

# Dry-Heat Inactivation Kinetics of Naturally Occurring Spore Populations

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Twenty-three soil samples were collected from areas of the United States where major spacecraft assembly and launch facilities are in operation. Soil samples were treated with ethyl alcohol, ultrasonic energy, and gross filtration. The resultant suspensions consisted of viable, naturally occurring bacterial spores and were used to inoculate stainless-steel strips. The strips were suspended in a forced air oven and assays were made at 5-min intervals for the number of viable spores. Most survivor curves were nonlinear. Subsequently, spore crops of heat-sensitive and heat-resistant soil isolates were found to have linear survivor curves at 125 C which were unaffected by the presence or absence of sterile soil particles from the parent sample. When two spore crops, one of which was heat-resistant and the other heat-sensitive, were mixed, the resultant nonlinear curves were unaffected by the presence or absence of sterile parent soil. Therefore, the survivor curves obtained originally with the soils were the result of heterogeneous spore populations rather than of protection afforded by soil particles in our test system. These results question the rationale both of assuming logarithmic death and of using decimal-reduction values obtained with subcultured standard reference spores in the derivation of dry-heat sterilization cycles for items contaminated with naturally occurring spore populations.

The National Aeronautics and Space Administration (NASA) requires that spacecraft which may impact Mars or other planets of biological interest be sterilized (NASA, Outbound Planetary Biological Contamination Control, NASA Policy Directive 8020.10, 7 September 1967). The problems associated with the delivery of a sterile capsule to a particular planet are manifest and cannot be fully covered in this paper so that reference is made to the more detailed reports of Bruch (2, 3), Craven et al. (5), Favero (8), Hall (11), and Light et al. (13).

The basic approach that will be used to produce a sterile spacecraft involves manufacturing, testing, and assembling the hardware under rigid environmental controls, enclosing it in a hermetically sealed canister, and subjecting this unit to a terminal dry-heat sterilization cycle. Once sealed and heated, the biological barrier (canister) cannot be broken to determine that the spacecraft is in fact sterile. Therefore, sterility of the spacecraft is expressed as a low probability which must be established entirely by inference.

The candidates most likely to survive the terminal sterilization cycle would be heterogeneous populations of bacterial spores. Rosebury (16) states that members of the genus *Bacillus* are

widely distributed in nature, and, although they are frequently recovered from surfaces of the healthy human body, they are not indigenous to humans but are merely transient organisms whose ultimate origin is soil. Therefore, as a contaminated spacecraft enters its terminal sterilization cycle, the organisms of major concern with regard to thermal resistance will be bacterial spores of soil origin.

Presently, dry-heat sterilization cycles for spacecraft are based upon the thermal inactivation characteristics of *B. subtilis* var. *niger* (*B. globigii*) spores. Craven et al. (5) have stated that many workers in the field of spacecraft sterilization technology accept the spores of this microorganism to be representative of the more heat-resistant organisms likely to be found on spacecraft. They further pointed out that a representative organism is necessary because of the magnitude in variations of thermal resistance among spore populations found in and on space hardware. In addition, the "logarithmic death rate model" of spore populations is assumed in subsequent calculations (12), since survivor curves of the standard reference organism are essentially straight-line functions on a semilog scale.

Rather than employ a standard reference or-

ganism, it was thought that the use of naturally occurring spore populations (i.e., mixed populations of spores employed directly without intermediate isolation and subculture on conventional laboratory media) might yield more representative and therefore pertinent information regarding the dry-heat inactivation kinetics to be encountered in actual practice. Even though methods are available for efficient removal and recovery of surface contamination (8, 10), collection of spores directly from spacecraft surfaces in numbers sufficient for dry-heat resistance tests to be conducted is impossible. Assays on several unmanned spacecraft have shown the bacterial spore level to be approximately  $10^4$  to  $10^5$  per spacecraft (J. R. Puleo, *personal communication*). Since spores, including those found on spacecraft, are mainly of soil origin, soils collected in the vicinities of manufacture and assembly of spacecraft would be the next most logical source of naturally occurring spore populations for heat studies.

The objectives of these studies were to formulate a method for obtaining spore populations from soil without the use of heat shock (4, 7) or subculture and to observe the inactivation kinetics of these populations found in soil samples collected from various areas of the United States where spacecraft components are manufactured or assembled.

#### MATERIALS AND METHODS

**Soil samples.** One-hundred-gram amounts of each soil sample were dried in loosely capped 1-liter Erlenmeyer flasks at 50 C for 48 hr. A 100-ml amount of 95% ethyl alcohol was added to each dried soil, and the suspensions were insonated at maximum power (25 kHz) for 30 min in an ultrasonic bath (Sonogen LTH60-3 transducerized tank; Sonogen A-300 generator; Branson Instruments, Inc., Stamford, Conn.). A single flask was placed on the center of the tank bottom with the level of the bath fluid, 0.3% Tween 80 (Hilltop Research, Inc., Miamiville, Ohio) in distilled water, slightly above the level of ethyl alcohol in the flask to ensure optimum transfer of ultrasonic energy. The temperature of the bath fluid was maintained from 4 to 20 C during the insonation period. After insonation, each suspension was filtered through a sterile linen towel to remove large particles and then stored at 4 C in a tightly capped bottle. These treatments yielded suspensions of finely dispersed soil and bacterial spores, free from viable fungi, actinomycetes, and vegetative bacteria. No isolations have been made from these suspensions other than gram-positive, sporeforming rods. Also, extended storage of bacterial spore crops in ethyl alcohol, as opposed to distilled water, has been shown to have no effect on viability or heat resistance (7).

**Assay system.** Dry-heat inactivation kinetics of pure and mixed spore populations were determined by inoculating sterile stainless-steel strips [0.5 by 0.5 inch (1.27 by 1.27 cm); coldroll, type 302, no. 4 finish, 22

gauge] with 0.05 ml of an ethyl alcohol suspension. The strips were then dried under vacuum for 16 hr over silica gel. For each heating interval, three strips were suspended in a forced-air dry-heat oven (model no. 625, Precision Scientific Co., Chicago, Ill.) at 125 C ( $\pm 0.5$  C). The time required to heat a strip to 125 C was 2.5 min as determined by a copper-constantan thermocouple attached to a control strip. This amount of time was added to each exposure interval. One set of samples was placed in the oven at each interval, and the temperature was monitored constantly with a recording thermometer. Immediately after removal from the oven, each strip was placed in a tube containing 10 ml of chilled (4 C), phosphate-buffered distilled water (BDW; 1) with several 3-mm glass beads and was insonated for 12 min in an ultrasonic bath. The suspensions were diluted appropriately with BDW and plated in triplicate with Trypticase Soy Agar (TSA; BBL). After the medium had solidified, 10 to 15 ml of TSA was overlaid on each plate to lessen spreading growth. One triplicate set of strips was used as a control (no heat), and six or more sets were heated for the desired times. Three uninoculated strips were processed as sterility controls, and counts of survivors were made after 24 and 48 hr of incubation at 32 C. All manipulations of sterile items, with the exception of transferring triplicate sets of strips from sterile petri plates into the oven and vice versa, were performed in class 100 horizontal laminar flow clean benches to eliminate background contamination (9).  $D_{125\text{ C}}$  values were determined from a best-fit regression line of the data points by using a least squares method. The variation of the data points around each regression line was measured by calculating a standard error of the estimate.

**Isolations and spore preparations.** Colonies were picked randomly from pour plates of heated and unheated strips of selected soils and streaked for isolation on TSA. Pure cultures were maintained on TSA slants at 4 C. Spore preparations were made of selected isolates in the following manner. A turbid suspension of cells in sterile BDW was heat-shocked in a water bath at 80 C for 15 min. The suspension was then lightly swabbed onto TAM Sporulation Agar (Difco) supplemented with 20  $\mu\text{g}$  of  $\text{MgSO}_4$  per ml and 80  $\mu\text{g}$  of  $\text{CaCl}_2$  per ml. After 2.5 hr of incubation at 32 C, growth was swabbed onto a fresh plate of medium. This procedure was performed twice. After the last incubation period, growth from the final plate was swabbed onto three plates of fresh medium and incubated at 32 C for 24 to 48 hr. Most isolates exhibited maximum sporulation at these times with only a few requiring up to 96 hr of incubation. Growth from the three final plates was harvested in approximately 20 ml of chilled BDW and insonated for 90 sec with a Biosonic III Ultrasonic Probe (Bronwill Scientific, Rochester, N.Y.) at 60% maximum intensity. The suspension was then centrifuged for 15 min at 4 C ( $8,590 \times g$ ; Servall SS-1, Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant fluid was discarded, and the cellular debris layer of the pellet was removed by gentle washing with 5 ml of chilled BDW. The remainder of the pellet, consisting mainly of spores, was resuspended and washed twice more in the same man-

ner. Simple staining with crystal violet and microscopic examination were used to determine if more washings were necessary. The pellet was then washed three times with 95% ethyl alcohol and resuspended for storage at 4 C. Subsequent heat inactivation studies were performed in the same manner as described for the ethyl alcohol-soil suspensions.

**Model spore population.** A system to simulate the survivor curve of a mixed, naturally occurring spore population was developed by using one heat-sensitive and one heat-resistant aerobic mesophilic isolate from soil sample X (Phoenix). Clean spore crops were prepared, and  $D_{125\text{ C}}$  values of each ethyl alcohol suspension were determined in the presence and absence of sterile soil from the parent sample to determine the characteristics of the respective survivor curves. The two isolate suspensions were then mixed in a proportion similar to the heat-sensitive and heat-resistant populations in the original soil. Sterile parent soil was added to a portion of this mixture to approximate the spore per unit volume of the original soil sample (0.3 g/ml). Dry-heat assays were performed in the same manner as the comparative  $D_{125\text{ C}}$  survey of soils.

**RESULTS**

During preliminary testing of the heating system, the soil sample collected in Phoenix (sample X) consistently produced a nonlinear survivor curve (Fig. 1), indicating a decrease in inactivation rate with time. In general, the survival data from the 23 soil samples tested subsequently did not appear linear, and the shapes of the survivor curves were similar to that of sample X.

The sample containing the most resistant spores was the one collected in Phoenix. To examine the possibility that the nonlinear survivor curves of this sample could have been due to protective effects offered by the small amount of soil particles present, various subcultured spore suspensions of heat-sensitive and heat-resistant isolates suspended in ethyl alcohol were tested in the presence and absence of sterile parent soil. Survivor curves

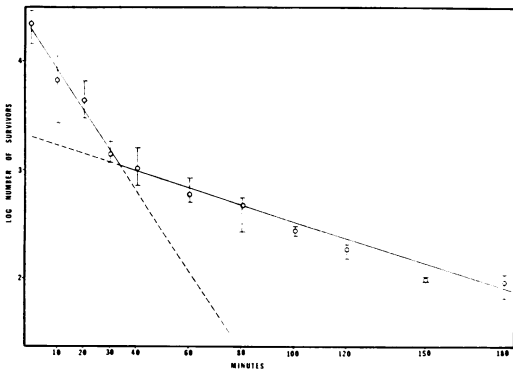


FIG. 1. Survival of naturally occurring spores in soil sample X at 125 C. Composite data from three experiments.

TABLE 1.  $D_{125\text{ C}}$  values of spore isolates and variation of data around best-fit lines

Sample description	$D_{125\text{ C}}$	Standard error of estimate	Coefficient of variation
	<i>min</i>		%
Sporulated isolate X-1 from soil X, heated without soil.....	8.6	0.088	2.2
Sporulated isolate X-1 from soil X, heated with soil.....	7.1	0.059	1.5
Sporulated isolate XA from soil X, heated without soil.....	52	0.011	0.4
Sporulated isolate XA from soil X, heated with soil.....	49	0.055	2.2
<i>B. subtilis</i> var. <i>niger</i> heated without soil.....	16	0.036	0.7

at 125 C were found to be linear, and the  $D_{125\text{ C}}$  values were unaffected by the presence of added soil (Table 1). Consequently, the soil per se did not seem to alter dry-heat resistance by physical protection. Also, the nonlinear survivor curves of the soil samples did not appear to result from technique-induced error since pure spore cultures of *B. subtilis* var. *niger*, *B. cereus* T, *B. subtilis* 5230, and several soil isolates have consistently yielded linear survivor curves at 125 C in our test system.

By expressing the standard error of the estimate for each line as a coefficient of variation, it was possible to compare the variation from a straight line in the soil suspension tests with the variation measured in like tests on spore isolates. These data are presented in Tables 1 and 2. It is evident that the coefficients of variation for the soil suspension tests are markedly higher than those for spore isolates, the mean value of the suspensions being more than five times the mean value of the isolates. The consistency in the shape of the 24 survivor curves for the soil suspensions coupled with the large coefficients of variation for straight lines fit to these data indicated that the survivor curves were not linear.

In an effort to define more clearly the nature of the nonlinear survivor curves observed with the soil samples, a model system described above was developed by using heat-sensitive and heat-resistant aerobic mesophilic isolates from soil sample X. Clean spore crops were prepared, and survivor curves of both suspensions were found to be linear and unaffected by the presence of added soil (isolates X-1 and XA, Table 1). When the two suspensions were mixed in the presence

TABLE 2.  $D_{125\text{ C}}$  values of naturally occurring spore populations in soil and variation of data around best-fit lines

Sample	Sample source	$D_{125\text{ C}}$	Standard error of estimate	Coefficient of variation
		min		
A	Cape Kennedy, Fla.	11	0.414	11.4
D	The Boeing Co., Seattle, Wash.	14	0.318	10.6
E	Jet Propulsion Laboratory, Pasadena, Calif.	12	0.248	10.3
G	McDonnell Aircraft Corp., St. Louis, Mo.	18	0.168	6.5
H	McDonnell Aircraft Corp., St. Louis, Mo.	16	0.158	8.7
I	McDonnell Aircraft Corp., St. Louis, Mo.	30	0.139	4.7
J	Langley Research Center, Hampton, Va.	16	0.214	8.9
L	Marshall Space Flight Center, Huntsville, Ala.	18	0.147	6.2
M	Marshall Space Flight Center, Huntsville, Ala.	23	0.157	6.5
N	General Electric Co., Valley Forge, Pa.	17	0.195	9.7
O	General Electric Co., Valley Forge, Pa.	30	0.120	4.8
Q	General Electric Co., Valley Forge, Pa.	12	0.428	17.9
S	TRW, Redondo Beach, Calif.	19	0.152	4.4
T	TRW, Redondo Beach, Calif.	15	0.141	4.2
U	TRW, Redondo Beach, Calif.	23	0.097	3.9
AA	Manned Space Flight Center, Houston, Tex.	12	0.249	6.9
AB	Manned Space Flight Center, Houston, Tex.	15	0.216	8.3
AC	Manned Space Flight Center, Houston, Tex.	10	0.274	7.4
AD	Manned Space Flight Center, Houston, Tex.	13	0.232	6.7
BA	Hughes Aircraft Co., Culver City, Calif.	21	0.100	3.5
CC	Goddard Space Flight Center, Beltsville, Md.	12	0.241	20.6
DA	Martin-Marietta Corp., Denver, Colo.	13	0.216	6.2
DB	Martin-Marietta Corp., Denver, Colo.	17	0.224	10.7
X <sup>a</sup>	Phoenix, Ariz.	38	0.141	4.2

<sup>a</sup> Values based on three experiments; all others based on single experiments.

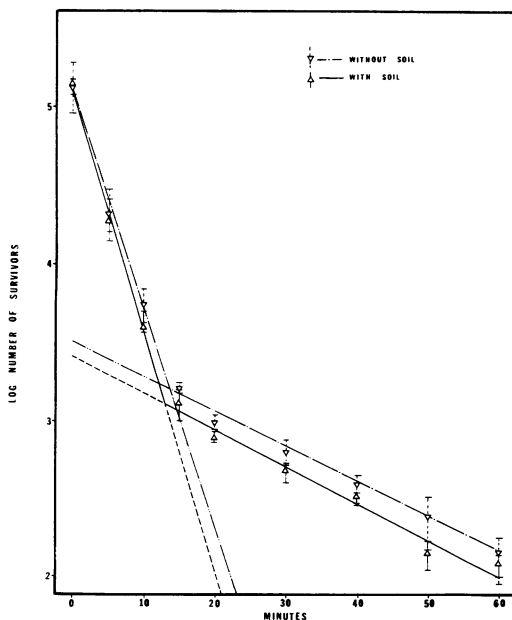


FIG. 2. Survival of a mixture of isolate X-1 and isolate XA spores in the presence and absence of parent soil. Composite data from four experiments.

and absence of soil and tested in parallel, the survivor curves were shown to be nonlinear and unaffected by the presence of soil (Fig. 2).

## DISCUSSION

During comparison of  $D_{125\text{ C}}$  values of naturally occurring spore populations in soils and respective subcultured spore isolates, another factor was noted which may significantly affect the current rationale behind calculation of spacecraft sterilization cycles. No spore isolates from heated soils were obtained which exhibited resistances equal to or greater than the portion of the survivor curves from which they were isolated. Consequently, it appeared that the subculture of the naturally occurring spores significantly lowered their dry-heat resistances. Whether these losses in dry-heat resistance were due to unsatisfied nutritional requirements or differences in environmental conditions or both is not known. However, studies by many other investigators have shown that the heat stability of spores may be altered significantly by manipulation of cultural conditions (14). Lowering of dry-heat resistance by subculture has also been noted with gram-negative organisms such as salmonellae (15). It must be reemphasized here that current space-

craft sterilization cycles are calculated on the basis of a standard reference spore population, i.e., *B. subtilis* var. *niger*. However, there is no evidence available to suggest that this organism or its thermal inactivation characteristics are in any way associated with actual microbial contaminants on spacecraft. Qualitative and quantitative microbiological assays conducted on many spacecraft while in residence at Cape Kennedy (Apollo and Mariner) have failed to reveal a single instance of contamination by *B. subtilis* var. *niger*.

Ernst (6) reviewed the thermal inactivation kinetics of bacterial spores and pointed out several pitfalls which may be encountered in the interpretation and subsequent extrapolation of death rate constants (*D* values). When studying the nonlogarithmic survival of mixed spore populations in soils, he found, as our observations also indicate, that isolates from the later phases of the survivor curves were less heat-resistant than the original tests would have indicated. From this observation, he assumed a mechanistic approach by stating that the more heat-resistant spores were "undoubtedly protected in some way in the soil menstroom." Based on this assumption, he concluded that the slope of a straight line passing through the point of extinction (obtained by end-point determinations) of a population would represent the largest possible *D* value of the unprotected spores. The findings in our study, however, indicate that when end-point data are used and logarithmic death of a naturally occurring spore population is assumed, there can be a significant error in estimating the time required to achieve a high probability of sterility (Fig. 3). This figure was adapted from a hypothetical curve presented by Ernst (6) in his illustration of the protective effects by soil.

The controversy regarding calculation of sterilization cycles in general can be appropriately described as a classical argument between two basic schools of thought. One school explains deviations from logarithmic survival in terms of inherent heterogeneity within a population, whereas the other school attributes these deviations to factors in operation within the lethal period. With particular regard to dry-heat inactivation of bacterial spores, members of either school would expect to observe nonlinear survival when dealing with mixed populations in soil. However, it remains that such nonlinear survival has been and is today explained entirely in terms of protective effects by the soil menstroom. This philosophy continues by reasoning that, when dealing with relatively low levels of surface contamination such as encountered with spacecraft, the protective effects of soil may be neglected with concomi-

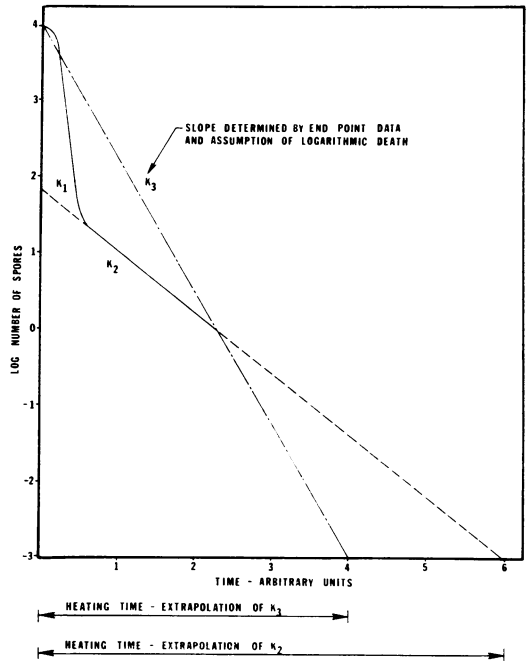


FIG. 3. Inactivation of a hypothetical spore population based on laboratory observations (6): calculated heating times necessary to effect a 7-log reduction.

tant assumption of logarithmic death. Data presented in this report indicate such an assumption to be workable provided that the probability of contamination by a particular population is not extrapolated below  $10^0$ . However, in extrapolation below measurable range, for example, to  $10^{-8}$ , the greatest possible *D* value of the population becomes critical and may not be accurately reflected by an end-point determination. Therefore, in dealing with sterilization of naturally occurring spore populations, it is recommended that assumption of logarithmic death in conjunction with a standard reference organism may well constitute an invalid model in certain applied situations, particularly in the field of spacecraft sterilization.

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