

ATCC 29669 Spores Show Substantial Dry Heat Survivability

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ABSTRACT

Bacillus sp. ATCC 29669 was isolated from microbial fallout in clean rooms during the assembly of the Viking Spacecraft missions to Mars, making it a potential contamination concern for outbound space missions. Spores from this bacterial strain were found to be thirty times more resistant to dry heat than *B. atrophaeus*. Spore inactivation rates under vacuum controlled humidity were faster than rates obtained under ambient humidity. Inactivation rates for these heat resistant spores are important considerations for planetary protection implementation where temperature, time and humidity conditions are used to estimate the effectiveness of dry heat microbial reduction (DHMR) procedures.

INTRODUCTION

The heat inactivation of *Bacillus atrophaeus* (ATCC 9372) had been studied to obtain lethality rates and their temperature dependence at the extended temperature range of 125°C to 170°C [1, 2]. These experiments were conducted with both controlled (vacuum, no humidity) and ambient humidity (40-60%) to determine at which temperature the effect of humidity becomes negligible. In the course of these studies, questions arose about the behavior of heat resistant spores, also known as "hardies."

During Viking assembly and sterilization, it was recognized that spores with greater thermal resistance than *B. atrophaeus* could survive the proposed heating cycles. Investigations carried out in the 1970's sought to understand and quantify this phenomenon [3]. The presence of these hardies led to the four-log restriction for the bio-burden reduction on spacecraft parts. A survey of air particulate fallout deposition from clean rooms and assembly areas was conducted to identify naturally occurring spores with high-heat tolerance [4]. Puleo *et al.* collected 234 dry heat resistant isolates from Teflon ribbon fallout studies at the Kennedy Space Center, plus 1296 isolates from the Viking mission planetary quarantine work for a total of 1530 isolates [5]. One isolate that ex-

hibited exceptional heat tolerance was named CK4-6 and deposited at the American Type Culture Collection as strain ATCC 29669 [6]. Early work with this strain demonstrated that it was much more resistant than *B. atrophaeus* to heating under low humidity conditions [7,8,9].

The purpose of this study was to evaluate the lethality rate constants, the D-values and the effect of humidity on a representative hardy spore over an extended temperature range for which there is very little data. At the inception of the current study, numerous resistant strains were screened. One previously described strain was shown to have exceptional heat resistance [10]; however, low sporulation yields and an unacceptably slow growth rate [11] excluded it from this study. Other strains examined showed significant clumping, undermining the premise that one colony forming unit derives from a single spore, which would present additional difficulties in data interpretation [11]. Thus, ATCC 29669 was selected for this study based on its relative ease of handling and exceptional heat resistance.

This paper presents preliminary results on the dry heat resistance of spores from ATCC 29669. D-values and survivor ratios are presented at 125°C and 170°C. These temperatures were selected to parallel the earlier work on *B. atrophaeus*. This data could be used to refine mathematical predictions of spore inactivation at high temperatures, with or without controlled humidity.

MATERIALS AND METHODS

In these experiments, stainless steel vessels were inoculated with a spore suspension, dried and then heated in a constant temperature bath for specified time durations under either vacuum or ambient humidity conditions. Then, the spores were recovered, plated and processed to determine the number of survivors.

Preparation of Spore Stock Suspension. ATCC 29669 was obtained from the American Type Culture Collection (Manassas, VA). Spores from this strain were prepared in a large batch by growing on an agar surface

in 19 stainless steel pans, equivalent in surface area to 380 standard Petri dishes. Sporulation was carried out on Nutrient Sporulation Medium supplemented with divalent cations. The medium consisted of Nutrient Broth 8 g (Difco), 0.25 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 16 g agar (BD, Difco), 0.5 mM each CaCl_2 , MnCl_2 and FeSO_4 [12]. After 2 weeks of growth, the spores were harvested by scraping the surface of the agar and rinsing with deionized water. The spores were suspended in deionized water at 4°C and washed by centrifugation multiple times over a seven day period [13]. Between washes the spore suspension was gently shaken at 4°C. The washed pellets were suspended in 50 mL of deionized water and then heat shocked at 80°C for 15 minutes. The final product was aliquoted and stored in capped glass serum bottles at 4°C.

Thermal Spore Exposure Vessel (TSEV). The TSEV consists of a blind end stainless steel tube, 1.27 cm internal diameter, 10.16 cm long, machined to a wall thickness of 0.025 cm [Fig 1]. They were previously described by Kempf, *et al.*, [14].

TSEV inoculation. The TSEVs were inoculated by spotting 50 μL of the spore suspension onto the internal bottom surface of each TSEV, producing 3×10^6 recoverable colony forming units. The inoculated TSEVs were loosely capped and dried for a minimum of two days in a desiccator jar containing a silica gel desiccant (EMD Chemicals) prior to use. Eight identically inoculated TSEVs (five heated and three unheated controls) and a temperature reference TSEV were used in each exposure run.

Silicone oil bath. The TSEVs were heated in a constant high temperature bath (model 6330; Hart Scientific, American Fork, UT) filled with a high temperature silicone oil (Hart bath fluid #5617, Dow Corning Silicone Oil Type 710, Hart Scientific, American Fork, UT). Bath lids were modified to suspend the TSEVs in the bath oil, keeping the open end of the TSEV above the fluid level. The bath temperature was calibrated against a reference thermometer (Model 1521 handheld thermometer with a platinum resistance thermometer, Hart Scientific, American Fork, UT) and was maintained to within $\pm 0.05^\circ\text{C}$ of the target temperature during the exposures. All thermocouples were calibrated by immersing them in the bath with the reference thermometer at the temperature at which they were used.

Ambient humidity exposures. For the exposures at ambient humidity, a butyl rubber tube stopper was placed on each of the eight inoculated TSEVs. The loose fitting stopper covered the tube opening permitting air exchange and preventing pressure changes. The five inoculated TSEVs and a reference TSEV containing a thermocouple were held in the modified lid of the bath. The thermocouple touched the bottom of the TSEV to record the actual temperature to which the spores were being exposed. All six TSEVs were immersed in the

bath simultaneously, and the time-temperature history of the reference TSEV and the bath were recorded at either one second or sixty second intervals with an Agilent 34970 data logger (Agilent Technologies, Inc., Santa Clara, CA). The temperature profile was recorded using a LabView software program (version 6.0, National Instruments, Austin, TX). For exposures lasting 12 hours or longer, temperature data were collected every 60 seconds, but for shorter exposures, the time-step was increased to every second. At the conclusion of the nominal exposure time, the modified lid with TSEVs was removed from the oil bath and placed into an ice bath to rapidly cool the TSEVs to below 20°C. The same exposure temperatures were used for both ambient humidity and controlled humidity. The time durations were selected to provide countable survivors to determine measurable survivor ratios. The laboratory room ambient temperature and humidity were recorded with a hygrometer (model TM121, Dickson, Addison, IL) to document the ambient experimental conditions at the time the TSEVs were prepared and during the heated exposures. On all days measured, the relative humidity was bounded between 36% and 66%. Measuring the temperature and relative humidity established that neither parameter exceeded a range for a typical climate controlled room.

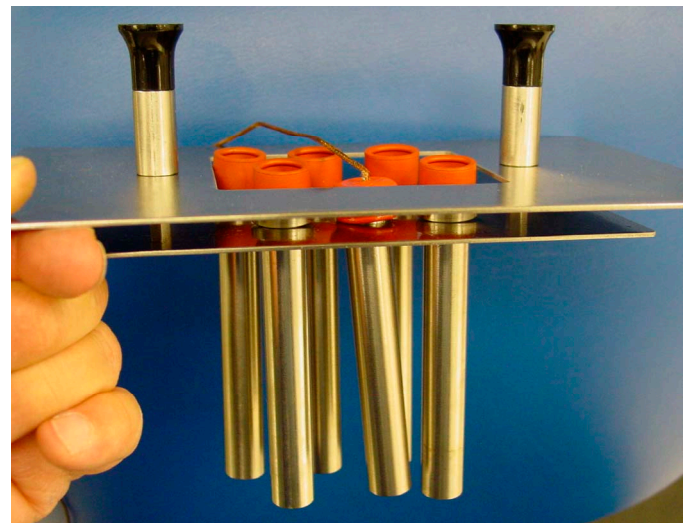


Figure 1. A picture of the thermal spore exposure vessels (TSEV) and carrier platform (modified bath lid) as configured for uncontrolled humidity exposures. A thermocouple wire can be seen in the front center TSEV. The thermocouple touched the inside bottom of the surrogate tube and was used to track the exact time and temperature experienced by the spores.

Controlled humidity exposures. For controlled humidity exposures, a silicone rubber septum (ST-495, Specialty Silicone Products, Inc., Ballston Spa, NY) covered the openings of the inoculated TSEVs. The septa material was obtained as a 3 mm thick silicone rubber sheet, and the individual seals were hand punched with a coring tool. Each TSEV was sealed with an aluminum crimp-seal (20 mm; Wheaton Science Products, Inc.).

Millville, NJ). The TSEVs were placed into the modified lid, and a manifold that held six syringe needles was placed over the lid. The needles were inserted into the TSEVs through the septa. The side-hole needles (22 gauge, side-hole, Hamilton Company, Reno, NV) penetrated the septa without coring or clogging, permitting the TSEVs to be reliably evacuated by a vacuum pump (TriScroll 300, Varian Vacuum Technologies, Lexington, MA). The TSEVs were evacuated to 147 Pa (1.10 torr) prior to heating (measured by a calibrated absolute pressure gauge model FA160, Wallace & Tiernan, Belleville, NJ) [Fig 2]. Immersion, data collection, and ramp-downs were carried out as described for the ambient exposures. After the controlled humidity exposures were completed, the septum and crimp-seal were removed under aseptic conditions (returning the inside of the vessel and spores to ambient pressure and humidity) and a sterile rubber tube stopper was placed on each TSEV until the spores were recovered and processed.

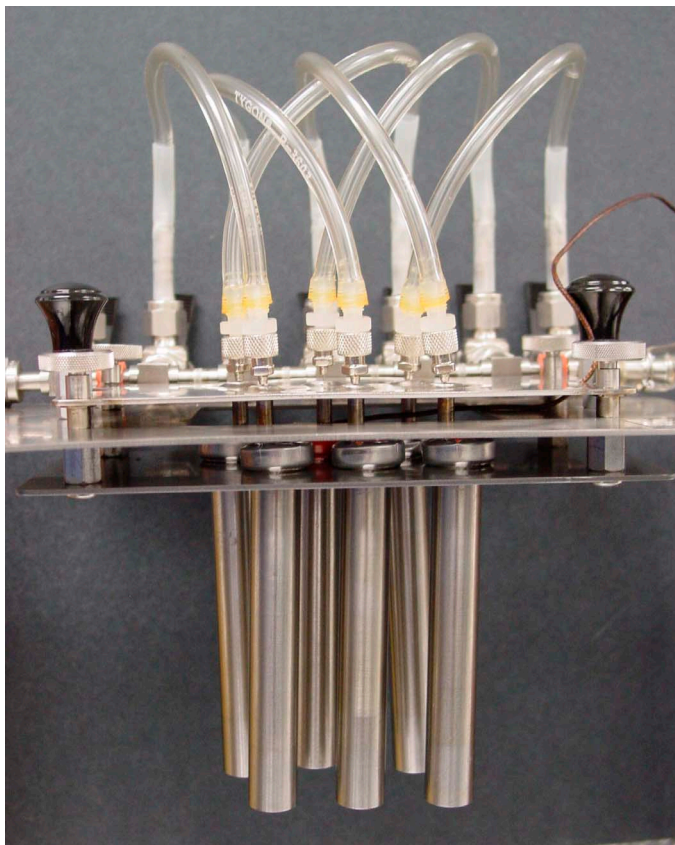


Figure 2. A picture of the thermal spore exposure vessels (TSEV) and carrier platform as configured for controlled humidity (vacuum) exposures. Spores were deposited and dried on the inside bottom of the tubes. A surrogate tube containing a thermocouple is used to track the time and temperature of the exposures.

Post-exposure processing and plating of spores.

The spore recovery and plating procedure [2,14,15] was used both for the five exposed TSEVs and the three unexposed TSEVs serving as positive controls. After the

vessels had returned to room temperature, the TSEVs were processed to recover the viable spores. Two mL of sterile deionized water was added to each TSEV, and it was sonicated for 2 minutes (25 kHz; minimum of 0.35 W/cm², Series 8500 Advanced Ultrasonic Generator, Branson Ultrasonics Corp., Danbury, CT). After sonication, the 2 mL volume was aseptically transferred to a screw-cap glass test tube. This operation was repeated three additional times to collect as many spores as possible. The wash volume collected in the test tube was approximately 8 mL. The collected wash volume was measured by pipette and the final volume was brought to 10.0 mL by adding sterile deionized water. Ten-fold serial dilutions of this collected volume were made in sterile deionized water. Four 2.0 mL aliquots from each dilution tube were plated using the pour plate method and tryptic soy agar (TSA) (BD, Difco) as the growth medium. The plates were incubated for 5 days at 32°C. After incubation, the colonies were counted and the number of colony forming units (CFU) for each of the eight exposure vessels was calculated. The average and the standard deviation for total CFUs were calculated for both the five heated and the three unheated control vessels.

Exposure data collection and processing.

The exact time and temperature of each exposure was tracked by the thermocouple-data-logger-LabView system. This information was used to calculate the equivalent time of the exposure. The equivalent time is the time at temperature corrected for spores killed during ramp up and ramp down in the time temperature profile. A procedure similar to that of Pflug [16] was used and described by Kempf *et al.* [14]. Before and after each run, the reference TSEV with the thermocouple was calibrated in the pre-equilibrated silicone oil bath against the Hart 1521 reference thermometer.

RESULTS AND DISCUSSION

Bacillus sp. ATCC 29669 spores showed substantial dry heat survival in all of the experiments conducted. Spores of this strain were more resistant to dry heat than spores of the standard strain used as a dry heat indicator, *Bacillus atrophaeus* ATCC 9372. In the figures and table presented, spores of both of these strains nearly always show greater survival under ambient relative humidity conditions than under vacuum-controlled humidity conditions.

Experimental results, as presented in the graphs, plot the survivor ratio of the dry heat exposed spores against the exposure time. The slopes of the lines are the lethality rates. The lethality rates were calculated in the following way: The spore population remaining after heat exposure (N_t) was divided by the population of the unheated controls (N_0) to obtain the survivor ratios (N_t/N_0). The natural logs of these survivor ratios were plotted versus the equivalent time. The lethality rates were obtained from a linear regression of the logarithm