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# Thermal Death of *Bacillus subtilis* var. *niger* Spores on Selected Lander Capsule Surfaces

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Dry-heat sterilization of planetary lander capsules requires a knowledge of the thermal resistivity of microorganisms in the environment to which they will be subjected during sterilization of the space hardware. The dry-heat resistance of *Bacillus subtilis* var. *niger* spores on various lander capsule materials was determined at 125 C. Eight surface materials were evaluated, including a reference material, stainless steel. Survivor curves were computed, and decimal reduction times (D values) were obtained by a linear regression analysis. In four tests on stainless steel, the average value of D at 125 C was 17.07 min. The D values for the other seven materials tested ranged from 18.64 min on magnesium surfaces to 20.83 min on conversion-coated magnesium. Of the materials evaluated, the results indicate that there is only a significant difference in the thermal resistance of *B. subtilis* var. *niger* spores on conversion-coated magnesium and conversion-coated aluminum from that on the reference material, stainless steel. The differences in D values for all the test surfaces may be the result of variations in test procedures rather than the effect of the surfaces on the thermal resistivity of the spores.

Dry-heat sterilization processes are the only processes being considered for the sterilization of planetary lander capsule subsystems (1, 3, 4). A dry-heat sterilization process for a lander capsule incorporates various considerations such as the thermal characteristics of the final assembly, the total microbial load and its distribution, and the thermal resistance of the microbial population.

The determination of the thermal resistance of microorganisms is a complex problem that must include consideration of the physiological condition of the cells, their distribution, the physical characteristics of the environment, the type of heat (dry or wet) to which the cells are exposed, and other experimental factors. For analytical purposes, the distribution of the total microbial burden of a lander capsule can be considered in three categories in which each time-temperature relationship necessary to inactivate microorganisms is different. The first category comprises those microorganisms contained within the interior of space hardware components; the second, the microbial load existent upon exposed surfaces; and the third, those microorganisms occluded between the mated surfaces of the lander capsule. To establish a dry-heat sterilization cycle, the time-temperature variables of each category must be known.

This paper presents the results of a study to determine the dry-heat resistance of bacteria spores upon lander capsule surface materials The investigation encompassed a spectrum of materials to provide definition of any variances in the thermal resistivity of spores that might be caused by different materials. A test method was developed to simulate the situation that may be encountered by the presence of microorganisms on exposed metallic surfaces during dry-heat sterilization of lander capsule hardware.

## MATERIALS AND METHODS

**Preparation of spore suspension.** Spores of *Bacillus subtilis* var. *niger* were grown on TAM (BBL) agar supplemented with $MgSO_4$ (20 $\mu$g/ml) and $CaCl_2$ (80 $\mu$g/ml). After 24 hr of incubation at 41 C, a loopful of inoculum was suspended in 20 ml of phosphate buffer (85.0 mg of $KH_2PO_4$/liter; *p*H 7.0) and heat-shocked in a water bath at 80 C for 15 min. A 1-ml amount of this fluid was spread over a TAM agar plate (150 by 25 mm) and incubated at 41 C for 24 hr. The procedure was repeated three times. A 1-ml amount of the final heat-shocked cell suspension was distributed over each of 30 TAM agar plates. These plates were incubated at 41 C for 24 hr, at which time microscopic examination revealed over 95% sporulation. The spores were harvested from the agar by agitating the surface growth with glass beads and sterile distilled water and by collecting the spores by centrifugation (5,000 rev/min for 15 min) at 4 C. The cell mass was washed five times in cold phosphate

buffer, suspended in 95% ethyl alcohol, and then stored under refrigeration.

**Preparation of test samples.** A list of the lander capsule surfaces used and several of their material characteristics are presented in Table 1. Test strips of each material were inoculated with a 100-$\mu$liter pipette containing a known quantity of *B. subtilis* var. *niger* spores from a vortex-mixed, ethyl alcohol suspension and were air-dried in a class 100 (2) laminar airflow clean bench. After drying, the test strips were placed in a vacuum desiccator containing active (heated at 175 C for 2 hr) silica gel. The desiccator was evacuated to a 24-inch negative pressure maintained for 16 hr.

**Method of thermal exposure.** The test strips were suspended from a support rod within a preheated oven (125 ± 0.5 C) The. temperature of the test strips was monitored by means of a thermocouple brazed to a control test strip, and a constant rate of mechanical air circulation (2 ft/sec) was maintained within the oven throughout the test area. A sterile stainless-steel strip was also suspended from the support rack as a sterility control. Six inoculated test strips and two control strips (biological and thermal) were exposed to each test-exposure period. The time necessary to heat strips of different test materials to 125 C was quantitatively measured (*see* Table 1). The initial assay was conducted after 13 min of heat exposure, thus ensuring that all test strips had reached 125 C. Subsequent assays were conducted after 23, 33, 43, 53, and 63 min of heating, which included the 13-min heat-up period.

TABLE 1. *Element composition and test-heating properties of inoculated-surface materials*

| Material | Composition | Time required to heat test strip from 21 to 125 ± 1 C (min)[a] |
|---|---|---|
| Stainless steel (Type 302) | 70.38% Fe, 18% Cr, 8% Ni, 2% Mn, 1% Si, 0.15% C, 0.45% P, 0.03% S | 8.5 |
| Aluminum alloy (6061-T6) | 97.7% Al, 1.2% Mg, 0.6% Si, 0.2% Cr, 0.3% Cu | 7.0 |
| Conversion-coated (Dow 7) magnesium | 95.8% Mg, 3% Al, 1% Zn, 0.2% Mn (% MgCrO unknown) | 7.5 |
| Magnesium alloy (AZ31B-H24) | 95.8% Mg, 3% Al, 1% Zn, 0.2% Mn | 7.5 |
| Titanium alloy (6A14V) | 90% Ti, 6% Al, 4% V | 9.0 |
| Cat-A-Lac black paint (436-1-B) over stainless steel (type 302) | Epoxy resin base, primary and secondary amines, inert pigments, talc, mica, carbon[b] | 10.5 |
| Conversion-coated (Iridite 14) aluminum alloy | Chromium hydroxide, chromium oxide, iron, aluminum hydroxide and silicone compounds[b] | 11.5 |
| 24-Karat gold-plated electroless nickel over aluminum (6061-T6) | 24-Karat gold (purity unknown) | 11.5 |

[a] Strips (1.0 by 3.0 × 0.020 inches) were suspended from a Teflon-coated, aluminum support rack.

[b] Percentage composition of these compounds is proprietary information.

**Determination of surviving spores.** After the desired time of exposure to dry heat, the test strips and control strips were removed from the oven and immediately placed in bottles containing 22 ml of sterile, prechilled (4 C) phosphate buffer. The bottles were placed in an ultrasonic bath (Branson ultrasonic bath: generator, A-300; tank, LT-88; power control, PC-30) and treated at 25 kc/sec for 12 min. An 11-ml sample of the phosphate buffer was aseptically transferred to a 99-ml phosphate buffer dilution blank, and serial dilutions were made. Viable spores were determined by the pour-plate method in triplicate, using Trypticase Soy Agar (TSA; BBL). The test strips were also plated directly on TSA by an agar overlay method. All plates were aerobically incubated at 32 C for 72 hr, at which time colony counts were made. The total count was calculated from the mean value of the various dilutions and from the number of viable spores remaining on the test strips.

**Statistical treatment of data.** To calculate the decimal reduction time (D value)—that time at a specific temperature which results in a 90% reduction in the number of viable cells within a population—the results from each type of surface were subjected to standard linear regression analysis. A computer code was used that converted the test data to a semi-logarithmic (X, Y) file and then computed and plotted: (i) the regression line ($Y = b_0 - b_1X$ where $1/b_1$ was the D value in minutes); (ii) the 95% confidence limits for this D value; and (iii) the $R^2$ term (the measure of the proportion of total variation about the mean $\bar{Y}$ explained by the regression).

## RESULTS

Three inoculated strips each, of two different materials, were exposed to the experimental conditions during each test sequence. Stainless steel was chosen as a standard reference material because of its previous use as a test material in dry-heat sterilization studies (5). Figure 1 presents the results from four tests in which stainless steel was used. The D value varied from 15.93 to 18.38 min for these four tests. Figure 2 presents the results ($D_{125\,C}$ = 19.96 min) on aluminum surfaces. Figure 3 presents a survivor curve with a $D_{125\,C}$ value of 20.83 min for the same spore suspension exposed on conversion-coated

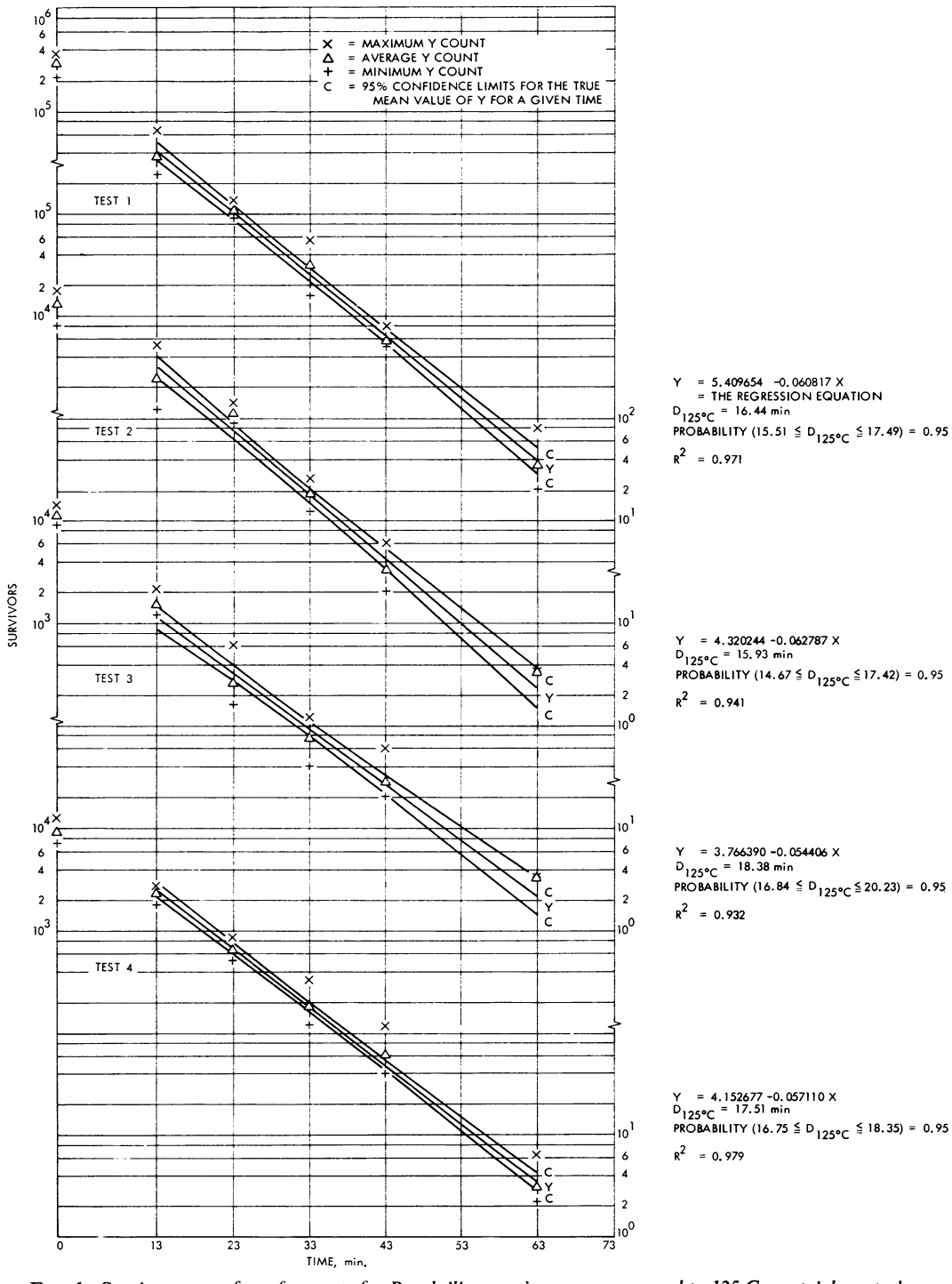


FIG. 1. *Survivor curves from four tests for B. subtilis var. niger spores exposed to 125 C on stainless steel.*

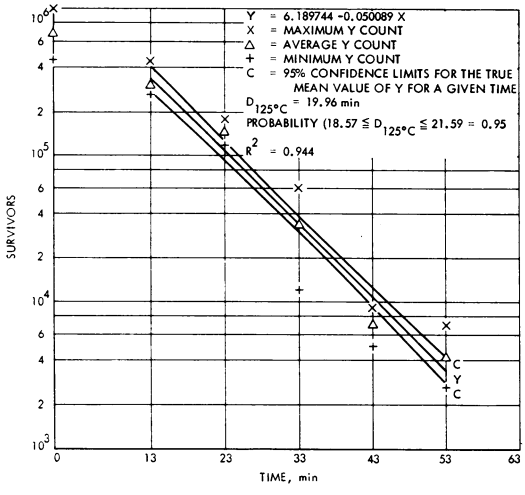


FIG. 2. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on aluminum alloy.*

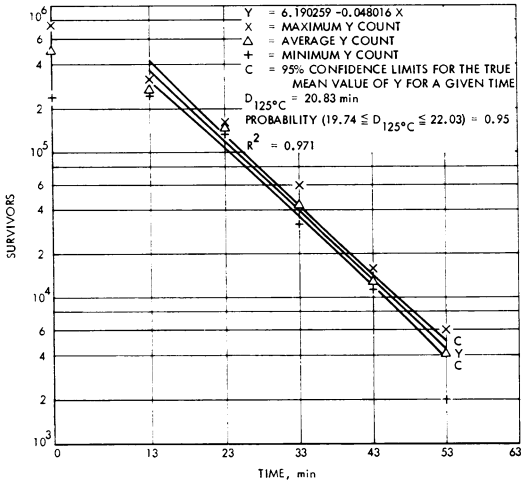


FIG. 3. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on conversion-coated (Dow 7) magnesium alloy.*

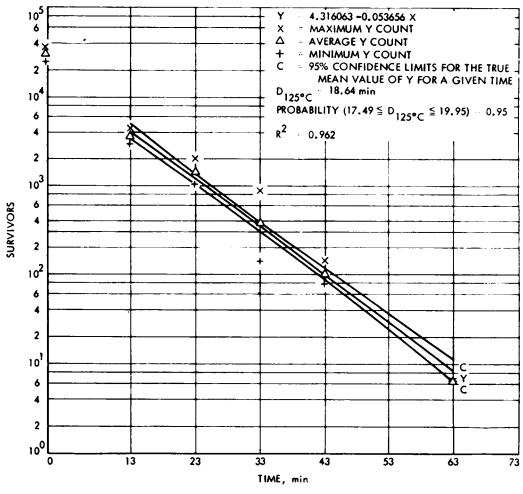


FIG. 4. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on magnesium alloy.*

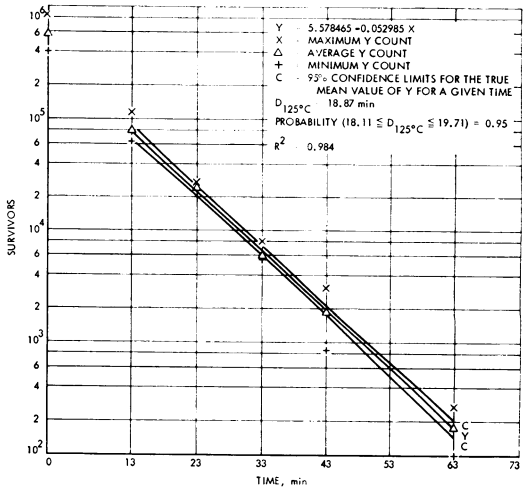


FIG. 5. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on titanium alloy.*

(Dow 7) magnesium. The data derived from similar tests on magnesium and titanium alloys are presented in Fig. 4 and 5 in which the decimal reduction times are 18.64 and 18.87 min, respectively. Figure 6 presents a survivor curve ($D_{125\,C}$ = 19.33 min) for spores exposed on Cat-A-Lac black-painted stainless steel. The results for conversion-coated (Iridite 14) aluminum and gold-plated aluminum are shown in Fig. 7 and 8. The D values were 20.68 min on Iridite 14 and 19.51 min on gold-plated aluminum.

## DISCUSSION

The difference in the D values obtained from the four tests on the reference material stainless steel and from the conversion-coated magnesium was statistically significant. The first test, in which the results of the regression analysis are used, shows that the D value for the conversion-coated (Dow 7) magnesium was outside the upper 95% confidence limits for the D values of all four reference tests. Further, the confidence limits for the D value on the conversion-coated magnesium surface do not even overlap the confidence limits for the D values from tests 1, 2, and 4. Finally,

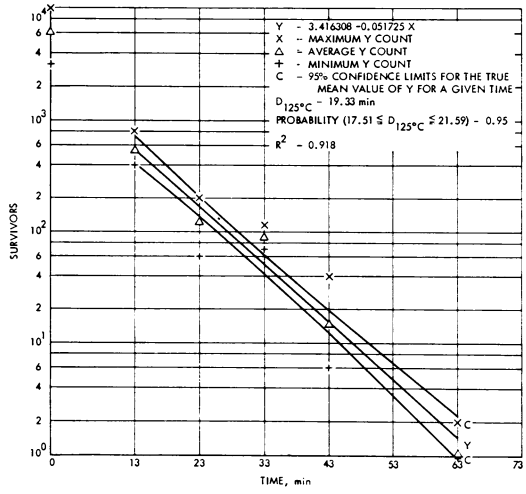


FIG. 6. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on Cat-A-Lac black-painted stainless steel.*

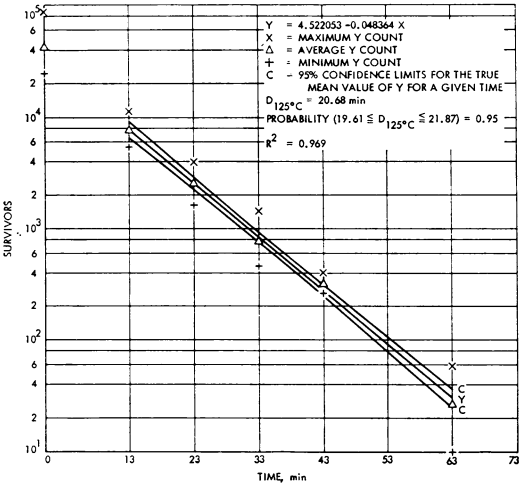


FIG. 7. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on conversion-coated (Iridite 14) aluminum alloy.*

the D value for the conversion-coated magnesium was also outside the upper 99% confidence limit established for the mean of the four reference tests (i.e., by student's t-test). In a similar manner, the D value for conversion-coated (Iridite 14) aluminum was greater than the upper 95% confidence limits for the four stainless-steel D values. As with the Dow 7-coated magnesium, the confidence limits for the D value from the test on conversion-coated aluminum do not overlap the confidence limits for the D values of tests 1, 2, and 4 for stainless steel.

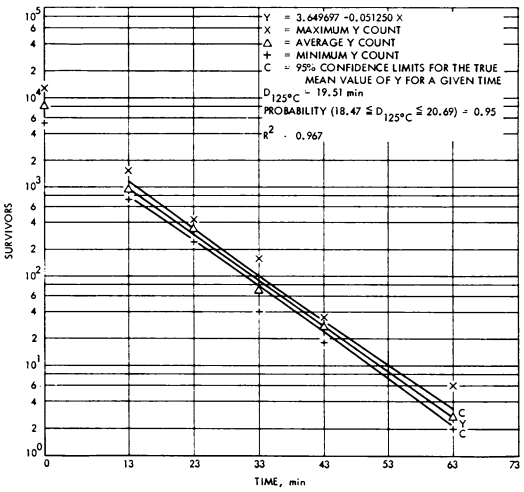


FIG. 8. *Survivor curve for B. subtilis var. niger spores exposed to 125 C on gold-plated aluminum alloy.*

The variations in D values between the various test surfaces may result from slight variations in test procedures rather than from differences in thermal resistivity of the spores on these surfaces. The variation in D values in the stainless-steel reference tests tends to substantiate this possibility.

It was concluded that, regardless of the varying D values, the survivor curves derived from all tests (Fig. 1–8) indicate that death of the spores closely follows a monomolecular reaction expressed by a logarithmic death rate. This conclusion is reflected in the $R^2$ term which was always greater than 0.91.

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