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Novel strains of *Moorella thermoacetica* form unusually heat-resistant spores

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Abstract Two strains of *Moorella thermoacetica*, JW/B-2 and JW/DB-4, isolated as contaminants from autoclaved media for chemolithoautotrophic growth containing 0.1% (wt/vol) yeast extract, formed unusually heat-resistant spores. Spores of the two strains required heat activation at 100 °C of more than 2 min and up to 90 min for maximal percentage of germination. Kinetic analysis indicated the presence of two distinct subpopulations of heat-resistant spores. The decimal reduction time (D_{10}-time=time of exposure to reduce viable spore counts by 90%) at 121 °C was determined for each strain using spores obtained under different conditions. For strains JW/DB-2 and JW/DB-4, respectively, spores obtained at ~25 °C from cells grown chemolithoautotrophically had D_{10}-times of 43 min and 23 min; spores obtained at 60 °C from cells grown chemoorganoheterotrophically had D_{10}-times of 44 min and 38 min; spores obtained at 60 °C from cells grown chemolithoautotrophically had D_{10}-times of 83 min and 111 min. The thickness of the cortex varied between 0.10 and 0.29 μm and the radius of the cytoplasm from 0.14 to 0.46 μm. These spores are amongst the most heat-resistant noted to date. Electron microscopy revealed structures within the exosporia of spores prior to full maturity that were assumed to be layers of the outer spore coat.

Keywords Heat-resistance · Endospores · Sporulation · D_{10}-time · *Moorella thermoacetica* · Spore ultrastructure

This manuscript is dedicated to Professor Gerhard Gottschalk on the occasion of his 65th birthday

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Introduction

Heat-resistant endospores of thermophiles can be troublesome as contaminants in research laboratories as well as in industrial processes, such as those in the food-canning industry. Yeast extract is often used as a component of media, and frequently contains bacteria and/or spores (which can also cling tenaciously to glassware and butyl rubber septae) which are assumed to be killed or inactivated by routine autoclaving of media. However, many bacterial endospores are activated to germinate by changes in temperature (Foerster 1983), such that autoclaving at 121 °C for 15 min or less may serve only to activate some spores of thermophilic bacteria, rather than killing them, as we have observed with some batches of yeast extract (unpublished results; Wiegel 1986). Uninoculated medium which is then incubated at elevated temperatures of 50 °C and above often yields cultures of contaminating thermophiles (Hyun et al. 1983).

Many gram-type positive (Wiegel 1981) bacteria are able to form endospores during adverse conditions. Thermophilic bacteria often form endospores which are quite heat-resistant (Donnelly and Busta 1980; Hyun et al. 1983). There is a correlation between the maximum growth temperature of *Bacillus* strains and the heat resistance of their spores (Warth 1978). Also, a correlation between temperature of sporulation and heat resistance of spores has been observed (Khoury et al. 1987; Williams et al. 1954; Condon et al. 1992). Innate properties of endospores, such as moisture content, dipicolinic acid content, and cortex layer thickness, as well as external conditions (media composition, pH, salinity, temperature) are thought to contribute to heat resistance (Gerhardt and Marquis 1989). Thus, mineral concentration and dehydration of spores immobilize, and thus stabilize, molecules and structures, making them more heat-resistant. The spore coat and spore cortex have important roles in the dehydration of the spore core as well as in the concentration of minerals, especially calcium, possibly via a contractile or osmotic mechanism (Gould and Dring 1975; Lewis et al. 1960).

Here we report on two strains of endospore-forming bacteria which were isolated from 0.1% (wt/vol) yeast-extract-containing media designed for chemolithoautotrophic growth studies (Wiegel et al. 1981) that had been autoclaved at 121 °C for 45 min. To determine the heat activation requirement and heat resistance of these spores, solid culture techniques were used to quantitate survival after heat treatment for various time periods and to determine the decimal reduction times (D_{10}-time=time required to reduce viable counts to 10%, i.e., time after which 90% of activated spores are unable to germinate and produce colonies on solid media). Spores were obtained at ~25 °C after chemolithoautotrophic growth and at 60 °C after chemoorganoheterotrophic and chemolithoautotrophic growth in order to investigate heat resistance for spores obtained under different sporulation conditions. The temperature and pH ranges that allowed growth, doubling times for growth, and sporulation frequency were investigated, fermentation analysis was performed, and the morphology of the spores was studied using electron microscopy. Based on these data and 16S rRNA gene sequence comparisons, the two strains were placed into the species *Moorella* (basonym *Clostridium*) *thermoacetica*.

Materials and methods

Cultivation and sporulation conditions

Strains JW/DB-2 and JW/DB-4 (ATCC number BAA-48) were isolated in our laboratory and kept at –75 °C in 40% (vol/vol) glycerol-containing anoxic media under a gas atmosphere of oxygen-free nitrogen. Vegetative cells were grown in prereduced media containing, per liter of deionized H_20, 2 g yeast extract, 0.5 g KH_2PO_4, 1 g Na_2HPO_4, 0.4 g NH_4Cl, 0.3 g $(NH_4)_2SO_4$, 0.09 g $MgSO_4 \cdot 7H_20$, 0.45 g NaCl, 10 ml reducing solution (1.25 g Na_2S+1.25 g cysteine-HCl per 100 ml), 5 ml trace element solution (Freier et al. 1988), 0.5 ml vitamin solution (Freier et al. 1988), and 1 ml of 0.1% (wt/vol) resazurin. Anoxic media were prepared with nitrogen gas using standard anaerobic techniques (Ljundahl and Wiegel 1986), autoclaved at 121 °C for 45 min, adjusted with 2 M HCl to pH~6.5–6.8 (25 °C), incubated at 60 °C for 6 h and autoclaved again in order to kill any spores that had been activated to germinate by the first autoclave cycle. For chemoorganoheterotrophic growth, anoxic twice-autoclaved glucose in water was added to media prior to use at a final concentration of 1% (vol/vol) unless otherwise noted. For chemolithoautotrophic growth, glucose was substituted for by 1.5 g $NaHCO_3$ l^{-1}, and after final autoclaving, the nitrogen gas was replaced with sterile 80% (vol/vol) H_2 and 20% (vol/vol) CO_2 under pressure (10–20 psi). The pH (25 °C) was adjusted with CO_2 to 6.5–6.8. Agar shake-roll tubes were made with chemoorganoheterotrophic growth media solidified with 3% (wt/vol) agar. Three different populations of spores were obtained: cells were grown to the mid-exponential phase at 60 °C in liquid medium and then placed either at ~25 °C (chemolithoautotrophically grown) or at 60 °C (chemoorganoheterotrophically and chemolithoautotrophically grown) for 2–3 weeks to allow sporulation. All spore suspensions were concentrated by centrifugation in sterile, anoxic Hungate tubes at 3500×g for 30 min, then resuspended in chemolithoautotrophic growth media kept under oxygen-free nitrogen gas. This was also the medium in which heating was performed. Greater than 90% of the spores were noted to be phase-bright and were subjected to heating trials without purification from vegetative debris. Initial spore counts were determined for the suspensions used in heating experiments by averaging eight fields using a counting chamber (Hausser Scientific Partnership, Horsham, Penn.). Initial counts were: grown chemolithoautotrophically with sporulation at 25 °C: strain JW/DB-2, 2.2×10^8 ml^{-1}; strain JW/DB-4, 2.1×10^8 ml^{-1}; grown chemoorganoheterotrophically with sporulation at 60 °C: strain JW/DB-2, 1.2×10^8 ml^{-1}; strain JW/DB-4, 9.8×10^7 ml^{-1}; grown chemolithoautotrophically with sporulation at 60 °C: strain JW/DB-2, 3.9×10^7 ml^{-1}; strain JW/DB-4, 4.1×10^7 ml^{-1}.

Heat resistance of spores

Boiling water and glycerol were used to heat spore aliquots to 100 °C and 121 °C, respectively, by direct immersion of inverted Hungate tubes (ensuring that the rubber stoppers were covered). Duplicate tubes containing media with inserted thermometers were used to ascertain lag time and desired temperature. In order to set the t_0 value for viable spore counts, spores heated to 121 °C were first heated for 3 min at 100 °C. For spores of strain 2 obtained at 60 °C, spores were heated at 100 °C for 5 min prior to heating at 121 °C. Heat-treated spore aliquots were cooled on ice and inoculated into liquid or agar-containing chemoorganoheterotrophic growth medium which was kept at 60 °C in a water bath. Serial tenfold dilutions were made up to 10^{-8} in three runs for each time point (see

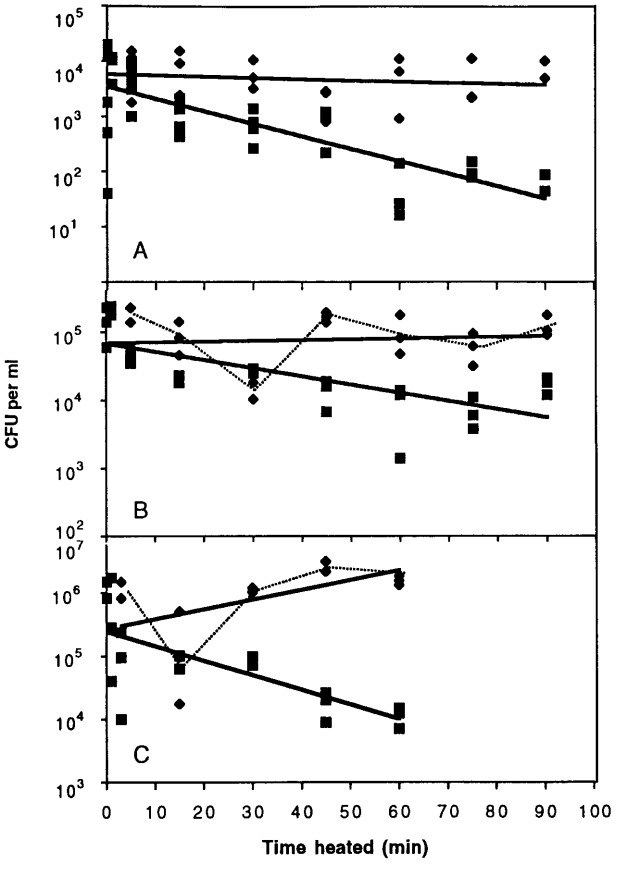

Fig. 1A–C Inactivation curves for spores of strain JW/DB-2. Data shown for spores obtained at **A** 60 °C after chemoorganoheterotrophic growth, **B** 60 °C after chemolithoautotrophic growth, and **C** ~25 °C after chemolithoautotrophic growth. Spores were heated to 100 °C (◆) or 121 °C (■). Spores were concentrated (as described in Materials and methods), and heated in either boiling water or glycerol bath for the times noted. Final colony counts were made after a 3-week incubation at 60 °C in agar shake-roll tubes. D_{10}-times at 121 °C were calculated to be 44 min (**A**), 83 min (**B**), and 43 min (**C**). The *dashed lines* represent the differential heat-activation requirements of the spore subpopulations, as noted in the Discussion

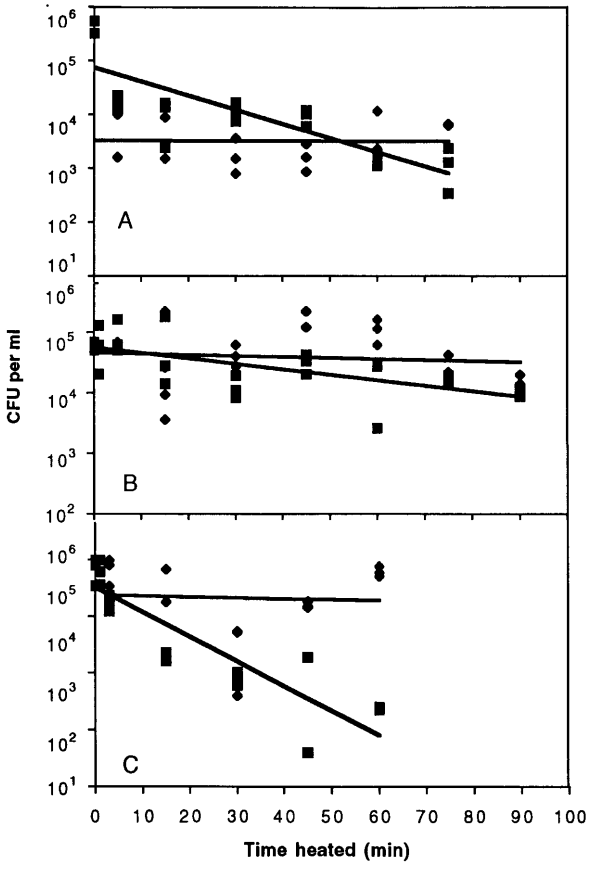

Fig. 2A–C Inactivation curves for spores of strain JW/DB-4. Data shown for spores obtained at **A** 60 °C after chemoorganoheterotrophic growth, **B** 60 °C after chemolithoautotrophic growth, and **C** ~25 °C after chemolithoautotrophic growth. Spores were heated to 100 °C (◆) or 121 °C (■). Conditions were otherwise as described in Fig. 1 and Material and methods. D_{10}-times at 121 °C were calculated to be 38 min (**A**),111 min (**B**), and 23 min (**C**)

Figs. 1, 2) to inoculate agar shake-roll tubes. The tubes were incubated at 60 °C and colonies were counted after 3 weeks.

Determination of heat-activation requirement

Aliquots of spores obtained at ~25 °C from chemolithoautotrophically grown cultures were heated at 100 °C for various time periods and inoculated into dilution series of agar shake-roll tubes. The tubes were incubated at 60 °C and colonies were counted after 3 weeks.

Growth temperature and pH range determination

Temperature ranges and optimum temperatures for growth were determined in chemoorganoheterotrophic media at pH 6.7 in a shaking Temperature Gradient Incubator (Scientific Industries) with a gradient in 30 steps between 40 and 75 °C. The pH ranges and optimum pH for growth at 60 °C were determined in chemoorganoheterotrophic media with pH (25 °C) values ranging from 4.0 to 10.0.

Fermentation analysis

Cultures grown chemoorganoheterotrophically on 0.5% (wt/vol) glucose were analyzed for fermentation products using the Beckman 110 high-pressure liquid chromatograph, as described previously (Svetlitshnyi et al. 1996).

Electron microscopy

Spore suspensions were prepared according to the method of Kellenberger et al. (1958), embedded in Epon 812, thin-sectioned and placed on 300-mesh, Formvar-coated copper grids. Freeze-substitution fixation was performed using propane-plunge-frozen spores, which were then placed in 2% (wt/vol) osmium tetroxide in acetone at –80 °C for 48 h, then gradually warmed to ~25 °C and embedded in Spurr's resin, thin-sectioned and placed on Formvar-coated copper grids. All sections were post-stained with uranyl acetate and lead citrate. Freeze-fracture platinum replicas were made of propane-plunge-frozen spore suspensions and placed on naked carbon grids. The JEOL 100CX II TEM was used to make electron photomicrographs.

16S rRNA gene sequence analysis

Partial 16S rRNA gene sequences comprising 597 nucleotides at the 5′ end of the gene were determined for both strains as described previously (Svetlitshnyi et al. 1996). The 597 nucleotides between positions 39 and 615 (*Escherichia coli* positions) were compared with all available 16S rRNA gene sequences of low G+C gram-positive bacteria. Pairwise comparisons of the partial sequences of the two strains found them to be identical and to have 99.8% and 99.5% similarity with the previously determined 16S rRNA gene sequences of *M. thermoacetica* and *M. thermoautotrophica*, respectively. In this same region the 16S rRNA gene sequences of *M. thermoacetica* and *M. thermoautotrophica* share 99.3% similarity.

Results and discussion

Physiological properties such as temperature and pH range for growth, fermentation data and morphology of vegetative cells are all consistent with known parameters of *Moorella thermoacetica* (Table 1). Comparison of the partial 16S rRNA gene sequences indicated that the two strains (JW/DB-2 and JW/DB-4) are identical in the partial 16S rRNA gene sequence, though physiological differences were observed, and are strains of *M. thermoacetica* (99.8% similarity), with a close relationship (99.5% similarity) to *Moorella thermoautotrophica*. The strains were placed into *M. thermoacetica* instead of *M. thermoautotrophica* based on the significantly lower frequency of sporulation (maximum of 60% vs >95% for *M. thermoautotrophica*), and the closer 16S rRNA sequence similarity (Table 1). The spores of the type strain of *M. thermoacetica* (ATCC 35608) have been reported to have D_{10}-times at 121 °C of around 15 min (Fontaine et al. 1942) and no other strains of *Moorella* have been reported to have heat resistance above this (no data available on *M. thermoautotrophica*). The D_{10}-times at 121 °C for strains JW/DB-2 and JW/DB-4, respectively, were 43 min and 23 min when grown chemolithoautotrophically with sporulation at ~25 °C; 44 min and 38 min when grown chemoorganoheterotrophically with sporulation at 60 °C;

Table 1 Selected properties of the strains compared with other *Moorella* isolates. For the novel strains: Cell size was measured from electron micrographs of vegetative cells prepared by negative staining with 2% uranyl acetate on formvar-coated copper grids. Temperature range was determined at pH 6.7. The pH range was determined at 60 °C. Fermentation balance was at 60 °C, and pH 6.7. Doubling time was determined at optima of pH and temperature with 1% glucose. Information on *Moorella thermoacetica* and *Moorella thermoautotrophica* from Wiegel et al. (1991)

	JW/DB-2	JW/DB-4	*M. thermoacetica* Ljd	*M. thermoautotrophica* 701/5
Vegetative cell size [diameter×length (μm)]	0.4–1×2–3	0.4–1×2–3	0.6–1×2.5–4	0.8–1×3–6
Temperature [growth (no growth)]				
Maximum	65 °C (68 °C)	67 °C (69 °C)	65 °C	66 °C
Minimum	47 °C (45 °C)	48 °C (47 °C)	51 °C	42 °C
Optimum	58 °C	59 °C	60 °C	60 °C
pH (25 °C range)	5.5–8.3	5.25–8.7	5.7–7.65	4.8–7.3
Acetate formed per glucose utilized	2.51	2.74	2.5–2.9	2.5–2.9
Doubling time (h)	7	5	6–8	5

Table 2 Comparisons of reported D_{10}-times for bacterial endospores

Species	Sporulation temperature (°C)	Heating temperature (°C)	D_{10}-time (min)	Reference
Moorella thermoacetica JW/DB-4	60	121	111[c]	Byrer et al., this work
Moorella thermoacetica JW/DB-2	60	121	83[c]	Byrer et al., this work
Clostridium thermosaccharolyticum	55	124	72.5	Xezones et al. (1965)
Clostridium difficile 88	37	100	32.5[c]	Nakamura et al. (1985)
Clostridium thermohydrosulfuricum 39E[a]	69[b]	121	11	Hyun et al. (1983)
Desulfotomaculum nigrificans 7946	55	121	5.6[c]	Donnelly and Busta (1980)
Bacillus stearothermophilus 12980	55	121.1	3.3	Feeherry et al. (1986)
Clostridium thermosulfurogenes 4B	60[b]	121	2.5[c]	Hyun et al. (1983)
Clostridium thermocellum LQRI	60[b]	121	0.5[c]	Hyun et al. (1983)

[a]Renamed *Thermoanaerobacter ethanolicus* 39E
[b]Optimum growth temperature
[c]Heat-treated by closed-tube method; others were by thermoresister method

and 83 min and 111 min when grown chemolithoautotrophically with sporulation at 60 °C (Figs. 1, 2). When spores were heated at 100 °C for up to 60 or 90 min, there was little change in the final colony counts. Spores obtained at ~25 °C from chemolithoautotrophically grown cultures of strain JW/DB-2 actually showed a trend towards increasing colony-forming units. Thus, D_{10}-times for 100 °C could not be calculated (Figs. 1, 2). For any of the spores, low numbers of colony-forming units (from 0 to 50 CFU/ml) were obtained from suspensions heated for less than 1 min at 100 °C (data not shown).

Phase-contrast microscopy demonstrated ~60% sporulation after 2 weeks with chemolithoautotrophically grown cells, whether placed at ~25 °C or 60 °C to sporulate, and about ~15% sporulation after 3 weeks with chemoorganoheterotrophically grown cells kept at 60 °C. Thus, it appears that chemolithoautotrophic growth tends to precede more complete sporulation of cultures compared to chemoorganoheterotrophic growth.

The increased heat resistance of spores from these two isolates separates them from other strains in the genus and species. The increase in final colony counts of spores obtained from chemolithoautotrophically grown cultures of strain JW/DB-2 with continued heating at 100 °C likely represents the activation of distinct subpopulations of spores, with an overall higher percentage of spores only becoming activated (and thus able to germinate and grow into colonies) as heating proceeded. This pattern is represented by the dashed lines in Fig. 1B, C and is also noted with strain JW/DB-4 spores obtained at 60 °C, though in that strain the trend is less pronounced (Fig. 2A, B). Even those spores which do not show an increase in final colony counts with longer heating times are stable to heating at 100 °C – that is, for the times tested, colony counts do not decrease overall. However, in most cases, an intermittent decrease in colony-forming units is observed in the mid-range of the heating times, with an upswing at the longer heating times. These observations support the hypothesis of differential activation of subpopulations of spores by increased heating times, which is in agreement with the findings of Feeherry et al. (1987) with spores of *Bacillus stearothermophilus*. Thus, the reported optimal heat-activation times and D_{10}-times must be regarded as "apparent" or "average" values for what are evidently heterogeneous spore populations. Both chemolithoautotrophic growth conditions and sporulation at higher temperature seem to contribute to heat-resistance. In each strain, temperature of sporulation seems to play a larger role, supporting the findings of Condon et al. (1992) that some thermophiles form spores with greater heat-resistance at higher temperatures than they do at lower temperatures.

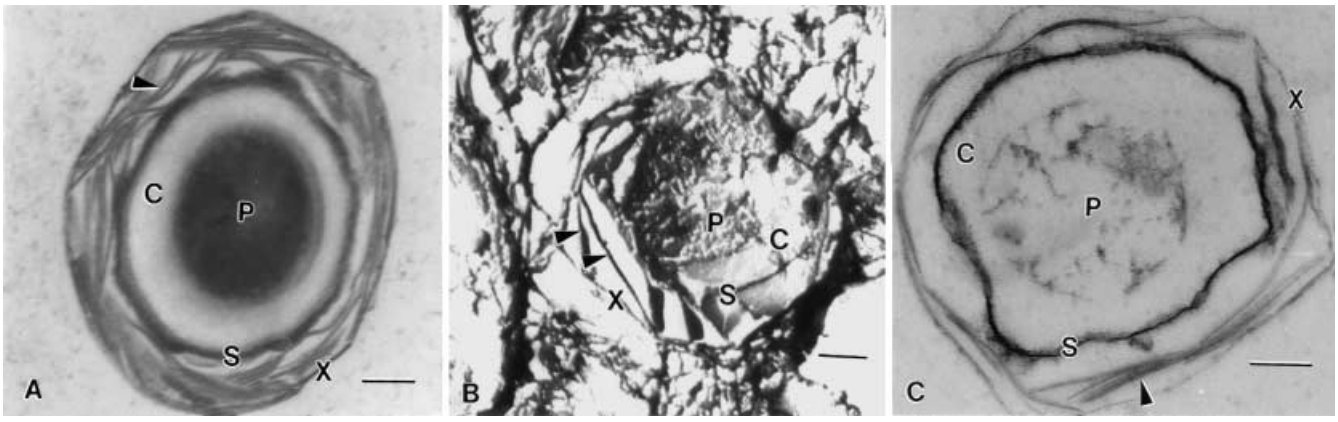

Fig. 3A–C Electron micrographs of spores of strain JW/DB-2. **A** Thin section of a spore prepared by the method of Kellenberger et al. (1958); **B** platinum replica of a spore prepared by freeze-fracture, and **C** thin section of a spore prepared by freeze substitution, as described in Materials and methods. *C* Spore cortex, *P* spore protoplasm, *S* spore coat, *X* exosporium. *Arrowheads* are lamellar structures that probably represent layers of the outer spore coat. *Bars* 0.2 μm

These strains' D_{10}-times at 121 °C are amongst the longest reported for bacterial endospores (Table 2). Some differences in technique may make direct comparisons of the data difficult, e.g., Xezones et al. (1965) heated their spores in distilled water and Nakamura et al. (1985) heated their spores in phosphate buffer; while in other reported studies spores were heat-tested, as in this work, in culture/sporulation medium. The reported D_{10}-time noted for *Clostridium difficile* by Nakamura et al. (1985) was after heat-treatment at 100 °C, while that reported by Xezones et al. (1965)for *Clostridium thermosaccharolyticum* was at 124 °C. All reported investigations used solid-culture colony counts to determine D_{10}-times.

When examined by electron microscopy, structures were noted between the exosporium and spore coat of spores prior to full maturity (Fig. 3), after which point the exosporium was virtually indistinguishable and the structures no longer noted. The structures were seen regardless of chemical fixation, freeze-substitution, or freeze-fracture-replica preparation. As in a previous report of similar findings (Cook et al. 1991), this was attributed to outer spore coat layers. Using electron micrographs of mature spores (n=10 for strain JW/DB 2, n=4 for strain JW/DB 4), the volumes of the cortex and the cytoplasm were determined according to Hyun et al. (1983), assuming a spherical spore. The thickness of the cortex ranged from 0.10 to 0.29 μm with an average of 0.15 μm for strain JW/DB-2, and from 0.11 to 0.21 μm with an average of 0.16 μm for strain JW/DB-4. The radius of the cytoplasm ranged from 0.18 to 0.46 μm with an average of 0.37 μm, and from 0.14 to 0.25 μm, with an average of 0.19 μm, respectively. According to Hyun et al. (1983), the ratio of the volumes of the cortex to cytoplasm should correlate with the degree of heat resistance. The cortex volume was calculated by subtracting the cytoplasm volume from the volume of cortex plus cytoplasm. Hyun et al. (1983) found for spores from clostridial species with D_{10}-times (at 121 °C) of 0.5, 2.5 and 11 min, ratios of 1.4, 1.6 and 6.6, respectively. For spores of strain JW/DB-2, which were sporulated at ~25 °C after chemolithoautotrophic growth and which exhibited a D_{10}-time (121 °C) of 43 min, the ratios ranged from 1.4 to 8.3 (average=3.13). For spores of strain JW/DB-4 which were sporulated at ~25 °C after chemolithoautotrophic growth and which exhibited a D_{10}-time (121 °C) of 23 min, the ratios ranged from 2.88 to 9.65 (average=5.97). Only to some extent, and not in a directly proportional correlation, do these ratios follow the postulated (Hyun et al. 1993) trend of an increasing D_{10}-time with an increasing ratio. We also measured the spore coat thickness: 0.01 to 0.075 μm (average 0.05 μm) for strain JW/DB-2, and 0.03 to 0.06 μm (average 0.04 μm) for strain JW/DB-4. The significantly larger variations of the thickness of the cortex and the calculated ratios for these strains are likely due to the presence of differently heat-resistant subpopulations of spores, which was also indicated by the inactivation kinetics. Again, a comparison of the data from the two strains does not yield a strong correlation between the thickness of the cortex or the coat and the D_{10}-times.

It makes sense intuitively that those organisms best adapted to extreme conditions will better survive environmental stress. Measures necessary to ensure sterile media in laboratories working with thermophiles or thermobiotic soil and sediment samples often include autoclaving times of longer than 20 min, which for strains such as these may actually result in increased heat resistance due to full activation of several subpopulations of endospores. As uses of thermophilic bacteria increase, more heat-resistant contaminants will likely be isolated. A better understanding of these extreme organisms will allow for contamination prevention and optimization of environmental conditions for the desired bacteria. The unique ultrastructural features of these spores are intriguing, raising the question of whether they contribute to the spores' unusual heat-resistance.

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