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Expression, Localization and Modification of YxeE Spore Coat Protein in *Bacillus subtilis*

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YxeE is a spore coat protein of *Bacillus subtilis*. We have previously reported that YxeE can be extracted from *yabG* mutant spores. In the present study, we analysed the expression and localization of YxeE. Northern analysis detected the transcript of *yxeE* from sporulating cells 4 h after the onset of sporulation, and revealed that the synthesis of *yxeE* mRNA was dependent on expression of the SigK RNA polymerase and the GerE regulator. Immunoblotting with anti-YxeE antiserum detected YxeE from sporulating cells 4 h after the onset of sporulation. YxeE was detected in the extracts from mature spores of *yabG* mutant strain but not in those from wild-type spores. On the other hand, fluorescence microscope observations showed that YxeE–green fluorescent protein (GFP) is located at the surface of both wild-type and *yabG* spores. Immunoblot analysis with anti-GFP antiserum showed that YxeE–GFP was not digested in *yabG*-disrupted strain and that only the GFP portion remained in the wild-type *yabG* background. We conclude that YxeE is a substrate of YabG protease.

Key words: protein modification, processing, protease, spore coat.

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Bacteria such as *Bacillus subtilis* change their vegetative cells to spores in response to specific environmental conditions such as nutritional insufficiency (*1*, *2*). The spore layers of *B. subtilis* consist of coat, cortex and core. *Bacillus subtilis* sporulation initiates a developmental process with formation of an asymmetric septum that divides the bacterium into two compartments, the mother cell and prespore (Stages 0–II). The prespore is engulfed by the mother cell and becomes the forespore (Stage III) that in turn develops cortex structures (Stage IV) and spore coat structures (Stage V). During the subsequent process, the forespore develops resistance to heat, chemicals, lytic enzymes and radiation. This period is termed maturation (Stage VI). The mother cell eventually lyses and the mature spore is released (Stage VII); the released spore is called the free spore or the dormant spore. Each stage of spore formation in *B. subtilis* is defined according to the morphological features of the sporulating cell.

Endospore formation involves a series of temporally and spatially ordered changes in cell morphology and gene expression. Genes involved in sporulation are mostly transcribed by RNA polymerase, which contains sporulation-specific sigma factors (*1*). Two sigma factors, SigF and SigG, are active specifically in the prespore and forespore, respectively, whereas SigE and SigK direct transcription in the mother cell (*1*). A small DNA-binding protein, SpoIIID, activates or represses transcription of many SigE- and/or SigK-dependent genes; the *spoIIID* gene is transcribed by SigE-containing RNA polymerase in the mother cell (*1*, *3–5*). GerE is another small DNA-binding protein, which also activates or represses transcription of many SigK-dependent genes. The *gerE* gene is transcribed by SigK-containing RNA polymerases in the mother cell (*1*, *3*, *5*). All of the proteins known to be involved in spore coat development are synthesized by SigE- and/or SigK-containing RNA polymerases in the mother cell (*1*, *2*, *6*).

Spore coat formation is the result of a complex, highly controlled process of macromolecular assembly (*6*). The spore coat acts as a barrier, protecting the spore from damage and providing resistance to chemicals and lytic enzymes. More than 40 polypeptides must be properly assembled in the developing forespores to generate the spore coat (*7*, *8*). These proteins are synthesized in the mother cell and then assembled into a thick layer. Several types of post-translational modification are found in coat proteins, including glycosylation, proteolytic processing and crosslinking (*7*). SodA, OxdD (YoaN), YabG and Tgl are particularly important for post-translational modifications. SodA (superoxide dismutase) is required for the assembly of the coat protein CotG into the insoluble spore matrix (*9*), while OxdD (YoaN) is an oxalate decarboxylase that associates with the spore coat structure (*10*). Tgl (transglutaminase) is a spore coat protein that is involved in the modification of some spore coat proteins in *B. subtilis* (*11*, *12*). YabG is a protease in the spore coat of *B. subtilis* (*13*, *14*). The *yabG* gene is transcribed by SigK-containing RNA polymerase and encodes a protease involved in the modification of CotF, CotT, SafA (YrbA), SpoIVA, YeeK and YxeE during sporulation (*13*, *14*). In this study, we report the characterization of YxeE in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media and General Techniques—The *B. subtilis* and *Escherichia coli* strains

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and plasmids used in this study are listed in Table 1. The *B. subtilis* strains are all derivatives of strain 168 and were constructed by transformation with plasmid DNA. Constructed gene configuration was confirmed by polymerase chain reaction (PCR). The *E. coli* strain JM109 was used for the production of plasmids. The oligonucleotides used for PCR amplifications are listed in Table 2.

We used a Campbell-type single-crossover recombination method to construct a series of insertion mutants of *B. subtilis* (*15–17*). Segments of the *yxeE* gene were PCR-amplified (primers listed in Table 2), cut with *Hind*III and *Bam*HI at primer-based sites, and inserted into the *Hind*III–*Bam*HI-digested pMutin3 vector to generate plasmid pYXEE5E (Table 1) (*16*). Segments of the *yabG* gene were PCR-amplified (primers listed in Table 2), cut with *Hind*III and *Bam*HI at primer-based sites and inserted into the *Hind*III–*Bam*HI-digested pCAT5 vector to obtain plasmid pYABG5C (*17*). These plasmids were used to transform *B. subtilis* strain 168 by a Campbell-type single-crossover recombination method with selection for erythromycin resistance

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

Strains	Genotype/description	Source, reference or construction
Bacillus subtilis		
168	*trpC2*	1A1 (*Bacillus* Genetic Stock Center)
SGF602C	*trpC2 sigF*::*cat*	(*17*)
SGE603C	*trpC2 sigE*::*cat*	(*17*)
SGG604C	*trpC2 sigG*::*cat*	(*17*)
SGK605C	*trpC2 spoIVCB (sigK)::cat*	(*17*)
GERE5E	*trpC2 gerE*::pMutin3	(*24*)
YABG5E	*trpC2 yabG*::pMutin3	(*11*)
YABG5C	*trpC2 yabG*::*cat*	This work (pYABG5C, 168)
YXEE5E	*trpC2 yxeE*::pMutin3	This work (pYXEE5E, 168)
YXEE5EYABG5C	*trpC2 yxeE*::pMutin3 *yabG*::*cat*	This work (YXEE5E, YABG5C)
COTE5E	trpC2 *cotE*::pMutin3	(*18*)
YRBA5E	trpC2 *safA (yrbA)*::pMutin3	(*11*)
S6D5E	trpC2 *spoVID*::pMutin3	(*11*)
YXEE8G	*trpC2 yxeE-gfp cat*	This work (pYXEE8G, 168)
YXEE8GYABG	*trpC2 yabG*::pMutin3 *yxeE-gfp cat*	This work (YXEE8G, YABG5E)
YXEE8GCOTE	*trpC2 cotE::pMutin3 yxeE-gfp cat*	This work (YXEE8G, COTE5E)
YXEE8GYRBA	*trpC2 safA (yrbA)::pMutin3 yxeE-gfp cat*	This work (YXEE8G, YRBA5E)
YXEE8GS6D	*trpC2 spoVID::pMutin3 yxeE-gfp cat*	This work (YXEE8G, S6D5E)
Escherichia coli		
JM109	*relA supE44 endA1 hsdR17 gyrA96 mcrA mcrB + thi*Δ*(lac-proAB)/F′(traD36 proAB + lacIq lacZ*Δ*M15)*	(*21*)
Plasmids		
pMutin3	*bla erm lacZ lacI Pspac*	(*16*)
pYXEE5E	*bla erm yxeE′-lacZ lacI Pspac-′yxeE*	This work
pCAT5	*cat*	(*17*)
pYABG5C	*cat yabG′*	This work
pMALEH6	*bla lacI tac promoter malE His6*	(*18*)
pMYXEE1A	*bla lacI tac promoter malE yxeE His6*	This work
pGFP7C	*gfp cat*	(*11*)
pYXEE8G	*yxeE-gfp cat*	This work

Table 2. **Oligonucleotide primers used in this study.**

Name	Sequence	Added site	Resulting construct or products
YXEE330RT7	5′-<u>TAATACGACTCACTATAGGGCGAG</u>CAGCCGGTTTTCTACTGTA-3′	T7	*yxeE* RNA probe
YXEE3	5′-TGAGGATCCGAACCCTTATCAATATTACAG-3′	*Bam*HI	pMYXEE1A
YXEE364R	5′-TTCCTCGAGAAACCGCCTTCCCCTG-3′	*Xho*I	pMYXEE1A
YABG69	5′-CGAAAGCTTGGAATAGAGCAAACAAGCAA-3′	*Hind*III	pYABG5C
YABG220R	5′-GAGGGATCCATTCATTCTGCTCTCATCT-3′	*Bam*HI	pYABG5C
YXEE20	5′-ATCGGATCCACAGCCCTCAGCTGCCT-3′	*Bam*HI	pYXEE8G
YXEE364R	5′-TTCCTCGAGAAACCGCCTTCCCCTG-3′	*Xho*I	pYXEE8G, *yxeE* RNA probe
PMT353RD	5′-AGCATTAGTGTATCAACAAGCTGGGG-3′		primer extension product

Oligonucleotides used for PCR amplifications are listed. The T7 promoter sequence is underlined. PCR products were digested with restriction enzymes at the primer-derived sequences and inserted into restriction enzyme-digested plasmids to generate the plasmids listed in Table 1.

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(0.5 μg/ml erythromycin) or chloramphenicol resistance (5 μg/ml chloramphenicol) to generate strains YXEE5E and YABG5C, respectively (Table 1) (*15–17*). The chromosomal DNA of YXEE5E was introduced into YABG5C to construct the double-mutant YXEE5EYABG.

Oligonucleotide primers YXEE20 and YXEE364R were used to amplify the *yxeE* gene fragment from the *B. subtilis* strain 168 chromosome (Table 2). The PCR product was digested at the *Bam*HI and *Xho*I sites introduced by the primers, and then inserted into *Bam*HI/*Xho*I-digested pGFP7C to create plasmid pYXEE8G (Table 1). Strain 168 was transformed with this plasmid by a single-crossover recombination with selection for chloramphenicol resistance (5 μg/ml) to generate strain YXEE8G (Table 1). The chromosomal DNA of YXEE8G was introduced into strains YABG5E, S6D5E, YRBA5E and COTE5E to construct the YXEE8GYABG, YXEE8GS6D, YXEE8GYRBA, YXEE8GCOTE strains, respectively. The original *yxeE* gene was replaced by the *yxeE–gfp* fusion in these strains.

To construct a series of plasmids, DNA fragments encoding YxeE were PCR-amplified (see Table 2 for primers), digested with restriction enzymes and inserted into *Bam*HI–*Xho*I-digested pMALEH6 (which contains a 6x His tag) to generate the recombinant plasmid pMYXEE1A (Table 1) (*18*). The plasmid was integrated into the chromosomal DNA by homologous recombination.

Bacillus subtilis strains were grown in Difco sporulation (DS) medium (Difco Laboratories, Detroit, MI, USA) (*19*). The conditions for sporulation of *B. subtilis* were as previously described (*20*). In this study, each developmental stage was indicated by T_n, where n was measured in hours after the onset of sporulation. Recombinant DNA techniques were carried out according to standard protocols (*21*). Preparation of competent cells, transformation and preparation of chromosomal *B. subtilis* DNA were carried out as previously described (*22*).

RNA Preparation and Northern Analysis—Total RNA was prepared from *B. subtilis* cells as described previously (*23*). Northern analysis, hybridization and detection were performed using the DIG Northern Starter Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to our previous report (*24*). RNA probes for northern hybridization were synthesized using T7 RNA polymerase with PCR products as templates. The 0.3 kb probe for *yxeE*, corresponding to nucleotides 20–330 downstream of the translation initiation codon of *yxeE*, was prepared by PCR using primers YXEE20 and YXEE330RT7 (Table 2). RNA probes specific for *yxeE* were labelled with the Roche digoxigenin labelling system as previously described (*24*).

Mapping of the 5′ Terminus of yxeE mRNA during Sporulation—Total RNA was extracted from the cells of the strain YXEE5E growing in DS medium 6 h after the onset of sporulation. In strain YXEE5E, the *yxeE* promoter region is fused to the promoter-less *lacZ* gene of pMutinT3 (*16*). The RNA sample was subjected to primer extension assays with a digoxigenin-end-labelled primer (PMT353RD) specific for the sequences on either side of the *Bam*HI site and the *lacZ* gene of pMutinT3. Therefore, this primer, PMT353RD, can detect *yxeE*-specific transcription. RNAs for primer extension analysis were prepared according to a previously described procedure with some modifications (*23*). Primer extension was performed with the 5′-digoxigenin-labelled primer PMT353RD, as described previously (*24*). The RNAs and the oligonucleotide primers were hybridized at 60°C for 1 h. SuperScript II reverse transcriptase (Invitrogen, CA, USA) was added and the mixture was incubated at 42°C for 1 h. DNA ladders for size markers were created using the same 5′-digoxigenin-labelled primers using the dideoxy chain termination method (TAKARA Bio, Ohtsu, Japan). The primer extension products were resolved on DNA sequencing gels, and detected as recommended by DIG detection system (Roche Molecular Biochemicals, Mannheim, Germany).

Phase-Contrast and Fluorescence Microscopy—Aliquots of the cultures in DS medium of strains harbouring the *yxeE–gfp* fusion were transferred to a microscope slide. Fluorescence from the green fluorescent protein (GFP) was observed under a BX51 fluorescent microscope with a GFP mirror cube unit (Olympus, Tokyo, Japan). The images were captured with a CoolSNAP ES/OL cooled charge-coupled device camera (Roper Scientific, Trenton, NJ, USA), and processed with RS Image Express version 4.5 (Roper Scientific, Trenton, NJ, USA), as previously described (*11*).

Preparation of Spores—The *B. subtilis* strains were grown in DS medium at 37°C as described previously (*17*). Spores were harvested 18 h after the cessation of exponential growth (T_{18}) and washed once with 10 mM Tris–HCl (pH 7.2). The spore samples were then prepared according to a previously described procedure with some modifications (*17*). To remove cell debris and vegetative cells, the pellets were suspended in 0.1 ml lysozyme buffer [10 mM Tris–HCl, pH 7.2 with 1% (w/v) lysozyme] and incubated at 37°C for 10 min. Complete protease inhibitor cocktail (Roche) was added to the lysozyme buffer. The pellets were then washed repeatedly with buffer (10 mM Tris–HCl, pH 7.2 and 0.5 M NaCl) at room temperature. After this treatment, more than 99% of the spores were refractive, and almost no dark or grey spores were visible by phase-contrast microscopy. Furthermore, the samples contained neither vegetative cells nor cell debris (data not shown). The pellets were then washed with buffer (10 mM Tris–HCl, pH 7.2) at room temperature. The pellets were suspended in 10 mM Tris–HCl, pH 7.2.

Solubilization of Proteins from Mature Spores—Spore proteins were solubilized in 0.1 ml of loading buffer [62.5 mM Tris–HCl, pH 6.8, 10% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue] and boiled for 5 min (*17*). A Bio-Rad RC DC protein assay kit was used to measure the protein quantities in the samples, as described previously (*25*). The proteins were separated by 14% SDS–PAGE and visualized by staining with Coomassie brilliant blue R-250 (CBB).

Preparation and Solubilization of Whole Proteins from Sporulating Cells—The sporulating samples were prepared according to a previously described procedure with some modifications (*20*). Cells were grown in DS medium and samples were harvested at appropriate times during sporulation. Harvested cells of each sample (10 ml) were

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washed with 10 mM Tris–HCl (pH 7.6). The pellets were suspended in 100 μl of 10 mM Tris–HCl (pH 7.6). Hundred micro litres of 0.4 mg/ml lysozyme in 10 mM Tris–HCl (pH 7.6) (final concentration 0.1 mg/ml lysozyme) was added to the samples, mixed and incubated at 37°C for 10 min. Proteins were solubilized in 0.2 ml of loading buffer [62.5 mM Tris–HCl, pH 6.8, 10% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue] and boiled for 5 min. The proteins were separated by 14% SDS–PAGE, as previously described (*20*).

Purification of Recombinant MalE-YxeE and GFP Proteins from E.coli—*Escherichia coli* cells were transformed with recombinant plasmids pMYXEE1A and pGFP7C. The resulting transformants were grown at 37°C for 3 h in 200 ml of Luria broth supplemented with ampicillin (50 μg/ml). The cultures were then supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated for an additional 3 h at 37°C. The His-tagged recombinant proteins MalE-YxeE and GFP were purified by affinity chromatography on Ni-NTA agarose beads (Qiagen, Hilden, Germany), and were further purified by electro-elution from the resulting SDS–PAGE, as previously described (*20*).

Preparation of Antisera Against MalE-YxeE and GFP—We prepared antisera against MalE-YxeE and GFP as follows. The purified proteins (1 ml of 0.2 mg/ml) and 16 mg of killed *Mycobacterium tuberculosis* cells (Difco) were mixed with 2 ml of complete Freund's adjuvant (Difco). Three millilitres of each emulsion was injected into healthy rabbits. After 2 weeks, the rabbits were injected with solutions of the purified proteins in incomplete Freund's adjuvant (Difco). Two weeks after the second immunization, antiserum was isolated from each rabbit as previously described (*20*).

SDS–PAGE and Immunoblotting—Protein samples were analysed by 14% SDS–PAGE as described previously (*17*). Immunoblotting was performed using rabbit immunoglobulin G (IgG) against YaaH, MalE-YxeE or GFP, as described previously (*18*). In agreement with a previous report (*18*), we found that the anti-*E. coli* MalE antiserum did not react with any spore proteins of *B. subtilis* (data not shown). The anti-GFP antiserum did not react with any of the *B. subtilis* spore proteins.

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 previously described (*26*). The cultures were heated at 80°C for 30 min or were treated with lysozyme (250 μg/ml final concentration) at 37°C for 10 min. After the cultures were serially diluted with distilled water in steps of 100-fold, appropriate volumes of the dilutions 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—To study the effects of the germination temperature, purified spores were heat-activated at 80°C for 20 min, cooled and suspended in 10 mM Tris–HCl (pH 7.2) buffer to an optical density of 0.5 absorbance units at 600 nm. Spores were then mixed with either L-alanine (10 mM) or AGFK (10 mM L-asparagine, 10 mM D-glucose, 10 mM D-fructose and 10 mM potassium chloride). Germination was monitored for up to 120 min by measuring the decrease in optical density at 660 nm of the spore suspension at 37°C (*27*).

RESULTS

Sporulation-Specific Expression of the yxeE Gene—In this study, developmental stage of sporulation was indicated by T_n, where *n* was measured in hours after the onset of sporulation. Aliquots were collected from T_0 to T_8 cultures. To determine the expression pattern and the transcription unit of the *yxeE* gene, total RNAs were analysed by northern hybridization (Fig. 1A). A 0.3 kb transcript was first detected in T_4 cells by a probe specific for *yxeE*. No *yxeE* mRNA was detected in any of the sigma factor mutants or in *gerE*-deficient cells at T_6 (Fig. 1A). The locus and probe of *yxeE* gene used in this experiment are shown in Fig. 1B. The size of the transcript was consistent with the estimated length of *yxeE* mRNA. *yxeE* mRNA was monocistronically transcribed. To further analyse the dependency of *yxeE* expression on sigma factors, the start point of its transcription was mapped by primer extension analysis (Fig. 1C). We performed primer extension analysis using a synthetic oligonucleotide primer and RNA isolated from sporulating YXEE5E cells (see MATERIALS AND METHODS section). The size of the transcript indicated that transcription of *yxeE* started at a G residue 27 nt upstream of the proposed start codon of *yxeE* (Fig. 1C and D). The nucleotide sequences of -35 and -10 regions of the *yxeE* promoter were similar to the consensus sequence of the -35 (mACm) and -10 (CATA—Ta) promoter region recognized by RNA polymerase containing SigK (Fig. 1D) (*28*). The dependency of *yxeE* expression on GerE indicates that one or more GerE-binding sites are located near the *yxeE* promoters. Indeed, two putative GerE-binding sites are present near the *yxeE* promoter sequence (Fig. 1D) (*29*). These results indicate that the expression of *yxeE* is dependent on SigK-containing RNA polymerase and is positively controlled by GerE in the mother cell compartment.

Properties of yxeE-Deficient Spores—The vegetative growth of the *yxeE*-deficient cells in DS medium was the same as those of wild-type cells (data not shown). Mature spores of the *yxeE* mutant harvested at T_{18} had resistance to heat and lysozyme, which was similar to the observations with wild-type spores (data not shown). Germination of *yxeE* spores in L-alanine and in a mixture of L-asparagine, D-glucose, D-fructose and potassium chloride was also similar to that of wild-type spores (data not shown).

We analysed the protein composition of spores prepared from wild-type and mutant strains of *yxeE* by SDS–PAGE and CBB staining (see MATERIALS AND METHODS section). The protein profile of *yxeE* mutant spores was similar to that of wild-type spores (data not shown).

YxeE is a Substrate for YabG—We analysed the proteins extracted from the wild-type, *yxeE, yabG* and the *yabG*/*yxeE* spores by immunoblotting with the anti-YxeE antiserum. We used purified spores obtained from T_{18} cultures. Spores were prepared in the presence of a protease inhibitor cocktail to prevent protein

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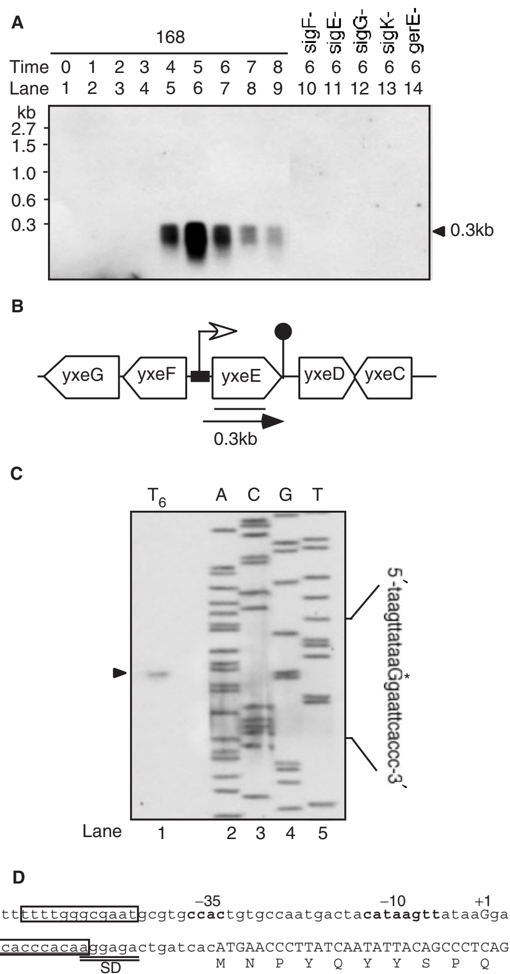

Fig. 1. **Northern analysis and primer extension analysis of *yxeE* mRNA.** (A) Total RNA was prepared from sporulating cells, and mRNAs were detected by northern hybridization using probes specific for *yxeE*. The arrowhead indicates the position of *yxeE* mRNA hybridized with the digoxigenin-labelled RNA probe. Transcripts of *yxeE* in strain 168 (lanes 1–9), and *sigF* mutant (lane 10), *sigE* mutant (lane 11), *sigG* mutant (lane 12), *sigK* mutant (lane 13) and *gerE* mutant (lane 14) cells were analysed by northern hybridization. The time (h) after the onset of sporulation is shown. (B) The position (indicated by thick bar) of the probe used in this experiment, the promoter for *yxeE* (bar with open arrow), and the terminator (lollipop) are shown. The filled arrow indicates the length and direction of mRNA hybridizing with a digoxigenin-labelled RNA probe. (C) RNA was prepared from sporulating cells of YXEE5E 6 h after the onset of sporulation. Primer extension analysis and sequencing were performed with the digoxigenin-labelled primer. Primer extension product is marked with an arrowhead and the transcription start site of the *yxeE* gene is marked by a capital letter with an asterisk. Lane 1 shows the primer extension products. The lanes labelled A, C, G and T contain the products of DNA sequencing reactions with the primer (lanes 2–5). (D) The nucleotide sequence of the promoter region of *yxeE* and the predicted amino-acid sequences of the N-terminal portion of YxeE are shown. Upstream of the coding region, the -35 and -10 sequences recognized by the sporulation-specific transcription factor SigK RNA polymerase are shown in bold. The experimentally determined transcription start site (+1) is indicated by an uppercase letter. A putative ribosome-binding site of the *yxeE* gene is doubly underlined (SD). Putative GerE-binding sites are boxed.

degradation (see MATERIALS AND METHODS section). We then extracted the spore proteins and performed immunoblot analyses to detect YxeE (Fig. 2A). YxeE is estimated to be 14,574 Da based on its primary amino acid sequence (Japan Functional Analysis Network of *B. subtilis* database; http://bacillus.genome.jp). YxeE was not detected in the wild-type, *yxeE* or *yabG*/*yxeE* spores (Fig. 2A, lanes 1–3). In the *yabG* spores, a 21 kDa band was detected (Fig. 2A, lane 3). We concluded that YxeE was a substrate for YabG protease. As a control, YaaH was analysed using anti-YaaH antiserum, and was detected in extracts from the wild-type, *yxeE, yabG* and *yabG*/*yxeE* spores (Fig. 2B). YaaH is involved in germination, and is expressed by SigE-containing RNA polymerase during sporulation (*27*). YaaH has been reported to exist as a 50 kDa monomer in wild-type cells (*11*). Visual analysis indicated that the densities of the 50 kDa YaaH band in each lane were equal (Fig. 2B), so we used YaaH as a loading control in the following experiments.

Detection of YxeE Protein in yabG Mutant Cells—To examine the synthesis and accumulation of the YxeE proteins, we prepared protein samples from T_0 to T_8 and T_{18} cultures. We performed immunoblotting with anti-YxeE antiserum (Fig. 3). In protein samples from wild-type cells, YxeE was detected in samples from T_4 to T_8 cultures, but was hardly detected in samples from T_{18} cultures (Fig. 3, lanes 1–6); whereas, in protein samples from *yabG* mutant cells, YxeE was detected in samples from T_4 to T_8 and T_{18} cultures (Fig. 3, lanes 7–12). In contrast, YaaH was detected in samples from T_2 to T_8 and T_{18} cultures in extracts from the wild-type spores and *yabG* spores (Fig. 3). These results indicate that most YxeE was digested before T_{18} in the wild-type cells.

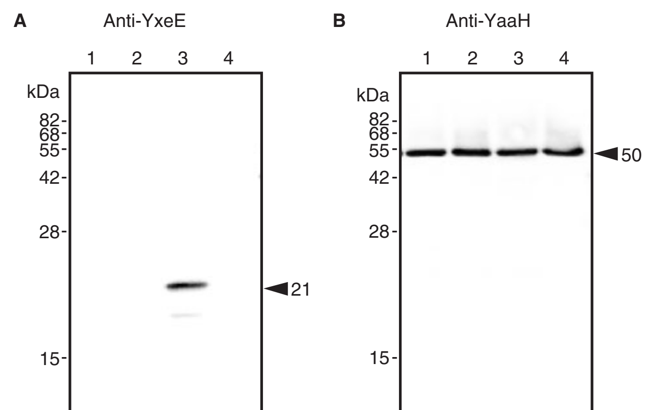

Fig. 2. **Immunoblot analysis of YxeE proteins extracted from purified spores.** Cells were cultured for 24 h in DS medium at 37°C. Purified spores were prepared in lysozyme buffer containing a complete protease inhibitor cocktail (Roche). A portion (10 μg) of protein solubilized from wild-type cells (lane 1), YXEE5E spores (lane 2), YABG5C spores (lane 3) and YXEE5EYABG spores (lane 4) was resolved by 14% SDS–PAGE, and subjected to immunoblot analyses with anti-YxeE (A) and anti-YaaH (B) antisera. The arrowheads show the position of each protein.

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Fig. 3. **Immunoblot analysis of YxeE extracted from sporulating cells.** Wild-type (lanes 1–6) and YABG5E (lanes 7–12) strains were grown in DS medium at 37°C. Whole protein samples were solubilized from the sporulating cells. The samples were resolved by 14% SDS–PAGE, and immunoblot analyses were performed with anti-YxeE antiserum. Arrowheads show the positions of YxeE (upper panel) and YaaH (lower panel). YaaH was analysed as a loading control using anti-YaaH antiserum (lower panel). The number of hours after the end of the exponential growth phase is shown.

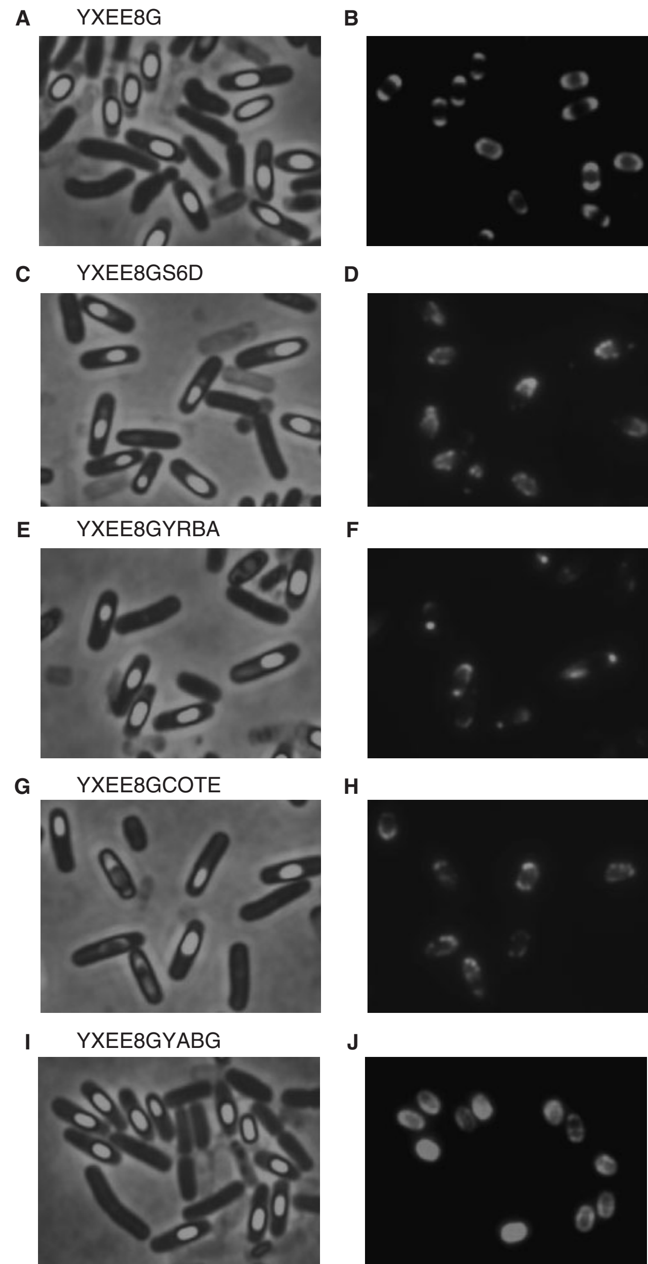

Fig. 4. **Detection of YxeE–GFP fusion in T_{18} cells.** YXEE8G (A and B), YXEE8GS6D (C and D), YXEE8GYRBA (E and F), YXEE8GCOTE (G and H) and YXEE8GYABG (I and J) strains harbouring the *yxeE–gfp* fusion were grown in DS medium at 37°C. The cells were collected 18 h after the onset of sporulation and analysed by phase-contrast microscopy (A, C, E, G and I) and fluorescence microscopy (B, D, F, H and J).

Localization of YxeE–GFP during Sporulation—We constructed a gene encoding the YxeE–GFP fusion protein to examine the localization of YxeE. We introduced the *yxeE–gfp* fusion into the chromosomes of the wild-type and the *spoVID*, *safA*, *cotE* and *yabG* mutant strains. SpoVID, SafA and CotE are required for the proper assembly of many coat proteins (*26*, *30*, *31*). The resulting transformants were grown in DS medium at 37°C and analysed at T_{18} (Fig. 4). Both free spores and forespores are present in the cultures at T_{18}, as shown by phase-contrast microscopy observations (Fig. 4). In the wild-type transformants, the fluorescence of YxeE–GFP was detected around the edges of free spores and forespores but not in the mother cell compartment (Fig. 4B). The fluorescence of YxeE–GFP was concentrated at both polar ends of free spores and forespores (Fig. 4B). Few free spores were observed in T_{18} cultures of the *spoVID*, *safA* and *cotE* mutants with phase-contrast microscopy, because SpoVID, SafA and CotE are essential proteins for spore coat formation (*26*, *30*, *31*). In the *spoVID*, *safA* and *cotE* mutant transformants, the fluorescence of YxeE–GFP was abnormally detected in the mother cell compartment and/or around the edges of the forespores (Fig. 4D, F and H). The level of YxeE–GFP fluorescence was slightly reduced in the *spoVID*, *safA* and *cotE* mutant strains compared with the wild-type strain. These results indicate that YxeE is synthesized in the mother cell compartment and its assembly in the spore coat is mediated by SpoVID, SafA and CotE. In the *yabG* mutant, the fluorescence of YxeE–GFP is located around the edges of free spores and forespores but is not concentrated at the polar ends (Fig. 4J).

The Fluorescence of YxeE–GFP Remains at the Spore Coat after 7 Days Incubation—We studied the stability of GFP at the spore coat. Strains YXEE8G and YXEE8GYABG were incubated in DS medium at 37°C for 7 days. We analysed the cells using fluorescent microscopy (Fig. 5). Almost all YXEE8G and YXEE8GYABG spores were released from mother cells (Fig. 5A and C). The fluorescence of YxeE–GFP was detected around the edges of free spores and was concentrated at both polar ends of spores in the YXEE8G strain (Fig. 5B). In the 7-day spores of YXEE8GYABG, YxeE–GFP fluorescence was partially condensed at the edges of the spores; however, the localization was a little different from that of YxeE–GFP at T_{18} (Fig. 4J and 5D). These results showed that GFP was functional for 7 days in both the wild-type and the *yabG* spores.

YxeE–GFP is Processed by YabG—To investigate the YxeE–GFP fusion proteins processed by YabG,

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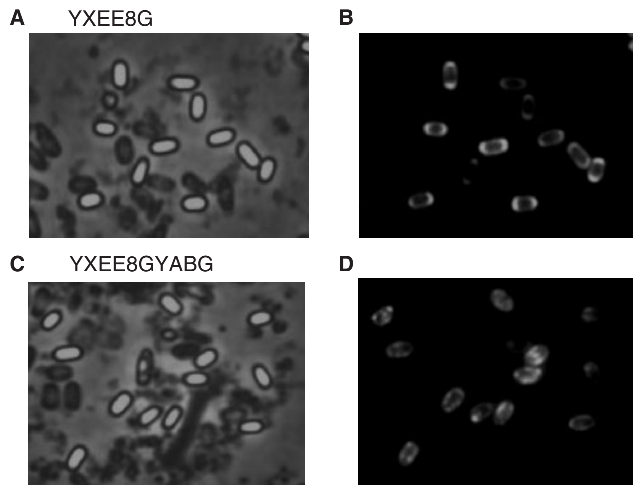

Fig. 5. **Detection of YxeE–GFP fusion in 7-day spores.** YXEE8G (A and B) and YXEE8GYABG (C and D) strains harbouring the *yxeE–gfp* fusion were incubated in DS medium at 37°C for 7 days. The cells were analysed by phase-contrast microscopy (A and C) and fluorescence microscopy (B and D).

we performed immunoblotting with anti-YxeE and anti-GFP antisera (Fig. 6). Cells were grown in DS medium at 37°C and analysed at T_{18} (Fig. 6, lanes 1–6). Using anti-YxeE antiserum, 21 kDa bands were detected in the *yabG* mutant cells (Fig. 6A, lane 2). This is in agreement with the previous result (Fig. 2A). A 43 kDa band was detected at low levels in the YXEE8GYABG strain (Fig. 6A, lane 6), which corresponded to the predicted molecular sizes of YxeE and GFP fusion proteins. The band of YxeE was not detectable in the wild-type, YXEE5E, YXEE5EYABG and YXEE8G spores (Fig. 6A, lanes 1, 3–5). Using anti-GFP antiserum, a 29 kDa band was detected in the YXEE8G spores (Fig. 6B, lane 5), which corresponded to the predicted size of GFP. In the YXEE8GYABG spores, a 43 kDa band was detected (Fig. 6B, lane 6), which was also in agreement with the predicted molecular size of the YxeE–GFP fusion protein.

The modification of some spore coat proteins proceeds steadily in mature spores under appropriate conditions (*2*). The localization of YxeE–GFP after 7 days of cultivation differed from that of YxeE–GFP in T_{18} cells (Fig. 4J and 5D). Therefore, we analysed whether the protein modification of YxeE and YxeE–GFP occurred during the 7-day culture period. Cells were collected and spore proteins were analysed by SDS–PAGE and immunoblot analyses (Fig. 6, lanes 7–12). Using anti-YxeE antiserum, a 21 kDa band was detected in samples of T_{18} spores and 7-day spores of YABG5C (Fig. 6A, lanes 2 and 8). The YxeE band was not detected in the wild-type, YXEE5E, YXEE5EYABG and YXEE8G spores (Fig. 6A, lanes 7, 9–11). A 43 kDa band was still detectable in the 7-day culture sample of the YXEE8GYABG cells (Fig. 6A, lane 12). This molecular size corresponded to the predicted molecular size of the YxeE–GFP fusion protein. The 43 kDa band was also detected with anti-GFP antiserum (Fig. 6B, lanes 6 and 12). The density of the

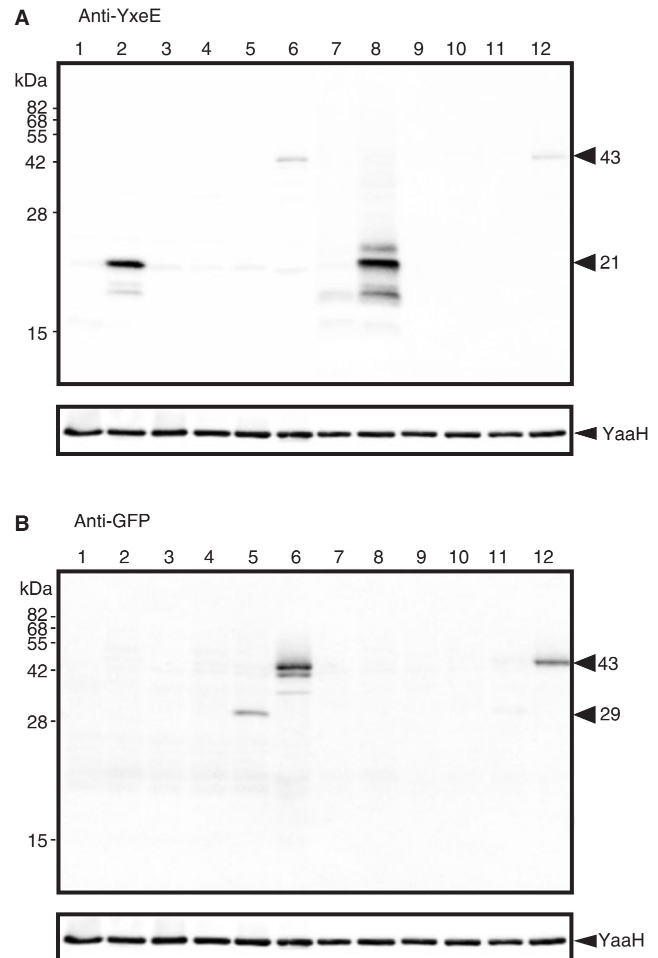

Fig. 6. **Immunoblot analysis of YxeE and GFP extracted from purified spores.** Wild-type (lanes 1 and 7), YABG5C (lanes 2 and 8), YXEE5E (lanes 3 and 9), YXEE5EYABG (lanes 4 and 10), YXEE8G (lanes 5 and 11) and YXEE8GYABG (lanes 6 and 12) strains were incubated in DS medium at 37°C, and the spores were purified 18 h after the onset of sporulation (lanes 1–6) and 7 days (lanes 7–12). Proteins were solubilized as described in the MATERIALS AND METHODS section. The samples were resolved by 14% SDS–PAGE, and immunoblot analyses were performed with anti-YxeE (A) and anti-GFP (B) antisera. The arrowheads show the position of each protein. YaaH was analysed as a loading control using anti-YaaH antiserum (lower panel).

43 kDa band was reduced after the 7-day culture period. We suggest that YxeE–GFP was partially digested by some proteases in YXEE8GYABG. The 29 kDa band that corresponded to the intact size of GFP, was detected using anti-GFP antiserum in T_{18} spores but not in 7-day spores of YXEE8G (Fig. 6B, lanes 5 and 11). We assumed that the GFP in the YXEE8G 7-day spores was modified and incorporated into insoluble material. No bands were detected with anti-GFP antiserum in the wild-type, YABG5C, YXEE5E or YXEE5EYABG spores (Fig. 6B, lanes 7–10). These results show that YxeE–GFP is stable in the mature spores of *yabG* mutant, and that GFP was modified by YabG or other modification enzymes in YXEE8G.

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DISCUSSION

We have previously detected YxeE proteins with molecular masses of 6–24 kDa in purified wild-type spores using liquid chromatography and mass spectrometry (LC-MS/MS) (*17*). In this study, YxeE protein with a molecular mass of 21 kDa was detected from T_4 to T_8 cells by immunoblot analysis with anti-YxeE antiserum (Fig. 3); however, this protein was not detected with anti-YxeE antiserum in T_{18} wild-type spores (Fig. 2A). The LC-MS/MS method is highly sensitive and a small quantity of YxeE protein and its fragments were detected in the purified spores. YxeE protein in T_{18} spores was probably digested and/or cross-linked with other materials, although no fragments of YxeE were detected with anti-YxeE antiserum (Fig. 2A). However, the 21 kDa band of YxeE was detected in the protein extracts from T_4 to T_{18} *yabG* mutant cells with anti-YxeE antiserum (Fig. 4, lanes 7–12). The processed form of SafA was detected in the wild-type spores, whereas the intact form was detected in the *yabG* spores (*14*). We have previously reported that SafA is a substrate of YabG protease *in vitro*. Immunoblot analysis with anti-SpoIVA, anti-CotF and anti-CotT antisera did not detect SpoIVA, CotF and CotT, respectively, in the wild-type spores (*11*, *14*); however, they were detected in the *yabG* spores in our previous study (*11*, *14*). Expression of the *yxeE* gene was dependent on the SigK RNA polymerase and the GerE regulator (Fig. 1). These results indicate that YxeE is a spore coat protein that is synthesized in the mother cell. YxeE is likely to be proteolytically processed by YabG in the wild-type cells.

We confirmed that YxeE–GFP fusion proteins also localize to the spore coat in T_{18} YXEE8G cells (Fig. 4A and B). The YxeE–GFP fluorescence is detected at the polar cap regions of released spores and forespores of wild-type strain, in agreement with previous reports (*32*). In contrast, the YxeE–GFP fluorescence was abnormally distributed in the forespores of *spoVID*, *safA* and *cotE* mutants (Fig. 4C–H). The coat assembly with YxeE–GFP is independent of *cotE* (*32*). YxeE is synthesized in the mother cell compartment and attaches to the forespore surface in the presence or absence of CotE; however, the localization of YxeE–GFP in *cotE* spores differed from that in wild-type spores (Fig. 4B and H). We suggest that CotE and/or some CotE-dependent spore coat proteins are required for the correct localization of YxeE–GFP in spores.

The fluorescence of YxeE–GFP was observed all over some T_{18} forespores of *yabG* mutants (Fig. 4J). We suggest that YabG protease is involved in the correct localization of YxeE–GFP, and that YxeE–GFP is therefore evenly dispersed in the spore coat of the *yabG* mutant cells. YxeE–GFP in the YXEE8G cells was stable after 7 days of cultivation and the fluorescence distribution of YxeE–GFP was similar to that of T_{18} YXEE8G cells (Figs 4B and 5B). On the other hand, 7 days of cultivation altered the fluorescence of YxeE–GFP in the *yabG* mutant (Fig. 5D). The fluorescence of YxeE–GFP in the *yabG* mutant was partially reduced and irregularly maintained. These results indicate that YxeE–GFP is digested and/or moved in the spore coat during the 7-day culture period.

The results of immunoblotting with anti-YxeE and anti-GFP antisera showed that YxeE–GFP was cleaved at or near to the junction between YxeE and GFP by YabG protease (Fig 6, lane 5). We suggest that the YxeE portion of the YxeE–GFP is also processed by YabG, like the authentic YxeE protein, and that the GFP portion is active and its fluorescence can be detected in T_{18} YXEE8G spores (Fig. 4B). In T_{18} YXEE8G spores, the GFP portion was detected with anti-GFP antiserum (Fig. 6B, lane 5). However, after 7 days culture, the GFP portion could not be detected with anti-GFP antiserum in the YXEE8G spores (Fig. 6B, lane 11). These results show that the GFP portion is cross-linked to some insoluble materials during the long incubation period, although the fluorescence activity of GFP is maintained. Here, we are not able to discuss which spore coat protein caused this modification. On the other hand, we found partial proteolysis of YxeE–GFP in the 7-day YXEE8GYABG spores with anti-GFP antiserum (Fig. 6, lane 12). The localization of YxeE–GFP in the 7-day spores of YXEE8GYABG differs from that in T_{18} cells (Figs 4J and 5D). Therefore, we assume that coat materials are incompletely fixed in T_{18} forespores and spores.

SafA is a substrate of YabG and its processing is followed by the synthesis of YabG at T_5 in the developing forespores (*14*). We have reported that the modification of SpoIVA and SafA with *de novo* synthesis of coat-modification enzymes is almost complete at T_{18} (*14*). Although we have found that YabG is involved in the cross-linking of GerQ in purified spores (*11*), the temperature-dependent modification of GerQ occurs in the absence of YabG (*11*). The cross-linking of GerQ mediated by Tgl occurs after mother cell lysis (*12*). We conclude that the rearrangement of some spore coat proteins proceeds independently of *de novo* protein synthesis after the localization of spore coat proteins and lysis of the mother cell.

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