Relative Frequency Distribution of $D_{125 \, ^{\circ}C}$ Values for Spore Isolates from the Mariner-Mars 1969 Spacecraft

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Bacterial spore crops were prepared from 103 randomly selected aerobic mesophilic isolates collected during a spore assay of Mariner-Mars 1969 spacecraft conducted by the Jet Propulsion Laboratory. $D_{125 \, ^{\circ}C}$ values, which were determined by the fractional-replicate-unit-negative-most-probable number assay method using a forced air oven, ranged from less than 5 min to a maximum of 58 min. Subsequent identification of the 103 isolates indicated that there was no relationship between species and dry-heat resistance. A theoretical dry-heat survival curve of the "population" was nonlinear. The slope of this curve was determined almost exclusively by the more resistant organisms, although they represented only a small portion of the "population."

Spacecraft designed to land on or impact Mars and other planets of biological interest are required to be sterilized before launch to preserve possible ecological systems and prevent misinterpretation of exobiological tests. Dry heat is the chosen method of sterilization, and heating cycles considered in recent years are based on the thermal inactivation kinetics of *Bacillus subtilis* var. *niger* spores. The following assumptions have been made to support the use of the spores of this organism as an index of the heat resistance of spores found on spacecraft (3).

(i) The level of dry-heat resistance for *B. subtilis* var. *niger* spores represents the average or typical heat resistance of spores found on spacecraft.

(ii) The thermal inactivation kinetics of naturally occurring spore populations on spacecraft will be logarithmic, comparable to pure spore crops of *B. subtilis* var. *niger*.

These assumptions are tenuous at best. There is no evidence to suggest that *B. subtilis* var. *niger* spores are representative of dry-heat resistance levels exhibited by the types of spores actually found on spacecraft. In addition, *B. subtilis* var. *niger* has never been detected on spacecraft at Cape Kennedy (8–10; J. R. Puleo, personal communication). The rationale for using average or typical heat resistance of spores thought to occur on spacecraft as a criterion for establishing sterilization cycles is, in our opinion, neither logical nor valid. Rather it would seem that emphasis should be placed on the highest heat resistance values for spores detected on spacecraft located in their final assembly and testing environments.

It has been shown previously (2) that naturally occurring spore populations (i.e., spores obtained directly from soil without intermediate isolation and subculture on artificial culture media) which comprise the types of contamination anticipated on space hardware exhibit nonlogarithmic inactivation. These results would be expected, since naturally occurring spore populations are composed of organisms having a wide range of heat resistance.

The objective of this study was to obtain a relative frequency distribution of heat resistance of spores associated with spacecraft at Cape Kennedy and also to determine if there is a relationship between species and degrees of resistance.

The Mariner-Mars 1969 spacecraft, the source of isolates used in this study, did not require sterilization. However, location and manufacturing and assembly and testing procedures were similar to those scheduled for spacecraft requiring sterilization in future Mars landing missions.

**MATERIALS AND METHODS**

Spore isolates and preparation of spore crops. Mesophilic aerobic isolates picked randomly from...
spore assays conducted by the Jet Propulsion Laboratory, Pasadena, Calif., on the Mariner-Mars 1969 spacecraft were received from Cape Kennedy and streaked for isolation on Trypticase Soy Agar (TSA; BBL). The isolates were grown on TAM Sporulation Agar (Difco) supplemented with 20 μg of magnesium sulfate per ml and 80 μg of calcium chloride per ml at 32 C by using a method described previously (2). A total of 103 spore crops were prepared, titered, and used in this study.

**Assay system.** A multiple replicate-unit testing system was used in the following manner to determine heat resistance. For each spore crop, each of 33 sterile stainless-steel strips (0.5 by 0.5 inch (1.27 by 1.27 cm) coldroll, type 302, no. 4 finish, 22 gauge) was inoculated with approximately 10⁶ spores suspended in 95% ethyl alcohol by using a dropper pipette (Scientific Products, no. B1186-1, 0.025 ml).

The coefficient of variation in spore deposition was 9.5% as determined by plate count. The strips were then dried under vacuum for 16 hr over silica gel. Three unheated strips from each set were processed in a manner previously described (2) and plated in triplicate to obtain N₀ (control) values. The remaining 30 strips from each set were suspended in six quintuplicate sets in a forced air oven (model no. 625, Precision Scientific Co., Chicago, Ill.) at 125 ± 0.5 C. The oven was modified by the addition of six ports (1 by 5 inches (2.54 by 12.7 cm)) on the top. Hangers for inserting samples were constructed of bronze rod, fiber glass sheeting, and no. 30 aluminum alligator clips, each capable of holding a maximum of six quintuplicate units of strips. The time required for a stainless-steel strip to reach 125 C in this system was 1.5 min as determined by copper constantan thermocouples attached to control strips. Insertion of the six hangers into the oven was staggered at 5-min intervals to minimize the temperature lag. Hangers were removed at 40-min intervals (plus 1.5 min come-up time) and were allowed to cool for 2 min in a horizontal laminar flow cabinet (4) before processing. Heating of strips and all other operations in this study, with the exceptions of incubation and plate counting, were performed in a vertical laminar flow clean room (72 ± 2 F; 42 ± 2% relative humidity).

**Recovery of spores.** N₀ (unheated control) values were determined by plating with TSA supplemented with 0.1% (w/v) soluble starch (BBL) and 0.2% (w/v) yeast extract (BBL). After solidifying, the plates were overlaid with 10 to 15 ml of molten medium to lessen spreading growth. After cooling, the heated strips for the most probable number (MPN) assay were placed in individual tubes containing 9 ml of broth made according to the formula of TSA minus agar [TSA-B: Trypticase (BBL), 15 g/liter; Phytone (BBL), 5 g/liter; NaCl, 5 g/liter] and supplemented with the same concentrations of soluble starch and yeast extract as the solid medium. Plates were incubated for 48 hr before counting, and visible growth in the tubes was recorded after 14 days at 32 C. Uninoculated strips were assayed as sterility controls.

Preliminary tests were conducted to compare N₀ values based on colony counts from pour plates (supplemented TSA) and those based on an MPN system (supplemented TSA-Bi). Serial 10-fold dilutions were prepared from three inoculated strips and plated in triplicate with the solid medium. In addition, 1-ml portions from each dilution were pipetted into five tubes containing 9 ml of the broth. Spores were removed from the strips for plate counting by a method described previously (2) employing ultrasonic energy. Spore counts obtained by both methods were comparable (Table 1). D₁₂₅ c values were calculated by the fractional-replicate-unit-negative (FN) technique and the data analysis method of Stumbo, Murphy, and Cochran as suggested by Pflug and Schmidt (7).

### Table 1. Comparison of the pour plate and most-probable-number (MPN) techniques for enumerating spores

| Spore Isolate | No. of spores per strip | MPN 95% confidence range |
|---------------|------------------------|-------------------------|
|               | Pour plate | MPN<sup>a</sup> | MPN 95% confidence range |
| 1             | 1.0 × 10⁶ | 9.4 × 10⁴ | 2.8 × 10³–2.2 × 10⁶ |
| 2             | 2.5 × 10⁶ | 1.4 × 10⁶ | 4.0 × 10³–3.4 × 10⁶ |
| 3             | 2.2 × 10⁶ | 1.7 × 10⁶ | 5.0 × 10³–4.6 × 10⁶ |
| 4             | 1.7 × 10⁶ | 2.3 × 10⁶ | 7.0 × 10³–7.0 × 10⁶ |
| 5             | 1.6 × 10⁶ | 2.3 × 10⁶ | 7.0 × 10³–7.0 × 10⁶ |

<sup>a</sup> Most probable number.

![FIG. 1. Frequency distribution of D₁₂₅ c values for 103 spore isolates from Mariner 1969.](image_url)
Identification of isolates. The spore crops prepared from the 103 isolates were streaked for isolation on TSA to confirm purity. Cultures were maintained on TSA slants and were transferred to fresh medium 24 hr before inoculation into test media. All incubations, unless stated otherwise, were conducted at 35 C.

Growth at 50 C was determined by placing inoculated Brain Heart Infusion (BBL) slants in a water bath located inside a constant-temperature incubator. Anaerobic growth and gas production in glucose broth, anaerobic production of gas from nitrate, and growth in 10% NaCl broth were determined by the methods of Smith et al. (11). Production of indole and reduction of nitrate to nitrite were detected with Indol-Nitrite Medium (BBL) and the assay methods described by Smith et al. (11). With MR-VP Medium (BBL), the Voges-Proskauer test was performed by the "standard methods" of the American Public Health Association (1) technique. The utilization of citrate test employed Simmons Citrate Agar (BBL) slants.

Production of acid from mannitol was observed by using Phenol Red Broth Base (BBL) containing 0.5% of the carbohydrate. Readings were made after 24, 48, and 72 hr of incubation. The ability of the organisms to hydrolyze starch and gelatin was measured by the technique of Oxborrow and Favero (6). Isolates were identified by the scheme of Smith et al. (11).

RESULTS

Three spore crops of differing resistance levels (mean $D_{125}$ C values of 6, 16, and 34 min) were tested simultaneously on four consecutive days to determine the reproducibility of the FNMMPN system. The coefficients of variation for these mean values were found to be 9, 8, and 6% respectively.

Figure 1 shows the frequency distribution of $D_{125}$ C values obtained with the 103 spore isolates. The values ranged from less than 5 to a maximum of 58 min with the distribution skewed toward the lower range. A compilation of species and $D_{125}$ C values is shown in Table 2.

![Fig. 2. Survival curves based on a frequency of $D_{125}$ C values for 103 spore isolates from Mariner 1969.](image-url)
Bacillus pulvifaciens, B. firmus, B. licheniformis, B. pantothenticus, B. polymyxa, and B. pumilus, in decreasing order, were the species found most frequently. As can be seen by the ranges of D values within each group, there was no definite relationship between species and heat resistance.

**DISCUSSION**

A total of 10^4 bacterial spores were estimated to be on the Mariner-Mars 1969 spacecraft (R. H. Green, personal communication). Assuming that the naturally occurring spore population on the spacecraft had the frequency distribution of $D_{235}$ values indicated by this representative sampling of the population, a survival curve would have the form shown in Fig. 2. In the figure, the curve has been extended to a probability of survival of 10^{-8}. As noted previously, recent proposals for spacecraft sterilization cycles have been based on the thermal inactivation of B. subtilis var. niger spore cultures which have reported $D_{235}$ values on open surfaces ranging from 10 to 30 min (3) with the majority being around 15 min. Viewing the data presented here, several workers have suggested that the B. subtilis var. niger values constitute valid bases for subsequent extrapolations, since the mean D value of the 103 isolates (18.8 min) corresponds closely with the mean value of B. subtilis var. niger cultures. As can be seen in Fig. 2, this would be unrealistic since the slope of the hypothetical survival curve is determined almost exclusively by the more resistant organisms even though they represent only a small portion of the population.

It is emphasized that the thermal resistance data as presented should be considered as relative and not absolute, since past observations have shown that subculturing of naturally occurring spores may result in significant loss of dry-heat resistance (2) and that the culture medium employed may drastically affect resistance levels (7). Studies of Mariner-Mars 1969 isolates conducted by Wardel et al. (personal communication) showed considerably higher $D_{235}$ values when compared to the observations presented here. The liquid sporulation medium they employed (SSM-10 supplemented with tryptophan and methionine) was described by Lazzarini and Santangelo (5) and in our experience has consistently yielded spore crops of higher dry-heat resistances when compared to crops prepared with conventional sporulation media. Consequently, the D values as reported (Table 2) are most likely rather low estimates of the resistances to be found in nature. Even so, the distribution of species and resistance values indicates that B. subtilis var. niger is not representative of the types of bacterial spore contamination found in the environment of concern.

Another important factor to consider in the interpretation of these data is that resistance may be greatly affected by the moisture level in spores during exposure to heat (7). A closed or partially closed heating system (such as mated spacecraft surfaces) may restrict water loss during heating thereby decreasing lethality. The heating system described in this report was one in which the water loss was not mechanically restricted.

Much of the controversy in the field of spacecraft sterilization technology stems from the fact that spacecraft are composed of heat-labile hardware, which necessitates the relatively low temperature constraint of 125 C for the terminal sterilization cycle. With cycle time as the only available variable, the final slope of a survival curve becomes critical in subsequent extrapolations. Coupled with the observation that subculturing appears to lower resistance to dry heat, the data presented here question the use of an arbitrarily selected spore crop as reference for such extrapolation to determine a specified probability of sterility. Overall confidence levels would be increased if terminal sterilization cycles were based on the highest heat resistance values obtained directly from spore populations associated with spacecraft located in their final assembly and testing environments.

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