

Morphological Study of Heat-Sensitive and Heat-Resistant Spores of *Clostridium sporogenes*, Using Transmission Electron Microscopy

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ABSTRACT

To investigate the primary structural determinants affecting heat resistance of *Clostridium sporogenes* spores, electron micrographs of heat-sensitive ($D_{121^{\circ}\text{C}} = 0.56$ min) and heat-resistant ($D_{121^{\circ}\text{C}} = 0.93$ min) spores of *C. sporogenes* were taken with a transmission electron microscope. The mean thickness (\pm standard deviation [SD]) of coat layers and cortex regions of heat-sensitive spores were 82.9 ± 14.5 and 86.0 ± 22.7 nm, while those of heat-resistant spores were 106.9 ± 45.7 and 111.7 ± 32.1 nm, respectively. The thickness of coat ($P = 0.031$) and cortex ($P = 0.006$) showed statistically significant differences, suggesting that heat-resistant spores have a thicker coat and cortex than do heat-sensitive spores. The mean sizes (\pm SD) of cores were 467.0 ± 88.7 nm for heat-sensitive spores and 460.2 ± 98.5 nm for heat-resistant spores, respectively, which showed no statistically significant differences. The ratios (\pm SD) of the core size to the sporoplast size were 0.84 ± 0.05 for heat-sensitive spores and 0.80 ± 0.07 for heat-resistant spores, respectively, showing statistically significant differences ($P = 0.030$), which indicated that the ratio is negatively related to heat resistance. Accordingly, the structural components of heat-sensitive spores were severely damaged by heat treatment, whereas those of heat-resistant spores were unlysed under the same conditions. Based on the structural analyses of spores, it was elucidated that the thickness of coat layer and cortex region are significantly correlated with heat resistance of *C. sporogenes* spores, and that cortex region plays a major role in protecting the spore from heat damage.

Bacterial sporulation is a process to form spores, a dormant cell type, in response to adverse conditions such as starvation (5, 18, 19) and/or to quorum sensing (21). Through sporulation, spore-forming bacteria such as *Clostridium* and *Bacillus* develop structural components including the spore core, cortex region, and coat layers, and thereby have mechanisms to protect themselves from intrinsic or extrinsic stresses (5, 16).

The spore core is the analogue of the protoplast of a growing cell and includes DNA, ribosomes, and tRNA (20). A key factor determining heat resistance of spores is water content in the core (1). Water makes up approximately 30 to 50% of the wet weight of the core, depending on the species, which is lower than water content (75 to 80% of wet weight) in the protoplast of a growing cell (6), indicating that the low water content in the core increases the heat resistance of the spore. Pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) also influences the heat resistance of spores. Most DPA is present as the Ca^{2+} chelate form (Ca-DPA) and is located only in the core (6). Since the DPA is partially responsible for reduction of water content in the core, spores lacking DPA are more sensitive to heat (20). Furthermore, the molar ratio of Ca to DPA has been found to be positively related to heat resistance (12). The spore coat and cortex have been known to function in pro-

tecting the core from damages and thereby contribute to resistance. The role of those two structural components protecting the core is particularly important because the core contains ribosomes and a nucleoid involving genetic information (22). The spore coat is a proteinaceous multilayered structure and composed of highly cross-linked polypeptides (5). While the coat has been considered to play little or no role in heat resistance, it is responsible for the spore's resistance to chemicals and to exogenous lytic enzymes that can degrade the spore cortex (20). The cortex is made of a peptidoglycan and occupies as much as half the spore volume (9, 17, 20). Since the cortex is essential for the reduction of the water content of the spore core, this component is usually considered to be necessary for heat resistance of the spore, even though mechanisms contributing to the heat resistance remain unknown (20).

C. sporogenes PA 3679, a nonpathogenic, putrefactive, and spore-forming anaerobe, is an important microorganism used as a surrogate for modeling thermal inactivation of *C. botulinum*. The ease of monitoring the presence of the organism through off-odor and gas formation has made *C. sporogenes* an excellent surrogate microorganism for developing thermal processing conditions (7, 11, 15). A process that insufficiently sterilizes *C. sporogenes* allows *C. botulinum* to survive, grow, and produce toxin (3). Thus, refined understanding of the heat resistance of *C. sporogenes* spores and its mechanisms are therefore important

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and helpful in establishing sterilization processes applicable to inactivate *C. botulinum*. Since structural components, including the spore core, cortex region, and coat layer, have been suggested to contribute to the resistance of spores as described above, it can be speculated that those components may cause a large variation of heat resistance of spores, depending on their morphological peculiarities and chemical compositions. Under conditions of insufficient heat treatment for inactivating spores, spores can survive and persist in the final product, which may occur when spores are much more resistant to heat treatment than expected (3, 13, 25). To address the correlation of structural components and heat resistance of spores, much work on the heat resistance of spores has been carried out with the genus *Bacillus*, whereas only a little work has been done with *Clostridium* spores (20). Besides, only few electron microscope studies on the morphology of *Clostridium* spores in connection with heat resistance have been performed. The objective of this study was to investigate *Clostridium* spores' resistance to heat damage. Therefore, we examined transmission electron microscopic appearances of heat-sensitive and heat-resistant spores of *C. sporogenes*, comparing thickness of structural components before and after heat treatment, and attempted to elucidate roles of the structural components in enhancing heat tolerance.

MATERIALS AND METHODS

Microorganism. Eight batches of *Clostridium sporogenes* PA 3679 spores were obtained from the Center for Technical Assistance of the former National Food Processors Association (Dublin, Calif.). Each spore batch suspended in phosphate buffer (pH 7.0) was divided into cryogenic sterile vials (Fisher Scientific, Pittsburgh, Pa.) and kept in a freezer (-20°C) until use, and used as a stock suspension. The initial concentration of the stock suspension was approximately 10^7 CFU/ml and was determined by the enumeration procedure described below.

Measurement of *D*-value. The *D*-value (time required for a 10-fold reduction in viable spores) of different batches of *C. sporogenes* spore was determined using the multiple-point method (4). Test spore suspension diluted with phosphate buffer (pH 7.0) was carefully injected into a glass capillary tube with an inner diameter of 1.8 mm and an outer diameter of 3 mm (Corning, Inc., Corning, N.Y.), using a pipette, and then the open ends of the tubes were heat sealed. The tubes were immersed completely in an oil bath (Thermo Electron Corp., Waltham, Mass.) and heated at 121°C for different time intervals. After heating, the tubes were removed from the oil bath, cooled immediately in a crushed ice water bath, and washed in 70% ethyl alcohol. Both ends of tubes were cut aseptically; suspension was flushed out with 2 ml of sterile 0.1% peptone water.

To enumerate spore survival, treated samples were 10-fold serially diluted in sterile 0.1% peptone water and pour plated onto tryptone-peptone-glucose-yeast extract medium, consisting of 50 g of tryptone, 20 g of yeast extract, 5 g of peptone, 4 g of dextrose, 1 g of sodium thioglycolate (all from Difco, Becton Dickinson, Sparks, Md.), and 15 g of agar in 1 liter of distilled water (24). Solidified plates were incubated for 2 days at 32°C in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, Mich.) containing an atmosphere of 95% nitrogen and 5% hydrogen (Oxarc, Inc., Spokane, Wash.), and then the colonies were manually counted. The number of colonies was calculated from

dual platings, and the number of survivors was calculated from duplicate determinations. Survivor curves were plotted to determine *D*-value. *D*-values were obtained by taking the reciprocal of the slope from linear regression of the survivor curves. To confirm the *D*-values obtained, two independent experiments were conducted.

Preparation of heat-treated *C. sporogenes* spore for electron microscopy. Suspensions of heat-sensitive and heat-resistant spores, selected based on their $D_{121^{\circ}\text{C}}$ -values, were placed into a Pyrex glass test tube with a screw cap (25 by 150 mm), immersed completely in an oil bath, and heated at 121°C for 5 min to cause damage to the structural components of the spores. After heating, the tubes were removed from the oil bath, cooled immediately in a crushed ice water bath, and kept in a refrigerator (4°C) prior to fixation for transmission electron microscopy.

Transmission electron microscopy of *C. sporogenes* spores. Specimen preparation was carried out according to a protocol for transmission electron microscopy provided by the Electron Microscopy Center at Washington State University (Pullman), with minor modifications, as follows.

Heat-treated and untreated spores of *C. sporogenes* were fixed with $2\times$ fixative (6% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) for 2 h at ambient temperature. The cell monolayers were postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C . The samples were then dehydrated with a serial concentration of ethanol (30, 50, 70, 95, and 100%), embedded in Spurr's resin and polymerized overnight at 60°C . The polymerized samples were sectioned with a diamond knife in an ultramicrotome, stained with uranyl acetate and lead citrate, and observed using a JEOL JEM-1200 EX TEM (JEOL USA, Inc., Peabody, Mass.) with AnalySIS imaging software (Soft Imaging Systems, Lakewood, Colo.).

Measurements of spore structural components. To ensure consistency and avoid variation in the plane of sectioning, only clear and well-defined spore cross-sections were selected and examined by transmission electron microscopy. At least 20 transmission electron micrographs were taken for each spore sample, and the thickness of the coat layers and cortex regions, and the diameters of cores were measured using AnalySIS imaging software. The mean values of thickness measured at four different sites of the coat and the cortex and that of horizontal and vertical diameters of the core were taken as representative values of the structural components of each spore. All calculated values were statistically analyzed for differences in structural components.

Statistical analysis. Data were presented as means and standard deviations (SD). The significance of differences was determined by one-way analysis of variance with Tukey's multiple-comparison module of the Minitab statistical software, version 12.11 (Minitab, Inc., State College, Pa.), and differences with $P < 0.05$ were considered statistically significant.

RESULTS

Relationship between heat resistance and structural components of *C. sporogenes* spores. The thermal inactivation patterns of eight spore batches were examined using spore suspensions (approximately 10^7 CFU/ml) of *C. sporogenes* and showed $D_{121^{\circ}\text{C}}$ -values ranging from 0.3 to 1.0 min (data not shown). The quite different responses to heat treatment led us to investigate whether there are detectable differences in shape and size of structural components, including coat layers, cortex regions and cores, of the spores.

TABLE 1. Comparison of structural component sizes of heat-sensitive and heat-resistant spores of *C. sporogenes*

Spore	Coat layer (thickness) ^a	Cortex region (thickness) ^a	Core (diameter) ^a	Ratio of core to sporoplast ^b	D-value ^c
Heat-sensitive spore	82.86 ± 14.50 A ^d	86.04 ± 22.68 A	467.00 ± 88.71 A	0.84 ± 0.05 A	0.56 ± 0.008 A
Heat-resistant spore	106.89 ± 45.73 B	111.67 ± 32.13 B	460.17 ± 98.48 A	0.80 ± 0.07 B	0.93 ± 0.004 B

^a Thickness and size in nanometers were taken as a mean ± SD from 20 micrographs.

^b The ratio was calculated as the core size divided by the combined size of the core plus cortex, and the indicated values were obtained from 20 micrographs.

^c D-values in minutes were determined at 121°C, and the indicated values were obtained from two independent experiments.

^d Mean values in the same column that are not followed by the same letter are significantly different ($P < 0.05$).

To observe the shapes of the structural components, along with the transmission electron microscopic appearances, two batches were selected based on their heat tolerance, a heat-sensitive spore batch having a $D_{121^{\circ}\text{C}}$ -value of 0.56 min and a heat-resistant spore batch having a $D_{121^{\circ}\text{C}}$ -value of 0.93 min (Table 1). Using a transmission electron microscope, it was observed that the heat-resistant spores had much thicker layers of coat and cortex than did the heat-sensitive spores (Fig. 1).

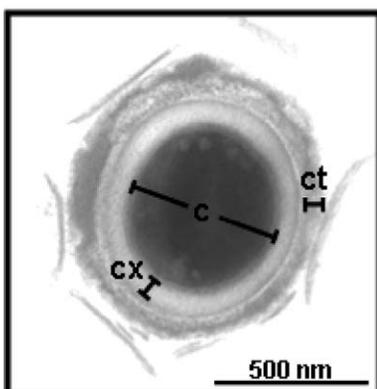
To ensure this observation, 20 transmission electron micrographs were taken from specimens of heat-sensitive and heat-resistant spores, respectively, and the thickness of the coat layers and cortex regions and the sizes of cores were measured and compared, as shown in Table 1. The thickness of coat layers of heat-sensitive spores ranged from 58.2 to 111.4 nm, with a mean of 82.9 nm (\pm SD of 14.5). On the other hand, heat-resistant spores had thicker coat layers, ranging from 64.3 to 277.0 nm, with a mean of 106.9 nm (SD of 45.7). The differences were statistically significant ($P = 0.031$). The thickness of cortex region of heat-sensitive spores varied from 41.7 to 128.6 nm, with a mean of 86.0 nm (SD of 22.7), while those of heat-resistant spores were measured in the range of 67.7 to 178.7 nm with a mean of 111.7 nm (SD of 32.1). Statistical analysis showed that there was a statistically significant difference ($P = 0.006$) between thickness of the cortex region of heat-sensitive and heat-resistant spores, which means that heat-resistant spores had thicker cortex regions than did heat-sensitive spores, like their coat layers. These results indicate that thickness of the coat layer and cortex region are positively related to heat resistance. Meanwhile, cores of heat-

sensitive spores ranged in size from 290.2 to 620.0 nm, with a mean of 467.0 nm (SD of 88.7). Similarly, cores of heat-resistant spores had a mean of 460.2 nm (SD of 98.5; range of 305.6 to 705.6 nm), showing no statistically significant difference between heat-sensitive and heat-resistant spores. The ratio of the core size to the sporoplast size (the combined size of the core plus cortex) was also determined to check whether there were detectable differences. The mean ratios were 0.84 (SD of 0.05) for heat-sensitive spores and 0.80 (SD of 0.07) for heat-resistant spores, respectively, and found to be significantly different ($P = 0.030$), which indicates that the ratio is negatively related to heat resistance.

Collectively, it was likely that the thickness of spore coat and cortex region, rather than size of core, contributes to heat tolerance of *C. sporogenes* spores. In addition, based on the statistical analyses of measurements, it is clear that although the coat layer is statistically correlated with heat resistance of *C. sporogenes* spores, the cortex region may play a larger role in protecting the spores from heat damage.

Thermal inactivation of *C. sporogenes* spores. The fact that the spore batch showing a higher $D_{121^{\circ}\text{C}}$ -value has thicker coat layer and cortex region led us to check whether its structural components are more resistant to heat damage. In the case of heat-sensitive spores, it was found that the heat-treated spores used above to measure $D_{121^{\circ}\text{C}}$ -values exhibited a relatively rough surface resulting from partial disruption of the coat layer and shrunk shape (data not shown). In contrast to the heat-treated spores, however, untreated

Heat-sensitive spore



Heat-resistant spore

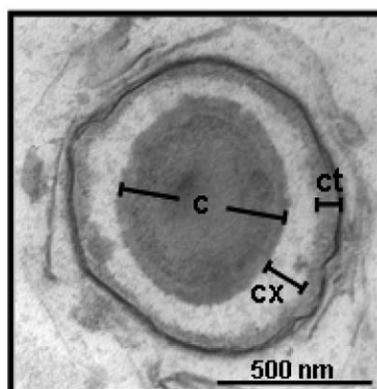
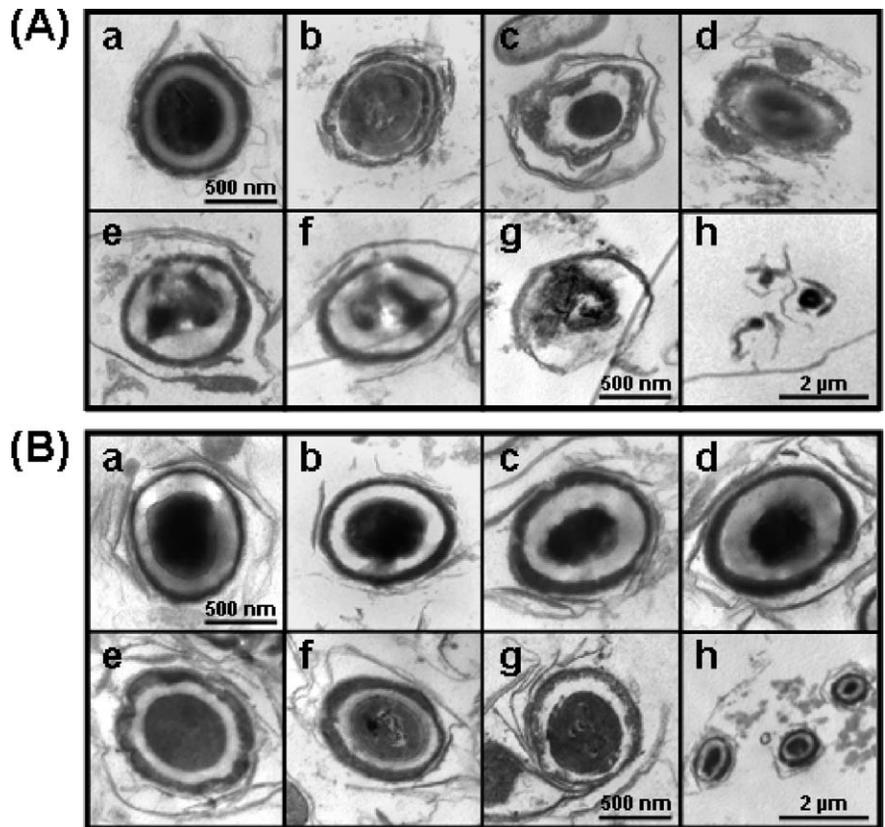


FIGURE 1. Representative transmission electron micrographs of heat-sensitive and heat-resistant spores of *C. sporogenes*. Structural components measured: ct, coat layers; cx, cortex region of spore; and c, the spore core. The scale bars in electron micrographs represent 500 nm. Each micrograph was taken at $\times 60,000$ magnification.

FIGURE 2. Transmission electron micrographs of sectioned spores of *C. sporogenes* after heat treatment. Micrographs of heat-sensitive (A) and heat-resistant (B) spores of *C. sporogenes* before (a) and after (b through h) heat treatment at 121°C for 5 min are shown. Close-up views of individual spores (a through g) were taken at $\times 60,000$ magnification (bar = 500 nm), while representative micrographs (h) were taken at $\times 10,000$ magnification (bar = 2 μm).



spores had a smooth surface, as shown in Figure 1 (left panel). To support this observation, therefore, the shapes of the structural components of untreated spores and the spores heat damaged at 121°C for 5 min were further observed and compared via transmission electron microscopy. From the transmission electron microscopic appearances of varying heat-damaged heat-sensitive spores, it was found that heat treatment caused severe destruction of the structural components of the spores, exhibiting decoating and cortex lysis, and subsequently resulted in extensive damage to the core in any case, as presented in Figure 2A.

In contrast to heat-sensitive spores, however, the structural components of heat-treated heat-resistant spores remained to be unlysed, which in turn allowed most of the cores to be less or not damaged, as shown in Figure 2B. Therefore, it was clarified that the thicker coat layer and cortex region of the heat-resistant spore effectively protected its core where genetic materials are distributed, which is consistent with the $D_{121^\circ\text{C}}$ -value results shown previously.

DISCUSSION

Setlow (20) reviewed various studies on the structural components of *Bacillus* spores and suggested that while the coat layer is responsible for spore's resistance to chemicals and to exogenous lytic enzymes, but not for heat resistance, the cortex region is considered to contribute to heat resistance. However, since a small heat shock protein participates in spore coat formation in *Bacillus* (8), it could be speculated that somehow the coat layer may function as primary barrier, protecting spores from heat damage, as the

cortex region does. In this study, therefore, the roles of the three major structural components, the coat layer, cortex region, and core, in protecting *C. sporogenes* spores from heat damage were examined regarding their thickness and sizes.

To test whether the structural components participate in heat resistance, $D_{121^\circ\text{C}}$ -values of eight spore batches were examined and found to be distributed in a broad range of 0.3 to 1.0 min in our preliminary study. Such a wide range of heat resistance might arise from unnoticeable experimental errors or variations in the culture condition that would affect morphological peculiarities and chemical compositions of the spores. It is not discussed in depth herein, but experiments testing the influence of various culture conditions on heat tolerance are currently being carried out.

In any case, since it can be speculated that disparate intracellular structures caused such a difference in heat tolerance, we attempted to observe appearances of the structural components of spores by using transmission electron microscopy. Multiple transmission electron micrographs of two spore batches showing remarkably different degree of tolerances to heat—in terms of $D_{121^\circ\text{C}}$ -value—were taken and examined to determine if there were any differences in the structural components. Transmission electron microscopic and statistical analyses clarified that heat-sensitive spores have thinner coat layers and cortex regions than do heat-resistant spores, showing noticeable differences, as expected, which suggests, somewhat surprisingly, that the spore coat's multilayered structure composed of highly cross-linked polypeptides may function (at least partially) in protecting spores from heat damage. This speculation

was further investigated in this study to examine the role of each structural component in protecting itself from heat damage, using transmission electron microscopy. The micrographs showed that the thicker coat layer and cortex region of heat-resistant spores are less or not damaged by heat treatment, compared with heat-sensitive spores that were noticeably damaged. These results are in agreement with our observation that spores associated with thicker coat layer and cortex region were more resistant to heat.

The core of a heat-resistant spore possesses lower water content than the protoplast of a growing cell (6), which has been known to contribute to heat resistance. Novak et al. (14) reported that the size of the core of *C. perfringens* showed a negative correlation with the heat resistance of spore. Similarly, in *Bacillus* spores, an inverse correlation between the heat resistance and the core-to-sporoplast (in the original report, the sporoplast was defined as the structures bounded by the outer pericortex membrane) volume ratio was found (1). Together with the fact that small molecules found in spores, i.e., DPA and Ca, are involved in resistance to heat (6), it was interesting to test whether the size of the spore core would have a positive relationship with the heat resistance of *C. sporogenes*. However, somewhat distinct from *C. perfringens* and *Bacillus* spp., the core sizes of heat-sensitive and heat-resistant spores measured were not significantly different in this study. Meanwhile, it was found that the ratios of the core size to the sporoplast size is negatively related to heat resistance, which is in agreement with an earlier report of Beaman et al. (1). The dehydrated state of the spore core primarily confers the property of heat resistance upon a spore (2, 6), which in turn depends for its maintenance on the cortex (5). Furthermore, heat treatment has been found to induce heat shock proteins in diverse microorganisms (10). Taken together, therefore, it can be concluded that the small molecules themselves and reduced water content of the spore core participate in heat resistance, regardless of the size of the core. In addition, the structural components, the coat layer, cortex region, and core, are probably a series of barriers protecting genetic information in a spore and (at least partially) act in concert with each other to achieve heat resistance.

Meanwhile, the dehydration procedure of conventional transmission electron microscopy technique is known to cause a potential limitation in interpretation of measurements. Moreover, the resolution limitation of the technique can obscure spore structures, and thereby make it difficult to obtain accurate size distribution data (23). However, since all specimens used in this study were fixed, dehydrated, and stained under the same procedures, it is clear that all comparative data analyzed herein represent the evidences on the role of each structural component, regardless of dehydration artifacts.

In this study, it was demonstrated that the structural components, including the coat layer and cortex region, were significantly correlated with heat tolerance of *C. sporogenes* spores. It is not surprising that spores have a multitude of barriers to protect themselves. Spores have to survive for long periods, protecting themselves from multiple

intrinsic and/or extrinsic stress resources (19, 20). Thus, it seems likely that spores use multiple mechanisms to make it easy to prevent damage caused by those various environmental stresses. However, the underlying mechanisms as to how the structural components collaborate to enhance heat resistance are less clear. To better dissect the mechanism and understand the role of each component, it is necessary to introduce a genetic approach. It is expected that the mechanism of heat resistance of a spore may be completely explained using relevant loss-of-function mutant strains that lack a structural component.

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