Dry-Heat Resistance of Bacterial Spores Recovered from Mariner-Mars 1969 Spacecraft

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The dry-heat resistances of 70 bacterial spore isolates recovered from Mariner-Mars 1969 spacecraft were determined and expressed as D values (decimal reduction times). Fifty per cent of the spore isolates had D values of 60 min or less at 125 C. Of organisms with D values greater than 60 min, four were selected for a study of the effect of sporulation medium and suspension menstrum on dry-heat resistance. Both sporulation medium and suspension menstrum were found to affect significantly the dry-heat resistance of the bacterial spores tested.

It is imperative that an understanding of the dry-heat resistance of microorganisms on space-craft that require sterilization, i.e., landers for an extraterrestrial life-detection mission, be acquired (6). An important step toward obtaining this necessary knowledge for the determination of a sterilization cycle for such a spacecraft was made by the prelaunch recovery of spore isolates from the Mariner-Mars 1969 flybys. During the Mariner-Mars 1969 Microbiological Monitoring Program at Cape Kennedy, Fla., 70 spore isolates were recovered from the craft and their dry-heat resistance was tested. In addition, samples of environmental fallout were subjected to dry-heat testing. This program served to further the knowledge of the dry-heat resistance of microorganisms accumulating on flight hardware during assembly. A similar program is planned for the Mariner-Mars 1971 mission.

A major difficulty in defining the dry-heat resistance of microbial populations in spacecraft assembly areas rests in the inability to work with a natural population because of the low population densities present (8). Hence, a study of the heat resistance of organisms from such areas requires that the organisms be cultured to produce sufficient numbers for testing. It has been observed that the D value [a D value (or decimal reduction time) is the duration of exposure at a given temperature necessary to reduce a microbial population by 90%] for a microorganism may vary according to the microorganism's inherent thermal resistance and environmental influences acting on that resistance (7). Of these environmental influences, the chemical milieu of the organism prior to dry-heat treatment is of importance in determining its dry-heat resistance (1). Therefore, a separate study was undertaken to define the effect of certain sporulation media and storage menstrum on the dry-heat resistance of bacterial spores isolated from Mariner 1969.

MATERIALS AND METHODS

Spore recovery and selection. Bacteria were collected from Mariner 1969 by the swab-rinse method, as defined by NASA (5). The samples were heat-shocked at 80 C for 15 min prior to quadrant streaking on Trypticase Soy Agar (TSA; BBL). Colonies were removed from the TSA plates and, from these, 70 organisms capable of sporulation in a synthetic sporulation medium (SSM; 4), were selected for dry-heat resistance testing (phase I). Four isolates capable of satisfying the criteria of D values at 125 C (D125 c) in excess of 60 min when sporulated on SSM and of 95% or greater sporulation in both SSM and TAM sporulation agar (Difco; supplemented with 80 μg of CaCl2 per mg and 20 μg of MgSO4 per mg) were chosen for further study (phase II).

Culture and sporulation technique. Isolated colonies were removed from TSA streak plates, added to Trypticase Soy Broth (TSB; BBL), and incubated at 37 C until visible turbidity occurred. For phase I testing, 2 ml of the TSB suspension was inoculated into a flask containing 250 ml of SSM. (Phase II included inoculation onto a TAM agar plate.) The flask (or plate) was then incubated at 37 C until spores constituted 95% or more of the cells (as determined by microscopic examination of a stained preparation) at which time the spores were harvested. (For the TAM-grown isolates, harvesting consisted of washing the spores from the agar surface with sterile distilled water. The SSM spore cultures were harvested by centrifugation.) The suspension was centrifuged (10,400 × g for 15 min), resuspended,
and washed eight times in sterile distilled water. After the final washing, the spore pellet was re-suspended and divided equally to form both 95% ethanol and sterile distilled water suspensions which were then stored at 4°C.

Dry-heat resistance testing. Only SSM sporulated-95% ethanol-suspended organisms were dry-heat tested in phase I. Phase II involved testing of TAM- and SSM-cultured organisms suspended in both 95% ethanol and sterile distilled water. Three stainless-steel coupons (1 by 2 inch, 2.54 by 5.08 cm; 24 gauge, type 304, no. 4 finish) were inoculated with 20 µl of a suspension containing between 10⁶ and 10⁷ viable spores for each spore tested. During phase I studies, the inoculated coupons were air-dried and placed in a vacuum dessicator (at 24 inches of mercury, negative pressure), containing silica gel, for approximately 16 hr. Phase II coupons were air-dried, placed in sterile petri dishes, and allowed to equilibrate to room conditions (43 to 44% relative humidity, 22°C) for approximately 16 hr prior to heat testing. The identical room conditions were present during dry-heat testing, with equilibration and testing being performed in a segment of the Sterilization Assembly and Development Laboratory at the Jet Propulsion Laboratory. This facility provided temperature and humidity control by circulating air through a system of heating and cooling coils. The system used a water spray and cooling coil to assure that air leaving the cooling coil was at saturation. The air then underwent a reheating function to provide air at the specific temperature and humidity conditions required for the room. After the equilibration period, the coupons were placed on trays in a dry-heat oven and exposed to 125°C (+0.5) for designated time intervals. The oven was a mechanical convection oven modified by installing removable sliding trays into the door (Fig. 1). To minimize temperature fluctuations in the oven, the trays were individually removed and replaced without opening the door. Thermocouples were mounted at 23 oven locations (with an additional thermocouple exposed to ambient conditions), and the leads were connected to a 24-point recorder for continuous temperature monitoring during an experiment. After a 10-min temperature come-up time, coupons inoculated with TAM-grown spores were removed and assayed at 30-min intervals, whereas those with SSM-grown spores were removed at 1-hr intervals. This variation in assay intervals was established because of the differences in heat resistance that resulted from sporulation on the two different media.

The Mariner 1969 Microbiological Monitoring Program included a study to determine the heat resistance of the microbial burden collected on stainless-steel strips exposed in the Assembly Operations (AO) Building and the Explosive Safe Facility (ESF), at the Air Force Eastern Test Range (AFETR), Cape Kennedy. Upon collection, the strips were exposed to given temperatures (125 or 115°C) for designated intervals, at which time groups of eight were removed from the dry-heat oven and subjected to a microbiological assay.

Assay procedure and data handling. After a designated heat exposure of the spore isolate, the three coupons were removed from the dry-heat oven tray with sterile forceps and placed individually into three flasks each of which contained 20 ml of sterile 0.1% peptone water. The flasks, partially immersed in an aqueous solution of 0.1% Tween-80, were then treated in an ultrasonic bath for 12 min at 25 kHz. After this treatment, 10-fold serial dilutions were made in sterile 0.1% peptone water. Dilutions were then plated in triplicate by the pour-plate method by using TSA.

**FIG. 1.** Modified dry-heat oven (National Appliance Co.).

**FIG. 2.** Frequency distribution of Mariner 1969 spore isolates. * Ten to 14.3% of isolates had D125°C values >180 min. † Calculated by separate summation and division by 70 of upper and lower D value 95% confidence limits ≤t min.
TABLE 1. Analysis of variance of Mariner 1969 spore isolate D_{125} C values

| Source of variation | Effect on heat resistance |
|---------------------|---------------------------|
| Replication         | 0                         |
| Strain (A)          | +                         |
| Sporulation medium (B) | +                       |
| Suspending menstruum (C) | +                     |
| A × B               | 0                         |
| A × C               | 0                         |
| B × C               | 0                         |
| A × B × C           | 0                         |

a D_{125} C value is duration of exposure at 125 C necessary to reduce a microbial population by 90%.

b +, no significant effect on dry-heat resistance; +, significant effect on dry-heat resistance (P < 0.01).

Plates were incubated for 48 hr at 32 C, and the dilutions yielding 30 to 300 colony-forming units per plate were counted and recorded. The resulting data were subjected to a computerized linear regression analysis which generated a survivor curve and calculated the reciprocal of the slope of the curve (or D value) and the 95% confidence limits about the D value. An analysis of variance and Duncan multiple-range tests were performed on phase II data (2).

Strips collected in the AO and ESF areas were aseptically placed into a flask containing 50 ml of sterile TSB. All flasks were incubated at 32 C for 48 hr and examined for growth. Results were recorded as growth or no growth.

Isolate Identification. Identification of the phase II isolates was performed as described by Bergey's Manual (7th ed.).

RESULTS AND DISCUSSION

Figure 2 shows a cumulative relative frequency distribution of D_{125} C values (expressed in minutes) for the Mariner 1969 isolates sporulated in SSM and suspended in 95% ethanol prior to heat testing. The graph gives a measure of the likelihood that bacterial spores accumulating on spacecraft surfaces under Mariner 1969 assembly conditions would not exceed a specified D value. For example, 20 to 30% of the spores accumulating on the spacecraft were found to have D_{125} C values of 30 min or less; 49 to 57%, 1 hr or less; and 86 to 90%, 3 hr or less. In general, the survivor curves for those spores with D_{125} C values greater than 180 min were not satisfactorily explained by linear regression analysis; i.e., the resulting R (SQ) terms were quite low. [R (SQ) refers to the measure of the proportion of total variation about the mean explained by linear regression.]

Identification of the four phase II isolates (see above) indicated that isolate number 1 was Bacillus cereus, whereas isolates 2, 3, and 4 were classified as B. licheniformis. Identification was not pursued to the subspecies level; however, because of differences in heat resistance of isolates 2, 3, and 4, it was believed that more than one variety of B. licheniformis was tested.

Four D_{125} C determinations were made for each phase II isolate test condition (a total of 64 heat-resistance tests). An analysis of variance was performed on the data to determine the significance of the sources of variation present in the experiment (Table 1). No effect on D_{125} C due to replication was noted, thus indicating the ability to repeat significantly D value estimates for an organism handled in a specified way. Differences in strain, sporulation medium, and suspension menstruum were all seen to affect D values. A strain-sporulation medium interaction that affected heat resistance was also noted; i.e., for all four organisms tested a significantly higher D_{125} C value was observed when the sporulation was performed with SSM rather than TAM (P < 0.05; Table 2).

Table 3 summarizes the media-menstruum effects on the four Mariner isolates. The far right-hand column of this table indicates a significantly higher D_{125} C value for the ethanol versus the water-suspended spores; however, this observation did not hold for all strain-media combinations. For example, the ethanol and water suspended-TAM cultured isolates, with the exception of isolate number 4, had a similar D_{125} C.

Table 4 shows the results of determinations of thermal extinction points of environmental fallout strips from the AFETR. No growth was observed from the assay of strips exposed to 125 C for 90 min. However, growth was still present after 60 min of exposure, indicating

| Sporulation medium | D_{125} C value (min) from Mariner isolate |
|--------------------|-------------------------------------------|
|                    | 1          | 2          | 3          | 4          |
| SSM                | 73.94_{de} | 124.09_{t} | 67.73_{de} | 120.74_{t} |
| TAM                | 47.53_{abe}| 59.53_{bcd} | 42.38_{ab} | 37.21_{a}  |

a D_{125} C value is duration of exposure at 125 C necessary to reduce a microbial population by 90%. D values shown are averages of eight tests.
b Mean D_{125} C values subscripted with any identical letters are not significantly different, whereas those with dissimilar letters are significantly different (P < 0.05).
TABLE 3. Summary of media-menstrua effects on D_{125} C values of Mariner 1969 spore isolates

| Suspending menstruum | D_{125} C value (min) from Mariner isolate<sup>b</sup> |
|----------------------|---------------------------------------------------|
|                      | TAM | SSM | TAM | SSM | TAM | SSM | TAM | SSM | Avg |
| 95% Ethanol Sterile distilled water | 53.41<sub>bede</sub> 83.28<sub>f</sub> | 41.64<sub>b</sub> 64.60<sub>def</sub> | 61.37<sub>ede</sub> 57.68<sub>bede</sub> | 135.80<sub>e</sub> 122.38<sub>de</sub> | 47.89<sub>bde</sub> 36.87<sub>ab</sub> | 70.58<sub>def</sub> 64.87<sub>de</sub> | 55.48<sub>bede</sub> 18.93<sub>a</sub> | 133.20<sub>b</sub> 108.28<sub>e</sub> | 80.13<sub>x</sub> |

<sup>a</sup> D_{125} C value is duration of exposure at 125 C necessary to reduce a microbial population by 90%. D values shown are averages of four tests.

<sup>b</sup> Mean D_{125} C values subscripted with any identical letters are not significantly different, whereas those with dissimilar letters are significantly different (P < 0.05).

TABLE 4. Dry-heat resistance of bacterial populations collected on stainless-steel fallout strips at Air Force Eastern Test Range, Cape Kennedy

| Time (min) | No. of strips with viable organisms<sup>a</sup> (8 total) | Time (min) | No. of strips with viable organisms<sup>a</sup> (8 total) |
|------------|-----------------------------------------------------|------------|-----------------------------------------------------|
| 125 C      | AO        | ESF       | 115 C      | AO        | ESF       |
| OT         | 7         | 8         | OT         | 7         | 8         |
| T<sup>b</sup>     | 3         | 8         | T<sup>b</sup>     | 8         | 5         |
| T + 10     | 4         | 7         | T + 15     | 6         | 7         |
| T + 15     | 5         | 6         | T + 60     | 1         | 4         |
| T + 20     | 3         | 5         | T + 90     | 3         | 6         |
| T + 25     | 4         | 6         | T + 120    | 0         | 4         |
| T + 30     | 4         | 8         | T + 135    | 1         | 2         |
| T + 35     | 8         | 7         | T + 150    | 1         | 3         |
| T + 40     | 3         | 7         | T + 180    | 1         | 2         |
| T + 45     | 5         | 8         | T + 210    | 0         | 0         |
| T + 60     | 5         | 3         | T + 240    | 0         | 1         |
| T + 90     | 0         | 0         | T + 300    | 0         | 1         |

<sup>a</sup> An average viable count per strip of approximately 10 for Assembly Operations building (AO) and 20 for Explosive Safe Facility (ESF) was found.

<sup>b</sup> T = Zero time at temperature.

a probable extinction point between 60 and 90 min of heat treatment. At 115 C, the extinction point for the AO strips appeared to be between 180 and 210 min, whereas that for the ESF strips was in excess of 300 min. The results of this study provided an indication of the dry-heat resistance of noncultured microbial populations recovered from spacecraft assembly areas. No attempt was made to compare quantitatively the dry-heat resistance exhibited by fallout strip populations with that of media-sporulated organisms. In

general, more than one type of microorganism was present on the fallout strips, whereas purified spore suspensions were formed from the SSM and TAM cultures. In addition, the low number of viable microorganisms detected on the fallout strips (see footnote Table 4) made questionable any expression of the data in terms of D values.

The U.S. Public Health Service at Phoenix, Ariz., has classified and dry-heat tested 103 Mariner 1969 bacterial spore isolates (9). Spores were cultured by using TAM agar and found to have substantially lower heat resistance than the 70 SSM-cultured spores discussed in the present study. Such findings would be in agreement with the sporulation medium effects on heat resistance reported above. Work by the Phoenix group (M. Favero, personal communication) suggests that spores harvested from SSM retain a dry-heat resistance more closely approximating their natural dry-heat resistance, i.e., resistance before culturing, than do spores cultured on TAM.

The present study of the dry-heat resistance of bacterial spores recovered from the surfaces of flight craft provides some of the information necessary for the formulation of a dry-heat sterilization cycle for unmanned spacecraft. In addition to a knowledge of the dry-heat resistance of microorganisms on such a craft, a thorough understanding of other factors must be acquired, e.g., the level of microbial contamination and the temperature profile of the craft (3).

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