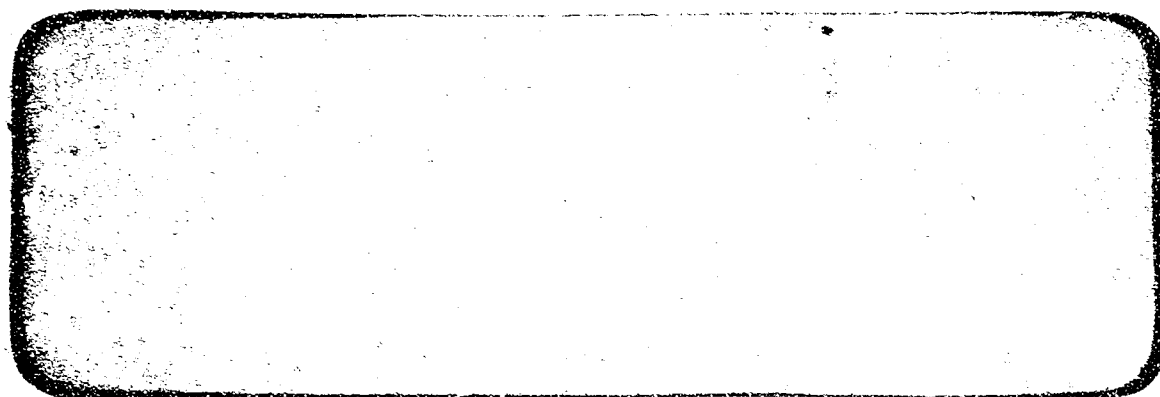


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## Effect of Cell Moisture on the Thermal Inactivation Rate of Bacterial Spores

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Thermal inactivation rates were determined for two strains of *Bacillus subtilis* var. *niger* spores after equilibration to various relative humidity (RH) levels. In these tests, small thin stainless-steel squares were each inoculated with a drop of spore suspension and equilibrated to 11, 33, or 85% RH. Following equilibration, the squares were placed on a hot plate preheated to 108, 125, 136, 164, or 192 C for various exposure times and then assayed for surviving organisms. The results revealed that spores of the A strain of *B. subtilis* were least resistant if preequilibrated to 11% RH and most resistant if preequilibrated to 85% RH. The same trend was obtained at all temperatures except 192 C, at which, no difference was noted, probably because the rapid kill time approaches the heat-up time of the stainless-steel square. The B strain of *B. subtilis* spores showed an opposite RH effect; that is, the cells preequilibrated to 11% RH were the most resistant. Because the two strains of spores were grown on different media, further studies were conducted at 136 C after subculturing the cells on different media. When the B strain was subcultured on the A strain medium, the pattern was reversed; the cells preequilibrated to low RH were then least resistant. Although it was not possible to reverse these cells to the original pattern by subculturing on the original B strain medium again, the pattern was altered to the point that there was no significant difference in heat resistance of these cells regardless of the preequilibration RH. The same result was obtained when the A strain was grown on the B strain medium; that is, the thermal resistance could not be reversed, but it was altered from the point where the low RH equilibrated cells were least resistant initially to the point where there was no significant difference in any of the cells regardless of what RH was used for preequilibration. The thermal resistance of spores seemed to be dependent on (i) the medium on which the spores are grown, (ii) the RH on which they are exposed before heating, and (iii) some genetic characteristic of the cell.

Interest in sterilization by low-temperature dry heat has increased within the past 7 or 8 years because of its consideration for sterilizing interplanetary spacecraft. Until recently, the effect that cell moisture plays on the thermal inactivation rate has been given little attention. The most notable studies were conducted by Murrell and Scott (2, 3), who investigated the effect of cell moisture content on the rate of microbial death at dry-heat temperatures from 70 to 120 C. In the first of these studies, the microorganisms were dried at various relative humidity levels (RH), then heat-treated in closed vials at ambient, but obviously low, RH. All the bacterial spore species tested showed that preequilibration to an RH of 80 to 90% produced spores most resistant to dry heat, but spores preequilibrated to greater or lesser RH values were less resistant. In the second study, the spores were preequilibrated and heated

at the same RH in closed vials. These results showed that spores equilibrated and tested at 20 to 40% RH were the most resistant to heat inactivation, but spores at RH levels above or below this amount had lower heat resistance.

The present investigation was undertaken to determine the effect of cell moisture content on the thermal inactivation of spores exposed to temperatures generally higher than those used by Murrell and Scott, i.e., 108 to 192 C.

### MATERIALS AND METHODS

*Bacillus subtilis* var. *niger* has been used for over 20 years as a test organism at Fort Detrick. During this time, cultures of the organism were given to many other laboratories for test purposes. It has been an especially valuable agent in disinfection and sterilization studies, and many publications based on its use as a test organism have appeared. It has been im-

plied that the numerous cultures are essentially the same; however, no comparative tests have been made to determine whether there are now differences in heat resistance or other properties in strains long separated.

Thus, two strains of *B. subtilis* var. *niger* spores were used in this study. Both originated from single colony isolates from Fort Detrick strains. However, the one used in this laboratory (strain A) was grown on Trypticase Soy Agar (TSA, BBL) for 7 days, harvested, washed, and suspended in sterile distilled water; the other (strain B) was grown in a laboratory at the National Center for Urban and Industrial Health, Cincinnati, Ohio on spore medium containing glucose, 0.25%; Casamino Acids, 0.25%; yeast extract, 0.25%;  $MnSO_4 \cdot H_2O$ , 0.001%;  $FeSO_4 \cdot 7H_2O$ , 0.0014%; and agar, 1.5%. These cells were grown, harvested, and washed in a manner similar to that used for strain A. All spore suspensions were heat-shocked at 60°C for 30 min before use in order to kill the less resistant vegetative cells that might be present.

Thermal exposure was conducted on a fabricated hot plate (Fig. 1) made of a 4 by 5 by  $\frac{1}{4}$  inch (10.3 by 12.7 by 0.62 cm) aluminum plate set on well-distributed heating elements and fixed in an asbestos base. The hot plate was suspended by nonheat-conductive material in a Transite box. A Transite hood was placed on top of the hot plate to assure more even heat distribution and to reduce heat radia-

tion. Stainless-steel squares, 0.5 × 0.5 × 0.01 inch (1.3 by 1.3 by .03 cm), were used as the test surface. The technique of placing the contaminated steel squares on the heated aluminum plate was chosen because it provided a means of bringing the organisms up to the test temperature in minimal time.

Each stainless-steel square was contaminated with a single equal drop of aqueous suspension containing approximately  $10^8$  *B. subtilis* spores per ml. The inoculated squares were placed in desiccator jars containing saturated solutions of lithium chloride, magnesium chloride, or potassium chloride above which 11, 33, and 85% RH, respectively, is maintained at 25°C. The organisms were equilibrated for 48 to 72 hr.

After equilibration, 42 of the contaminated stainless steel squares were placed on the preheated hot plate; of these, 14 each were equilibrated to 11, 33, and 85% RH. No attempt was made to control the humidity surrounding the hot plate, but it was obviously low; e.g., even 100% RH air at 25°C will be reduced to 3% RH when heated to 108°C. After various exposure times, test squares were removed and immediately placed in sterile water blanks containing 0.01% nonionic detergent and 0.3 to 0.4 g of coarse white sand. The detergent and sand facilitated removal of spores from the stainless steel. To enumerate the surviving spores, pour plates were prepared with tryptose agar, and the colonies were counted after incubation at 37°C for 48 hr.

Temperatures used in this study were 108, 125, 136, 164, and 192°C. They were determined by the melting points of Tempilstik (Tempil Corp., New York, N.Y.) markings on stainless-steel shims. A number of shims were marked with two Tempilstiks, one that melts at 1 degree below the desired temperature and one that melts 1 to 2 degrees above that temperature, so that one Tempilstik sample was melted and the other was not in all tests. To ensure that the temperature did not rise above that desired, it was also monitored by thermocouples. For that reason, the temperatures noted are plus or minus about 1°C. The Tempilstik-marked shims were placed at the corner and central locations on the hot plate to assure that an even heat distribution was obtained. The air temperature a few millimeters above the hot plate was only several degrees below the hot plate temperature; however, the temperature fell rapidly above this height.

After the initial tests at all five temperatures, further tests were conducted at 136°C to see whether the cause of the marked difference in results with the two strains could be determined. First, both strains of *B. subtilis* var. *niger* spores were subcultured on TSA and then harvested and cleaned as before; both strains were equilibrated to the same three RH levels used above, and the death rates were measured at 136°C. Second, both strains were grown on spore media and tested as before. Finally, a third test was run on both strains of spores that had been grown on the spore media in the second test and then grown on TSA; these cells were harvested and tested as before.

The data for all tests represent the average of four samples obtained from two samples in each of two tests.



FIG. 1. Hot plate used for thermal exposure.

## RESULTS AND DISCUSSION

The death rates plotted on a semilogarithmic scale all showed straight-line functions from which  $D$  values or decimal reduction times could be readily calculated. Bar graphs showing the  $D$  values for each strain of organism tested, at the three RH levels and five temperatures, are presented in Fig. 2 and 3. The  $D$  values are expressed in seconds, but a different time scale was necessary for each temperature because of the great variation from one temperature to the next. Figure 2 shows that strain A spores equilibrated to 11 and 85% RH were, respectively, the least and the most resistant to thermal inactivation. This was true for all temperatures except 192°C, where no difference was noted, probably because the rapid death time at this temperature approaches the time required to heat up the stainless-steel square surfaces; therefore, the usefulness of the test procedure is limited to temperature somewhat below 192°C. Figure 3 shows the  $D$  values for strain B of *B. subtilis* var. *niger* spores equilibrated to the same three RH levels and five temperatures. In this case, the effect of RH on the organism was the reverse of the observed with strain A. The cells equilibrated to the low RH were more resistant than those equilibrated to the high RH at

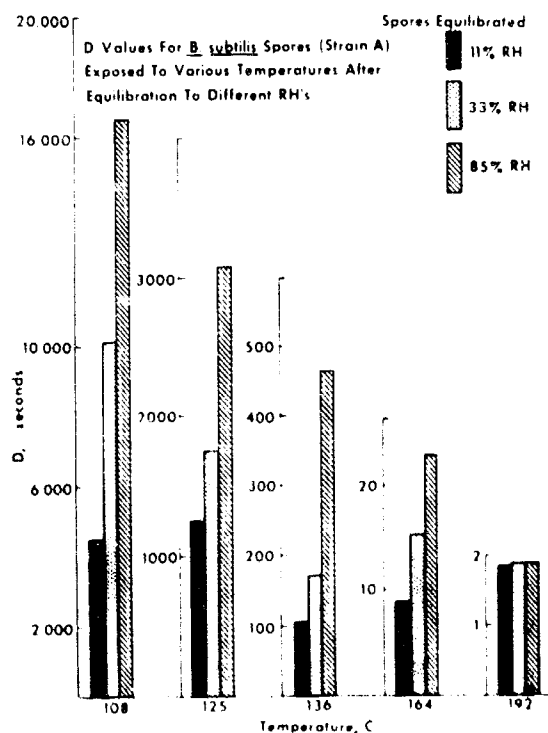


FIG. 2.  $D$  values for *B. subtilis* spores (strain A) exposed to various temperatures after equilibration to different RH levels.

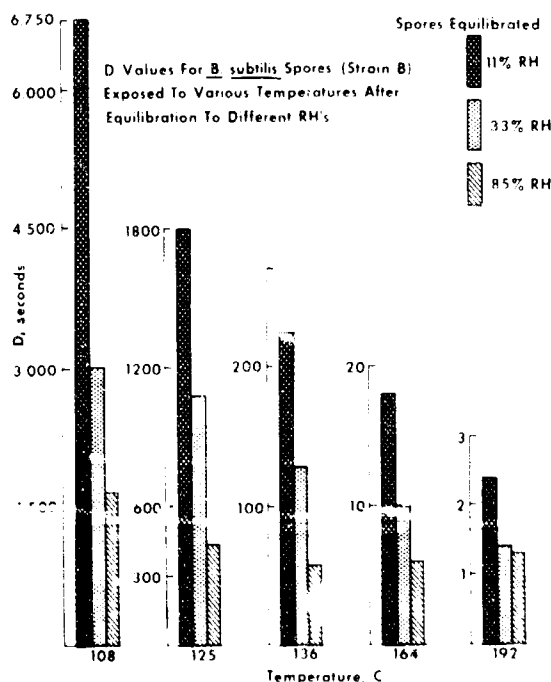


FIG. 3.  $D$  values for *B. subtilis* spores (strain B) exposed to various temperatures after equilibration to different RH levels.

each temperature. Again, at 192°C, the death time was very short, and the difference in resistance among the three RH levels is not significant.

Because the variation in results with the two strains may have resulted from the fact that they were grown on different media, the two strains were subcultured, first on TSA, then on the strain B spore media, and then on TSA again; they were tested after growth on each medium. Figure 4 shows the  $D$  values for strain A grown on the different media and tested only at 136°C. Regardless of which medium the cells were subcultured on, those equilibrated at the low RH were always more susceptible to heat sterilization than were those equilibrated to the high RH. Statistical analysis showed that the  $D$  values at 11 and 85% RH were not significantly different for strain A spores when grown on spore medium; however, the  $D$  values for the 11 and 85% RH were significantly different whenever the cells were grown on TSA medium.

Figure 5 shows the  $D$  values for the B strain spores grown on the different media. Of great interest here is the reversal of the resistance at the various RH levels. That is, B strain spores originally grown on spore media were more resistant when equilibrated to the low RH than when equilibrated at high RH, but the reverse was true when this strain was grown on TSA. Analysis showed that the  $D$  values for the low and high RH tests

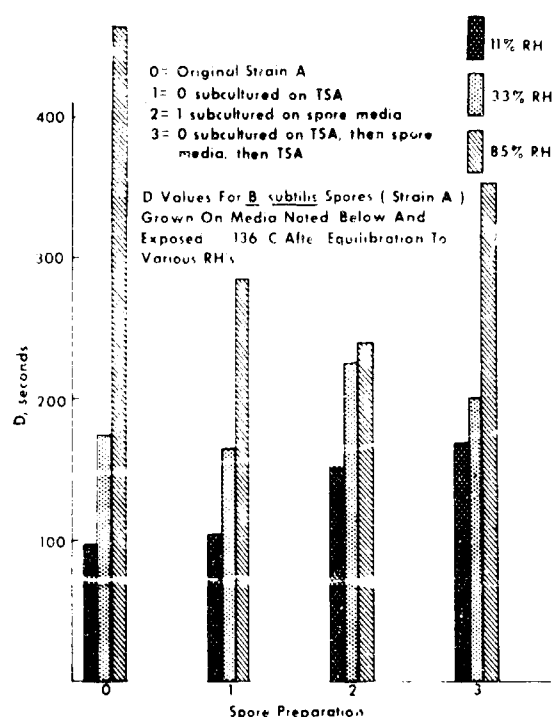


FIG. 4. *D* values for *B. subtilis* spores (strain A) grown on media noted and exposed to 136° C after equilibration to various RH levels.

were significantly different in both cases. Growing the cells on spore media after growth on TSA did not give a pattern similar to that of the original B strain, however, low and high RH *D* values were not significantly different. Growth on TSA did produce spores that had significantly different *D* values for the low and high RH preequilibrations.

Both *B. subtilis* strains used in this study were initially isolated from single bacterial colonies. Why the *D* value pattern of the B strain was reversed when grown on TSA but failed to revert to the original pattern when grown on the spore agar is not clear. It appears that we are dealing with a genetic characteristic because the colonial color and formation did appear to change somewhat with successive transfers on the various media. This phenomenon occurred although the seed was obtained by removing a loop full of spores from 10 to 15 typical colonies on an agar surface each time the spores were grown. Close inspection of the data obtained with the A strain of *B. subtilis* spores and the data obtained by Murrell and Scott (2) in their studies with *Clostridium botulinum* spores shows a similar trend of increased thermal resistance after equilibration to higher RH values. Both of these studies were performed without controlling the RH during the heating process.

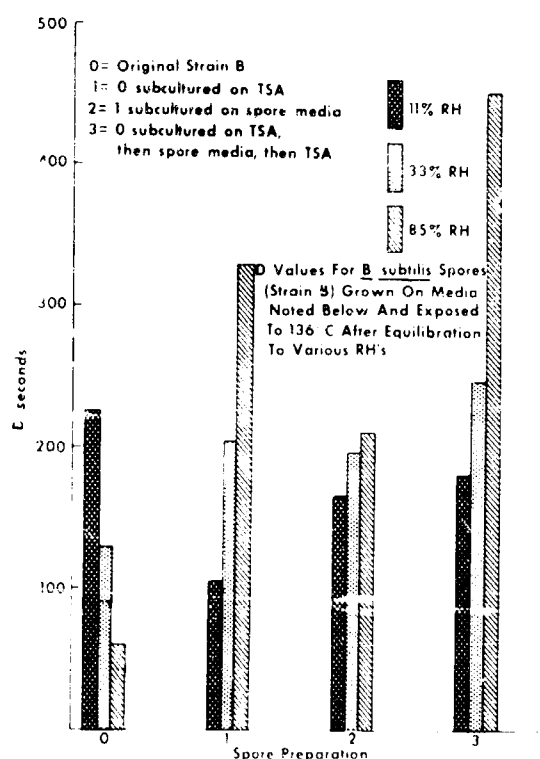


FIG. 5. Values for *B. subtilis* spores (strain B) grown on media noted and exposed to 136° C after equilibration to various RH levels.

Three important facts stand out from this study. One is that broad generalities should not be made about the heat resistance of spores as a result of their preliminary exposure to any specific RH. Second, the medium on which the organisms are grown appears to have some bearing on the subsequent dry heat resistance of the cells. Third, the RH to which the cells are exposed before heat sterilization plays an important role in their subsequent rate of kill.

The equilibration of the cell to various RH levels does more than just vary the moisture content of the cell; it causes a more "permanent" change in thermal resistance. Water freely moves in and out of the cell (1), yet the *D* value pattern at the various RH levels is the same for either strain of *B. subtilis* spores, whether tested at 108 or 164° C (Fig. 2 and 3).

If the amount of cell moisture is the primary factor in regulating the heat inactivation rate, one would expect to find no difference in *D* values for the cells equilibrated at 11, 33, and 85% RH at the lower temperature (e.g., 108° C). At this temperature, the *D* values are so high that most of the water molecules have ample time to evaporate from the cell certainly long before sterilization is accomplished. Yet no deviation from linearity was observed in the log per cent survival versus

time plots from beginning to end. This indicates that the important factor regulating the *D* values in the system used here is not the amount of moisture but where and how it is bound in the cell.

Spores heated in a dry atmosphere rapidly lose most of their moisture; therefore, the rate of their inactivation by heat appears to be controlled by a relatively few water molecules left in the cell. If one plots the *D* value as a function of the RH for the three humidities used, the curves in general are not linear. The curves of Murrell and Scott (2), likewise, are not linear when plotted as a straight function of *D* value versus RH. This phenomenon indicates that the rate of heat inactivation of the cell is not a simple function of the RH.

At this time, there is no clear explanation of the mechanism by which the cell is inactivated by dry heat. It is evident that heat inactivation is a complicated mechanism that hinges on the medium on which the cell was initially grown, the presence or

absence of water molecules in certain internal cell locations, and, probably, some genetic characteristic of the cell.

#### ACKNOWLEDGMENTS

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