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Dry-Heat Resistance of Selected Psychrophiles

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The dry-heat resistance characteristics of spores of psychrophilic organisms isolated from soil samples from the Viking spacecraft assembly areas at Cape Kennedy Space Flight Center, Cape Canaveral, Fla., were studied. Spore suspensions were produced, and dry-heat D values were determined for the microorganisms that demonstrated growth or survival under a simulated Martian environment. The dry-heat tests were carried out by using the planchet-boat-hot plate system at 110 and 125°C with an ambient relative humidity of 50% at 22°C. The spores evaluated had a relatively low resistance to dry heat. D(110°C) values ranged from 7.5 to 122 min, whereas the D(125°C) values ranged from <1.0 to 9.8 min.

In the exploration of distant planets, it has been recognized that there is a definite need to prevent their contamination (9). For this reason, the National Aeronautics and Space Administration has, for the past several years, supported research in planetary quarantine. This involves many fields, including spacecraft sterilization (9). All possible factors related to sterilization should be investigated to assure that planetary exploration by terrestrial spacecraft does not alter the ecology of the planet under investigation or interfere with the search for life on other planets.

In an earlier paper on the characteristics of psychrophilic microorganisms (8), it was noted that this important microbial subpopulation may have been excluded from the microbial monitoring program. Although it is generally accepted that psychrophiles may not be the most heat-resistant group of microorganisms, they do possess physiological characteristics that may favor their growth in environments similar to those of the outer planets. Also, it is known that some sporeformers possess the ability to grow at low temperatures, and this group may include some of the more heat-resistant microorganisms.

The objective of this investigation was to determine the resistance of selected psychrophiles to dry heat, the lethal agent that was employed by the National Aeronautics and Space Administration to sterilize the Viking Lander.

For this investigation, psychrophiles are defined as microorganisms that grow and form visible colonies on solid media at 3°C within 7 to 10 days and will not grow above 24°C.

MATERIALS AND METHODS

Microbial cultures. Spore suspensions were prepared from 15 pure cultures of psychrophilic spore-forming organisms isolated from soil samples obtained from the Viking spacecraft assembly areas at Cape Kennedy Space Flight Center, Cape Canaveral, Fla. The cultures that were used had survived an exposure to a simulated Martian environment. The isolation of the psychrophilic organisms was carried out at 3°C.

Preparation of spores. The spores used in this investigation were allowed to germinate and grow on AK-2 sporulating agar (Baltimore Biological Laboratory, Cockeysville, Md.). The medium was supplemented with 0.8 ml of sterile 10% calcium chloride per 100 ml before the plates were poured.

Pure cultures of the desired organisms were washed with a sterile 0.07 M phosphate buffer (pH 7.0) and then heat-shocked at 80°C for 15 min. Portions of the heat-shocked suspension were inoculated onto the surface of supplemented AK plates with a sterile cotton swab. Plates were incubated at 3°C for 7 to 10 days or until sporulation occurred. A heavy suspension of spores in sterile, distilled water was prepared from the surface colonies of the AK plates. This suspension was heat-shocked at 80°C for 15 min and used to inoculate 10 fresh plates of AK agar. These plates were incubated for 3 to 5 days at 7°C. Each plate was checked for sporulation by preparing smears and examining the stained smears microscopically. Any plate where the relative number of cells per smear showing spores was not at least 90% after 7 days was discarded.

The growth from each plate was collected by using cold (<10°C), sterile, distilled water. The suspension was subjected to sonic treatment by placing it in an ultrasonic bath for 30 min at 25 kHz/s (Sonogen A, Branson Instruments Co., Stamford, Conn.) to break up spore clumps. The tank fluid was

0.3% (vol/vol) Tween 80 and water. The temperature of the bath was monitored and maintained at 5 to 10°C by the addition of ice. The spore suspension was then centrifuged at 2,400 rpm at 4°C in a refrigerated centrifuge. The spores were washed six times in sterile phosphate buffer and then rinsed twice in 95% ethanol, both by centrifugation. The spore preparations were titered and adjusted to ca. 10^8 spores per ml and then examined by the Schaeffer and Fulton modification of the Wutz staining method (6). The final suspension was then stored in sterile 95% ethanol at 3°C.

D-value determination. The objective of these studies was to compare data gathered on the survival characteristics of different psychrophilic spores under identical test conditions. All operations, prior to incubation of the plates, were carried out in a laminar downflow clean room. The clean room was maintained at 22°C and 50% relative humidity.

The procedures used in this investigation are those described by Drummond and Pflug (6, 7), in which stainless-steel planchets supported the spore deposits and rectangular copper boats supported the stainless-steel planchets during heating.

Every heating trial utilized 24 permanently numbered rectangular copper boats, each containing two planchets (0.5 by 0.5 inches [ca. 1.27 by 1.27 cm]) laid flat in a shallow groove running the length of each boat. The copper boats were cleaned with sterile, 95% ethyl alcohol and arranged in numerical order in four rows of six boats each on a sterile, stainless-steel sheet.

A 0.01-ml portion of spore suspension was deposited on each planchet. The boats containing inoculated planchets were transferred to a second sterile tray in the order in which they were to be later placed on a hot plate. Hot-plate position was determined by the use of a table of random numbers.

After the spores had been deposited, they were allowed to condition (dry and equilibrate) for 20 to 24 h in the laminar downflow clean room maintained at 22°C and 50% relative humidity. During this period, the boats and planchets were shielded from direct airflow by placing a sterile, stainless-steel tray over them; however, air could circulate over the planchet, since all sides were left open.

Using a table of random numbers, we assigned 21 of the boats, in groups of three, heating times, and the remaining three were used as nonheated controls. Heating schedules and hot-plate placement diagrams were made for each experiment. The copper boats with the planchets were placed on the hot plate and heated for the designated time. Upon completion of the heating time, the boat was immediately removed from the hot plate and placed on a cooling plate for a minimum of 3 min. All heating times were completed before the planchets were processed for spore recovery.

The two planchets from a boat were both transferred spore side down into a dry, sterile, prelabeled 125-ml Erlenmeyer flask. Based on the estimated number of survivors, 25 to 50 ml of 0.07 M phosphate buffer was added. Each flask was then subjected to sonic treatment at 25 kHz/s for 2 min. After sonic treatment, the flask was removed, and 1.0 ml of suspension was transferred to a 25- or 50-ml dilution blank. After mixing by shaking, 0.1-, 1.0-, and 10.0-ml portions were each plated in duplicate. The second dilution step was eliminated when planchets from longer heating times were processed. The 10-ml portions were plated with 15 ml of 1.5-strength Trypticase soy agar; all other volumes were plated with 20 ml of single-strength Trypticase soy agar. An estimated D value was used as the basis for selecting the heating time and the volumes to be plated, so that countable plates would be obtained at each time interval. The last heating time was selected to yield ca. 10^2 spores per two planchets.

The plates were inverted and incubated for 3 to 5 days at 21°C and counted with a Bactronic counter (New Brunswick Scientific Co., New Brunswick, N.J.).

Data analysis. The estimated number of surviving spores at each heating time was recorded on special data sheets and punched on data cards. The cards were sent to the University of Minnesota Biomedical Computer Center for analysis. The computer program carried out a least-squares regression analysis (not including N_0) to estimate a D-value and y-intercept (Y_0) for each time-temperature treatment combination. The intercept ratio was calculated by using the Y_0 and N_0 (N_0 = initial population).

In all experiments, the spores were heated at 110° and 125°C. Heating was carried out on a specially designed hot plate. The temperature of the hot plate was electronically monitored by thermocouples attached to two special copper boats. The thermocouples were connected to a temperatue-recording potentiometer.

Expected sources of variation were the physical characteristics of the boats, the position of the boats on the hot plates, the order of deposition, and the order of treatment. The experimental design included a randomization scheme designed to reduce the chance of systematic effects due to these sources.

RESULTS

The results of this investigation are summarized in Table 1. D_{110} values ranged from 7.54 (K-3-27) to 122 min (K-12-33), whereas the D_{125} values ranged from <1 (K-3-27 and K-11-123) to 9.78 min (K-3-110). The intercept ratio (IR) (IR = $\log Y_0/\log N_0$) is a gross measure of the shape of the survivor curve during the initial portion of the heat treatment (14). If the IR value is greater than 1.0, the curve is concave downwards (the curve may be thought to have a shoulder). If the IR value is less than 1.0, the initial portion of the curve is concave upwards. The IR value is used to help characterize the survivor curve and to test whether the line is linear through N_0. A linear line through N_0 has an IR value of 1.0. IR values ranged from 0.83 to 1.1, with spores K-1-6 and K-2-38 showing IR values of 0.99 and 1.0 at 110°C.

Eight of the 15 samples at 110°C had IR val-

TABLE 1. *Summary of D-value data for pure-culture psychrophilic spores isolated from Cape Canaveral soil when conditioned at 50% relative humidity at 22°C.*

Spore code	Heating temp (°C)	Log N_0	D-value (min)	95% Confidence limits		Z value (°C)[a]	IR
				Lower	Higher		
K-1-6	110	6.64	30.10	28.89	31.42	17.12	0.99
	125	6.63	4.01	3.55	4.62		1.07
K-1-8	110	6.67	21.28	20.15	22.53	17.61	1.02
	125	6.68	2.99	2.81	3.21		1.10
K-2-38	110	4.49	11.30	10.44	12.47	26.00	1.00
	125	4.49	1.83	1.01	2.02		1.04
K-3-27	110	6.59	7.54	6.49	9.01	–	0.87
	125	–[b]	–	–	–		–
K-3-89	110	6.20	8.10	7.59	8.68	35.13	1.01
	125	6.16	3.03	2.39	4.11		0.96
K-3-110	110	6.21	43.37	33.55	49.57	23.08	0.96
	125	5.89	9.78	9.24	10.39		0.93
K-4-118	110	6.96	15.74	14.24	17.60	19.47	0.91
	125	6.96	2.67	2.24	3.29		0.83
K-7-137	110	6.51	22.74	20.32	25.04	19.01	1.04
	125	6.44	3.70	3.27	4.26		1.07
K-7-140	110	6.99	17.91	16.99	18.91	22.35	1.04
	125	6.97	3.82	3.52	4.17		1.10
K-8-42	110	7.00	34.16	32.39	36.14	18.36	1.03
	125	7.05	5.21	4.91	5.55		1.07
K-10-99	110	6.90	29.81	27.49	32.57	18.77	1.03
	125	6.90	4.73	4.35	5.18		1.04
K-10-113	110	6.70	33.09	31.67	34.65	17.69	0.92
	125	6.89	4.70	4.48	4.93		1.03
K-11-38	110	6.12	30.27	27.68	33.40	16.29	1.02
	125	6.14	3.63	3.33	4.04		1.08
K-11-123	110	6.53	12.175	10.80	13.86	–	0.90
	125	–	–	–	–		–
K-12-33	110	4.91	122.00	115.16	130.73	12.05	0.93
	125	6.76	6.94	6.56	7.36		1.08

[a] Z = Change in temperature to change D value by factor of 10.
[b] D values were less than 1.0; therefore, no other data could be computed.

ues of ≤1.0 (Table 1). Three had values of 0.99 to 1.01, denoting a linear response to the heating cycle. Spore K-3-27 had a much lower IR value at 110°C, denoting a fairly pronounced population decrease at the beginning of the heating period, followed by a longer, slower population decrease. The D_{110} value of spore K-3-27 was 7.5 min. The survivor curve for spore K-3-27 shows this deviation from linearity, as predicted by the IR value. The other spores, when heated at 110°C, had IR values of >1.0 or had a slower initial population decrease followed by a more rapid one. As indicated by the IR values (Table 1), none of these survivor curves has very pronounced shoulders. The IR values ranged from 1.02 to 1.04, denoting a fairly linear response by the remaining 7 spore suspensions.

The D_{125} values are shown in Table 1, and a typical survivor curve at 125°C is shown in Fig. 1. D_{125} values ranged from <1 min, for spores K-3-27 and K-11-123, to 9.78 min, for spore K-3-

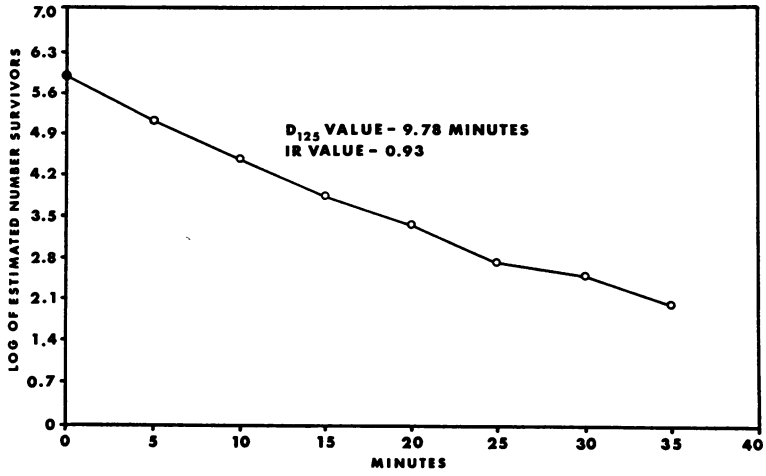

FIG. 1. *Survivor curve for spore K-3-110 that was heated at 125°C at an ambient relative humidity of 50%.*

110. *D* and IR values for the low-resistant spores K-3-27 and K-11-123 are not reported because of possible errors in the very short heating times that would have to be used with these extremely low-resistant spores. The IR values ranged from 0.83 to 1.10. The survivor curve for spore K-3-110 appears to be concave upwards and should have an IR value of <1.0, which it does (0.93, Table 1). Spore K-2-38 shows a survivor curve that is concave downwards and should have an IR value >1.0. An IR value of 1.04 is shown for spore K-2-39 (Table 1).

DISCUSSION

It has been demonstrated that psychrophilic sporeformers are present in the soil from the assembly areas of the Viking spacecraft (8) at the Cape Kennedy Space Flight Center. On primary isolation, many of these sporeforming organisms failed to grow at 32°C and, therefore, may have been excluded from the Viking microbial monitoring systems. For this reason, it was desirable that dry-heat studies be performed on representative psychrophilic sporeformers isolated during these investigations. The organisms selected for this investigation possessed characteristics that caused them to be of interest to planetary quarantine research. They are sporeformers and facultative anaerobes, capable of growth at low temperatures, and have demonstrated ability to grow in a simulated Martian environment (unpublished data).

This study was done primarily to explore the scope of the problem of survival of psychrophilic spores and to make a preliminary study of their heat sensitivity. The experimental conditions used during this investigation included heat treatment at 110 and 125°C, with preconditioning of spores performed at a relative humidity of 50% and 22°C.

Previous investigations on the effects of dry heat used either mesophiles or thermophiles that were isolated from areas remote from the spacecraft (18). The results of this study (Table 1) indicate that pure-culture isolates of psychrophilic spores are fairly sensitive to dry heat at 110°C and even more sensitive at 125°C. D_{110} values in this investigation ranged from 7.54 to 122 min, whereas D_{125} values ranged from <1 to 9.78 min. Pflug (7), who worked with *Bacillus subtilis* subsp. *niger*, has reported D_{110} values of 136 to 167 min and D_{125} values of 16.6 to 32.3 min. In our study, only spore K-12-33 approached these D_{110} values (Table 1). Spore K-12-33 had a D_{110} value of 122 min, whereas *B. subtilis* subsp. *niger* had a D_{110} value of ca. 151 min. At 125°C, none of the psychrophilic isolates showed *D* values approaching those of *B. subtilis* subsp. *niger*.

Among our psychrophilic isolates, the highest D_{125} value was 9.78 min (K-3-110). *B. subtilis* subsp. *niger* is now used as one of the standard dry-heat indicator organisms, due to its fairly high resistance to dry heat.

Recent work on the dry-heat resistance of bacterial spores has indicated a critical role for water (1-3, 12, 13). Drummond and Pflug (7) 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 term dry heat obviously implies the application of heat in the absence of water. A wet- or moist-heat sterilization cycle may be defined as one in which the

organism is in contact with an environment having an a_w of 1.0 or a water-saturated atmosphere. These conditions are met only when the organism is heated in contact with pure water or saturated steam. This definition of wet heat implies that dry heat is not an equally specific condition but, rather, a range of conditions. For this reason, the relative humidity throughout this investigation was controlled and kept at 50% at 22°C to standardize the procedures and, therefore, keep the relative humidity from becoming a variable. No attempt was made to study the effect of relative humidity on the dry-heat resistance of the psychrophilic spores. Further investigations should be conducted to establish the psychrophilic response to various dry-heat cycles at different relative humidities.

It has been established experimentally that the dry-heat resistance of psychrophilic spores isolated in the pure state is low. These same psychrophilic organisms when heated in their natural mixed populations or with soil particles of various sizes may react differently. The dry-heat sterilization cycle used for sterilizing the Viking Lander gives a high probability for selecting against psychrophilic sporeformers, even when they are present in large numbers.

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