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Occurrence of a Highly Heat-Sensitive Spore Subpopulation of *Bacillus coagulans* STCC 4522 and Its Conversion to a More Heat-Stable Form

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The profile of the survival curves, at different heating temperatures, of *B. coagulans* STCC 4522 sporulated at 52°C has been studied, focusing on the early moments of treatment. A highly heat-sensitive spore subpopulation that includes more than 90% of the total spore population has been found. This heat-sensitive spore fraction was inactivated after 2 s of treatment at 111°C. Its heat resistance was as much as 200-fold lower than that of the heat-resistant spore fraction ($D_{111°C}$ of 0.01 min for the heat-sensitive spore fraction compared with $D_{111°C}$ of 2 min for the heat-resistant fraction). The shape of the survival curve at 108.5°C was modified after a sublethal heat shock at 80°C for 3.5 h, resulting in a straight-line survival curve. The temperature of treatment also influenced the shape of the survival curves. The conversion of the highly heat-sensitive spore subpopulation to a more heat-stable form is discussed.

Heat destruction of bacterial spores is generally considered a single reaction ruled by first-order kinetics. The reaction is expressed by an equation of the type:

$$N_t = N_0 e^{-kt} \quad (1)$$

where N_t is the number of surviving spores after a treatment of t minutes at a constant lethal temperature in a suspension that initially contained N_0 spores and k is the velocity constant of the reaction.

With this assumption, the plot of the logarithm of the number of surviving spores versus time is a straight line known as the survival curve. Heat resistance is estimated from the slope of the survival curve as the D_t value. The D_t value (time required to reduce the number of viable spores by 90% at a constant temperature t) is mathematically related to equation 1 as follows:

$$D_t = 2.303/k \quad (2)$$

The development of methodologies designed to estimate microbial heat resistance from the slope of the straight line of the survival curve by the multipoint method has permitted the observation that survival curves, obtained from laboratory data, frequently deviate from linearity (5, 9, 19). Some investigators (1, 24, 26, 27) have concluded that thermal inactivation does not strictly obey first-order kinetics and have proposed alternative mathematical models.

Deviations are particularly frequent during early periods of exposure. These deviations in the first steps of the survival curve are known as shoulders and have been related to the heat activation of dormant spores (1, 27) and to heat damage repair mechanisms (9, 14).

Bacterial spores are in a dormant state, and a stimulus such as heat, high or low pH, or numerous combinations of chemical and physical treatments is often required to increase the rate and extent of germination (4). Heat activation of dormant spores is considered by most authors (1, 22, 25) to be a single reaction that is ruled, like heat destruction, by first-order kinetics. However, the relationship between both types of constant-rate kinetics, if any, is unknown. Shull et al. (27) postulated that heat activation had to precede thermal death of dormant spores. Abraham et al. (1) confirmed this hypothesis and concluded that the activation reaction was the limiting factor for thermal death. Rodriguez et al. (24) proposed a new model that assumed that heat activation and thermal death of dormant spores were two simultaneous and independent first-order reactions. These reactions would take place at the same time as heat inactivation of activated spores. In their model, these authors assumed the same heat resistances for activated and nonactivated spores. Sapru et al. (25) modified this model by assuming different heat resistances for the two types of spores.

Microscopic counts of spore suspensions are in many cases higher than plate counts at the first time of heating, being more than 10-fold greater on some occasions (10). This has led some authors (1, 3) to the conclusion that dormant spores can represent as much as 90% of the total spore population, so that differences between microscopic spore counts and plate counts could account for the dormant spores. This common observation led Pflug and Bearman (21) to propose the intercept ratio (IR) concept (IR is the ratio between the zero time intercept of the regression line and the log of the initial number of microorganisms in the survival curve). The IR describes the proportion of dormant spores in a spore suspension from data of the survival curves.

However, we have found that plate counts of a *Bacillus coagulans* STCC (Spanish Type Culture Collection) 4522 spore suspension without previous heating (N_0) were closer to the microscopic counts (Y_0) than to the plate counts obtained after heating the suspension for 2 s at 111°C (N_A). The difference in plate counts between N_A and N_0 could not be related to thermal death since *B. coagulans* STCC 4522 showed a $D_{111°C}$ of 2 min. The difference between Y_0 and N_A could not be related to a high proportion of dormant spores in this suspension since the count after activation by heat (2 s at 111°C) was lower than

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that without heating. It seemed likely that a very heat-sensitive spore subpopulation could account for the differences between N_0 and N_A. If this were the case, these spores would die in the early moments of the heat treatment, before the first samples of the survival curve were collected. Rodriguez et al. (24) postulated the possibility of the occurrence of a less heat-resistant (i.e., heat-sensitive) fraction of spores present in spore suspensions, but they did not find this in their *Bacillus subtilis* suspension. There are no available data in the literature on the kinetics of destruction of this hypothetical heat-sensitive spore subpopulation or on its influence on the profile of the survival curve.

The thermoresistometer TR-SC (7) provides a homogeneous distribution of the bacterial suspension in the menstruum of treatment in 0.2 s, and up to 32 samples per s can be collected. These characteristics make the thermoresistometer a useful instrument for investigating the shape of the survival curves at very high heat destruction rates.

The objective of this work was to investigate the possible presence of a heat-sensitive spore subpopulation in this *B. coagulans* spore suspension and, in this case, to determine its heat inactivation kinetics.

MATERIALS AND METHODS

Spore suspension. The strain of *B. coagulans* used in this investigation (STCC 4522) was isolated in the laboratories of AICV (San Adrián, Navarre, Spain) during a routine check of the sterility of canned asparagus.

Sporulation was carried out in Roux bottles of nutrient agar (Biolife, Milan, Italy) containing 500 mg of Bacto Dextrose (Difco, Detroit, Mich.) liter^{-1} and 3 mg of manganese sulfate (Probus, Barcelona, Spain) liter^{-1}. Roux bottles were inoculated with a young culture (24 h at 35°C) in nutrient broth (Biolife) and incubated for 5 days at 52°C.

After 80 to 90% of sporulation was obtained, spores were collected by flooding the surface of the culture with sterile citrate-phosphate McIlvaine buffer (pH 7) (11). After harvesting, spores were washed five times by centrifugation and resuspension in the same buffer to eliminate vegetative cells and debris. The spore suspension (ca. 2×10^8 spores ml^{-1} assessed by microscopic count) was always stored at 0 to 5°C in the same buffer until used. Heat resistance of the spore suspension did not change after storage under our conditions during the time the experiment was performed.

Spore counts. Microscopic spore counts were carried out by means of a Thoma chamber with a phase-contrast microscope (Nikon, Nippon Kogayu, Japan).

Plate counts were carried out, without previous heat activation and after appropriate dilutions of the sample in sterile McIlvaine buffer (pH 7), in nutrient agar (Biolife) with 500 mg of Bacto Dextrose (Difco) liter^{-1}. To detect the contribution of vegetative cells or germinated spores to the total counts of suspensions, plate counts were also performed after incubation of the spore suspension with 0.1 mg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) ml^{-1} for 2 h at 52°C.

Plates were incubated at 35°C for 24 h.

Heat treatments. Heat resistance determinations were carried out in a thermoresistometer (TR-SC) that was designed and built in our laboratory as described elsewhere (8). The instrument was improved for this investigation by fitting the rack of the revolving fraction collector of the TR-SC (7) with a photoelectric cell (OMRON Corp., model E32). This photoelectric cell allowed the control of the opening time of the sampling valve. With this improvement, up to five samples per s were taken with the fraction collector in the first seconds of the heat treatment. The experiment could be prolonged after automatically sampling by collecting samples by use of the manual mode. In this way, experiments including manual and automatical sampling modes can be performed to obtain the same volume of sample for each treatment time. The precision of automatic sampling was the same as that of the manual mode (coefficient of variation [CV], <1%).

In heat resistance determinations, the thermoresistometer TR-SC containing 350 ml of McIlvaine buffer (pH 7) was stabilized at the selected temperature. Once the preset temperature had attained stability (±0.05°C), the menstruum was inoculated with 0.2 ml of the spore suspension. After inoculation, one 0.1-ml sample for each treatment time was placed in a test tube containing melted sterile nutrient agar (Biolife) with 500 mg of Bacto Dextrose (Difco) liter^{-1} and plated immediately. Survival curves of figures correspond to one single experiment. However, their reliability was high because the levels of precision of sampling as well as counting methods were also high (CV, <1% and <5%, respectively). Nevertheless, the more significant experiments (see Fig. 1B and 4) were repeated three times. CVs of D_t values of heat-sensitive as well as heat-resistant fractions were lower than 10%.

Incubation and survivor counting. Incubation of plates for survivor counting was carried out at 35°C for 24 h to provide damage repair of heat-injured cells. Previous experiments showed that longer incubation times did not influence the survivor count. Survivor counts of plates with a high density of CFU were carried out with an improved Image Analyzer Automatic Counter (Protos Analytical Measuring Systems, Cambridge, England) as described by Condon et al. (9).

D_t and z values. Decimal reduction times (D_t values) were calculated from the slope of the regression line plotted with the values of the straight portion of the survival curve. The first straight portion was used for the heat-sensitive fraction, and the second straight portion was used for the heat-resistant fraction.

z values (degrees of increase in the temperature of treatment for the D_t value to drop 1 log cycle) were determined from the regression line obtained by plotting log D_t values versus their corresponding heating temperatures (decimal reduction time curves [DRTC]).

Comparison of slopes of survival curves and DRTC were carried out as described by Steel and Torrie (28). Correlation coefficients (r_0) and 95% confidence limits (CL) were calculated by use of the appropriate statistical package (StatView SE+Graphics; Abacus Concepts, Inc., Berkeley, Calif.)

RESULTS

Microscopic observations of clean suspensions diluted 1:10 showed a homogeneous population of mature phase-bright spores, released from sporangia. The percentage of vegetative cells or phase-dark spores in the suspension was negligible. Furthermore, incubation of the spore suspension with lysozyme before plating did not modify the spore counts.

Figure 1 shows survival curves at 111.0°C of our strain of *B. coagulans* in McIlvaine buffer (pH 7). The survival curve in Fig. 1A is the result of a typical experiment obtained with the manual sampling mode, in which the first sample was taken after 5 s of treatment. The curve showed an initial shoulder followed by the exponential death kinetics. The microscopic spore count (Y_0), the plate count without previous heating (N_0), and the plate count of the first point of the survival curve (N_A) are depicted in this figure to illustrate our findings.

Figure 1B shows the survival curve obtained under the same conditions but with the automatic sampling mode in the first 5 s of treatment at a speed of 5 samples per s. The death rate in the first few seconds of treatment is shown amplified in Fig. 1C. A quick inactivation of a heat-sensitive spore fraction which comprises more than 90% of the total population is evident within the first 2 s of treatment (Fig. 1B and C). The first plate count in Fig. 1B matched the microscopic (Y_0) and plate (N_0) counts depicted in Fig. 1A. The shoulder occurred after the first slope of the survival curve corresponding to the heat-sensitive fraction, and then the logarithmic death of the heat-resistant fraction took place.

The profiles of the survival curves changed with the heating temperature. Figure 2 shows survival curves obtained by coupling the manual and automatic sampling modes at 101.0, 103.3, 105.6, and 108.5°C in McIlvaine buffer (pH 7). The whole survival curves are shown in Fig. 2A, and the first portions, which were amplified, are shown in Fig. 2B. As demonstrated by these survival curves, the lower the temperature of treatment, the lower the extension of the first slope, which was negligible at 101.0°C. The first plate counts were, however, similar for all survival curves.

The thermodependence of D_t values of the heat-sensitive and heat-resistant populations was quite different, as shown in Fig. 3. The equation of the regression line of the DRTC corresponding to the heat-sensitive population was as follows: log $D_t = 21.192 - 0.208 \cdot T$ (°C) ($r_0 = 0.92$; 95% CL for the slope, 0.136 to 0.280). However, the equation of the regression line for the heat-resistant population was the following: log $D_t = 12.741 - 0.112 \cdot T$ (°C) ($r_0 = 0.99$; 95% CL for the slope, 0.106 to 0.119). The z value corresponding to the thermosensitive spores (5°C) was significantly lower ($P \leq 0.05$) than that of thermoresistant spores (9°C).

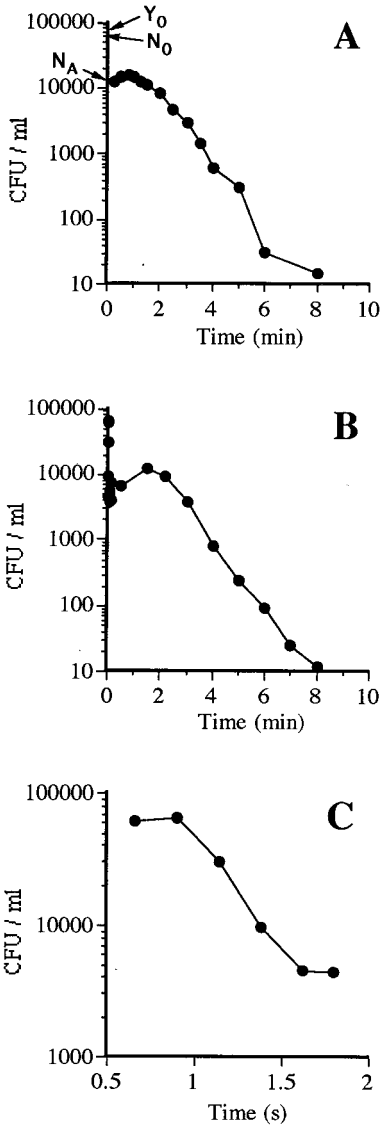

FIG. 1. Survival curve of *B. coagulans* at 111.0°C in McIlvaine buffer (pH 7). (A) Full survival curve resulting from the use of only the manual sampling mode; (B) full survival curve resulting from the use of both automatic and manual sampling modes; (C) first seconds of the survival curve resulting from the use of the automatic sampling mode.

The heat resistance of the heat-sensitive population increased after a sublethal heat shock, but that of the heat-resistant spores did not. Figure 4 shows the survival curves of *B. coagulans* at 108.5°C in McIlvaine buffer (pH 7) before and after a sublethal heat shock of 3.5 h at 80°C in distilled deionized water. This heat shock caused a modification of the profile of the survival curve in its first portion, resulting in a full straight-line survival curve.

DISCUSSION

The profile of the survival curve shown in Fig. 1A is common to most of the published survival curves corresponding to bacterial spore suspensions subjected to heat treatment. The initial shoulder is followed by a first-order inactivation kinetics pattern, and the microscopic count (Y_0) is approximately 10-fold higher than the first plate count in the survival curve (N_A). However, when studying in detail the first portion of the survival curve at this temperature (111.0°C), a quick inactivation of the majority of the bacterial spores present in this suspension (more than 90%) was shown to occur prior to the shoulder (Fig. 1B and C).

Survival curves with two slopes or a sigmoidal profile have occasionally been published (15–17, 20) and have usually been related to the coexistence of spores of different species in the same suspension (24, 29) or to the presence of vegetative cells and germinated spores (24). The phase-contrast microscope observation of our suspension showed a homogeneous population of phase-bright spores. No vegetative cells or phase-dark spores were observed. Lysozyme incubation did not modify the plate counts or the profile of the survival curves. Furthermore, when a new spore suspension was obtained from a single colony isolated from the former suspension, the same two subpopulations were observed. Therefore, our spore suspension was composed of two subpopulations of *B. coagulans* phase-bright spores with very different heat resistance values ($D_{111°C}$ of 0.01 min for the heat-sensitive fraction compared with a

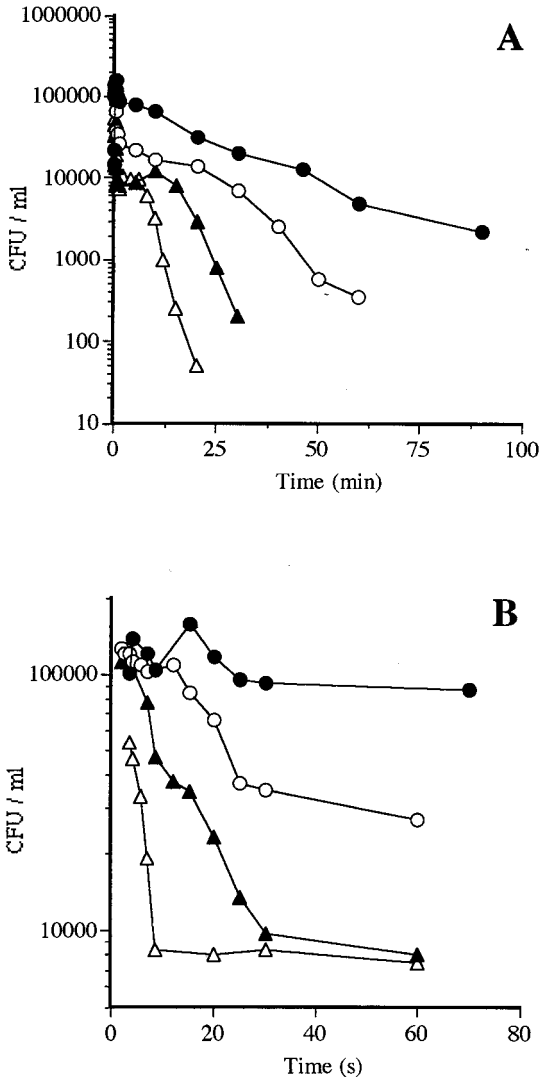

FIG. 2. Survival curves of *B. coagulans* at 101.0°C (●), 103.3°C (○), 105.6°C (▲), and 108.5°C (△) in McIlvaine buffer (pH 7). (A) Full survival curves; (B) first seconds of treatment.

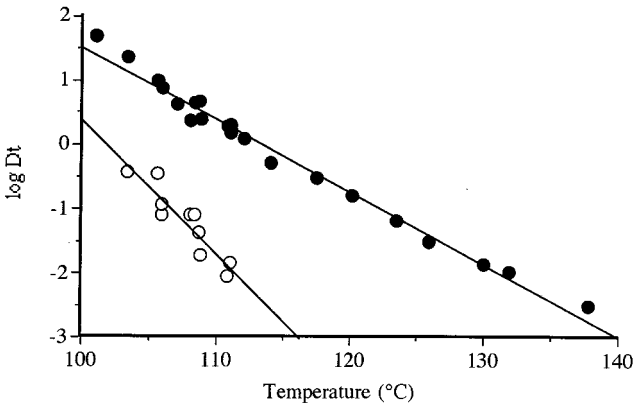

FIG. 3. Decimal reduction time curves of *B. coagulans* in McIlvaine buffer (pH 7). Symbols: ●, heat-resistant subpopulation; ○, heat-sensitive subpopulation.

$D_{111°C}$ of 2 min for the heat-resistant fraction). The heat-sensitive fraction of spores could be present in other suspensions of *B. coagulans* and of *B. subtilis*, *Bacillus licheniformis*, and *Bacillus stearothermophilus*. We have also observed in spore suspensions of these microorganisms that microscopic counts were much higher than the plate counts of the first sample of the survival curves (data not shown).

Rodriguez et al. (24) developed a mathematical model to explain the heat inactivation of bacterial spores which included the existence of a fraction of spores with a very low heat resistance. However, they did not find this in their spore suspension, probably because of the low sampling speed of their methodology. These authors assumed first-order inactivation kinetics for this heat-sensitive subpopulation and no change in its heat resistance during the heat treatment. However, there are no available data in the literature about its heat resistance (D_t and z values) or about its inactivation kinetics that could allow such an assumption. The profile of the survival curve of our suspension of *B. coagulans* at 111.0°C (Fig. 1B) was similar to that hypothesized by Rodriguez et al. (24) in their model. This suspension should be composed of a subpopulation of dormant spores with high heat resistance ($D_{111°C}$ = 2 min) and a subpopulation of spores with reduced thermal resistance ($D_{111°C}$ = 0.01 min). The heat resistance of the thermosensitive subpopulation was still several orders of magnitude higher than that reported for vegetative cells of aerobic spore-forming bacteria (6).

As deduced from Fig. 1B and C, at 111.0°C, the heat-sensitive spore fraction comprised more than 90% of the total spore population. However, the proportion for both fractions seemed to change with treatment temperature, and at 101.0°C, the heat-sensitive fraction was nearly negligible (Fig. 2). The survival curve at this lowest temperature was almost a straight line from the beginning of the thermal treatment (Fig. 2A). As demonstrated by the first portions of the survival curves plotted in Fig. 2A and 1B, the first plate counts (N_A) were similar at all temperatures and matched the microscopic counts (Y_0) and the plate counts without previous heating (N_0).

This behavior could be explained if the z value of the heat-sensitive fraction were much smaller than that of the heat-resistant fraction. In this way, differences in heat resistance of both fractions would decrease when the temperature was reduced, and a temperature would be reached (which should be 101.0°C in this case) where the D_t values would be the same. At this temperature, the survival curve would be a single straight line. Figure 3 shows the DRTC of both spore fractions. The heat-sensitive fraction had a smaller z value (5°C) than that of the heat-resistant fraction (9°C). However, when the $D_{101°C}$ values for both fractions were calculated from their respective DRTC equations, the heat-sensitive fraction was still less than 10-fold more heat resistant than the heat-resistant fraction. The difference in z values between both subpopulations did not seem to be responsible for the straightness of the survival curve at 101.0°C. Furthermore, the percentage of the heat-sensitive subpopulation decreased as the heating temperature lowered. This decrease cannot be explained solely as a result of a change in the z value of both subpopulations.

The different shape of the survival curves at different temperatures (Fig. 2) could then be explained in terms of the model proposed by Rodriguez et al. (24). The heat-resistant fraction could be composed of dormant spores susceptible to heat activation. During heat treatment, heat activation and thermal death of these dormant spores could take place simultaneously. Also, the thermal death of the heat-sensitive spores could occur at the same time. The profile of survival curves would then comprise the activation of dormant spores and the inactivation of heat-resistant and heat-sensitive spores at each heating time. The relationship between the constant-rate kinetics of these processes is expected to be different at different temperatures (24) and will be the shape of the survival curves. In our survival curve at 108.5°C (Fig. 2A), most of the heat-sensitive spores (more than 90%) could have been killed before the dormant spores were activated. At 103.3°C (Fig. 2A), activation of dormant spores could be a relatively faster process than their thermal destruction. Consequently, the level of CFU per plate at which the shoulder occurs increases, shortening the line corresponding to the first slope of the survival curve. The rate of heat activation/heat inactivation could be higher the lower the temperature, and a temperature could be reached (which should be 101°C in this case) at which inactivation of heat-sensitive spores would be completely hidden. The survival curve would be a straight line since all dormant spores would be activated at the beginning of the heat treatment.

If this were the case, plate counts after a sublethal heat activation treatment (80°C, 3.5 h) should be approximately twice those obtained before activation (N_0). They should account for the heat-sensitive subpopulation plus the dormant spores fully activated at the moment. However, plate counts

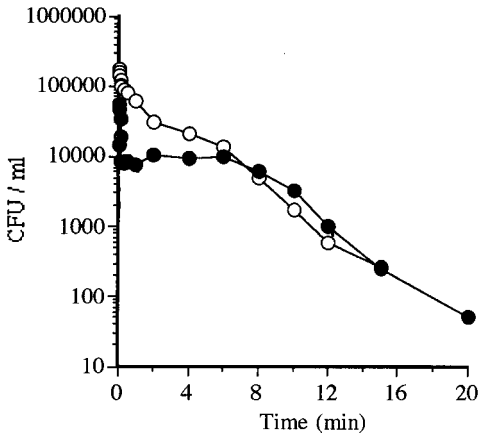

FIG. 4. Survival curves of *B. coagulans* at 108.5°C in McIlvaine buffer (pH 7) before (●) and after (○) a sublethal heat shock for 3.5 h at 80°C in distilled deionized water.

remained the same before and after the sublethal heat treatment (data not shown). Moreover, Fig. 2B demonstrates that if any activation of dormant spores takes place, almost all the population would be activated in 10 s at 101.0°C but only approximately 10% would be activated at 108.5°C in the same period of time. This observation contradicts all published data (1, 24, 27, 30) that suggest first-order kinetics for heat activation, i.e., the proportion of heat-activated spores should be related to the amount of heat provided to the spore suspension and, for the same period of time (10 s), this amount of heat should be greater at higher temperatures.

There is still another possible explanation for this behavior. The heat-sensitive spores could transform into heat-resistant spores by thermal adaptation during heating. This hypothesis is in agreement with the observations of different authors who described an enhancement of spore heat resistance during the early moments of treatment (2, 3, 13, 17). But, the mechanisms that induce this increase in heat resistance are still unknown.

A more detailed observation of the first portion of survival curves in Fig. 2B shows the changes in the death rate of the heat-sensitive fraction during heating. The change in the slope occurred at higher plate count levels when the heat temperature was lowered. This level was approximately 10,000 CFU/ml at 108.5 and 105.6°C and approximately 40,000 CFU/ml at 103.3°C. At 101.0°C, if any change occurred, it appeared at an approximately 100,000-CFU/ml level.

A very similar change in the profile of inactivation curves with heating temperatures has been observed in several enzymes (12, 31, 32). This behavior has been related to a two-step irreversible denaturation process through an intermediate form that is less sensitive to temperature (18, 23). Differences in heat resistance between heat-resistant and heat-sensitive subpopulations in our bacterial suspension could be due to biphasic inactivation of some enzymes within the spores. The heat-sensitive spores could have these enzymes in the less heat-resistant form, changing to the more heat-resistant form upon heating.

However, this increase in heat resistance might also be a physical occurrence, similar to breaking dormancy by heat shock itself and not involving enzymes. Heat-sensitive spores could be immature spores with a high degree of hydration of their protoplast. The sublethal heat treatment would induce an expulsion of water from the protoplast, as a result of either a coat contraction, as has been previously observed with an electron microscope after a heat shock (22), or an expansion of the cortical peptidoglycan (3).

Whatever the mechanism is, the proportion of heat-sensitive spores which would experience this heat adaptation would be higher at lower temperatures, since this would take place before these heat-sensitive spores were destroyed by heat. For our suspension, temperatures over 105°C would not allow for any heat adaptation of heat-sensitive spores. As a result, they would be killed very quickly, following first-order kinetics (Fig. 2B). The shoulder at these temperatures would then account only for the activation of dormant spores. Below 105°C, heat-sensitive spores would be able to adapt to heat. This would be shown by a first slope in the survival curve corresponding to the heat inactivation of heat-sensitive spores (not yet thermally adapted), followed by a second portion with a less-pronounced slope, corresponding to the thermally adapted cells. The shoulder would represent the activation of dormant spores, followed then by the first-order inactivation kinetics of the entire heat-resistant subpopulation. This profile can be observed in the survival curve corresponding to 103.3°C (Fig. 2A). The lower the temperature, the higher the number of heat-sensitive spores that would be able to adapt to heat before being killed. In this way, at low temperatures the activation of dormant spores would be hidden by the higher number of heat-sensitive spores being adapted to heat. At 101°C, most heat-sensitive spores could attain the heat-resistant state, and therefore, the full survival curve follows an exponential rate (Fig. 2A).

If the adaptation also occurs after exposure to a sublethal heat shock, all of the heat-sensitive spores could have accomplished this enhancement of heat resistance. In this case, the heat resistance of all the spores present in the suspension would be the same at any temperature. This would lead to a straight-line survival curve even at high temperatures of treatment. Our results (Fig. 4) agree with this explanation.

Overall, our results demonstrate that a suspension of phase-bright spores may contain subpopulations of varying heat resistance. The heat-sensitive spores may adapt to heat and become as heat resistant as the heat-resistant spore fraction present in the suspension. The proportion of heat-sensitive spores able to adapt to heat will depend on the temperature of treatment. Then the survival curve will comprise the inactivation kinetics of the spores with different levels of heat resistance, the kinetics of thermal adaptation of the heat-sensitive spores, and the kinetics of activation of dormant spores. All of these processes should be taken into account when developing mathematical models which try to explain the inactivation kinetics of bacterial spores and the shape of survival curves.

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