 10% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 0.05% [wt/vol] bromophenol blue) and boiled for 5 min (29). The proteins were separated by 14% SDS-PAGE and visualized by Coomassie brilliant blue R-250 staining, as described previously

(48). Immunoblotting analysis was performed using a rabbit immunoglobulin G antibody against GFP, as described previously (26).

Visualization of GFP and RFP. Following sporulation in DS medium, aliquots of cultures harboring chromosomal *gfp* and *rfp* fusions were transferred to microscope slides. The fluorescence of the GFP and RFP fusion proteins were observed under an Olympus BX51 fluorescent microscope fitted with mirror cube units for GFP (U-MGFPHQ) and RFP (U-MWG2) (Olympus, Japan). An UPlanApo 100× oil iris 3Ph objective lens and a U-TV1X-2 camera adapter were used. The images were captured using a cooled charge-coupled-device camera (CoolSNAP ES/OL, Roper Scientific) and analyzed using RS Image Express processing software, version 4.5 (Roper Scientific). The exposure time for image capture of GFP fusions was 0.1 to 2 s. The exposure time for image capture of YeeK-RFP was 1 s. Overlay images of GFP and RFP were generated

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence[a]	Added site	Resulting construct[b]
APRE20	5′-AAAGAATTCGGATCAGCTTGTTGTTTGC-3′	EcoRI	pRFP3RP
APRE1090R	5′-AAGGAATTCCAAGATATGTTGCAGTGCTT-3′	EcoRI	pRFP3RP
CGEAM490	5′-CCAGGATCCAACACTTGAGAGTGAAACA-3′	BamHI	pCGEA8G
CGEA398R	5′-GGACTCGAGGAAAAGAACGTAACGCTTTC-3′	XhoI	pCGEA8G
COTA86	5′-ACAGGATCCTAAGTCACCATGGAGGAATG-3′	BamHI	pCOTA8G
COTA1538R	5′-TCGCTCGAGTTATGGGGATCAGTTATATCC-3′	XhoI	pCOTA8G
COTE59	5′-AAAGGATCCCAATGCACCAACACCA-3′	BamHI	pCOTE8G
COTE542R	5′-CTTCTCGAGTCTTCAGGATCTCCCACTA-3′	XhoI	pCOTE8G
COTT3	5′-GTTGGATCCTAGGATTACCCTTTGAATGAAC-3′	BamHI	pCOTT8G
COTT246R	5′-CCTCGAGTAACCGTAACCGTAACCTCCCCCAT-3′	XhoI	pCOTT8G
DRED3	5′-CGCCTCGAGGGACAACACCGAGGACG-3′	XhoI	pRFP3K
DRED681R	5′-GTCAAGCTTGCTCTACTGGGAGCCGG-3′	HindIII	pRFP3K
SIGE16	5′-ACTAAGCTTACGGTTGACGCACCTC-3′	HindIII	pSIGE5E
SIGE208R	5′-TATGGATCCAGACGCAAAATGCGTTC-3′	BamHI	pSIGE5E
SIGF24	5′-TAAAAGCTTCGGCAAAAACGCTCAGCT-3′	HindIII	pSIGF5E
SIGF207R	5′-TTAGGATCCCGATGCAGCCGATCT-3′	BamHI	pSIGF5E
SIGG121	5′-GAAAAGCTTGTAAACGGGAACTT-3′	HindIII	pSIGG5E
SIGG315R	5′-GTAGGATCCTGCGGATCTCTCCGA-3′	BamHI	pSIGG5E
SIGK16	5′-AGGAAGCTTCGCAGCGCTCGGCTT-3′	HindIII	pSIGK5E
SIGK228R	5′-TCGCTCGAGAAGTTACCATTGTGGATTC-3′	BamHI	pSIGK5E
SPCM189	5′-GCGAGTACTTGACAAATTATATGGTGATCTGTATAAT-3′	ScaI	pKMR5
SPC813R	5′-GTTGATATCGTAAGCACCTGTTATTGCAA-3′	EcoRV	pKMR5
PMT353RD	5′-AGCATTAGTGTATCAACAAGCTGGGG-3′		Digoxigenin end-labeled
PUC1697	5′-AAAGATATCTAGGTGAAGATCCTTTTT-3′	EcoRV	pKMR5
PUC723R	5′-TTAAGTACTCATGAGCGGATACATATT-3′	ScaI	pKMR5
YABG69	5′-CGAAAGCTTGGAATAGAGCAAACAAGCAA-3′	HindIII	pYABG5C
YABG220R	5′-GAGGGATCCATTCATTCTGCTCTCATCT-3′	BamHI	pYABG5C
YABG236	5′-AATGGATCCGCCAAGATTATAAGCTG-3′	BamHI	pYABG8G
YABG869R	5′-AACCTCGAGTTGGACTTATAAGGCATACC-3′	XhoI	pYABG8G
YEEK7	5′-AATGGATCCGATACAAGACATATGTATGGC-3′	BamHI	pYEEK8G
YEEK434R	5′-CTTCTCGAGTTATAACCGTCTTTTCCCCA-3′	XhoI	pYEEK8G, pYEEK3RP
YHCN51	5′-CCCGGATCCTATGACTGGCTGCGGTGT-3′	BamHI	pYHCN8G
YHCN566R	5′-TTCCTCGAGTCAGCGTTAGGGAATACAC-3′	XhoI	pYHCN8G
YEEKM400	5′-GAAGGATCCATGGAAGCGGATGTTGATG-3′	BamHI	pYEEK3RP
YEEK280RT7	5′-<u>TAATACGACTCACTATAGGGCGA</u>CATCATTTTCATGGT GCATGC-3′		*yeeK* RNA probe

[a] T7 promoter sequences are underlined.
[b] PCR products were digested with restriction enzymes at primer-derived sequences and inserted into restriction enzyme-digested plasmids to generate the plasmids listed in Table 1.

by RS Image Express processing software, version 4.5. The contrast and tone balance of the overlay images of GFP and RFP were adjusted by CANVAS 8 software (Deneba Systems Inc.).

RESULTS

Sporulation-specific expression of the *yeeK* gene of *B. subtilis*. To confirm the expression profile of the *yeeK* gene, total RNA was isolated from *B. subtilis* 168 and analyzed by Northern hybridization. Figure 1A shows a single mRNA species of approximately 0.5 kb that hybridized to a *yeeK*-specific probe, and this species was first detected 5 h after the onset of sporulation (i.e., T_5). The *yeeK* gene is 435 bp long, consistent with the size of *yeeK* mRNA detected by Northern analysis (Fig. 1A). Accumulation of *yeeK* transcripts was not altered in a mutant strain of *yeeI* (a gene upstream of *yeeK*) (data not shown). A possible terminator of *yeeI* (5′-AGCAGGCTGTTATGGCCTGCT-3′) was identified 107 nt upstream of the initiation codon of *yeeK* (Fig. 1C). A possible terminator of *yeeK* (5′-ACACTCTTGCAGATCAAGAGTGT-3′) was identified 22 nt downstream of the termination codon of *yeeK* (data not shown). These data indicate that the *yeeK* gene is

monocistronically transcribed. An RNA polymerase containing sporulation-specific sigma factors transcribes many of the genes induced during sporulation. To identify the sigma factor likely to be involved in the transcription of *yeeK*, we performed Northern analysis with RNA obtained from sigma factor-deficient mutants (Fig. 1A). We were unable to detect *yeeK* mRNA in *spoIIAC*, *spoIIGAB*, *spoIIIG*, and *spoIVCB* mutants (deficient in SigF, SigE, SigG, and SigK, respectively). Transcription of *yeeK* was repressed in a strain mutant for *gerE*; GerE is a regulator involved in SigK-dependent gene expression (16, 52). These results indicate that *yeeK* transcription requires SigK and GerE.

Localization of the putative *yeeK* promoter. To precisely identify the location of the *yeeK* mRNA 5′ end, primer extension analysis was performed using RNA from sporulating cells carrying the *yeeK*::*pMut* (YEEK5E) construct. The *yeeK* mRNA 5′ end mapped to a G residue 27 nt upstream of the *yeeK* AUG codon (Fig. 1B). We searched for DNA sequences upstream of the *yeeK* transcription start site that were similar to the consensus sequences recognized by SigK and GerE (16, 54). Sequences located 10 and 32 nt upstream of the transcrip-
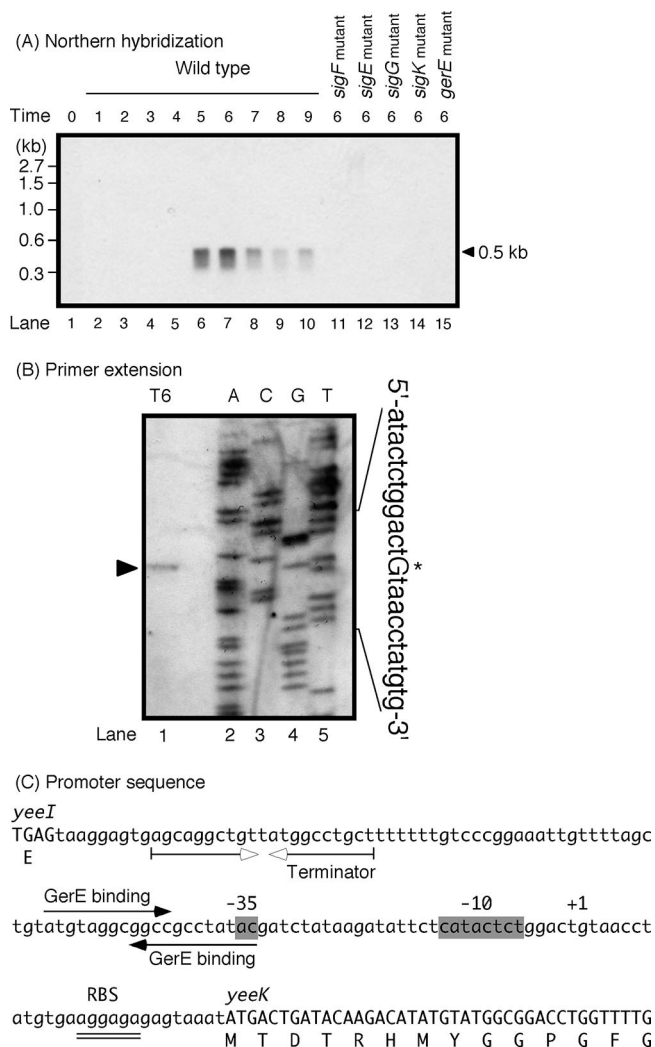
(A) Northern hybridization

(B) Primer extension

5'-atactctggactGtaacctatgtt-3'

(C) Promoter sequence

yeeI
TGAGtaaggagtgagcaggctgttatggcctgctttttttgtcccggaaattgttttagc
E

Terminator

GerE binding   -35   -10   +1
tgtatgtaggcggccgcctatacgatctataagatattctcatactctggactgtaacct
GerE binding

RBS   yeeK
atgtgaaggagagagtaaatATGACTGATACAAGACATATGTATGGCGGACCTGGTTTTG
                    M T D T R H M Y G G P G F G

FIG. 1. Northern blot and primer extension analysis of yeeK mRNA. (A) Total RNA was prepared from sporulating cells, and each mRNA was exposed to Northern hybridization using probes specific to yeeK. The arrowhead indicates the position of hybridization between the mRNA and the digoxigenin-labeled RNA probe. Lanes 1 to 10 correspond to total RNA (10 μg) isolated from strain 168. The length of time (in hours) after exponential growth ceased is shown at the top. Transcription of yeeK in the sigF (lane 11), sigE (lane 12), sigG (lane 13), sigK (lane 14), and gerE (lane 15) mutant strains was also analyzed at stage T_6 of sporulation by Northern hybridization. (B) Total RNA prepared from wild-type cells at stage T_6 of sporulation was hybridized with a digoxigenin-labeled primer specific to yeeK. The lanes labeled A, C, G, and T show DNA sequencing reactions with the appropriate primers. The primer extension product is indicated with an arrowhead, and the transcription start site upstream of yeeK is shown with an asterisk and a capital letter. The primer extension product of the wild-type strain is shown in lane 1. (C) The nucleotide sequence of the 5' region of yeeK (in capital letters), the putative −35 and −10 regions (shaded), and the transcription start site (+1) are shown. Filled arrows indicate putative GerE binding sites. Inverted open arrows indicate potential stem loop structures (i.e., transcriptional terminators) downstream of yeeI. A potential ribosome-binding site (RBS) is doubly underlined.

tion start site were very similar to the −10 and −35 consensus sequences recognized by SigK, and the spacing (17 nt) between the sequences was appropriate (Fig. 1C). Two possible GerE-binding sequences were identified upstream and overlapping
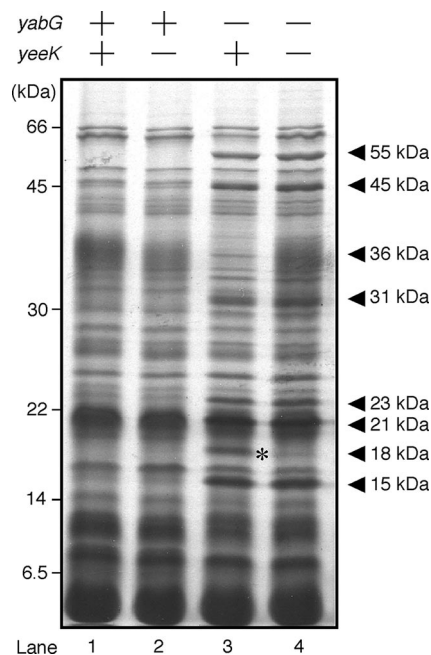

FIG. 2. SDS-PAGE analysis of proteins solubilized from mature spores. Spores were prepared at stage T_{18} of sporulation. Whole-protein samples were solubilized from the purified spores and analyzed by 14% (wt/vol) SDS-PAGE. Protein samples were prepared from the following strains: wild type (lane 1), yeeK mutant (lane 2), yabG mutant (lane 3), and yabG-yeeK double mutant (lane 4). Arrowheads indicate bands specifically found in yabG mutant spores. Asterisks indicate bands corresponding to the 18-kDa molecule of YeeK.

the promoter sequence (Fig. 1C). These findings further indicate that the SigK and GerE proteins in the mother cell compartment help to regulate the expression of yeeK.

Properties of yeeK mutant spores. We characterized the yeeK mutant phenotype (YEEK5E) to determine the function of YeeK in B. subtilis. The vegetative growth rate of the yeeK mutant in DS medium was similar to those of the wild-type strain (data not shown). Spores prepared from the yeeK mutant after 24 h of cultivation in DS medium at 37°C revealed resistance to heat and lysozyme as wild-type spores did (data not shown). Germination was similar in the wild-type and yeeK mutant spores after cultivation in L-alanine or a mixture of L-asparagine, D-glucose, D-fructose, and potassium chloride (data not shown).

YabG-dependent modification of YeeK. We previously showed that YabG is a coat-associated protease involved in the modification of coat proteins in B. subtilis spores (48, 49). Here, we analyzed the spore protein composition of wild-type (168), yeeK mutant (YEEK5E), yabG mutant (YABG5E), and yeeK-yabG double mutant (YEEK5EYABG) cells. Proteins were extracted from purified mature spores 18 h after the onset of sporulation (T_{18}) and stained with Coomassie brilliant blue dye after SDS-PAGE analysis (Fig. 2). The protein composition of yeeK mutant spores was visually similar to that of the wild type, and we were unable to identify bands attributable to the YeeK protein (Fig. 2, lanes 1 and 2). The protein composition of yabG mutant spores differed from that of wild-type spores, as previously described (Fig. 2, lanes 1 and 3) (48). A

band corresponding to a molecular mass of 18 kDa was detected in the *yabG* mutant but was lost in the *yeeK-yabG* double mutant (Fig. 2, lanes 3 and 4). The N-terminal amino acid sequence of this 18-kDa polypeptide has previously been determined and is identical to that of YeeK (48). These results suggest that YabG is involved in the modification of YeeK. A diffused band corresponding to a molecular mass of about 36 kDa, which was absent in YABG5E, was detected in the *yeeK-yabG* double mutant (Fig. 2, lanes 3 and 4). This 36-kDa polypeptide likely corresponds to CotG (41), which may be related in function to YabG and YeeK, although we were unable to determine the relationship between these proteins in the current study. We have previously reported that the 36-kDa band is present in *yabG* mutant spores (48). A possible explanation for this discrepancy is that a protein corresponding to the 36-kDa band is unstable under a certain condition and accidentally lost during spore preparation.

Proteolysis of YeeK-GFP. The YeeK protein has been identified in spore extracts from the *yabG* mutant, indicating that YeeK is digested by some protease(s) or is incorporated into the insoluble materials of wild-type spores (48, 49). We introduced an in-frame fusion of the *yeeK* gene and *gfp* into *B. subtilis* and monitored YeeK-GFP activity with immunoblotting and fluorescent microscopy. The native *yeeK* gene in this construct was replaced with a *yeeK-gfp* fusion, as described in Materials and Methods. The resistance and germination levels of wild-type (168) and YeeK-GFP spores were similar (data not shown). To confirm the molecular size of YeeK-GFP in mature spores, we detected YeeK-GFP via SDS-PAGE analysis and then immunoblotted with anti-GFP antiserum (26). The molecular sizes of the intact YeeK-GFP and the nonfusion GFP were estimated to be 45 and 28 kDa, respectively. A band corresponding to the intact polypeptide of YeeK-GFP (44 kDa in size) was detected in extracts prepared at T_5 (Fig. 3A and B). A band corresponding to a molecular mass of 29 kDa was identified in samples of *yabG* wild-type (YEEK8G) prepared at T_8, T_9, and T_{18} (Fig. 3A). This band was similar in size to GFP. A minor band corresponding to a molecular mass of 42 kDa was detected at T_7 and later times for the *yabG* mutant (YEEK8GYABG) (Fig. 3B), suggesting that at least two proteases are involved in the proteolysis of YeeK-GFP.

Identification of proteins involved in the assembly of YeeK-GFP. An in-frame fusion of *yeeK-gfp* was introduced into the chromosome of the wild type (YEEK8G) and the *cotE* (YEEK 8GCOTE), *safA* (YEEK8GSAFA), *spoVID* (YEEK8GS6D), and *yabG* (YEEK8GYABG) mutant strains (Table 1). The resultant transformants were grown in DS medium at 37°C, and cells were collected at T_{10}. In the wild-type strain, the fluorescence of YeeK-GFP was condensed at the spore poles (Fig. 4A). The spores of YEEK8G were bright when analyzed using phase-contrast microscopy, suggesting that YeeK-GFP did not impair spore maturation. Fluorescence of YeeK-GFP was not detected in the *spoIVA* mutant (data not shown). Irregular assembly of YeeK-GFP was observed for *cotE*, *safA*, and *spoVID* mutant spores, indicating that the CotE, SafA, and SpoVID proteins are required for the proper assembly of YeeK-GFP (Fig. 4B, C, and D). YeeK-GFP was distributed within the spore integument in the *yabG* mutant, suggesting that YabG also promotes the correct localization of YeeK (Fig. 4E).
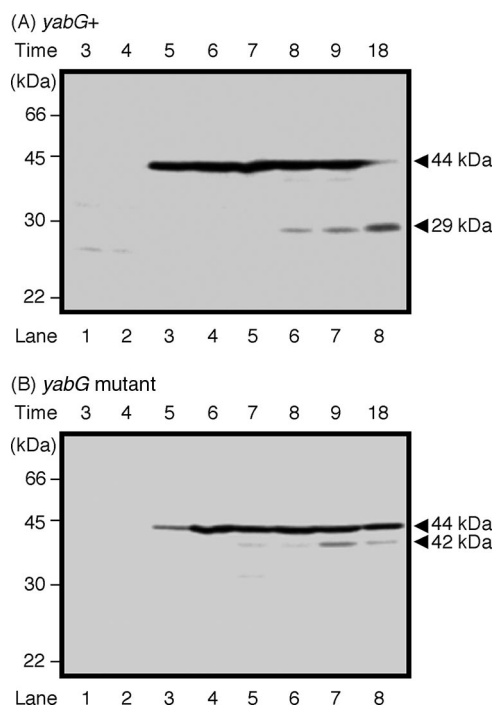

FIG. 3. Immunoblot analysis of YeeK-GFP extracted from sporulating cells. Wild-type (A) and *yabG* mutant (B) cells were harvested at 1-h intervals during stages T_3 to T_9 and T_{18} of sporulation. Whole-protein samples were solubilized from the sporulating cells and analyzed by 14% (wt/vol) SDS-PAGE. Immunoblotting analysis was performed using anti-GFP antiserum. Arrowheads indicate the positions of the YeeK-GFP protein (at 44 kDa) and degradation products (at 29 and 42 kDa). The length of time (in hours) after the end of the exponential growth phase is shown at the top.

Comparative analysis of the YeeK-RFP and GFP fusions of spore proteins. Immunoelectron microscopy is a conventional method used to determine the localization of spore coat proteins. However, this method requires special training and equipment. Many recent studies of protein localization in *B. subtilis* have exploited fluorescent microscopy using fluorescent proteins. It can be difficult to resolve the inner and outer spore coats by this method. We attempted to examine the localization of YeeK via comparative analyses of two types of fluorescent proteins (GFP and RFP). The GFP fusions used in this study were expressed in the presence of YeeK-RFP in *B. subtilis* (Fig. 5). The *yeeK-rfp* gene was introduced into the *aprE* region of the *B. subtilis* chromosome by a Campbell-type single-crossover recombination method, and expression of the genes was regulated by the *yeeK* promoter. The authentic genes encoding CgeA, CotA, CotE, CotT, YabG, YeeK, and YhcN were replaced with the respective *gfp* fusions. The localization profile of YeeK-RFP was consistent with that of YeeK-GFP (Fig. 5A). The CgeA, CotA, CotT, and YabG proteins are synthesized in the mother cell compartment, under the control of SigK (37). The *cge* operon, including the *cgeA* gene, influences the surface properties of spores; however, the localization of the Cge proteins remains unknown (39). A CotA-GFP fusion should indicate the position of the outer spore coat (23), and both CgeA-GFP and CotA-GFP fusions were detected outside of the YeeK-RFP fusion protein (Fig. 5B and C). The

(A) wild type

(B) *cotE* mutant

(C) *safA* (*yabA*) mutant

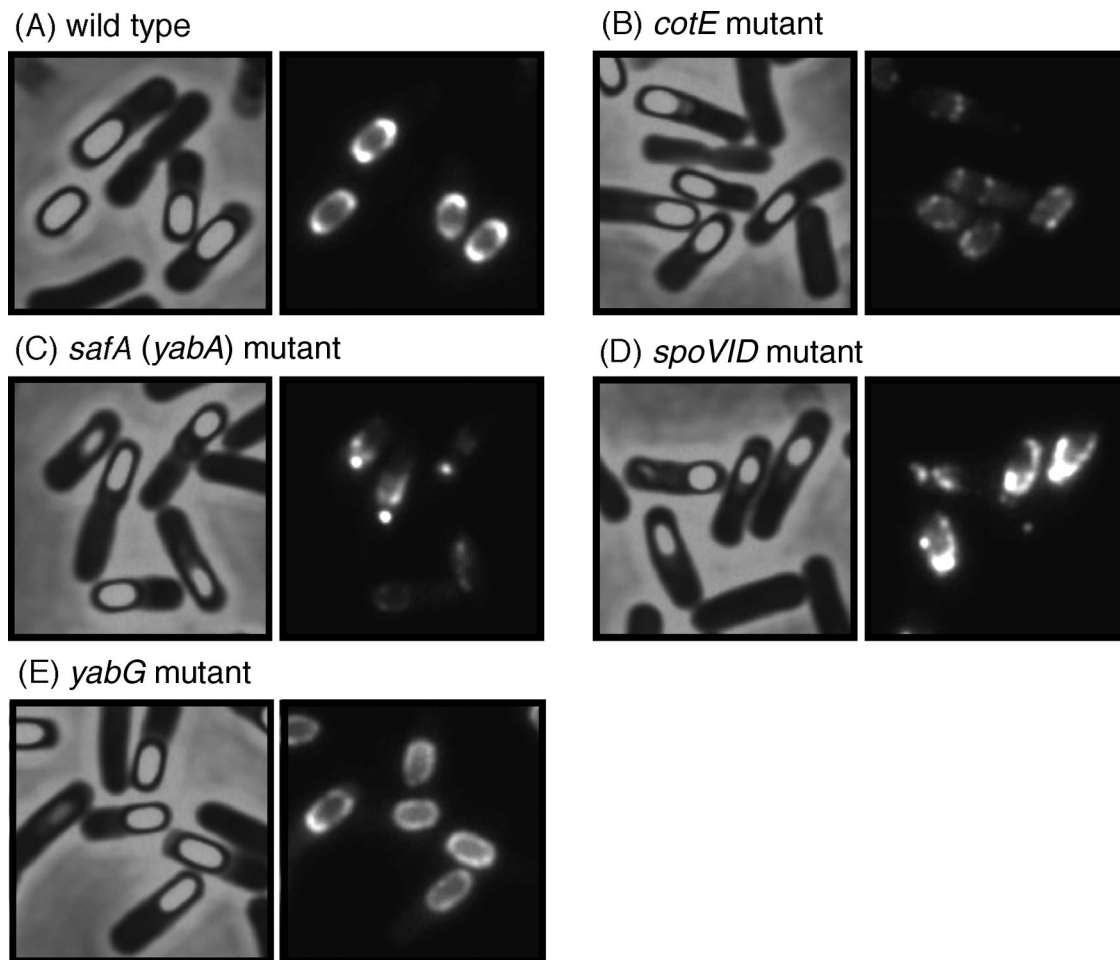## (D) *spoVID* mutant

(E) *yabG* mutant

FIG. 4. Detection of YeeK-GFP fusion proteins in sporulating cells. Cells expressing the YeeK-GFP fusion protein were allowed to sporulate in DS medium. Samples of sporangia from wild-type (A), *cotE* mutant (B), *safA* mutant (C), *spoVID* mutant (D), and *yabG* mutant (E) cells were obtained at stage T_{10} of sporulation and observed. Images resulting from phase-contrast (left panels) and fluorescence (right panels) microscopy are shown.

expression of CotE is controlled by a SigE-containing RNA polymerase, and CotE is thought to assemble into the outer coat (4, 15, 32, 55, 56). A CotE-GFP fusion was also detected outside of the YeeK-RFP fusion protein (Fig. 5D). A CotT-GFP fusion should indicate the position of the inner spore coat (6, 13, 23). The localization profiles of CotT-GFP and YabG-GFP were identical to that of YeeK-RFP (Fig. 5E and F). These results indicate that YeeK assembles into the inner spore coat in a manner similar to that employed by CotT and YabG. The integument of the *B. subtilis* spore is composed of the two coat layers, the outer spore membrane, and the cortex (2, 52). We analyzed a SigG-dependent spore protein (YhcN) that contained a putative signal peptide at the N-terminal end and potentially localized to the spore membrane or the cortex (3). The YhcN-GFP fusion was detected inside the YeeK-RFP fusion (Fig. 5G), indicating that YeeK was spatially separated from the SigG-dependent integument protein. All of the GFP fusions used in this study did not alter the localization profile of YeeK-RFP under fluorescent microscopy (Fig. 5). Except for YEEK3RPCOTE8G, all of the transformants carrying YEEK-RFP efficiently produced spores with normal resistance and germination properties (data not shown). YEEK3RPCO

TE8G produced lysozyme-sensitive spores under our experimental conditions (data not shown). Webb et al. also reported that free spores released from the sporangia of strain CW271 expressing the CotE-GFP fusion were somewhat abnormal (53). We analyzed the protein composition of the free spores of YEEK3RP and its derivatives by means of SDS-PAGE and then by Coomassie brilliant blue staining and found that the spores of YEEK3RPCOTE8G and YEEK3RPYABG8G were abnormal (data not shown). The protein profile of YEEK3R PCOTE8G was drastically changed, as was that of a *cotE*-disruption mutant. On the other hand, the degree of protein alteration in YEEK3RPYABG8G spores was visually lower than that of a *yabG*-disruption mutant. We mention that the localization profiles of CotE-GFP and YabG-GFP are likely different from those of CotE and YabG, although they do not disturb the assembly of YeeK-RFP.

DISCUSSION

Electron microscopy of *B. subtilis* spores revealed that the spore coat is composed of several distinct substructures (2, 52). Previous studies have attempted to identify these coat compo-
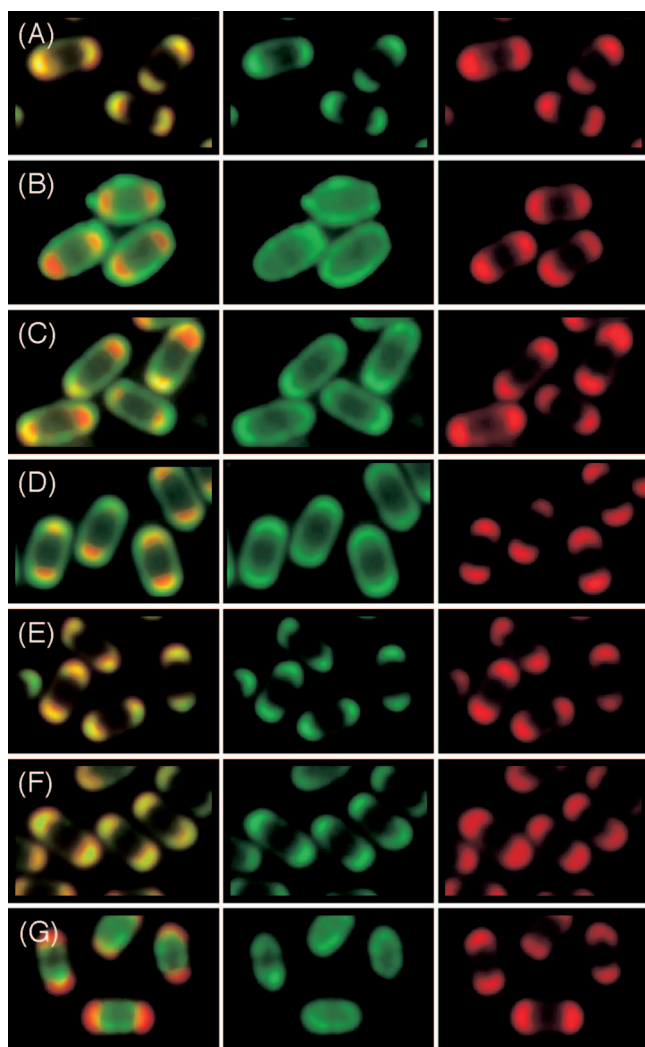
FIG. 5. Comparative analysis of YeeK-RFP and the GFP fusions of spore proteins. Cells expressing YeeK-RFP and YeeK-GFP (A), CgeA-GFP (B), CotA-GFP (C), CotE-GFP (D), CotT-GFP (E), YabG-GFP (F), and YhcN-GFP (G) were allowed to sporulate in DS medium. Mature spores were obtained at stage T_{18} of sporulation and analyzed. Green and red fluorescence overlays are shown (left panels). Green fluorescence (middle panels) and red fluorescence (right panels) microscopic images are also shown separately.

nents and to clarify the function of the spore coat and the molecular mechanisms of coat formation. Recent techniques (e.g., DNA arrays, protein mass spectrometry, and fluorescent microscopy) have enabled the identification of many spore proteins (18, 23, 29, 44). However, the molecular functions of most coat proteins remain unknown, as their respective mutants do not convey any apparent phenotypes. Consequently, we were unable to identify the function of YeeK in this study. Our *yeeK* mutant did not alter spore protein composition under the experimental conditions used in this study (Fig. 2); thus, the YeeK protein is probably not essential for spore resistance or germination. However, the unique amino acid sequence of YeeK suggests that this protein performs a specific function. The YeeK protein is composed of three types of repeat sequences (i.e., four repeats of GXPGY[or F]GM[or H]

in the N-terminal third of the protein, three repeats of GGY[or V]GGY[or S]P in the middle region of the protein, and four repeats of H[or Y]HHHHXG in the C-terminal third of the protein]. These motifs are specific for the YeeK protein, and we were unable to identify *yeeK* gene homologs in the genomic sequences of other *Bacillus* species by using BLAST searches (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). The closest neighbor of *B. subtilis* is *Bacillus amyloliquefaciens* FZB42, which does not carry a homolog of *yeeK*. On the other hand, the *yeeI* gene upstream of *yeeK* in *B. subtilis* 168 is conserved among *Bacillus* species. To determine if *yeeK* is an extraneous gene carried by *B. subtilis* 168, we searched for *yeeK* genes in other *B. subtilis* strains via PCR. A single band corresponding to *yeeK* was detected in each *B. subtilis* strain tested, with the exception of *B. amyloliquefaciens* (data not shown). We propose that a common *B. subtilis* ancestor acquired the *yeeK* gene after separating from the common *B. amyloliquefaciens* ancestor and that the *yeeK* gene has since been conserved among *B. subtilis* strains. Indeed, the YeeK protein or its peptide fragments are present in spores (Fig. 2, 3, 4, and 5). We speculate that YeeK promotes the survival of spores in certain environments.

Recent studies focused on interactions of the spore coat with other proteins (8, 22, 23). In addition, we remain interested in interactions of spore coat proteins and we would like to further investigate the role of YabG. The major proteins involved in coat assembly (SpoIVA, SpoVID, SafA, and CotE) are expressed during stage T_2 of sporulation, under the control of a SigE-containing RNA polymerase. YabG is expressed during stage T_4 of sporulation under the control of a SigK-containing RNA polymerase. The YabG protein is then assembled into the spore coat (48). Purified YabG protein cleaves SafA in vitro (49). However, there is no evidence indicating that YabG directly cleaves SafA and YeeK in the spore coat. YabG and also other proteases and enzymes are possibly involved in the YabG-dependent modification of spore coat proteins. Therefore, we call a hypothetical protease, which cleaves SafA, YeeK, and other coat proteins, "protease-X" below. Protease-X is possibly YabG and/or some protease(s) dependent on YabG. The degradation of SafA and YeeK-GFP in the presence of YabG begins during stages T_6 and T_8 of sporulation, respectively (48, 49) (Fig. 3A). Protease-X is most likely inactive when YabG is synthesized, and activation is delayed by some unknown factors. An active form of protease-X digests SafA, but not YeeK-GFP, in the spore coat during stage T_6 of sporulation (Fig. 3A). We speculate that YeeK-GFP is resistant to protease-X during stages T_6 and T_7, for conformational reasons, or because of protection by associated materials. The intact form of CotE is found in mature spores of *B. subtilis* 168 (i.e., the wild type), suggesting that protease-X does not digest CotE (28). These observations suggest that SpoIVA and SafA form basement layers for the assembly of CotE and other coat proteins, which are ultimately digested by protease-X when their functions have been performed. CotE is involved in the coat protein network and is required for the assembly of many SigE- and SigK-controlled coat proteins, including YeeK (23; this work). In addition, CotE is likely required for a longer period of time than are SpoIVA and SafA; therefore, CotE must be protected from protease-X. We showed in this and previous studies that the CotF, CotT, YeeK, and YxeE pro-

teins are also digested in the presence of YabG (26, 27, 48, 49). The CotT-GFP, YabG-GFP, and YeeK-GFP proteins colocalized with YeeK-RFP (Fig. 5). We examined the localization of CotF and YxeE using GFP fusions and found that CotF-GFP and YxeE-GFP colocalize with YeeK-RFP (data not shown). Furthermore, YabG and substrates of protease-X most likely colocalize within the inner coat and build an interactive network. We were unable to identify consensus sequences within substrates of protease-X; thus, we could not determine how protease-X recognizes its substrates. Some coat proteins, including YabG, are conserved among the endospore-forming bacteria of *Bacillus* and *Clostridia* (19). The YabG protein associates with essential coat proteins and is required for the proper localization of Tgl-GFP, YeeK-GFP, and YxeE-GFP in spores (26, 27). These results suggest that YabG plays an important role in spore formation, although a *yabG* mutant efficiently produces spores with normal resistance and germination properties. We have previously proposed that SpoIVA and SafA are digested by YabG when those two proteins are no longer needed for coat assembly (49). We cannot discuss the meaning of YeeK degradation because a *yeeK* mutant does not show any clear phenotypes.

The spore coat is composed of dozens of proteins that are arranged in an outer layer and an inner layer (11, 12, 13, 14, 47). The previous studies of protein localization in the *B. subtilis* spore coat have exploited fluorescent microscopy using only GFP. It can be difficult to resolve the inner and outer spore coats by this method. We examined the localization of YeeK via comparative analyses of GFP and RFP (Fig. 5). This is the first use of fluorescent proteins to show localization to different layers of the spore coat. Our method will be useful for analysis of the spore coat structures of *B. subtilis*.

ACKNOWLEDGMENT

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