Method for Collecting Naturally Occurring Airborne Bacterial Spores for Determining Their Thermal Resistance

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The ability to determine the thermal resistance of naturally occurring airborne bacterial spores associated with spacecraft and their assembly areas has been hindered by lack of an effective collecting system. Efforts to collect and concentrate spores with air samplers or from air filters have not been successful. A fallout method was developed for this purpose and tested. Sterile Teflon ribbons (7.6 by 183 cm) were exposed in pertinent spacecraft assembly areas and subsequently treated with dry heat. Thermal inactivation experiments were conducted at 125 and 113 C. Heating intervals ranged from 1 to 12 h at 125 C and 6, 12, 18, and 24 h at 113 C. Eight hours was the longest heating time yielding survivors at 125 C, whereas survivors were recovered at all of the heating intervals at 113 C. D125 values were calculated using the fractional-replicate-unit-negative technique of Pflug and Schmidt (1968) and ranged from 25 to 126 min. This variation indicated that the most probable number of survivors at each heating interval did not fall on a straight line passing through the initial spore population. However, the most-probable-number values taken alone formed a straight line suggesting logarithmic thermal destruction of a subpopulation of spores with a D125 value of 6.3 h.

One of the principal goals of unmanned exploration of the planets, such as Mars, is the search for life. The search for extraterrestrial life requires the use of life-detecting automated scientific instruments which must be delivered to the planet of interest by a lander capsule. To prevent the transport of terrestrial life to other planets, it is imperative that all viable entities associated with the life detection apparatus and lander capsule be destroyed prior to launch. This will be achieved by subjecting the lander capsule to a terminal dry-heat sterilization cycle.

Dry-heat sterilization cycles for spacecraft have been based upon the thermal inactivation characteristics of Bacillus subtilis var. niger (B. globigii) spores. The assumption has been made that this organism could be used as an "index" of the heat resistance of spores found on spacecraft. However, it has been pointed out that B. subtilis var. niger spores have never been detected on spacecraft (4, 17–20). Bond and co-workers (3, 4) have shown that naturally occurring bacterial spores in soil are significantly more resistant to dry heat than B. subtilis var. niger and that subcultured spores are often more sensitive to dry heat than naturally occurring spores. In addition, these investigations showed that spores isolated from the surfaces of actual flight spacecraft can be significantly more heat resistant than B. subtilis var. niger spores even when these isolates are subcultured. Consequently they suggested that naturally occurring airborne spores associated with spacecraft and their assembly areas comprise the index population rather than B. subtilis var. niger.

Although methods are available for the efficient removal and recovery of surface contaminants (8, 10), the collection of spores directly from spacecraft surfaces in sufficient numbers to be used for dry-heat resistance studies is most difficult, if not impossible. Several methods have been used to try to collect large numbers of spores in a laboratory, including: (i) membrane filters, (ii) large-volume air samplers, (iii) vacuum cleaner dust, and (iv) extraction from prefilter in ventilation systems. These techniques have been unsuccessful. The method of choice would be one that would collect high levels of airborne bacterial spores and collect them from environments to which spacecraft are exposed during assembly.

The objective of this study was to describe and evaluate a method, developed by M. Wardle, Jet Propulsion Laboratory, by which large numbers of naturally occurring airborne spores

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are collected on Teflon surfaces and subsequently are exposed to a thermal treatment to determine the resistance of the heterogenous airborne spore populations.

**MATERIALS AND METHODS**

Sixteen aluminum trays (17.8 cm wide, 188 cm long, and 102 cm high), each containing two 7.6- by 183-cm (0.139 m$^3$) ribbons of Teflon (5 mil FEP Dupont), intended to simulate spacecraft surfaces, were used as collection surfaces in the Low Bay area of the Manned Spacecraft Operations Building (MSOB), a class 100,000 clean room (17). This area was used for the assembly and testing of the Apollo spacecraft (18–20). The Teflon ribbons were cleaned by washing in hot tap water plus detergent (Haemo-Sol, Meinecke & Co., Inc.), rinsed three times in hot tap water, rinsed in distilled water, and drained dry. Ribbons were sterilized for 3 h at 175°C. New ribbons were preconditioned by two exposures to dry heat for 3 h each at 175°C prior to a third heating interval for sterilization.

Thirty-two sterile ribbons were exposed to intramural microbial fallout in the MSOB environment for 7 days, at which time each ribbon was rolled up and aseptically placed in sterile glass jars. Personnel were clean room clothing and sterile gloves and mask during handling of ribbons. Control and test ribbons were selected randomly, and eight of the 32 ribbons were used to determine the initial spore concentration ($N_0$). For each test, six test jars and three temperature control jars (each containing an uninoculated ribbon and a thermocouple) were heated at 125 ± 1°C or 113 ± 1°C in a forced-air oven (Precision Scientific model 625, Chicago, Ill.). Heating intervals ranged from 1 to 12 h at 125°C and 6, 12, 18, and 24 h at 113°C. Temperature in the oven was recorded constantly, and the heating-cycle interval was started when the temperature in the temperature control jars was the same as the oven temperature. The relative humidity of the assembly area (MSOB) was monitored constantly, and the relative humidity of the laboratory testing area was monitored during the heating intervals.

After the thermal treatment, 400 ml of sterile Trypticase soy agar (TSA) broth was added to each jar containing the test ribbon. The jars were incubated at 32°C under aerobic conditions and observed for the presence of growth at 7 days and weekly up to 28 days.

For each experimental run of 24 test ribbons, eight jars each containing a sterile Teflon ribbon were used as sterility controls. To these jars, 400 ml of sterile TSA broth was added aseptically and incubated with the test ribbons.

All laboratory procedures were performed in a horizontal laminar-flow clean bench (9) to eliminate background airborne contamination.

The test assay recovery medium was a broth made according to the formula of TSA minus agar (Trypticase [BBL], 15 g/liter; phytone [BBL], 5 g/liter; NaCl, 5 g/liter) supplemented with 0.2% (wt/vol) yeast extract (BBL) and 0.1% (wt/vol) soluble starch (BBL) (4); TSA plus yeast extract and soluble starch was used for determining total and spore counts on control ribbons. Each control ribbon was rolled up and placed in a sterile jar, and 400 ml of sterile buffered rinse solution containing 0.02% (wt/vol) solution of Tween 80 (polyoxyethylene sorbitan monolaurate; Hilltop Research, Inc., Miamiville, Ohio) was added. The control jars were placed in an ultrasonic bath (tank, LTH60-3; generator, A-300 Branson Instruments, Inc., Stamford, Conn.) containing a 0.3% (vol/vol) solution of Tween 80 ultrasonicated for 6 min at 25 kHz (11, 15, 19), removed from the tank manually, shaken 25 times, and then ultrasonicated for an additional 6 min. After ultrasonication, 5-ml portions were plated in duplicate for total aerobic mesophilic counts. Spore assays were performed by heat shocking duplicate 50-ml portions at 80°C for 25 min before plating. Culture plates were incubated at 32°C under aerobic conditions for 7 days, and colony counts were performed after 2, 3, and 7 days.

**Identification of Bacillus spp. heat survivors.** Organisms surviving the heat treatment were streaked for isolation on supplemented TSA. Cultures were maintained on supplemented TSA slants and transferred to fresh medium 24 h before inoculation into test media.

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 and anaerobic production of gas from nitrate were determined by the methods of Smith et al. (21). 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. (21). 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 h of incubation. The ability of the organisms to hydrolyze starch and gelatin was measured by the technique of Oxborrow and Favero (13). A computerized bacterial identification system (6) was employed to identify all bacterial heat survivors. This system was expanded for the genus Bacillus, and correct identifications were made for 68% of 72 Bacillus stock cultures tested from the American Type Culture Collection and the Nathan R. Smith culture collection.

"D" values were calculated by the fractional-replicate-unit-negative (FN-MPN) technique and the data analysis method of Stumbo, Murphy, and Cochran as suggested by Pflug and Schmidt (14).

**RESULTS AND DISCUSSION**

A statistical summary of the initial spore counts ($N_0$) of the control Teflon ribbons is presented in Table 1. The 95 and 99% confidence limits shown were derived after the original data were transformed logarithmically (7). The
variation in $N_0$'s and the associated confidence limits shown were based on the total control ribbons used for all the individual experiments conducted. Analysis of pertinent data indicates that the $N_0$ variation and confidence limits for each single experiment are even smaller.

A total of 42 thermal inactivation experiments were conducted at 125 C. A summary of these Teflon ribbon experiments, listed according to the various heating times, is shown in Table 2. Eight hours was the longest heating time yielding survivors. The $D_{125}$ values were calculated using the FN-MPN technique of Pflug and Schmidt (14) and ranged from 25 to 126 min.

Six thermal experiments were conducted at 113 C using heating times of 6, 12, 18, and 24 h. Techniques used were identical to the 125 C experiments except for the differences in temperature and heating intervals. Table 3 shows a summary of all Teflon experiments conducted at 113 C. Heat survivors were recovered at all heating intervals used. The $D_{113}$ values ranged from 170 to 451 min.

A total of 384 sterile Teflon ribbons were used as sterility controls during this study, and none was found to be positive for bacterial growth. This reflects the benefits gained by performing all procedures in a laminar flow system, where a controlled environment is maintained to prevent background microbial contamination.

The wide range of $D$ values observed in each of these experiments suggested that the FN-MPN values did not fit a straight line passing through the initial population, as would be expected if the population was homogeneous. However, it was apparent from a plot of these values (Fig. 1 and 2) that both at 125 and 113 C FN-MPN survivor values described straight lines representing $D$ values considerably higher than any calculated using the technique of Pflug and Schmidt (14). The best-fit lines through these two sets of four most-probable-number (MPN) values represent a $D_{125}$ value of 6.3 h and $D_{113}$ of 14.4 h, respectively. These

### Table 1. Statistical summary of Teflon ribbon control counts ($N_0$)

| Determination          | $N_0$ |
|------------------------|-------|
| Number of ribbons      | 384   |
| Mean                   | 229   |
| Minimum                | 20    |
| Maximum                | 2,400 |
| Standard deviation     | 242   |
| Standard error         | 12    |
| 95% Confidence limits  | 205–253 |
| 99% Confidence limits  | 197–261 |

### Table 2. Summation of results from Teflon ribbon experiments at 125 C

| Heating time (h) | No. of expt | Aerobic count | Aerobic spore count ($N_0$) | No. of survivors/total no. of ribbons | $D_{125}$ value (min) | Survivors (MPN) |
|------------------|-------------|---------------|-----------------------------|--------------------------------------|-----------------------|-----------------|
| 1                | 5           | $2.7 \times 10^4$ | $10^4$                     | 10/30                                | 25                    | 0.406           |
| 2                | 38          | $1.8 \times 10^4$ | $2.2 \times 10^4$           | 69/228                               | 43                    | 0.361           |
| 3                | 9           | $2.6 \times 10^4$ | $2.2 \times 10^4$           | 45/54                                | 52                    | 0.537           |
| 4                | 38          | $1.8 \times 10^4$ | $2.2 \times 10^4$           | 28/228                               | 74                    | 0.121           |
| 6                | 37          | $2.0 \times 10^4$ | $2.2 \times 10^4$           | 16/222                               | 104                   | 0.075           |
| 8                | 33          | $2.1 \times 10^4$ | $2.3 \times 10^4$           | 7/198                                | 126                   | 0.036           |
| 9                | 4           | $4.0 \times 10^3$ | $2.0 \times 10^3$           | 0/24                                 |                       |                 |
| 12               | 4           | $4.6 \times 10^3$ | $2.6 \times 10^3$           | 0/24                                 |                       |                 |

* Teflon ribbons were exposed at the Manned Spacecraft Operations Building.
* Samples were not heat shocked.
* Samples were heat shocked.

### Table 3. Summation of results from Teflon ribbon experiments at 113 C

| Heating time (h) | No. of expt | Aerobic count | Aerobic spore count ($N_0$) | No. of survivors/total no. of ribbons | $D_{113}$ value (min) | Survivors (MPN) |
|------------------|-------------|---------------|-----------------------------|--------------------------------------|-----------------------|-----------------|
| 6                | 6           | $4.1 \times 10^4$ | $3.3 \times 10^4$           | 33/36                                | 170                   | 2.485           |
| 12               | 6           | $3.4 \times 10^4$ | $2.6 \times 10^4$           | 19/36                                | 284                   | 0.751           |
| 18               | 6           | $3.4 \times 10^4$ | $3.1 \times 10^4$           | 8/36                                 | 351                   | 0.252           |
| 24               | 6           | $3.3 \times 10^4$ | $2.4 \times 10^4$           | 5/36                                 | 451                   | 0.149           |

* See footnotes to Table 2.
Method (11, 16) used for collecting airborne microbial fallout was the large variation found between strips on the number of airborne microorganisms collected. This was attributed to the small collecting surface (2.5 by 5.1 cm) available. This variation was greatly reduced by the Teflon ribbon method, as was shown by the close correlation obtained between ribbons (Table 1). The use of the Teflon ribbon technique provides a method to study the thermal inactivation kinetics of naturally occurring airborne spores.

One method of estimating the number of microorganisms in samples known to contain very low levels is the MPN technique. This technique utilizes statistically derived equations to yield the probable number of viable microorganisms in a series of replicate units in which a fraction of the units are determined to be sterile. This technique has been used in our heat studies to estimate the number of spores surviving exposure to various dry-heat cycles.

The MPN values derived from our heating experiments are used in two ways: (i) to calculate a D value for a specific spore population or (ii) to determine the decrease in a spore population associated with a specific heat cycle. In calculating what is called an FN-MPN D value, the findings are in agreement with previous experience in which naturally occurring heterogenous spore populations exposed to dry heat show an initial rapid decline in numbers followed by a much slower rate of destruction, which is characteristic of the most resistant portion of the initial population (2, 3).

Identifications were made of heat-resistant Bacillus spp. isolated from the thermal studies. A listing of these identifications is shown in Table 4. The B. lentus and atypical Bacillus group constituted the highest percentage of organisms recovered from either the 125 or 113 C studies. Of interest is the recovery of thermal-resistant actinomycetes (family Actinomycetaceae) from the Teflon ribbon experiments.

The use of Teflon ribbons proved to be a useful technique when employed to collect airborne microbial contaminants in spacecraft-associated environments. The small amount of area needed, the ease of manipulation required, and the large collecting surface (0.139 m²) available makes this system superior to others.

### Table 4. Identification of heat survivors from Teflon ribbons after exposure to dry heat at 125 and 113 C

| Identification | Temp |
|---------------|------|
|               | 125 C | 113 C |
| B. alvei      | 1     | 1     |
| B. brevis     | 9     | 6     |
| B. cereus     | 1     | 1     |
| B. circulans  | 4     | 0     |
| B. coagulans  | 1     | 0     |
| B. firmus     | 3     | 3     |
| B. laterosporus| 1   | 0     |
| B. lentus     | 23    | 16    |
| B. licheniformis| 9   | 9     |
| B. macerans   | 0     | 1     |
| B. megaterium | 3     | 0     |
| B. pantothenticus | 1  | 0    |
| B. polymyxax  | 4     | 0     |
| B. pumilus    | 2     | 2     |
| B. spheraicus | 13    | 0     |
| B. subtilis   | 11    | 9     |
| Atypical Bacillus* | 30 | 14 |
| Actinomycete  | 14    | 4     |
| Failed to grow on subculture | 4 | 0 |

Total number of survivors 134 66

* Atypical Bacillus = organism which, after being subjected to pertinent biochemical tests, did not conform to any specification.
the assumption is made that the population is homogenous and a semi-logarithmic survivor curve at a constant temperature is a straight line. The slope of this line formed by the \( N_0 \) and MPN points represents the rate of death or D value.

Our experience has shown that naturally occurring spore populations are not homogenous and do not form straight lines on a semi-logarithmic plot. Therefore, while the D value calculated using the FN-MPN technique has limited value in extrapolating the survivor curve of spores on heated Teflon ribbons, the MPN of survivors of a given heat cycle is a useful value. By subjecting a pertinent spore population to a specified heating cycle, measuring \( N_0 \) and the MPN of survivors, it is possible to determine the reduction of the population on the logarithmic scale caused by the heating cycle. When experiments of this type have been repeated a sufficient number of times to meet acceptable confidence levels, a valid estimate of the effect of the heat cycle on any spore population level can be made. The reduction would be comparable in any spore population composed of similar proportions of heat-resistant subpopulations.

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