J. Biochem. **133**, 475–483 (2003)
DOI: 10.1093/jb/mvg062

Transcriptional, Functional and Cytochemical Analyses of the *veg* Gene in *Bacillus subtilis*

Tatsuya Fukushima[1], Shu Ishikawa[1], Hiroki Yamamoto[1], Naotake Ogasawara[2] and Junichi Sekiguchi[*,1]

[1]Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda, Nagano 386-8567; and [2]Graduate School of Information Science, Nara Institute of Science and Technology, Ikoma, Nara 630-0101

Received December 30, 2002; accepted January 27, 2003

A *Bacillus subtilis veg* mutant exhibited a small reduction of absorbance, a large reduction of hexosamine release, and slow dipicolinic acid release from spores during germination with L-alanine as a germinant. But *veg* spores exhibited normal resistance to chloroform, 2-propanol, lysozyme, and heat. SDS–polyacrylamide gel electrophoresis of spore coat proteins revealed no difference in coat proteins between the wild type and the *veg* mutant. Northern and *veg-lacZ* fusion analyses indicated that the *veg* gene is transcribed in both the vegetative growth and sporulation phases, and primer extension analysis indicated an identical transcriptional start point in both phases. The upstream sequence suggests that *veg* is transcribed by $E\sigma^A$ RNA polymerase. Veg-GFP fusion protein was detected for vegetative cells and spores, but the fluorescence of mother cells disappeared completely in the late sporulation phase. Decoated spores containing Veg-GFP exhibited a strong green fluorescence in the core, but much weaker fluorescence in the cortex.

Key words: *Bacillus subtilis*, germination mutation, spore coat protein, *veg* gene, GFP.

Sporulation of *Bacillus subtilis* is regulated by sporulation-specific sigma factors. After initiation of sporulation, gene expression is controlled by σ^F in the forespore compartment and σ^E in the mother cell compartment. Later in sporulation, when the forespore has become engulfed by the mother cell, σ^F and σ^E are replaced by σ^G and σ^K, respectively (*1*, *2*). The coordinate functions of this cascade of sigma factors eventually transform the cell into a spore. σ^A is a major sigma factor in the vegetative growth phase. During sporulation, σ^A is also functional and thus called a house-keeping sigma factor (*1*, *2*).

The *veg* gene of *B. subtilis* encodes an 86-amino-acid polypeptide (*3*), but its function is not yet known and there is no significant motif in the amino acid sequence (Pfam database, Sanger Centre). This gene is, however, a useful tool for exploration of the mechanism of transcription, since it is strongly transcribed in cells growing in a variety of media (*4*). Previous reports indicated that *veg* is transcribed during the vegetative growth phase and that a σ^A consensus sequence is located upstream of the *veg* transcription start site (*3*, *4*). Losick and colleagues also reported that *veg* was transcribed by σ^A RNA polymerase during sporulation (*5*, *6*). The upstream and downstream genes of *veg* are *yabG* and *sspF*, which are transcribed by $E\sigma^K$ and $E\sigma^G$ RNA polymerases, respectively (*7*, *8*). *yabG* encodes a germination-specific protease consisting of 290 amino acid residues (*7*, *9*), and its disruption leads to an altered coat protein composition (*7*). *sspF* encodes a small acid-soluble spore protein (alpha/beta type SASP) consisting of 61 amino acid residues (*8*).

In this communication, we report the characterization of a *veg* mutant, localization of the Veg protein, and transcriptional profiles during sporulation.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Bacillus subtilis 168 *trpC* and *Escherichia coli* JM109 *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ (*lac-proAB*) [F′: *traD*36 *proAB lacI*^{q} *lacZ* ΔM15] or C600 *supE44 hsdR thi-1 leuB6 lacY1 tonA21* were used as cloning hosts. A *veg*-deletion strain (VEGdd) was constructed as follows. The upstream and downstream regions of the *veg* gene were amplified by PCR with veg-UF (5′-ctcctcgAGCTTGTTATTTTTGCGGGG; the underlining and lowercase letters indicate a *Xho*I site and tag sequence, respectively) as forward primer and veg-UR (5′-ggaggatcCGGACAACGTCTTCGCCATT; the underlining indicates a *Bam*HI site) as reverse primer, and with veg-DF (5′-aagaagcTTTTAACGGGCAGTGAACCT; the underlining indicates a *Hin*dIII site) as forward primer and veg-DR (5′-ctcctcgAGGATGAACATATCCATA; the underlining indicates a *Xho*I site) as reverse primer, respectively, and 168 DNA as a template. The PCR products were digested with the corresponding restriction enzymes, then ligated into *Hin*dIII and *Bam*HI-digested pMUTINT3 (*lacZ lacI bla erm*) (*10*). The resulting recombinant plasmid was used to transform *E. coli* cells, then plasmid DNA prepared with *E. coli* was used to transform *B. subtilis* 168. Upon double crossing-over integration, the plasmid was inserted into the chromosome, resulting in a *veg*-deficient strain

[*]To whom correspondence should be addressed. Tel: +81-268-21-5344, Fax: +81-268-21-5345, E-mail: jsekigu@giptc.shinshu-u.ac.jp

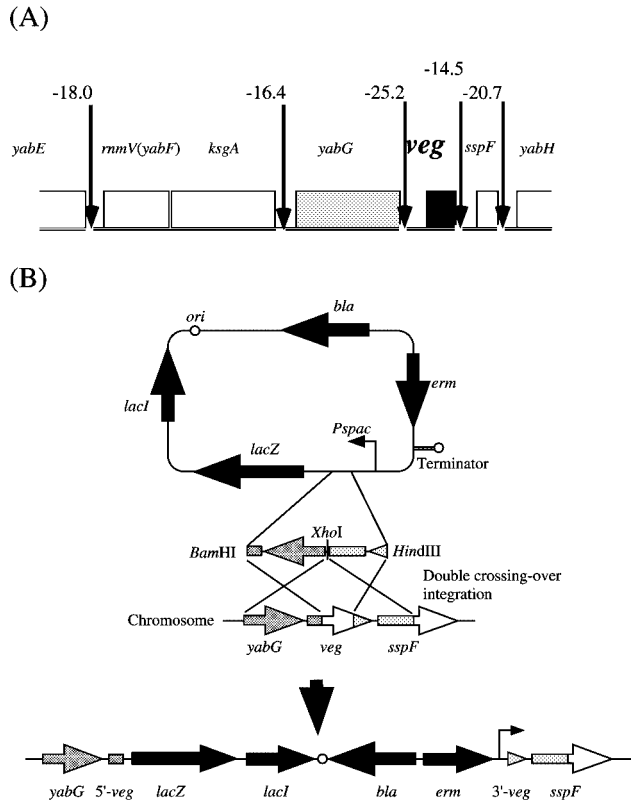

Fig. 1. **A map of *veg* and the neighboring genes (A), and construction of the VEGdd strain (B).** The arrows indicate the deduced rho-independent terminators with ΔG values (kcal/mol) (A). Disruption of the *veg* gene was carried out by insertion of a pMUTINT3 derivative into the *B. subtilis* chromosome. The gene order at the integration site is shown in panel B.

(VEGdd). Thus, the gene order in the chromosome is *yabG*-5′*veg-lacZ-lacI-ori-bla-erm*—3′*veg-sspF* (Fig. 1).

For complementation analysis of the *veg* mutation, a fragment containing the promoter, the Shine-Dalgarno (SD) sequence and the structural gene of *veg* was amplified by PCR with VEG-FW (5′-gccgGCGTACAGACATTCTAAGC; the lowercase letters indicate a tag sequence) as forward primer, VEG-TEMPD (5′-gccgGGATCCAAACAAAAGGTTCACTGCC; the underlining indicates a *Bam*HI site) as reverse primer, and 168 DNA as a template. The PCR product was blunt-ended with a Takara BKL Kit (Kyoto), then ligated into *Hinc*II-digested and dephosphorylated pGEM3Zf(+) (Ap^r, Promega), resulting in pGMPRveg. *Bam*HI- and *Xba*I-digested pGMPRveg DNA was ligated into the *Bgl*II and *Xba*I-digested DNA of pDHAFBΔP (*bla amyE*::(*cat lacI*)) derived from pDHAFB (*bla amyE*::(*cat lacI* Pspac)) (11, unpublished results). The resulting plasmid, pDHPRveg (*bla amyE*::(*cat lacI veg*)), was linearized with *Pst*I and then used for the transformation of VEGdd. After the double crossing-over event, a Cm^r transformant, AMPRvegdd, containing an intact *veg* gene in the *amyE* locus was constructed.

The nucleotide sequences of all PCR products in this manuscript were confirmed by sequencing with a DNA sequencer (Applied Biosystems, model 373A).

Construction of Translational GFP Fusion Strains—To prepare a VEG-GFP strain containing the *veg-gfp* translational fusion, three intermediate plasmids were constructed. A fragment containing a signal sequence, P_{spac} region, oid sequence and a multicloning site from pMUTIN4 was prepared by PCR with Spac-MunI (5′-gcgcCAATTGTACACAGCCCAGTC; the underlining indicates a *Mun*I site and the lowercase letters indicate a tag sequence) as forward primer, PM-RX2 (5′ccgCTCGAGGATTAAGTTGGGTAACGC; the underlining indicates a *Sma*I site) as reverse primer, and pMUTIN4 as a template. The PCR product was digested with *Mun*I and *Sma*I, followed by ligation to the *Eco*RI and *Sma*I sites of pDHAFB. Thus the resultant plasmid, pOIDAFB, contained a P_{spac}-oid-multicloning site upstream of the *amyE*-back region. A *gfp*-containing plasmid, pQBI63 (*bla gfp*) (*12*), was digested with *Xba*I and *Bam*HI, the digested DNA fragments were separated by agarose gel electrophoresis, and a *gfp*-containing fragment was extracted with a GenecleanII Kit (Funakoshi). This *gfp*-containing fragment was ligated with the *Xba*I and *Bgl*II-digested pOIDAFB. The resultant plasmid, pOIDGFP, was digested with *Hin*dIII and *Apa*I, and *gfp*-containing fragment was ligated to the corresponding sites of pMUTIN4, resulting in pM4GFP. VEG-GFP was constructed as follows. A fragment containing the 3′ region of the *veg* structure gene was amplified by PCR with ΔVG-U (5′-gccgAAGCTTC_{32}GCTTGATGGGAATTTAGG; the underlining indicates a *Hin*dIII site and the numbering is from the translational start codon of *veg*) as forward primer, VEG-GFP (gcgcTCTAGAA_{258}AATGCCACTGAGCTTGC; the underlining indicates a *Xba*I site and A_{258} corresponds to the complementary nucleotide of the 3′ terminus of *veg*) as reverse primer, and a chromosomal DNA as a template. The amplified DNA was digested with *Hin*dIII and *Xba*I, followed by ligation to the corresponding sites of pM4GFP. The resultant plasmid, pM4ΔvegG, was used for transformation of *B. subtilis* 168. The resultant VEG-GFP (*veg*::pM4GFP) was a single crossing-over recombinant and contained a *veg-gfp* translational fusion instead of *veg* in the chromosome.

Construction of Transcriptional GFP Fusion Strains—To construct a transcriptional *veg-gfp* fusion strain, VEGdg, a fragment containing the 3′-region and its downstream region of *veg* was amplified by PCR with ΔVG-U (gccgAAGCTTC_{32}GCTTGATGGGAATTTAGG; the underlining indicates a *Hin*dIII site and the numbering is from the translational start codon of *veg*) as forward primer, VEG-TEMPD (gcgcGGATCC$_{282}$AAACAAAAGGTTCACTGCC; the underlining indicates a *Bam*HI site) as reverse primer, and a chromosomal DNA as a template. The amplified fragment was blunt-ended with BKL kit (Takara), then ligated with *Eco*RV-digested and dephosphorylated pBluescriptII (SK$^+$), resulting pBLΔveg. After *Hin*dIII and *Eco*RI digestion of pBLΔveg, a fragment containing the 3′-region of *veg* was prepared by agarose gel electrophoresis. The fragment was ligated with the corresponding sites of pM4GFP, resulting in pM4veg-gfp. The pM4veg-gfp plasmid was used to transform *B. subtilis* 168 by single crossover, resulting in VEGdg (*veg*::pM4veg-gfp) containing the entire *veg* and *gfp* genes.

To construct a transcriptional *cotE-gfp* fusion strain, COTEdg, a fragment containing the 5′-region and its

upstream region of *cotE* was amplified by PCR with COTE-HP (gccgAAGCTT$_{-274}$CTGTTCTCTCTAAACACGG; the underlining indicates a *Hin*dIII site and the numbering is from the translational start codon of *cotE*) as forward primer, COTE-ER (gcgcGAATTC$_{16}$CCCTGTATTC-AGACATTCC; the underlining indicates a *Eco*RI site) as reverse primer, and a chromosomal DNA as a template. The amplified fragment was blunt-ended with a BKL kit (Takara), then ligated with *Eco*RV-digested and dephosphorylated pBluescriptII (SK$^+$), resulting in pBLcotE. After *Hin*dIII and *Eco*RI digestion of pBLcotE, a fragment containing the 5′-region of *cotE* was prepared by agarose gel electrophoresis. The fragment was ligated with the corresponding sites of pM4GFP, resulting in pM4cotE-gfp. The pM4cotE-gfp plasmid was used for transformation of *B. subtilis* 168 by single crossover, resulting in COTEdg (*cotE*::pM4cotE-gfp) containing a promoter (SigE) region followed by the 5′-region of *cotE* and the entire region of *gfp*.

Culture Conditions—*B. subtilis* and *E. coli* were cultured in LB medium (*9*) at 37°C. For sporulation, DSM medium (*13*) was used. If necessary, chloramphenicol (5 μg ml^{-1}), for *B. subtilis*, and ampicillin (50 μg ml^{-1}), for *E. coli*, were added to the medium.

Transformation of E. coli and B. subtilis—*E. coli* transformation was performed as described by Sambrook *et al*. (*14*), and *B. subtilis* transformation was performed by the competent cell method (*15*).

β-Galactosidase Assay—The assay was performed basically as described by Shimotsu and Henner (*16*). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from 2-nitrophenyl-β-D-galactopyranoside in 1 min.

Northern Blot and Primer Extension Analyses—Cells (15 A_{600}) cultured in DSM medium were harvested and suspended in 1 ml of chilled killing buffer (*17*). After centrifugation at 12,000 ×*g* for 1 min at 4°C, the pellet was suspended in 1 ml of SET buffer containing lysozyme (final, 6 mg/ml). After incubation for 10 min at 0°C, the suspension was centrifuged at 12,000 ×*g* for 1 min at 4°C. The pellet was used for RNA preparation with Isogen (Nippon Gene) according to the manufacturer's instructions. Agarose-formaldehyde gel electrophoresis was performed as described by Sambrook *et al*. (*14*). The transfer of RNAs onto a nylon membrane (Magnagraph, Micron Separations) was performed with a vacuum blotter (model BE-600; Biocraft). The DNA fragment used for preparing an RNA probe was amplified by PCR with M13(-21) and M13RV (Takara) as primers, and pGMveg DNA, containing the internal region of the *veg* gene, as a template. The amplified fragment was digested with *Hin*dIII, and then fragments were purified by phenol and chloroform treatment, followed by precipitation with ethanol. The RNA probe was prepared with a DIG RNA Labeling Kit (Roche Diagnostics), and Northern (RNA) hybridization was performed according to the manufacturer's instructions. Primer extension analysis was performed as described previously (*13*), using the VEG-PE primer (5′-TTGCTTTTAACGTCAGCC; the 5′ and 3′ ends corresponding to the complementary nucleotides at positions 73 and 56 with respect to the 5′ end of the *veg* gene).

Spore Germination and Outgrowth—*B. subtilis* 168 and disruptants were cultured on LB plates for 12 h at 37°C, then cells were inoculated into 5 ml of DSM medium and incubated for 4 h at 37°C. After centrifugation of the preculture, cells were cultured in 50 ml of DSM medium for 52–60 h (until t_{48}) at 37°C. After centrifugation of the culture, a mixture of free spores and sporangia was suspended in 10 ml of deionized water and washed by centrifugation basically as described by Nicholson and Setlow (*18*) until all the cell debris and vegetative cells had been removed. For routine experiments, the "spore washing" was repeated once daily for 2 weeks. The purified spores (0.3 A_{580}) were heat-activated at 70°C for 30 min, unless otherwise noted, then diluted with a 10 mM Tris-HCl buffer (pH 8.4). Germination was initiated by the addition of L-alanine to a final conc. of 10 mM or AGFK (L-asparagine, D-glucose, D-fructose, and KCl) to final conc. of 10, 1, 1, and 10 mM, respectively (*19*). At appropriate times (0, 30, 60, 90, and 120 min for routine experiments), the A_{580} of the mixture was measured and, if necessary, samples were taken and centrifuged in a microfuge. The supernatant (1 ml) was used for the measurement of released dipicolinic acid as described by Nicholson and Setlow (*18*). The rest of the supernatant was dried up with a concentrator (model CC-180; Tomy), followed by measurement of the released reducing groups by the method of Elson-Morgan, with *N*-acetylglucosamine as a standard (*18*). One half ml of a spore suspension (0.3 A_{580}) was mixed with 2.5 ml of LB medium or 2.25 ml of LB supplemented with 0.25 ml of L-alanine solution (10 mM L-alanine in 10 mM Tris-HCl, pH 8.4), and then germination following outgrowth at 37°C was monitored at A_{580}. To measure hexosamine release with AGFK as a germinant, the germinant was removed from spores by membrane filtration (0.30 μm diameter) after a 30-min incubation at 37°C. The spores were then washed and resuspended in the 10 mM Tris-HCl buffer, and the hexosamine released was measured as described above.

Determination of Solvent and Heat Resistance of Spores—Chloroform (final 10%), 2-propanol (final 10%), or lysozyme (final 250 μg/ml) was added to a spore suspension, followed by incubation for 10 min at room temperature or at 37°C in the case of lysozyme treatment. Heat treatment was performed at 80°C for 10 min. Treated spore suspensions were diluted and plated on LB agar. After a 12-h incubation at 37°C, the numbers of colonies (CFU) with or without the above treatments were counted. CFU/spore for VEGdd and 168 without treatment was 42% and 54%, respectively.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS–PAGE) of Spore Coat Proteins—To purify spores, urografin density gradient centrifugation was performed as described by Nicholson and Setlow (*18*). The purified spores (5 A_{580} amount) were solubilized in 0.1 ml of loading buffer [0.12 M Tris-HCl (pH 6.8), 4% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue], and boiled for 5 min, basically as described by Kodama *et al*. (*20*). The resulting samples were analyzed by SDS–PAGE (14% polyacrylamide) (*21*).

Phase and Fluorescence Microscopy of Vegetative and Sporulating Cells—Cells expressing green fluorescence were prepared as follows. A VEG-GFP strain containing

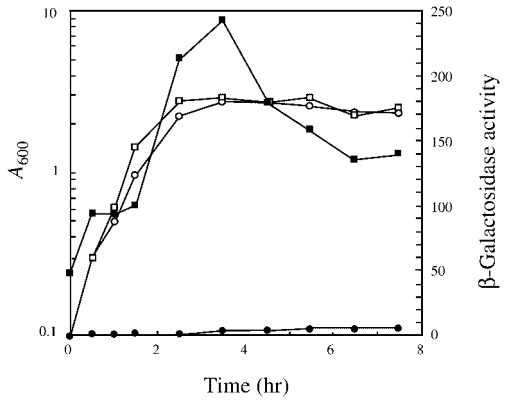

Fig. 2. **β-Galactosidase activity of the *veg-lacZ* transcriptional fusion strain (VEGdd).** Closed and open symbols are β-galactosidase activity and cell growth (A_{600}), respectively. Circles and squares are the wild-type and VEGdd strains, respectively.

the *veg-gfp* translational fusion, or VEGdg and COTEdg strains containing *veg-gfp* and *cotE-gfp* transcriptional fusions, respectively, were cultured in DSM at 37°C for various times. After centrifugation of their cultures, cells were washed and stained with 4,6-diamidino-2-phenylindole (DAPI; final concentration, 1.0 μg/ml; Wako). Phase and fluorescence microscopy was carried out as described previously (*12*). To determine localization of the LacZ protein during sporulation, cells (0.3 A_{580}) were washed and suspended in 100 μl of ultrapure water, and then a part of the cell suspension was stained with FDG (fluorescein-di-β-D-galactopyranoside; a final concentration, 0.33 mM) for 30 min at 37°C. Fluorescence microscopy of FDG-binding spores with an Olympus BX61 microscope was carried out with the dichroic mirror cube unit for FDG containing a band-pass (470 to 490 nm) excitation filter and a narrow-band-pass (510–550 nm) barrier filter.

Preparation of Decoating Spores—Spores were decoated basically as described by Makino *et al.* (*22*). Purified spores (3 A_{580} amount) were suspended in 1 ml of decoating solution 1 (2 M urea, 25 mM 2-mercaptoethanol, 50 mM Tricine-NaOH, pH 9.0) and incubated for 6 h at 37°C. After centrifugation of spores, the above procedure was repeated once. After centrifugation, treated spores were suspended in 1 ml of decoating solution 2 (30 mM SDS, 200 mM 2-mercaptoethanol, 50 mM Tricine-NaOH, pH 9.0) and incubated for 6 h at 37°C. After centrifugation of the spores, the procedure was repeated once. The partially decoated spores were washed several times with water, then suspended in 1 ml of decoating solution 3 (1 mg/ml trypsin, 50 mM MOPS-NaOH, pH 7.0) and inubated for 12 h at 37°C. After washing with water, decoated spores were suspended in ultrapure water.

Fluorescence Microscopy of the Decoated Spore—A 0.1-ml portion of the decoated spore suspension (0.01 A_{580}) was mixed with 2 μl of 3.3 mg/ml wheat germ agglutinin tetramethylrhodamine conjugate (WGA; Molecular Probes) and the solution was centrifuged at 15,000 rpm for 5 min at 4°C. The supernatant was added to 100 μl of ultrapure water. Fluorescence microscopy of WGA-binding spores with an Olympus BX61 microscope was carried out with the dichroic mirror cube unit for WGA containing a wide-band-pass (520 to 550 nm) excitation filter and a long-pass (580 nm) barrier filter.

RESULTS

By systematic genome functional analysis of *B. subtilis*, we found that a *veg*-disrupted spore exhibited very poor reduction of absorbance during germination with L-alanine (*19*). Therefore, we started analyzing the function of *veg* especially during sporulation and germination.

(1) Expression of the Veg Gene—A *veg-lacZ* fusion strain, VEGdd, was cultured in DSM medium, and β-galactosidase activity was determined. Strong activity was detected during not only the vegetative growth phase but also the sporulation phase (Fig. 2). To confirm the results, Northern hybridization analysis of mRNAs from the wild type was carried out. The *veg* probe hybridized to a 0.3-kb fragment throughout the vegetative and sporulation phases (Fig. 3A). Since the *veg* gene consists of 258 nucleotide residues, and the deduced rho-independent terminators are located upstream and downstream of the *veg* gene (Fig. 1), it was transcribed as a monocistronic mRNA (Fig. 3A). Previous reports also showed that *veg*

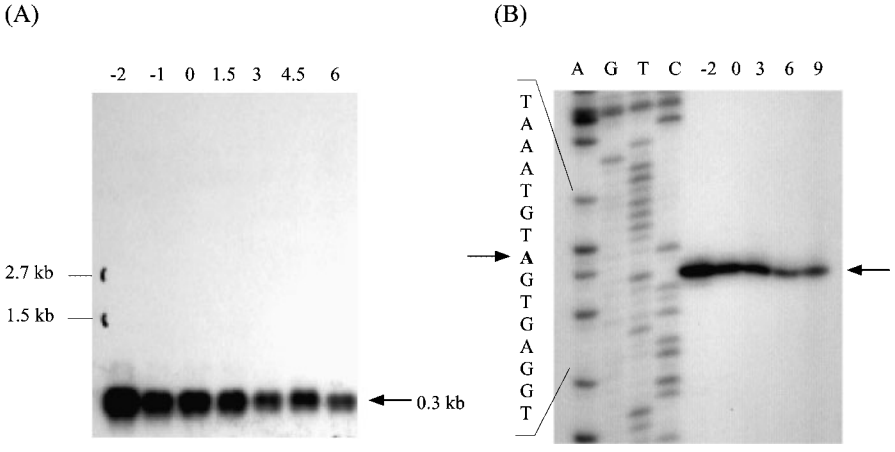

Fig. 3. **Northern blot analysis (A) and primer extension analysis (B) of transcription of the *veg* gene.** (A) The wild-type strain was grown at 37°C in DSM, and cell samples were taken at t_{-2} (-2), t_{-1} (-1), t_0 (0), $t_{1.5}$ (1.5), t_3 (3), $t_{4.5}$ (4.5), and t_6 (6). RNAs prepared from the cells were separated on a gel, and signals were detected with a DIG-labeled specific *veg* RNA probe. The positions of large and small rRNAs (2.7 and 1.5 kb, respectively) are shown by bars, and an arrow indicates the hybridization band at the 0.3 kb position. (B) RNAs from wild-type cells cultured in DSM to t_{-2} (-2), t_0 (0), t_3 (3), t_6 (6), and t_9 (9) were used as RNA samples. Signals were detected with ^{32}P-labeled primer VEG-PE. A dideoxy DNA sequencing reaction mixture with the same primer was electrophoresed in parallel (lanes G, A, T, and C). The nucleotide sequence of the sense strand is given beside the sequence ladder, and the arrow indicates the nucleotide at the transcriptional start site.

was transcribed during vegetative and sporulation phases, and the transcription initiation site during the vegetative phase was A as shown in Fig. 3B (*4*). To determine the transcription start point during the sporulation phase, primer extension analysis was performed with RNAs from 168 and the primer VEG-PE. An extended product was detected for both the vegetative growth and sporulation phases, and the products started at A, which was 15 bp upstream of the translational start codon, ATG (Fig. 3B). The upstream sequences, TTGACA and TtGTAcAAT (capital letters, consensus sequence), with spacing of 14 bp, were identical and very similar to the –35 and –10 consensus sequences, respectively, of sigma A (TTGACA and TGNTATAAT, respectively), with spacing of 14 bp (*8*).

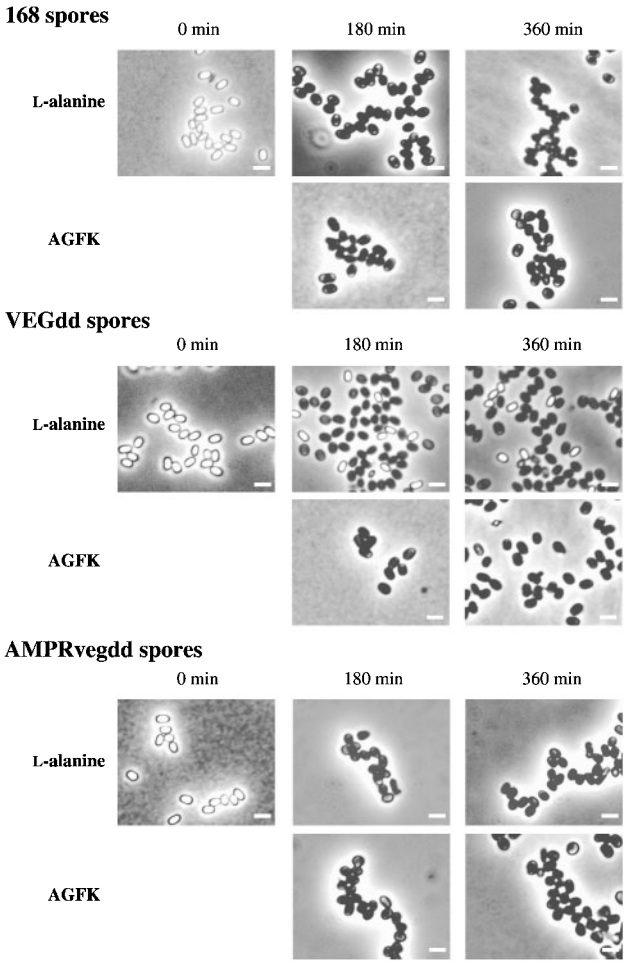

Fig. 4. **Phase-contrast microscopy of dormant and germinated spores of wild-type strain 168, *veg*-deficient strain VEGdd, and the complemented strain AMPRvegdd containing the intact *veg* gene at the *amyE* locus and the *veg*-disruption at the original *veg* locus, during germination in a buffer containing L-alanine or AGFK as germinant.** Time indicates the incubation period at 37°C. The bars indicate 2 μm.

(2) Morphology and Germination Profiles of Veg-Deficient Spores—The *veg*-deficient mutant VEGdd showed normal growth, cell separation and motility, and produced bright refractive spores in DSM medium (Fig. 4). The colony-forming ability (CFA) of spores of VEGdd after heat treatment at 80°C for 10 min was 27%, which was not significantly different from the level for the wild-type 168 spores (46%).

After heat activation, germination was measured by monitoring the decrease in the A_{580} of a spore suspension upon the addition of L-alanine or AGFK at 37°C (Fig. 5A). Spores of VEGdd responded to the germinant, and the A_{580} values of the spore suspensions with L-alanine slowly decreased by about 35% after 180 min, while those with AGFK decreased by about 45% (Fig. 5A). In contrast, the values for wild-type spores decreased rapidly, by 60% and 45%, with L-alanine and AGFK, respectively. It has been reported that *yabG*-deficient (YABGd) spores show normal germination with L-alanine and AGFK (*7*). Therefore, germination of the VEGdd spores with L-alanine was different from that of *yabG*-deficient spores after 180 min (Fig. 5A). During the incubation with L-alanine, the bright spores of VEGdd changed into two types: a major population consisting of phase-gray spores and a minor one of bright spores. After a 360-min incubation, the gray spores became dark, but bright spores were still observed. Swelling of the former VEGdd spores seemed to be poor with L-alanine (Fig. 4). With AGFK, all of the VEGdd spores became dark, similar to the wild-type spores (Fig. 4). Dipicolinic acid release by VEGdd with L-alanine was different from that by the wild type (63% of the wild-type level after 180 min), but the difference was smaller with AGFK (Fig. 5B). A great difference

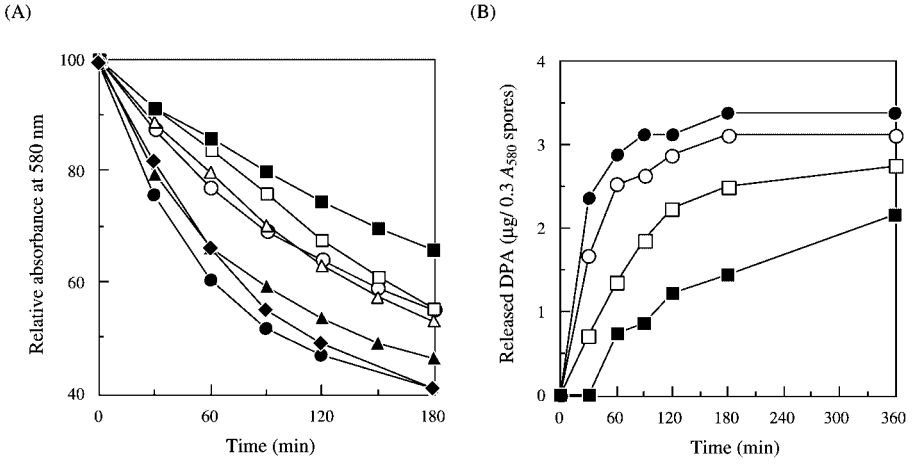

Fig. 5. **Spore germination of *B. subtilis* 168, VEGdd, AMPRvegdd, and VEG-GFP with L-alanine and/or AGFK.** (A) The germination of spores of *B. subtilis* strains was monitored at A_{580} at the indicated times after the addition of germinants and is expressed as the relative absorbance. (B) The released dipicolinic acid in the supernatants of the spore suspensions was measured. Closed and open symbols indicate germination with L-alanine and AGFK, respectively. Circles, squares, triangles, and diamonds indicate 168, VEGdd, AMPRvegdd, and VEG-GFP, respectively.

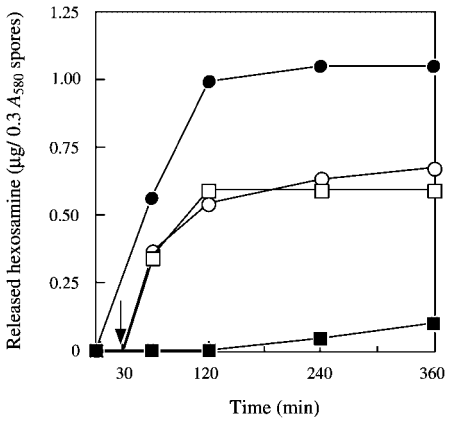

Fig. 6. **Hexosamine release from *B. subtilis* spores during germination.** Closed and open symbols indicate germination with L-alanine and AGFK, respectively. Circles and squares indicate 168 and VEGdd, respectively. For AGFK, spores were washed after 30 min of incubation (as shown by arrow), and then resuspended in the Tris-HCl buffer. Released hexosamine in the supernatants of the spore suspensions was measured.

between the VEGdd and wild-type spores was found in the release of polysaccharides containing hexosamine with L-alanine (Fig. 6). In the case of AGFK, high concentrations (1 mM) of glucose and fructose in the germinant solution affected the background level for hexosamine determination, thus the germinant was removed by centrifugation after 30 min of incubation, and the spores were resuspended in the buffer. Hexosamine release by the VEGdd spores was very similar to that by the wild-type ones (Fig. 6). A lack of hexosamine release with L-alanine has been reported for a mutant of *cwlD*, which has been deduced to be a cell wall-lytic *N*-acetylmuramoyl-L–alanine amidase gene (*23*). Although *cwlD* mutant spores did not show any outgrowth in a rich medium at 37°C (*23*), VEGdd spores showed normal outgrowth in LB + L-alanine (data not shown).

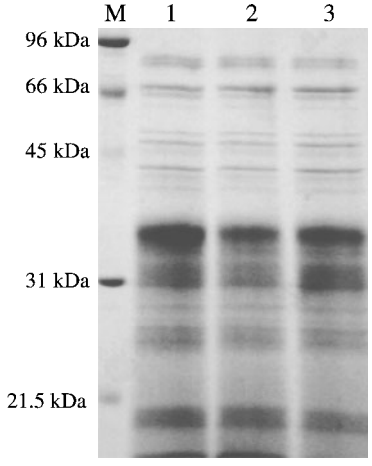

Fig. 7. **SDS–polyacrylamide gel electrophoresis of spore coat proteins from 168, VEGdd, and AMPRvegdd strains.** Coat proteins extracted from spores (1.67 A_{580}) were applied onto a gel as described in Materials and Methods. M, molecular mass marker proteins (Biorad); lane 1, AMPRvegdd; lane 2, VEGdd; lane 3, 168. The molecular sizes of the marker proteins are shown on the left.

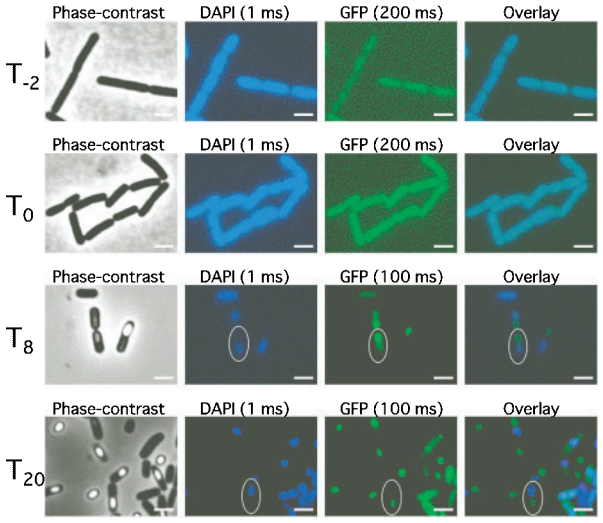

Fig. 8. **Localization of the Veg-GFP translational fusion protein.** The VEG-GFP strain containing the *veg-gfp* translational fusion was cultured in DSM medium at 37°C, and cells were collected at t_{-2}, t_0, t_8, and t_{20} followed by DNA staining with DAPI. Overlay is a DAPI image overlaid on a GFP image. Exposure time for DAPI and GFP detection are shown in parentheses. White circles indicate typical spore-forming cells in which mother cells and spores exhibit a weak (t_8) to almost no expression (t_{20}) and a strong expression (t_8 and t_{20}) of Veg-GFP, respectively. Bars indicate 2 μm.

(3) Complementation—The pDHPRveg plasmid containing the intact *veg* gene was inserted into the *amyE* locus of VEGdd. The resulting strain, AMPRvegdd, exhibited a normal germination profile with L-alanine (Figs. 4 and 5). Therefore, introduction of the intact *veg* gene into the *amyE* locus complemented the *veg*-deficient phenotype.

(4) SDS–PAGE Profile of Spore Coat Proteins and the Other Properties of the Veg-Deficient Mutant—VEGdd spores were resistant to chloroform, 2-propanol and lysozyme, the levels being similar to those of the wild-type spores (20%, 37%, and 26% CFU/spores for VEGdd, and 39%, 39%, and 44% for 168, respectively). Spore coat proteins of VEGdd, the *veg*-complementing strain AMPRvegdd, and the wild-type 168 were analyzed by SDS–PAGE (Fig. 7). The profile of VEGdd was very similar to those of AMPRvegdd and 168. Therefore, the *veg* mutation did not affect the protein profile of the spore coat.

(5) Localization of the Veg Protein during Sporulation—A *veg-gfp* translational fusion was constructed as described in Materials and Methods, and the resultant VEG-GFP spores were similar to the wild type in germination including absorbance and microscopic profiles (Fig. 5; data not shown). Since the *veg-gfp* gene fully complemented *veg* deficiency, it was a functional GFP fusion. Vegetative cells and spores of VEG-GFP exhibited strong fluorescence (T_{-2}, T_0, and T_{20} in Fig. 8), but the mother cell compartment exhibited weak fluorescence in the early sporulation phase and almost no fluorescence in the late sporulation phase, T_{20} (Fig. 8). These results indicate that the Veg protein is localized in spores.

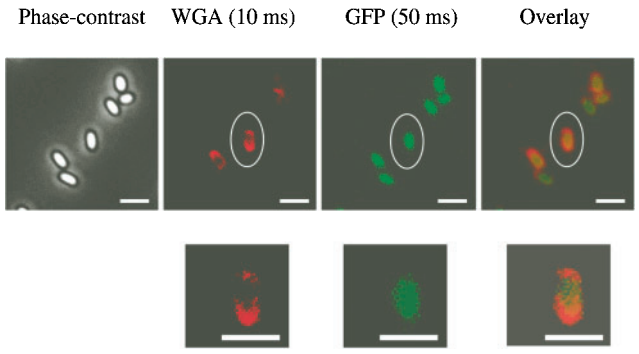

Fig. 9. **Localization of Veg-GFP in spores.** VEG-GFP spores prepared from a 48-h culture in DSM were decoated and then stained with WGA (wheat germ agglutinin tetramethylrhodamine conjugate). Overlay is a WGA image overlaid on a GFP image. Exposure time for WGA and GFP detection are shown in parentheses. Pictures in the second line are enlargements of spores circled in white. Peripheral and central regions, corresponding to cortex and core, exhibit strong fluorescence of WGA and GFP, respectively. Bars indicate 2 μm.

To further investigate the localization inside spores, VEG-GFP spores were stained with a fluorescent lectin (wheat germ agglutinin tetramethylrhodamine conjugate; WGA). However, spores exhibited weak fluorescence, because the spore coat is a strong barrier against staining. DAPI staining of spore DNA was also very poor. To improve the staining, spores were decoated before staining with WGA (Fig. 9). Strong red fluorescence caused by WGA was found in the peripheral layer of spores (probably cortex), and strong green fluorescence caused by GFP was found in the central region of spores (core) (Fig. 9). The location of GFP was very similar to that of DAPI staining (data not shown). These results indicate that the Veg protein was localized in the core region.

(6) Expression of Veg during Sporulation—Since the Veg-GFP protein had scarcely been found in the mother cell compartment after T_8, transcription of *veg* was investigated with the *veg-gfp* transcriptional fusion strain VEGdg. Green fluorescence was strong in the forespore compartment, but weak in the mother cell compartment (Fig. 10A). To eliminate the possibility of the instability of GFP in the mother cell compartment, the *cotE-gfp* transcriptional fusion strain COTEdg was constructed. Strong green fluorescence was found in the mother cell compartment (Fig. 10B), indicating the stability of GFP in this compartment. This result is supported by the fact that *cotE* is transcribed by RNA polymerases with mother cell-specific sigma factors (σ^E and σ^K) (*8*). These results suggest that *veg* is not transcribed in the mother cell compartment.

(7) Localization of a Reporter LacZ Protein Transcribed by σ^A RNA Polymerase—VEGdd is a transcriptional fusion of *veg* with *lacZ*, and thus localization of LacZ was determined with FDG. Strong fluorescence was found in vegetative cells (data not shown) and non-spore-forming cells (Fig. 10C). DNAs and membranes of spores and forespores after membrane fusion at the engulfment stage are not stained with DAPI and FM4-64 (membrane stain), respectively, but those in mother cells are easily

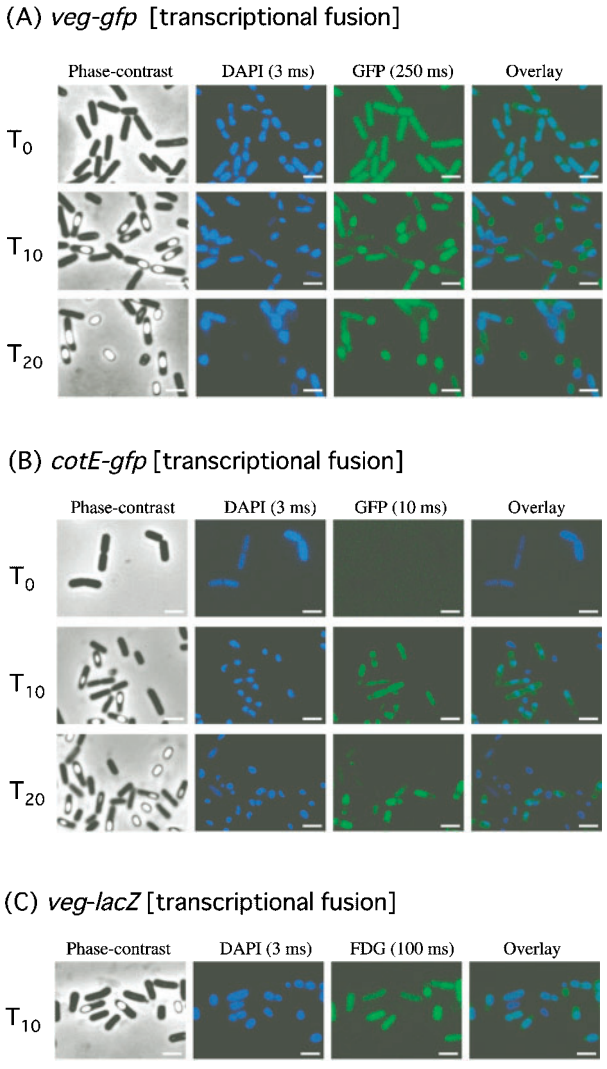

Fig. 10. **Localization of GFP and LacZ of the *veg-gfp* (A), *cotE-gfp* (B), and *veg-lacZ* (C) transcriptional fusion strains.** VEGdg (A) and COTEdg (B) strains were cultured in DSM medium at 37°C, and cells were collected at t_0, t_{10}, and t_{20} followed by DNA staining with DAPI. The *cotE* gene is transcribed by two mother cell–specific RNA polymerases, σ^E and σ^K RNA polymerases (*6*), and the *cotE-gfp* transcriptional fusion was a control of GFP production in the mother cell compartment. Overlay is a DAPI image overlaid on a GFP image. Panel C shows localization of LacZ of the VEGdd strain. Cells were cultured in DSM at 37°C and collected at t_{10} followed by DNA staining with DAPI and by LacZ staining with FDG. Overlay is a DAPI image overlaid on a FDG image. Bars indicate 2 μm.

stained (*24*). Moreover, P_{sigE}- and P_{sigK}-*lacZ* transcriptional fusion exhibited fluorescence in the mother cell compartment (data not shown). Previous reports by Lewis *et al*. (*25*) and Kodama *et al*. (*20*) also showed that FDG is permeable into mother cells. Figure 10C shows that neither spores nor mother cells exhibited fluorescence. These results support the inference that the *veg* gene is not transcribed in the mother cell compartment in spite of the presence of the σ^A promoter sequence.

DISCUSSION

The disruption of *veg* led to poor germination in the L-alanine-containing buffer, but this defect was not found in the AGFK-containing buffer (Figs. 4, 5, and 6). SDS–PAGE analysis of spore coat proteins did not show any difference in composition between *veg* and the wild-type spores (Fig. 7). Germination defects have often been found in mutants of the germination-specific cortex-lytic enzymes (*26*, *27*) and the muramic-δ-lactam biosynthetic enzymes (*23*, *12*). *sleB* and *cwlJ* are considered to encode germination-specific cortex-lytic enzymes (*26*, *27*). In particular, SleB is the major cortex-lytic enzyme, and *sleB*-minus spores completely lack the ability to release hexosamine-containing polysaccharides and show a poor decrease in refractivity during germination (*26*, *27*). Since these phenomena were similar to those observed for *veg*-deficient spores, we quantitated *sleB* and *cwlJ* transcripts of the wild type and *veg* mutant from t_3-$t_{7.5}$ by Northern blot analysis. There were no significant transcriptional differences between the wild-type and *veg* mutant: 2.2 kb and 0.47 kb transcripts were found with the *sleB* and *cwlJ* probes, respectively, and their intensity and time of appearance in the *veg* mutant were almost identical to those in the wild type (data not shown). Therefore, *veg* does not affect germination through *sleB* and *cwlJ* expression. The muramic-δ-lactam biosynthetic genes, *cwlD* and *pdaA* (*yfjS*), are transcribed by σ^E and σ^G, and σ^G RNA polymerases, respectively (*23*, *12*). Both defects affect germination severely, and they are found in a buffer containing L-alanine or AGFK. Since *veg* affected germination only in a buffer containing L-alanine, it is not likely that *veg* influences germination through *cwlD* and *pdaA*.

The *veg* gene encodes a small polypeptide (86 amino acid residues), which is assumed to be a typical cytoplasmic protein (PSORT and SOSUI databases). To determine the localization of Veg, a translational fusion strain, VEG-GFP, and two transcriptional fusion strains, VEGdg (*veg-gfp*) and VEGdd (*veg-lacZ*), were used with the transcriptional *cotE-gfp* strain COTEdg. The Veg-GFP protein was found in vegetative cells (Fig. 8). During the sporulation phase, it was accumulated in the forespore compartment but not in the mother cell compartment (Fig. 8). The *veg* transcription was almost abolished in the mother cell compartment (Figs. 10, A and C). Previously Losick and his colleagues reported that σ^A activity disappears after asymmetric septation (*28*). Ju *et al*. reported the sigma factor displacement from RNA polymerase during sporulation (*29*) and Lord *et al*. suggested that anti-σ^A factor may be synthesized or activated in sporulating cells at about the time of asymmetric septation (*30*). Recently Li and Piggot (*31*) reported that there is sufficient RNA polymerase including σ^A to drive P_{trpE}-*sacB'-'lacZ* expression in the prespore and in the mother cell both before and after engulfment. In our case, the fact that fluorescence was weak or absent in the mother cell compartment of the transcriptional and translational *gfp* fusion strains (Figs. 8 and 10) may be caused by specific regulator or anti-σ^A factor correlated with *veg*. In contrast, it is interesting that *veg* is active in the forespore compartment in spite of the presence of the σ^A promoter sequence.

The *veg* gene is conserved not only in various spore-forming bacteria [*Geobacillus* (*Bacillus*) *stearothermophilus, Oceanobacillus iheyensis, B. halodurans, B. anthracis, Clostridium thermocellum, C. perfringens, C. acetobutylicum*, and *Thermoanaerobacter tengcongensis*], but also various eubacteria (*Listeria monocytogenes, L. innocua, Staphylococcus aureus, Enterococcus faecium, Leuconostoc mesenteroides, Lactobacillus gasseri, and Oenococcus oeni*) (BlastP in NCBI and BlastN in the unfinished database of NCBI). Moreover, the gene order (*yabG-veg-sspF*) in the *B. subtilis* genome is conserved in *Oceanobacillus iheyensis* and *B. halodurans,* and partially conserved in *C. perfringens, C. acetobutylicum*, and *Thermoanaerobacter tengcongensis* (*yabG-veg*) (complete genomes in KEGG). The three genes are also neighbors in *G. stearothermophilus* and *B. anthracis* (microbial genomes in progress in TIGR). *Listeria monocytogenes, L. innocua, S. aureus, E. faecium, L. mesenteroides, L. gasseri, and O. oeni* do not possess significant orthologs of *yabG* and *sspF* (Blast server in NCBI). These results suggest that the *veg* gene has two functions: an unknown function during the vegetative growth phase, and a germination-related function. Since the *veg* gene and the neighboring spore gene are conserved in spore-forming bacteria, it is very likely that the function of *veg* is very important for germination in general.

The molecular mechanism of the *veg* function is still obscure. However, this is the first report to describe the cellular function of the well-known *veg* gene.

This research was supported by Grants-in-Aid for Scientific Research on Priority Areas Genome Biology (2) (12206005), and Scientific Research (B) (13460037) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

REFERENCES

1. Moran, C.P., Jr. (1993) RNA polymerase and transcription factors in *Bacillus subtilis and Other Gram-Positive Bacteria* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds) pp. 653–667, ASM Press, Washington, DC
2. Piggot, P.J. and Losick, R. (2002) Sporulation genes and intercompartmental regulation in *Bacillus subtilis and its Closest Relatives: From GENES to CELLS* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds.) pp. 483–517, ASM Press, Washington, DC
3. Moran, C.P., Jr., Lang, N., LeGrice, S.F., Lee, G., Stephens, M., Sonenshein, A.L., Pero, J., and Losick, R. (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol. Gen. Genet.* **186**, 339–346
4. Le Grice, S.F.J., Shih, C.-C., Whipple, F., and Sonenshein, A.L. (1986) Separation and analysis of the RNA polymerase binding sites of a complex *Bacillus subtilis* promoter. *Mol. Gen. Genet.* **204**, 229–236
5. Ollington, J.F., Haldenwang, W.G., Huynh, T.V., and Losick, R. (1981) Developmentally regulated transcription in a cloned segment of the *Bacillus subtilis* chromosome. *J. Bacteriol.* **147**, 432–442
6. Ollington, J.F. and Losick, R. (1981) A cloned gene that is turned on at an intermediate stage of spore formation in *Bacillus subtilis*. *J. Bacteriol.* **147**, 443–451
7. Takamatsu, H., Kodama, T., Imamura, A., Asai, K., Kobayashi, K., Nakayama, T., Ogasawara, N., and Watabe, K. (2000) The *Bacillus subtilis yabG* gene is transcribed by SigK RNA polymerase during sporulation, and *yabG* mutant spores have altered coat protein composition. *J. Bacteriol.* **182**, 1883–1888

8. Helmann, J. and Moran, C.P., Jr. (2002) RNA polymerase and sigma factors in *Bacillus subtilis and its Closest Relatives: From GENES to CELLS* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds.) pp. 289–312, ASM Press, Washington, DC
9. Takamatsu, H., Imamura, A., Kodama, T., Asai, K., Ogasawara, N., and Watabe, K. (2000) The *yabG* gene of *Bacillus subtilis* encodes a sporulation specific protease which is involved in the processing of several spore coat proteins. *FEMS Microbiol. Lett.* **192**, 33–38
10. Vagner, V., Dervyn, E., and Ehrlich, S.D. (1998) A vector for systematic gene inactivation in *Bacillus subtilis*. *Microbiology* **144**, 3097–3104
11. Yamamoto, H., Mori, M., and Sekiguchi, J. (1999) Transcription of genes near the *sspE* locus of the *Bacillus subtilis* genome. *Microbiology* **145**, 2171–2180
12. Fukushima, T., Yamamoto, H., Atrih, A., Foster, S.J., and Sekiguchi, J. (2002) A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis*. *J. Bacteriol.* **184**, 6007–6015
13. Yamamoto, H., Murata, M., and Sekiguchi, J. (2000) The CitST two-component system regulates the expression of the Mg-citrate transporter in *Bacillus subtilis. Mol. Microbiol.* **37**, 898–912
14. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning. A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
15. Anagnostopoulos, C. and Spizizen, J. (1961) Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**, 741–746
16. Shimotsu, H. and Henner, D.J. (1986) Modulation of *Bacillus subtilis* levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes. *J. Bacteriol.* **168**, 380–388
17. Völker, U., Engelmann, S., Maul, B., Riethdorf, S., Völker, A., Schmid, R, Mach, H., and Hecker, M. (1994) Analysis of the induction of general stress proteins of *Bacillus subtilis*. *Microbiology* **140**, 741–752
18. Nicholson, W.L. and Setlow, P. (1990) Sporulation, germination and outgrowth in *Molecular Biological Methods for Bacillus* (Harwood, C.R. and Cutting, S.M., eds.), pp. 391–450, John Wiley & Sons, Chichester
19. Sekiguchi, J., Fukushima, T., and Ishikawa, S. (2001) Selection and characterization of genes affecting germination in *Functional Analysis of Bacterial Genes. A Practical Manual* (Schumann, W., Ehrlich, S.D., and Ogasawara, N., eds.) pp. 211–213, John Wiley & Sons, Chichester
20. Kodama, T., Takamatsu, H., Asai, K., Kobayashi, K., Ogasawara, N., and Watabe, K. (1999) The *Bacillus subtilis yaaH* gene is transcribed by SigE RNA polymerase during sporulation, and its product is involved in germination of spores. *J. Bacteriol.* **181**, 4584–4591
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
22. Makino, S., Ito, N., Inoue, T., Miyata, S., and Moriyama, R. (1994) A spore-lytic enzyme released from *Bacillus cereus* spores during germination. *Microbiology* **140**, 1403–1410
23. Sekiguchi, J., Akeo, H., Yamamoto, H., Khasanov, F.K., Alonso, J.C., and Kuroda, A. (1995) Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis*. *J. Bacteriol.* **177**, 5582–5589
24. Sonenshein, A.L., Hoch, J.A., and Losick, R. (2002) *Bacillus subtilis and its Closest Relatives: From GENES to CELLS.* Color Plates, pp.549–550, ASM Press, Washington, DC
25. Lewis, P.J., Partridge, S.R., and Errington, J. (1994) σ factors, asymmetry, and the determination of cell fate in *Bacillus subtilis*. *Proc. Natl Acad. Sci. USA* **91**, 3849–3853
26. Moriyama, R., Hattori, A., Miyata, S., Kudoh, S., and Makino, S. (1996) A gene (*sleB*) encoding a spore cortex-lytic enzyme from *Bacillus subtilis* and response of the enzyme to L-alanine-mediated germination. *J. Bacteriol.* **178**, 6059–6063
27. Ishikawa, S., Yamane, K., and Sekiguchi, J. (1998) Regulation and characterization of a newly deduced cell wall hydrolase gene (*cwlJ*) which affects germination of *Bacillus subtilis* spores. *J. Bacteriol.* **180**, 1375–1380
28. Linn, T.G., Greenleaf, A.L., Shorenstein, R.G., and Losick, R. (1973) Loss of the sigma activity of RNA polymerase of *Bacillus subtilis* during sporulation. *Proc. Natl Acad. Sci. USA* **70**, 1865–1869
29. Ju, J., Mitchell, T., Peters, H., 3rd and Haldenwang, W.G. (1999) Sigma factor displacement from RNA polymerase during *Bacillus subtilis* sporulation. *J. Bacteriol.* **181**, 4969–4977
30. Lord, M., Barillà, D., and Yudkin, M.D. (1999) Replacement of vegetative σ^A by sporulation-specific σ^F as a component of the RNA polymerase holoenzyme in sporulating *Bacillus subtilis. J. Bacteriol.* **181**, 2346–2350
31. Li, Z. and Piggot, P.J. (2001) Development of a two-part transcription probe to determine the completeness of temporal and spacial compartmentalization of gene expression during bacterial development. *Proc. Natl Acad. Sci. USA* **98**, 12538–12543