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Increased Heat Resistance of *Geobacillus stearothermophilus* Spores Heat-Shocked During Sporulation

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Abstract The purpose of this study was to measure the heat resistance and core mineral content of *Geobacillus stearothermophilus* ATCC 7953 spores when the sporulating cells were exposed to heat shock at different times during sporulation. Heat shock during sporulation was found to increase the heat resistance of the spores produced subsequently. The spores heat shocked 2 h after the end of the exponential phase showed the highest heat resistance and a 30-fold increase in the d_{10} compared with the non-heat-shocked spores. The enhanced heat resistance was likely due to the increased mineral content observed in the spores heat shocked at t_7 or t_8.

Key words: Heat resistance, heat shock, sporulation, *Geobacillus stearothermophilus*

Bacterial endospores are interesting, as they show no detectable metabolism and are highly resistant to various physical insults such as heat, UV, radiation, and pH [16, 17]. This resistance is particularly important from a sanitation, clinical, and industrial aspect, as spores are difficult to eliminate and thus are sources of unwanted contamination [7, 9]. Spore survivability can also be important when used to monitor various sterilization and disinfection processes. Sterilization monitoring improves the assurance that medical devices and wastes have been adequately sterilized, and biological monitoring, such as the use of heat-resistant spores as indicators, is accepted as the most effective method for monitoring sterilization [1].

Thermophilic bacteria, especially *G. stearothermophilus*, are ideal candidates for monitoring moist-heat sterilization processes because of their high heat resistance. In particular, *G. stearothermophilus* ATCC 7953 has been widely employed as biological indicators for monitoring heat sterilization processes [4, 12].

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Accordingly, the purpose of this study was to evaluate the change in heat resistance of *G. stearothermophilus* ATCC 7953 when inducing heat shock during different periods of sporulation. Previous studies have already demonstrated that inducing heat shock during sporulation causes an increased heat resistance in the subsequently obtained spores of *Bacillus subtilis* [13], *Clostridium perfringens* [6], and *B. megaterium* [19]. Thus, if spores that are more heat resistant can be obtained by a simple procedure, such as administeration of heat shock during sporulation, then a biological indicator that is more resistant to the sterilization process could be obtained.

The water content, mineralization, and optimal growth temperature of sporulating organism are known to be the main factors contributing to the heat resistance of spores. As such, decreased water content and/or increased mineralization contribute to an increased heat resistance, whereas organisms with high growth temperatures are inherently more heat resistant [3, 5, 15].

Therefore, this study examined the extent of mineralization as one of the main factors determining the heat resistance of spores and is apparently related to the water content. In a previous study, increased mineralization has already been implicated, along with a decreased water content [2]. Furthermore, it has also been reported that continuous incubation of sporulating cells at an elevated temperature is correlated with increased mineralization of the spores [18]. Thus, since heat shock involves exposure to an elevated temperature, albeit for a brief period, it may correlate with the mineral content in spores heat shocked during sporulation. Therefore, the relationship between extent of mineralization and the heat resistance of *G. stearothermophilus* ATCC 7953 spores was investigated in this study, and it would appear that this is the first study on the mineral content in spores heat shocked during sporulation.

The *G. stearothermophilus* ATCC 7953 was first grown overnight at 55°C and 200 rpm agitation in a TSB medium (Difco, U.S.A.; 17.0 g pancreatic digest of casein, 3.0 g

enzymatic digest of soybean meal, 2.5 g glucose, 5.0 g NaCl, 2.5 g dipotassium phosphate, per liter of deionized water), then subcultured twice in the same medium. Thereafter, a liquid sporulation medium (0.18 g glucose, 0.4 g glutamic acid, 4 g yeast extract, 5 g peptone, 0.01 g NaCl, 0.01 g $MgSO_4$, 0.1 g $(NH_4)H_2PO_4$, 0.5 g KH_2PO_4, 0.01 g $CaCl_2$, 0.003 g $FeSO_4$, 0.01 g $MnSO_4$, per liter of deionized water) [21, 22] was inoculated with 1 v/v% of the TSB subculture in the late-exponential phase and cultured at 55°C and 200 rpm agitation to induce the sporulation of vegetative cells. To rule out any possibility of temperature change during the handling and inoculation of the cultures, all the cultures and media were handled outside the incubator under protection of water insulation maintained at 55°C.

The heat shock was applied at different times for each spore sample during sporulation, by placing the cultures in another incubator preset at 75°C, for 30 min. The heat shock was administered to each spore samples only once between 3–8 h after inoculation of the sporulation medium, hereby denoted as t_3-t_8. The start of sporulation was verified by a phase-contrast microscope, when nonrefractile endospores were observed within the mother cells in 15–30% of all cells at t_3. At t_5-t_6, 40% of the cells possessed refractile endospores. All cultures were incubated for a total of 28 h at 55°C with 200 rpm agitation. The resulting cultures contained 50–60% spores when observed under the microscope.

The spores were separated from the vegetative cells by repeated centrifugation (10 min at 5,000 ×*g*) followed by washing with deionized water, which was repeated four to five times. The washed cells were then checked under the microscope and all samples contained ca. 95% spores.

After being exposed to 115°C for different time periods in an oil bath, the direct viable counts were performed by spreading spore samples on TSA plates (TSB with 1.5% agar). All the plates were incubated for 48 h at 55°C.

For the mineral analysis, the spores suspended in deionized water were thoroughly probe sonicated on ice at 7 W output for about 2 h. Ten ml of nitric acid was then added to 0.5 ml of the spore lysate, and the solution was then heated at 100°C and 200 psi for 10 min, 100°C and 150 psi for 60 min, and 210°C and 350 psi for 10 min before being analyzed using an ICP-MS (Agilent 7500i, Agilent Technologies, U.S.A.) [8]. The concentrations of Ca^{2+}, Mn^{2+}, and Mg^{2+} were divided by the dry cell weight of spores.

Linear portions of survivor plots for the spores heat shocked during the exponential phase, t_3, t_4, and t_5, are shown along with a survivor plot for non-heat-shocked spores as the control (Fig. 1A). From the slopes of the exponential decline, the heat resistance clearly increased for all the spores that were heat shocked during sporulation.

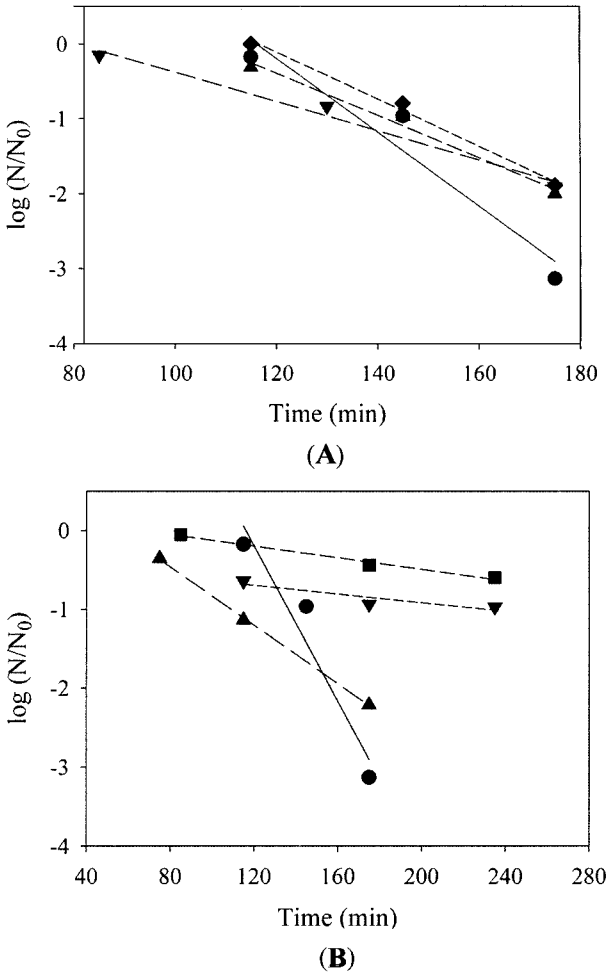

Fig. 1. Regression slopes of survivor curves representing heat resistance of spores heat shocked at different times.
Linear portions of each survivor curve are shown for **A)** spores heat shocked during exponential growth in sporulation medium, namely, at t_3 (▼), t_4 (▲), and t_5 (◆); and **B)**, spores heat shocked during the stationary phase in sporulation medium, namely, at t_6 (▲), t_7 (■), and t_8 (▼); along with non-heat-treated spores (con, ●).

The exponential decline intervals were also linearly regressed to calculate the decimal reduction time (d_{10}); i.e., the time required to kill 90% of the original cell population. Among the spores heat shocked during the exponential phase of sporulation, the spores heat shocked at t_3 showed the highest d_{10} value of 51 min. Thereafter, the heat resistance declined in the t_4 spores to a d_{10} value of 37 min, then to a d_{10} value of 32 min in t_5 spores. The spores that were not subjected to heat shock showed a d_{10} value of 20 min.

The survival plots for the spores heat shocked during the post-stationary phase, at t_6, t_7, and t_8, are shown in Fig. 1B. The spores heat shocked at t_7 and t_8 were particularly heat resistant, even failing to reach a −1 log reduction after more than 4 h under the given conditions. The spores heat shocked at t_8 exhibited the longest d_{10}, at 360 min, followed by the t_7 spores showing a d_{10} value of 270 min. As such,

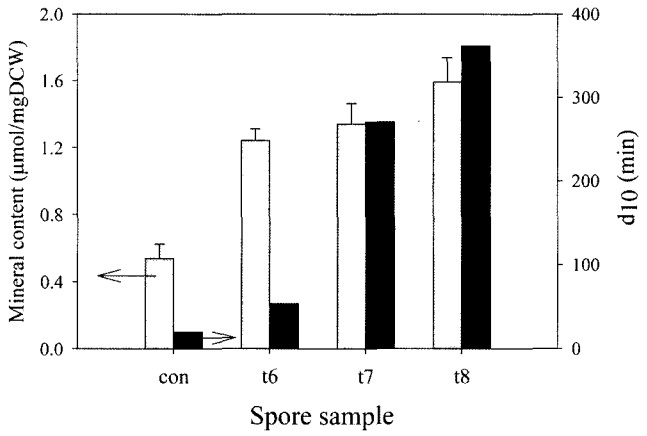

Fig. 2. Total Mn^{2+}, Mg^{2+}, Ca^{2+} content (shaded bars) and heat resistance (black bars) for spores heat shocked during sporulation.

this represents 30- and 20-fold increases in d_{10} for t_8 and t_7 compared with non-heat-shocked spores.

The total mineral content of Ca^{2+}, Mn^{2+}, and Mg^{2+} is shown in Fig. 2, along with the d_{10} values for the various spore samples. For the spores heat shocked after the stationary phase, the total mineral content was a good indication for the heat resistance of the given spore samples. However, for the spores heat shocked during the exponential phase, the mineral content did not reflect the heat resistance of the spores (data not shown). As such, differences in heat resistance were indicative of the temporal nature of sporulation, in which different stages of the sporulation are morphologically and biochemically distinct [10, 20]. The values were averaged from duplicate experiments, and the standard deviation was within 15% for all values.

The increases in the heat resistance of spores heat shocked during sporulation correlated well with the results of other studies, where the induction of heat shock at 1 or 2 h after the start of the stationary phase in a sporulation medium (corresponding to t_7 or t_8 in this experiment) resulted in spores with the highest heat resistance [13, 19]. Overall, the increased heat resistance of the heat-shocked spores indicated that applying heat shock during sporulation is a simple and effective method of increasing the heat resistance in biological indicator organisms.

Other stresses, such as acid shock [11], cold stress, ethanol, and puromycin treatments [14] during sporulation, have also been shown to increase the heat resistance of the spores. Yet, whether the increased heat resistance is due to the stress induced during sporulation or specific changes brought from the stress response itself remains to be elucidated.

In conclusion, this study applied heat shock to sporulating cells as a simple method of producing biological indicator organisms with a stronger heat resistance. In addition, their increased mineralization was identified as the likely cause of the elevated heat resistance of the spores heat shocked at the post-stationary phase.

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