Dry-Heat Resistance of *Bacillus subtilis* var. *niger* Spores on Mated Surfaces

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*Bacillus subtilis* var. *niger* spores were placed on the surfaces of test coupons manufactured from typical spacecraft materials (stainless steel, magnesium, titanium, and aluminum). These coupons were then juxtaposed at the inoculated surfaces and subjected to test pressures of 0, 1,000, 5,000, and 10,000 psi. Tests were conducted in ambient, nitrogen, and helium atmospheres. While under the test pressure condition, the spores were exposed to 125 °C for intervals of 5, 10, 20, 50, or 80 min, with survivor data being subjected to a linear regression analysis that calculated decimal reduction times. Differences in the dry-heat resistance of the test organism resulting from pressure, atmosphere, and material were observed.

One of the provisions set forth by the National Aeronautics and Space Administration (NASA) for unmanned planetary capsules designed to land on the Martian surface is that they be dry-heat sterilized in an inert gas environment before launch (7). The microbial burden of such a spacecraft may be categorized as follows: surface microorganisms, microorganisms located in the interior of materials, and microorganisms located between mated surfaces. It is important that the time/temperature relationships necessary to inactivate microorganisms in each of these locations be known to formulate a realistic dry-heat sterilization process that will not impair the performance of the spacecraft.

The work presented here was conducted with the aim of determining the dry-heat resistance of microbes located between mated surfaces.

MATERIALS AND METHODS

Test mechanism. A thermal joint conductance apparatus (Fig. 1) was used in this study to produce the temperature and pressures required. This apparatus consisted of a loading mechanism, heater elements and controls, and associated monitoring equipment. The loading mechanism was comprised of a balance-beam dead-weight loader and related support structures. A calibrated load cell measured the applied force. A pressure of as high as 10,000 psi could be applied by the loading mechanism. Because of the design of the dead-weight loading mechanism, no change took place in the applied load resulting from structural deformation or temperature change. The temperature of the heater block assemblies, between which mated surfaces were placed, was maintained by automatic controllers. One temperature controller was used for each heater block assembly, resulting in a temperature of 125 °C ± 0.2 °C.

Test surfaces. The following typical spacecraft materials were employed: stainless steel (347), magnesium (ZK60A, with Dow 17 coating), aluminum (2024), titanium (6A14V), silicone elastomer (Spaulding Rubber Co., Los Angeles, Calif.), and poly-carbonate plastic (Lexan, General Electric Co., Pittsfield, Mass.). Successful tests were not achieved with the latter two materials (see below). Test coupons were in the form of sheet material 1 inch (2.54 cm) square. The coupons were 22 to 26 gauge, depending on the material.

Preparation of test coupons. The metal coupons were washed in tap water with a nonionic detergent, rinsed in tap water five times, and then rinsed twice in distilled water. They were then dipped in isopropyl alcohol, followed by a dip in ethyl ether, and air-dried. The cleaned coupons were placed on aluminum trays and covered with aluminum foil. The trays were then placed in a dry-heat oven for 12 hr at 150 °C.

Preparation of test sample. Spore suspensions of *Bacillus subtilis* var. *niger* were prepared with a synthetic sporulation medium (6), by a method described earlier (10). A 10-μl liter sample of a 95% ethanol spore suspension containing between 10⁴ and 10⁵ viable spores was then inoculated onto a test surface with a micropipette (Eppendorf Micropipette, Brinkmann Instruments, Inc., Westbury, N.Y.). For the initial survey study, the inoculated coupons in sterile petri plates (100 by 15 mm) were placed in desiccator jars (one jar for each dry-heat exposure time) containing activated silica gel. The coupons were allowed to equilibrate for 16 hr (at 24 inches
of Hg absolute pressure) and, at the end of this time, were removed from the desiccator jars and dry heat-tested. The relative humidity (RH) of the room was recorded before and after each dry-heat test with a wet-dry bulb hygrometer (Taylor Instrument Corp., Rochester, N.Y.) and was found to vary between 25 and 50% RH over the testing program. In later studies dealing with varied atmospheres and aluminum mated to magnesium in a nitrogen atmosphere, the coupons were permitted to equilibrate to room conditions (21 to 23 C, 25 to 50% RH) for 16 hr before the test. The room RH was monitored with a Serdex B humidity-temperature recorder, (serial D1260, Bacharach Industrial Instrument Co., Pittsburgh, Pa.) and an electric hygrometer indicator (model 15-3001, Hygrodynamics Inc. Silver Spring, Md.). After the equilibration period, two inoculated coupons were placed between the heater blocks of the thermal joint conductance apparatus with their inoculated surfaces contiguous.

Test exposure. Through the use of thermocoupled coupons, it was determined that the test surfaces heated from 22 C to 125 C in less than 5 min. Five minutes was therefore used as the starting time for the materials tests. For the tests dealing with the effects of different atmospheres, a 10-min period was used. This increase was due to the fact that it took somewhat longer for the heater block temperature to equilibrate at 125 C after having the chamber evacuated to 1 torr and then purged with the desired gas [ambient air; extra-dry, high-purity nitrogen (Union Carbide Corp., Los Angeles, Calif.), or extra-dry, high-purity helium (Gardner Cryogenics, Bethlehem, Pa.)]. Mated coupons were exposed to test conditions for 5, 10, 20, 50, or 80 min.

Determination of survivors. After the desired temperature and pressure exposure, the two test coupons were removed from between the heater blocks, and each was placed into a flask containing 20 ml of sterile 0.1% peptone water. The flask was then placed in an ultrasonic bath (Sonogen Automatic Cleaner, model A-300, Bronson Instruments, Inc., Stanford, Conn.) and sonically treated at 25 kHz for a period of 12 min. After sonic treatment, a 1-ml amount was removed from the flask and transferred to a 9-ml dilution blank of 0.1% peptone water, and suitable 10-fold dilutions were made. Samples (1 ml) of the appropriate dilutions were plated in triplicate by using the pour plate method with Trypticase Soy Agar (BBL) as the plating medium. The plates were incu-
bated at 32 C for 72 hr, and colony-forming units were counted.

Data handling. Decimal reduction times (D values) for each test were calculated by a computerized linear regression analysis. Resulting D values were subjected to an analysis of variance and the Duncan multiple-range test (4).

RESULTS

An initial study was performed to survey a variety of spacecraft surfaces placed in a mated configuration at different applied pressures (0 to 10,000 psi) in ambient atmosphere. The purpose of these tests was to identify any possible pressure and/or material effects on heat resistance. Experiments with silicone elastomer and polycarbonate plastic were not successful because of poor recovery of the spores. Satisfactory tests were run with stainless steel mated to stainless steel, magnesium to magnesium, aluminum to aluminum, and titanium to stainless steel. With the exception of the aluminum mated to aluminum system, which possibly exhibited a material-caused enhancement of dry-heat resistance, no apparent pressure or material effect on the dry-heat resistance of B. subtilis var. niger spores was noted. (The range of D values for all tests was from 36 to 52 min.) However, when experiments were performed with aluminum mated to magnesium, a progressive increase in D125 c values from 37 min at zero applied pressure to 65 min at 10,000 psi was noted. Further tests in nitrogen (see below) were performed to elucidate the effect of pressure and material on heat resistance in the aluminum mated to magnesium system.

Another set of experiments, designed to study the effect of different gaseous environments on the heat resistance of spores in mated stainless-steel systems, yielded the results seen in Table 1. An analysis of variance was performed, showing that both gas and pressure (but not their interaction) had significant effects on dry-heat resistance (Table 2). For the higher pressures tested (5,000 and 10,000 psi), the heat resistance in ambient air was found to be significantly greater than that in nitrogen or helium (Table 1). The latter two gases appeared equivalent in their effect on heat resistance. A pressure-related increase in heat resistance was observed in ambient air when 0 and 5,000 psi applied-pressure data were compared (P < 0.01). No increase in heat resistance resulting from pressure was evident in the nitrogen or helium environments (P < 0.01).

In the initial material study, it was observed that the Al-Mg system had an effect on heat resistance that could be explained in terms of pressure or material or both. Therefore, a follow-up study was performed with this system.

### Table 1. D125 c values for Bacillus subtilis var. niger spores on mated stainless steel in selected atmospheres

| Atmosphere     | Applied pressure |
|----------------|------------------|
|                | 0 psi            | 1,000 psi | 5,000 psi | 10,000 psi |
| Air            | 27.98 BC        | 30.27 AB  | 39.65 A   | 40.67 A    |
| Nitrogen       | 17.05 C         | 19.58 BC  | 28.36 BC  | 25.97 BC   |
| Helium         | 18.73 BC        | 17.34 C   | 20.77 BC  | 24.30 BC   |

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| Nitrogen       | 17.05 C         | 19.58 BC  | 28.36 BC  | 25.97 BC   |
| Helium         | 18.73 BC        | 17.34 C   | 20.77 BC  | 24.30 BC   |

* D values are averages of three tests. Mean D125 c values subscripted with any identical letters are not significantly different (P < 0.01).

* Values expressed as minutes.

### Table 2. Analysis of variance for mated stainless-steel system in selected atmospheres

| Source of variation | F ratio* |
|---------------------|----------|
| Replication         | 0.184    |
| Gas (G)             | 34.98*   |
| Pressure (P)        | 10.00*   |
| G X P               | 0.824    |

* F ratios followed by an asterisk are significant (P < 0.01).

### Table 3. Comparison of D125 c values for Bacillus subtilis var. niger spores on Al-Mg and stainless steel-stainless steel mated surfaces in nitrogen

| Materials                        | Applied pressure |
|----------------------------------|------------------|
|                                  | 0 psi            | 1,000 psi | 5,000 psi | 10,000 psi |
| Aluminum mated to magnesium      | 44.26 ABC        | 37.02 BC  | 51.42 AB  | 56.92 A    |
| Stainless steel mated to stainless steel | 17.05 E         | 19.58 E   | 28.36 CDE | 25.97 DE   |

* D values are averages of three tests. Mean D125 c values subscripted with any identical letters are not significantly different (P < 0.01).

* Values expressed as minutes.

Tests of the Al-Mg and stainless steel-stainless steel systems in nitrogen yielded the results shown in Table 3. An analysis of variance (Table 4) indicated that material and pressure, but not their interaction, were significant in accounting for variance of heat resistance in the comparison of the two systems. For all pressures tested, material appeared to have a predominant effect on heat resistance, with the Al-Mg system exhibiting a significantly greater heat resistance than the stainless steel to stainless steel system (Table 3). A pressure effect was noted for the Al-Mg system when 1,000 and 10,000 psi data were compared (P < 0.01).
**TABLE 4. Analysis of variance for Al-Mg versus stainless steel-stainless steel mated systems in nitrogen**

| Source of variation | F ratio a |
|---------------------|-----------|
| Replication         | 0.617     |
| Material (M)        | 88.29*    |
| Pressure (P)        | 6.97*     |
| M × P               | 1.22      |

a F ratios followed by an asterisk are significant ($P < 0.01$).

**DISCUSSION**

Recent work on the dry-heat resistance of bacterial spores has indicated a critical role for water. Drummond and Pflug (3) showed that the relative humidity of the environment before and during dry-heat exposure may have profound effects on the heat resistance of bacterial spores. The present study imposed no humidity control of the room environment in which the spores were manipulated before heat exposure. However, within the range of pretest humidities experienced, no significant effect of these humidities on dry-heat resistance was apparent.

The present study concentrated on a joint-pressure range representative of that found on flight spacecraft. Over this range, significant pressure effects were sometimes observed. These instances saw higher pressures yielding higher $D$ values. The recent work of Drummond and Pflug (3) provided evidence that small increases in the relative humidity at test temperature (125 °C) can significantly enhance the dry-heat resistance of *B. subtilis* var. *niger* spores. The same investigators (2) have described differences in $D$ values in mated systems that could be correlated to moisture diffusion distances. In light of these findings, the pressure effects noted in the present study may be attributable to conditions whereby moisture release from the spores was retarded, thus providing a critical water reservoir at temperatures that resulted in spores of a greater dry-heat resistance. Evidence was acquired that certain of the materials (e.g., magnesium) exhibited a compressibility over the range of pressures employed that may have contributed to moisture retention between the mated surfaces. In an earlier study of mated systems, Angelotti et al. (1) reported $D_{125}$ c values of approximately 46 min for *B. subtilis* var. *niger* spores on stainless-steel washers mated with the application of a torque of 150 inch-lb in contrast to $D_{125}$ c values of approximately 8.6 min for spores on exposed stainless-steel surfaces. The mated and open surfaces were placed in sealed thermal death time tubes before heat exposure in a silicone bath, thereby preventing any contact with ambient air. Comparison of these results with the present data is not practical because of differences in the method of heat application and the difficulty of meaningfully converting torque into unit pressure.

The inert gases (nitrogen and helium) provided conditions whereby the spores tested in these environments showed lower heat resistance as compared to ambient air tests. These data may also be explained in terms of spore water content, with the dry, inert gases rendering a spore more desiccated (and hence less heat resistant) during heat exposure. Koesterer (5), working with an open surface system, also reported a greater heat resistance for *B. subtilis* spores in air as compared to helium. Work by Pfeil et al. (9) with *B. subtilis* spores on stainless-steel surfaces placed in hermetically sealed containers filled with air, nitrogen, or helium indicated that the heat resistance in the latter two gases was comparable. However, in contrast to the results reported here, these workers found the spores to be more heat-resistant in nitrogen and helium as compared to air. The two methods of heat application may account for the discrepancy.

The results of the material effect on $D$ values in the mated systems reported here are in agreement with an earlier study by Paik et al. (8) on the effect of materials in open systems. These workers found aluminum and magnesium to give a greater dry-heat resistance to spores as compared with stainless steel.

It should be pointed out that the $D_{125}$ c values obtained for mated systems in inert environments did not exceed the values stipulated by NASA (7) for sterilization of unmanned planetary spacecraft. The use of organisms other than *B. subtilis* var. *niger* should be investigated for further validation of this parameter, i.e., isolates from populations found on flight spacecraft.

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