INTRODUCTION

Spore-forming bacteria have complex life cycles that are characterized by formation of spores in environmental conditions that are deleterious to growth, such as a lack of nutrients (Cook et al., 1983; Santo and Doi, 1974). Spores are resistant to environmental stresses and when the conditions are again suitable for growth, the spores germinate to form vegetative cells, which then begin to proliferate. Spores are highly resistant to heat pasteurization and to various classes of bactericides. Bacterial spores must be under control in the food industry: food-poisoning bacteria such as Clostridium botulinum (Abgueguen et al., 2003) and Bacillus cereus (Granum et al., 1997), and food and beverage spoilage bacteria, such as Bacillus coagulans (Cosentino et al., 1997; Palop et al., 1999), Moorella thermoacetica (Prevost et al., 2010), and Thermoanaerobacter mathranii (Brown, 1994).

During sporulation, a vegetative bacterial cell forms a spore inside, starting with unequal division of the vegetative cell producing two cells of different sizes (Errington, 1993; Hitchins, 1978). Sporulation is one of the simplest differentiation of cells. To understand bacterial sporulation process, many studies have been performed using morphological and biochemical methods in conjunction with molecular genetics techniques. Sporulation is initiated by signal transduction within the phosphorylation system, a multicomponent control system (Perego, 1997). This is followed by successive expression of sporulation genes, a process known as the sigma cascade (Haldenwang, 1995).

In contrast, spore germination was investigated (Cook et al., 1983) through morphological observations (Santo and Doi, 1974; Vary and Halvorson, 1965) and biochemical approaches such as the measurement of enzyme activity (Hachisuka et al., 1958; Makino and Moriyama, 2002). Germination was shown to be a two-stage process (Foster and Johnstone, 1990).
consisting of a germination stage in which the spore emerges from dormancy and an outgrowth phase that proceeds until the cell in outgrowth phase undergoes first cell division. These studies also determined the enzymes (N-acetylmuramyl-L-alanine amidase, N-acetylmalamidase) that are expressed during each stage of the germination process (Foster and Johnstone, 1990; Hachisuka et al., 1958; Makino and Moriyama, 2002).

However, germinating spores undergo dramatic changes within few minutes to a few tens of minutes (Hachisuka et al., 1955), and a great variety of enzyme reactions occur during each stage. It was therefore extremely difficult to capture microscopic images of each germination stage in morphological examinations with the visualization techniques available at the time, such as electron microscopy and fluorescence microscopy. For this reason, no significant advances were made in the understanding of the mechanism of germination.

Microstructural analysis by electron microscopy has shown that spores consist of central core containing DNA, inner membrane, cortex, outer membrane, and coat proteins (Kozuka and Tochikubo, 1983; Plomp et al., 2005). However, the process of spore germination is not well understood because complicated and dramatic changes occur in the short duration of time. For this reason, details of the process from spore germination to outgrowth, which then proceeds to vegetative growth, remain unknown.

In this study, morphological changes at the time of germination were investigated in spores of *B. cereus* (Aronson and Fitz-James, 1976; Hashimoto and Conti, 1971) and *B. subtilis* using transmission electron microscopy (TEM) (Santo, L.Y. and Doi, RH., 1974). Samples were prepared by a standard sample preparation method and also by freeze-substitution fixation of spores during germination. Here, we report new insights into the surface and internal structure changes of spores that occur immediately after germination until outgrowth, which were observed through electron microscopic observations using freeze-substitution fixation.

**MATERIALS AND METHODS**

**Bacterial strains.** *Bacillus cereus* JCM 2152, which has an exosporium surrounding spores, and *B. subtilis* JCM 1465, which has no exosporium, were purchased from RIKEN (Japan).

**Preparation of spores.** The bacterial strains were cultured on a Soybean-Casein Digest (SCD) nutrient agar plate (Nissui Pharmaceutical Co., Ltd., Japan) at 30 °C for 5–7 days. The bacterial cells including spores were then harvested from the agar plate by scraping, and resuspended in sterile purified water. Cell suspension containing 80 % or more mature spores, as confirmed by phase-contrast microscopy, was used for further purification of spores. Cells including spores were harvested from the suspension by centrifugation at 5,200 × g for 3 min (Chitiban II; Merk Millipore, USA), washed three times and resuspended with 1.0-ml sterile purified water. The cell suspension was left for 24 h at 4 °C and the vegetative cells were then killed by heating at 80 °C for 20 min using a block heater (Cool Thermo Unit CTU-N Neo; Taitec Co., Japan). Immediately after heating, the suspension was stored at 4 °C. The suspension was diluted with sterile purified water to attain a spore count of 10^6 CFU/ml prior to use in the germination experiments.

**Germination experiments.** The spore suspension was inoculated into L-Broth (LB, Becton, Dickinson & Co., USA) adjusted to 50 % of its prescribed concentration (50 % LB) at pH 6.5 to attain cell concentration of 10^6 CFU/ml and cultured at 30 °C. In the present study, 50 % LB was used because it has been confirmed that the medium induced spore germination and promote progression to the outgrowth stage in both of the bacterial strains used in this study (Hachisuka et al., 1955). The spores were cultured from 1 min to 8 h to allow observation of germination at each stage.

**Observation of spore germination using scanning electron microscopy (SEM).** (chemical fixation) Spores were collected by dripping 0.1 ml of the spore suspension onto a membrane filter with a pore size of 0.2 µm (Isopore, Merk Millipore, USA) on a vacuum filtration system (Nalgene Labware) under gentle suction. Germination was induced by adding 0.1 ml of 50 % LB to the collected spores on the membrane filter. At fixed intervals, the spores were washed by a slow, continuous drip of 5.0 ml of 10 mM HEPES buffer solution (pH 7.0) (DOJINDO MOLECULAR TECHNOLOGIES, INC., Japan) at 4 °C. Washing was performed on the vacuum filtration system under gentle suction and care was taken to ensure that the samples did not dry out. After washing, the filter was removed and the samples were fixed with 2.5 % glutaraldehyde (NACALAI TESQUE, INC., Japan) in 10 mM HEPES buffer (pH 7.0) at 4 °C for 24 h. The samples on the filters were then washed with serial ethanol washes to remove water according to an established protocol (Baba and Osumi, 1978), and substitution was performed with t-butyl alcohol (NACALAI TESQUE, INC., Japan). The samples on the filters were dried in a freeze dryer (ES-2030; Hitachi, Ltd., Japan), and each filter was then cut into a suitable size, affixed to the aluminum specimen holder using double-sided adhesive tape with a gum needle, and allowed to dry at room temperature.
carbon tape, and observed under a scanning electron microscope (S-4300SE/N; Hitachi, Ltd., Japan). The samples were also subjected to elemental analysis using an energy dispersive x-ray (EDX) micro-analyzer (EMAX Energy; HORIBA Ltd., Japan).

**freeze substitution** The prepared spore suspensions (2.5 µl) were placed on a copper mesh (pore size 0.3 mm; Nisshin EM Co., Japan) held at the edge by negative-action tweezers (cross N type; Dumont, Switzerland). To this, 2.5 µl of 50 % LB solution (pH 6.5) was added and another sheet of copper mesh was immediately placed on top to hold the experimental solution between the two mesh sheets. The pairs of mesh sheets with the experimental suspensions were left at room temperature for the prescribed lengths of time, and then frozen in liquid nitrogen. Each pair of sheets was then placed in 100 µl of 0.5 % glutaraldehyde in acetone (NACALAI TESQUE, INC., Japan) cooled at -20 °C in a 1.5-ml microtube for 5–7 days. Then, the temperature was raised to 4 °C and sample fixing was performed for 3–6 h.

After fixing, the liquid was removed and 100 µl of 100 % acetonitrile was added to the tube. The sheets holding the samples were incubated in a microtube at room temperature for 15 min, the acetonitrile was removed, and 100 µl of 100 % acetone was added to further dehydrate the sample. After dehydration, the acetone solution was removed, 100 µl of t-butyl alcohol was added, and the samples that held on to the sheet were frozen at -20 °C and then dried in a freeze dryer (ES-2030; Hitachi Ltd., Japan).

Following freeze-drying, the mesh sheets were affixed to an aluminum SEM specimen holder using doublesided carbon tape, with the sides of the sheets that had held the liquid facing upward. After silver-vanadium coating using an ion sputter deposition system (E-1030; Hitachi Ltd., Japan), observations were made using SEM (S-4300SE/N; Hitachi Ltd., Japan). The samples were also subjected to elemental analysis using an energy dispersive x-ray (EDX) micro-analyzer (EMAX Energy; HORIBA Ltd., Japan) before silver-vanadium coating. The results are shown as average. Since the electron beam accelerating voltage in this study was 20 kV, setting this accelerating voltage is expected to provide measurement results in the depth range of several nm to several hundred nm.

**Observation of spore germination using transmission electron microscope (TEM).**

Spore samples were fixed in the same way as for SEM, and then washed with serial ethanol washes to remove water. After removal of water, 0.02 ml of the suspensions were mixed with 0.02 ml propylene oxide (Nisshin EM Co., Japan), dispensed into BEEM embedding capsules (Nisshin EM Co., Japan) and then dried in a desiccator for 15 min. An additional 0.02 ml propylene oxide was added and samples were dried for 15 min. This procedure was then repeated twice to substitute the ethanol with propylene oxide. Following propylene oxide substitution, 0.05 ml of resin (Q-615, Nisshin EM Co., Japan) were added and the samples were degassed with a sonicator (Branson 5200, Yamato Scientific Co., Japan) for 30 s. The samples were then infiltrated with resin in a desiccator under negative pressure at room temperature for 24–48 h. After resin substitution was complete, the BEEM capsules were heated in a block heater (Cool Thermo Unit CTU-N Neo, Taitec Co., Japan) at 40 °C for 24 h and then further heated at 70 °C for 48–72 h to harden the resin. Ultra-thin sections (70–90 nm) were cut from the capsules with a diamond knife (Sumi Knife, Okenshoji Co., Japan) using a microtome (Ultracut-S, Leica Microsystems, Germany) and collected on copper mesh (Sheet Mesh 150-A; Nisshin EM Co., Japan) for TEM observation. After drying, the mesh containing the collected sample was examined under a TEM (H-7650; Hitachi Ltd., Japan).

**Visualization of spores and cells in outgrowth phase at different stages using fluorescence-labeled anti-spore surface antibodies.** To 45 µl of the prepared spore suspensions, 5 µl of fluorescence-labeled anti-spore antibodies (fluorescent dye: Cy5® B. cereus antibody, Abcam Plc., UK) were added and the mixture was incubated at 4 °C for 24 h. The spore was harvested by centrifugation at 5,200 × g for 3 min (Chitiban II; Merk Millipore, USA), and resuspended in 0.5 ml of 10 mM HEPES buffer (pH 7) at 4 °C. The spore washed 3 times with the same buffer were finally resuspended in 0.5 ml of 10 mM HEPES buffer (pH 7) at 4 °C.

A glass-bottom dish (Iwaki & Co., Ltd., Japan) was prepared in advance by making the glass hydrophilic using an ion sputter deposition system (E-1030; Hitachi Ltd., Japan), and 100 µl of the suspension were added to the dish after washing. To this, 100 µl of 50 % LB, were added and differential interference observations of the germination process were done over time using a confocal laser scanning microscope (λex = 650 nm; λem = 670 nm, LSM 510 META, Carl Zeiss, Germany).

**Measurement of leakage of dipicolinic acid (DPA) from spores.** Chronological measurements of the optical density at 270 nm of the supernatant of spores after germination induction were made, as an index of the concentration of DPA (Kort et al., 2005). Spore suspensions at 10⁶–10⁷ CFU/ml in 10 mM HEPES buffer (pH 7) were added to an equal volume of LB at
30 °C to induce germination (Hachisuka, et al., 1955). After exposing the spores to the culture medium for 30 s, the spores were immediately collected by centrifuging the suspension at 5,200 × g for 3 min, and were then resuspended in 10 mM HEPES buffer (pH 7). The suspension was incubated at 30 °C and leakage of DPA over time was investigated by centrifuging the suspension and measuring the absorbance at 270 nm (A<sub>270</sub>) of the supernatant using SH-9000Lab (Hitachi Ltd., Japan). Values were average of two separate experiments.

**RESULTS**

Morphological changes in spores after germination induction observed under SEM, TEM, and confocal laser scanning microscopy

Chemical and freeze-substitution fixations were used to prepare samples for chronological SEM observations of spores that had been induced to germinate by exposure to 50 % LB. Morphological changes in *B. cereus* JCM2152 spores observed by SEM after chemical fixation are shown in Fig. 1. Figures 2 and 3 show SEM images of the spores of *B. cereus* JCM2152 and *B. subtilis* JCM1465 pretreated by freeze substitution,
respectively.

SEM observation of chemically fixed _B. cereus_ spores showed the presence of granular structures of approximately 20–30 nm in size covering the surface of the exosporium at 1 min after germination induction (Figs. 1C and D). These structures were not observed prior to the induction of germination (Figs. 1A and B). In samples prepared by freeze substitution, the granular structures were observed more clearly on the surface and spherical structures protruded from spores were also observed (Fig. 2B). At 15 min, the surface granular structures became clearer in the chemically fixed samples (Figs. 1E and F), and an increase in the size of the spherical structures was observed in the sample prepared by freeze substitution (Fig. 2C). At 30 min, the exosporium of the chemically fixed cells was still in place on the cell surface and the entire spore appeared considerably swollen (Figs. 1G and H). On the other hand, the sample prepared by freeze substitution showed many of spherical structures on the surface of the swollen spores (Fig. 2D). Similar spherical structures were observed in the _B. subtilis_ spores prepared by freeze substitution (Fig. 3).

SEM observation of chemically fixed _B. cereus_ spores at 60 min, spores had swollen to approximately twice of their initial volume (Figs. 1I and J), and no spherical structures were observed (Figs. 1C–J). The chemically fixed _B. subtilis_ spores showed similar morphological changes as that of the _B. cereus_ spores around this period (data not shown). A fissure was observed in the central part of some _B. cereus_ spores at 60 min (Figs. 1J, 2E, and F) and 180 min (Fig. 1L). Similar fissures were also observed on the chemically fixed _B. subtilis_ spores (data not shown). In addition, parts of the exosporium were observed on the surface of several _B. cereus_ spores fixed by freeze substitution (Fig. 2E). At 360 min, the cells in outgrowth phase had elongated to about 2 µm, and no exosporium was observed (Figs. 1M and N). In addition, hemisphere structures were observed at the end of the elongated cells (Fig. 1M). At 720 min, vegetative cells with a wavy irregular surface structure were observed (Figs. 1P and Q). To investigate the morphological changes at 360 min (Fig. 1M), chemically fixed samples were examined in detail by SEM. Figure 4 shows SEM images of _B. cereus_ JCM2152 spores at 360 min. Arrows and arrowheads indicate ring-shaped and discoid structures, respectively. Ring-shaped structures were found at the end of
the cell (Figs. 4A) and on a filter (Fig. 4F). Many ring-shaped structures were observed over the entire field of view of SEM (Fig. 4G, shown by arrow). Among some of discoid structures (inside is not transparent), 20-30 nm pores were observed at the center (Fig. 4C). The discoid structures and the structures in the process of decomposition were frequently observed (Figs. 4 D, E and G, shown by arrowhead). Both ends of the cells were flat in outgrowth phase (Figs. 4B and G).

These stages were also observed on *B. cereus* JCM2152 spores using confocal laser scanning microscopy after staining with fluorescence-labeled anti-spore antibodies. At 60 min, the fissure without red fluorescence was observed in the center of the spore (Fig. 5A). At 180 min, the cells did not emit red fluorescence, but a hemisphere structure emitting red fluorescence was observed at the end of the cells in outgrowth phase (Fig. 5B). The discoid structures emitting red fluorescence were also observed over the entire field of view (Fig. 5C), indicating the discoid structures were the spore-derived hemispherical structures detached from the cells.

TEM observation showed the cell wall of outgrowth cells germinated from the spores of *B. cereus* at 360 min...
min was clearly thinner than that of the cells at 720 min (Figs. 6A and B). The surface of the cell at 360 min in outgrowth phase looked smooth (Fig. 1N), whereas the surface of the cell at 720 min showed a wavy irregular surface structure (Fig. 1Q). The thickness of cell wall of the cell at 720 min was thicker than that of the cell at 360 min. The difference in the thickness might be ascribed to cell maturation (Ahn et al. 2018).

**Intracellular Ca and elemental analysis of leakages from spore during germination**

*Bacillus cereus* spores prepared by freeze substitution at 360 min were observed by SEM and the elements of the constituents (namely carbon (C), oxygen (O), nitrogen (N), phosphorus (P), sulfur (S), and calcium (Ca)) were analyzed by SEM-EDX elemental analysis (Fig. 7). A structure in which granular substances were densely arranged was observed on the entire surface of the spore. This structure was not observed in some circular areas on the surface of the spore. Inside the circular area without granular substances, the structure (crystalline proteins) of the exosporium were observed in a well-ordered fashion without physical damage (Fig. 7B). In addition, relatively large spherical structures having a smooth surface were observed on the spore surface. SEM-EDX elemental analysis showed that the elemental component ratio of the hemispherical and spherical structures (Figs. 7C and E) was almost same as that of the spore (Fig. 7D). SEM-EDX elemental analysis of Ca was done on both *B. cereus* and *B. subtilis* spores (Fig. 8). The amount of Ca in spores after swelling was less than 1/10 of that before swelling at 30 min (Figs. 8A, and B). Figure 9 shows changes of the optical density at 270 nm of the supernatant of germination-induced spores. The values increased immediately after germination induction and reached a plateau at 30 min.
DISCUSSION

Detailed observations of the morphological characteristics of the spore and cells in outgrowth phase were performed using TEM and SEM after chemical fixation and fixation following freeze substitution. While it is impossible to completely eliminate the effects of artifacts, SEM and TEM samples were carefully prepared to minimize the effects of artifacts, and reproducibility was confirmed. It has been reported that the artifacts produced during preparation of sample for SEM were less than those by chemical substitutions (Tsugukuni et al., 2008). In addition, confocal microscopy was performed to confirm that the images obtained from SEM and TEM observations were consistent.

During germination process, spore undergoes rehydration, release of DPA, cortex hydrolysis, and coat disassembly. This phase is accompanied by transition from a phase-bright spore to a phase-dark cell, as manifested by light microscopy (Sinai et al., 2015; Setlow, P., 2014). Bassi et al. (2009) used a carbon-coating SEM technique and X-ray microanalysis to observe the architecture of spores and to examine Ca in Clostridium tyrobutyricum spores. They confirmed that release of...
Ca after 1 h of germination treatment using L-alanine/L-lactate. In both B. subtilis and C. perfringens spores, it has been reported that Ca–DPA release allows partial core hydration and perhaps some cortex deformation (Paredes-Sabja et al., 2011). In this study, a decrease in spore Ca level and a leakage of DPA from the spores were observed within 30 min of germination induction (Figs. 8 and 9), suggesting an efflux of water from the spores.

According to Pandey et al. (2013), the time of outgrowth is the period between the end of germination and the beginning of vegetative growth of the cell. Outgrowth was usually shown to coincide with either the burst of the cell out of the spore coat or loss of the coat (Pandey, R. et al., 2013; Setlow, 2014). In B. cereus, both of the hemispherical structure at the end of the cell (Fig. 5B) and discoid structures on filter (Fig. 5C) emitted red fluorescence, suggesting that they are the same. The discoid structures on the filter (Figs. 4C, F and G) might be decomposed parts of the hemispherical structures (Fig. 1M) detached from the cells. According to the results obtained by SEM observations of samples prepared by freeze substitution, and confocal laser scanning microscopic observation, the cells of outgrowth phase elongated remaining with the spore coat of hemispherical structures at end of the cells, then the structure detached from the cell and degraded (Figs. 1M and 4). A small pore was observed in the

**FIG. 8.** Relative amount of Ca on spore surfaces before and at 30 min after germination induction determined using SEM-EDX analyzer. Spores of B. cereus JCM2152 (A) and B. subtilis JCM1465 (B) were examined for Ca before and at 30 min after germination induction using SEM-EDX analyzer after freeze substitution. Values were presented as mean ± SD for more than 3 different areas.

**FIG. 9.** Leakage of DPA from spores. Spores of B. cereus JCM2152 (●) and B. subtilis JCM1465 (▲) were induced to germinate by exposing to the culture medium for 30 s. Spores harvested by centrifugation were then resuspended in 10 mM HEPES buffer (pH 7) and incubated at 30 °C. Leakage of DPA from the spores was determined by measuring the optical density at 270 nm of the supernatant. Values were the averages of two separate experiments.
center of the spore coat of discoid structures (Fig. 4).

In order for the spores to swell (Figs. 1H, J, and K), which requires an influx of water (Hachisuka et al., 1955), the cortex must be broken down by enzymatic reaction (Makino et al., 2002). For this reason, influx and efflux of water may occur simultaneously. It might be speculated the influx of water might increase the pressure within the spore, resulting in the leakage of cellular contents from the spore through the spore coat and exosporium. The sample prepared by freeze substitution showed many of spherical structures on the surface of the swollen spores of both *B. cereus* (Fig. 2D) and *B. subtilis* (Fig. 3). According to the results obtained by SEM-EDX elemental analysis, components of the spherical structures were the same as those of the spore (Fig. 7).

It can be speculated that the spherical structure might be made of liquid with a different density from that of surrounding liquid which might allowed the liquid to form a sphere due to surface tension.

The spherical structures seem to be leaked from the spore. This study reports successfully captured images of the spore contents leaking from various parts of a spore (Figs. 2 and 3). As previously reported, SEM observation of chemically fixed spores could not observe similar images in this study. Therefore, it is presumed that similar SEM images will be obtained unless the spore samples have been prepared by this freeze substitution.

The exosporium of *B. cereus* is composed of two layers of crystalline proteins, as reported by Ball et al. (2008). Our results suggested that the leaked spore contents passed through the gaps in the crystalline structure of the exosporium without disrupting these layers of the crystalline proteins (Figs. 2 and 7).

In future work, we intend to clarify the biochemical changes accompanying the morphological observations occurring at each stage of germination that were observed and microscopically imaged in the present study.

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