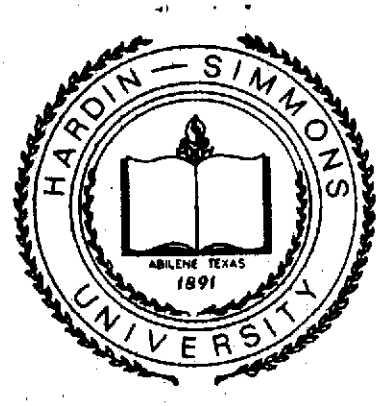

February, 1974

Report No. 3
July 1, 1973 - December 31, 1973

A STUDY OF PSYCHROPHILIC ORGANISMS ISOLATED FROM THE MANUFACTURE AND ASSEMBLY AREAS OF SPACECRAFT TO BE USED IN THE VIKING MISSION

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A STUDY OF PSYCHROPHILIC ORGANISMS ISOLATED FROM THE MANUFACTURE AND ASSEMBLY AREAS OF SPACECRAFT TO BE USED IN THE VIKING MISSION

Report No. 3 of Planetary Quarantine Activities
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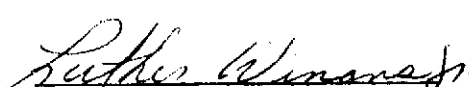

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February, 1974

A STUDY OF PSYCHROPHILIC ORGANISMS ISOLATED FROM THE MANUFACTURE AND ASSEMBLY AREAS OF SPACECRAFT TO BE USED IN THE VIKING MISSION

Presented at the

Semi-Annual NASA Spacecraft Sterilization
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FOREWORD

This third report summarizes work performed for the National Aeronautics and Space Administration by the Department of Biology at Hardin-Simmons University, supported by NASA Grant NGR 44-095-001 (Supplement No. 1), and covers the period of July 1, 1973 - December 31, 1973.

This report includes continued investigations of the ability of psychrophilic microorganisms to grow in some of the environmental conditions suggested for Mars, with particular attention given to the effects of moisture and nutrients on growth. Results of growth with the slide culture technique are presented (procedures given in H-SU Report No. 2) and indicate that this technique can be a rapid and sensitive technique for demonstration of microbial growth under various environmental conditions. Additional soil samples have been obtained from Cape Kennedy, and results of these assays at various low temperatures for psychrophilic populations are presented. The heat resistance of some of the psychrophilic sporeformers have been determined.

At the request of the Planetary Quarantine Office, psychrophilic organisms were isolated from the teflon ribbons at Cape Kennedy and characterization of these was begun. In addition, heat-survivors from the Cape Kennedy teflon ribbons are being investigated at H-SU, and partial characterizations of these are presented. The report also includes a description of work currently in progress and future work planned for the remainder of the contract period.

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A STUDY OF PSYCHROPHILIC ORGANISMS ISOLATED FROM THE MANUFACTURE AND ASSEMBLY AREAS OF SPACECRAFT TO BE USED IN THE VIKING MISSION

Introduction

This project was originally begun to characterize the psychrophilic microbial populations from soil samples associated with the Viking spacecraft. Earlier results have demonstrated that the samples from the manufacture area in Denver, Colorado possess very few or no spore-formers which are obligate psychrophiles, while samples from the assembly area at Cape Kennedy contain significant populations of psychrophiles, including sporeformers, which will not grow at 32°C. The procedures for these assays and the characterization of the populations are presented in H-SU Preliminary Report (January, 1973) and H-SU Report No. 2 (July, 1973).

Soil samples from both areas and pure cultures of psychrophilic sporeformers were subjected to an experimental Martian environment to determine their ability to grow under these conditions. Upon assay, survivors were incubated at 7°C to demonstrate the ability of low-temperature organisms to grow under these conditions. These pure cultures were also inoculated onto agar-coated slides to evaluate this procedure for determination of microbial growth in the experimental Martian environment (Report No. 2).

This third report presents a continuation of investigations begun earlier and several new ones which have begun as an outcome of previous results. This project period includes an investigation of the effects of moisture and nutrients on growth of selected isolates, and

presentation of results of the slide culture technique. After completion of this task, it was decided to redesign the apparatus for establishment of the experimental Martian environment primarily to more effectively purge the sample vessels, to allow for a more rapid and reliable closure of the vials, to reduce the moisture level of the samples, to allow more time for equilibration of the sample to the new environment, and to allow a more simplified method for alternation of various gas mixtures into the system. This new design will allow the use of additional atmospheres such as 99% CO_2 + 1% O_2. The presence of 1% O_2 was suggested by planetologists recently (telephone conversation with Dr. Hall), and we will use this mixture in addition to our original mixture of 80% CO_2 + 20% Argon. The redesigned apparatus has just recently been completed, and a continuation of this phase of our investigations will be initiated very soon.

Because the previous investigations have revealed the presence of sporeformers in Cape Kennedy soils which did not grow at 32°C on primary isolation, it was decided to assay fallout samples from Cape Kennedy. At the request of Dr. Hall during the July, 1973 AIBS seminar in Denver, plans were initiated to perform a run of the teflon ribbon experiments at Cape Kennedy with incubation of N_o samples and heat-treated ribbons at 7°C in an attempt to isolate psychrophilic sporeformers from the MSOB fallout samples. It was also decided to perform dry-heat studies on such isolates and on original psychrophilic sporeformers previously isolated from Cape Kennedy. This work was done at the University of Minnesota by Mr. Luther Winans under the supervision of Dr. I. J. Pflug.

At the time of collection of the teflon ribbon isolates from Cape Kennedy, new soil samples were obtained for determination of psychrophilic populations. In addition to the 7°C temperature of isolation, other low temperatures were used in performing counts of these organisms, and attempts were made to isolate these at sub-zero temperature. To perform this task, methods were attempted to prevent freezing of the medium or to allow the counting of colonies on the surface of frozen media. As reported here, the results were not encouraging for sub-zero counts, but this area is still under consideration for further study.

At the request of Dr. Hall, this project was expanded somewhat to include a study of sporeformers from Cape Kennedy which survived the simulated Viking dry-heat cycle. These studies include a detailed characterization of these isolates, and an evaluation of their ability to survive vacuum conditions. Preliminary results of these investigations and plans for the next project period are presented.

Procedures

Several procedures used in this project were presented in previous reports and will not be presented in detail.

Response of Original Soil Samples to the Experimental Martian Environment

The response of heat-shocked and non-heat-shocked soil samples from Denver and Cape Kennedy after 21 days in the experimental environment was presented in Report No. 2. This incubation was continued for 60 days, and those results are presented and discussed.

Slide Culture Technique

Pure cultures of psychrophilic isolates from the manufacture and assembly areas of the Viking spacecraft were inoculated into an artificial Martian soil and subjected to the experimental Martian environment. Results presented in the last report indicated that there was no increase or decrease in the population of these pure cultures after 21 days in this environment.

These pure cultures were also inoculated onto agar-coated slides for determination of growth based upon the slide culture technique as described by A. A. Imshenetsky at the 1972 COSPAR meeting in Madrid. This procedure was described in Report No. 2, but will be briefly repeated here. Standard microscope slides (25 x 75 mm) were cut in half longitudinally, cleaned, and sterilized. They were then covered with a thin layer of TSA (BBL) or artificial Martian soil extract (AMS)and allowed to dry overnight at 45°C. Multiple slides were inoculated with one of the 24 pure cultures until all had been inoculated onto both TSA and AMS coated slides. The inoculum size was determined by making suspensions which allowed 50 - 100 cells per microscope field at 1000X in 0.01 ml. of the suspension. The inoculated slides were then placed into sterile 16 x 150 test tubes containing a saturated solution of K_2SO_4, subjected to the experimental Martian environment, and heat-sealed. Duplicate slides of each sample were removed immediately and phase-contrast micrographs at 1000X were made and designated as Day 0. The remaining tubes were subjected to the freeze-thaw cycle for 10 - 14 days, with duplicate slides being removed and photographed as for the Day 0 slides. By making comparisons of the Day 0 and subsequent micrographs, determinations of growth of the selected pure cultures could be made.

Evidence of growth was considered positive upon the formation of microcolonies.

Response of Selected Microorganisms to Different Moisture and Nutrient Levels in the Experimental Martian Environment

Upon completion of the pure culture studies, a selected spore-former (K-1-6) and a gram-positive, non-sporeformer (K-11-1) were studied further to determine the effects of moisture and nutrients on their ability to grow in the Martian environment. These were selected because they are psychrophilic, facultative anaerobes, and demonstrated an ability to survive the conditions of the simulated Martian environment. The tube method was used in order to quantitate the results and to give a better understanding of the population changes that were occurring. The experimental conditions employed in this part of the investigation were divided into different groups which are presented in Table 1 (p. 6).

The test organisms were washed three times with phosphate buffer and were suspended in the buffer at a final concentration of approximately 10^7 cells/ml. For inoculations the stock suspension was diluted to 1:10 and 1:100. One milliliter of the 1:100 dilution, 0.1 ml of the 1:10 dilution, and 0.01 ml of the stock suspensions were inoculated into the experimental tubes to provide an inoculum of approximately 10^5 cells at the desired moisture level.

Psychrophilic (7^oC) Isolates from Cape Kennedy Teflon Ribbon Experiments

In September, 1973, Mr. Puleo of the JPL Planetary Quarantine Lab at Cape Kennedy agreed to perform one run of the teflon ribbon fallout samples from the MSOB according to standard procedures with exception

Table 1. Experimental groups to determine the effect of moisture and nutrient levels on growth in the simulated Martian environment

GROUP	NUTRIENT (1 gm/tube)	MOISTURE* (ml H_2O/gm soil)
I	Martian soil mixture	0.01 0.1 1.0
II	Martian soil mixture + 1% peptone (w/w)	0.01 0.1 1.0
III	Martian soil mixture + 1% TSB with yeast extract (w/w)**	0.01 0.1 1.0

*Moisture levels are considered as: low, less than 1% (w/w); intermediate, 10%; excess, greater than 10%

**Consists of dehydrated TSB with 3% yeast extract

that all incubations would be performed at 7°C instead of 32°C. This was done in an attempt to isolate psychrophilic organisms from fallout samples in a spacecraft environment at Cape Kennedy. All manipulations and incubations were performed at Cape Kennedy to prevent loss in shipping, and all isolates were forwarded to H-SU for study. The N_o determinations yielded only three isolates from the non-heat-shocked samples and seven from the heat-shocked samples. Test ribbons subjected to the 113°C dry-heat cycle yielded no organisms growing at 7°C.

Only limited investigations of the ten isolates from the N_o determinations have been initiated, but temperature studies have been performed and dry-heat studies are being written up soon for some of these. The temperature studies were qualitative only, and attempts were made to grow these at 0°C, 7°C, 24°C, and 32°C. Alcohol suspensions (spore crops) were attempted on all but one of these (a yeast), and those that yielded high titers (greater than 10^6/ml.) were taken to the University of Minnesota for dry-heat studies.

Psychrophilic Counts From Cape Kennedy Soil Samples

Soil samples were collected from Cape Kennedy on September 13, 1973. Sample sites are given in Table 2 (p. 8). These soil samples were diluted and plated according to the procedures given in H-SU Preliminary Report, except that temperatures used for incubation were 10°C, 7°C, 0°C, and -5°C. Countable plates incubated at 0°C were selected for further study. Colonies were transferred to five TSA slants and incubated at 0°C, 3°C, 7°C, 24°C, and 32°C, and the time required for growth to appear was recorded. The percentage of organisms growing at low temperature, but not at 32°C was determined.

Table 2. Sites from which Cape Kennedy soil samples were collected for psychrophilic bacterial counts

CODE	SOURCE
A	Bldg. AO - In front of high bay area (west side)
B	Complex 41 - Titan-Centaur Project
C	Area 60A - Fuel storage area
D	Bldg. M7-1469 - In front of high bay area (south side)
E	Bldg. AO - Main personnel entrance (east side)
F	Bldg. M7-1469 - In front of low bay area (north side) fill dirt
G	Bldg. M7-1469 - In front of low bay area (north side) native soil

Attempts to Count Bacteria at Sub-zero Temperatures

In this investigation a single soil sample was used (Sample K-1, H-SU Report No. 2). The sample was placed into a 1% peptone solution, one-half was heat-shocked, and one-half was untreated. These were then diluted and inoculated onto the surface of the following: (1) TSA, (2) TSA + 3% Agar, (3) TSA + 5% glycerol, (4) TSA + 7.5% glycerol, and (5) membrane filtered and placed on the surface of TSA. These plates were then incubated aerobically at 7°C, 3°C, 0°C, -5°C, and -10°C.

Heat Survivors from Cape Kennedy Teflon Ribbon Experiments

At the request of Dr. Hall, cultures of organisms which had survived the 113°C dry-heat cycle from the Cape Kennedy teflon-ribbon experiment were obtained from the JPL Planetary Quarantine Lab at Cape Kennedy. The 33 cultures were obtained on September 14, 1973 and returned to H-SU.

They were grown on TSA supplemented with 0.1% soluble starch and 0.2% yeast extract. Upon arrival at H-SU, the cultures were streaked onto supplemented TSA plates and determined to be pure and viable.

Identification of these isolates had been performed at Cape Kennedy immediately upon isolation according to the procedures given in Phoenix Report No. 39. This report also described the biochemical variability of Bacillus isolates following different methods of preservation. This phenomenon has also been observed at H-SU when identification procedures were performed on these same 33 heat-survivors in our lab.

In addition to performing required tests for identification, additional investigations were undertaken to more completely characterize these isolates. These studies are only partially complete at present, but the completed ones will be reported here.

1. All cultures were inoculated into supplemented TSA slants and placed into Brewer Anaerobe Jars with appropriate controls (Alcaligenes fecalis) to determine their oxygen requirements.
2. Thirty-two of the thirty-three isolates were inoculated into numerous different carbohydrates, and their reactions were recorded.
3. Supplemented TSB was diluted 10^0 - 10^{10}. These were inoculated with 0.1 ml. of suspensions, which had been standardized by use of the Spec 20, and were incubated at 24°C for 4 days. Samples were removed and turbidity of each dilution was measured by recorded transmitted light at 565 nm on the Spec 20.
4. Supplemented TSB was prepared and adjusted to various pH

ranges at 0.5 pH units from 5.0 to 9.5. These were inoculated with a standard suspension of the various isolates and incubated at 24°C for 4 days. Samples were removed, turbidity was determined on the Spec 20 at 660 nm, and pH curves were plotted for each organism.

5. Supplemented TSB was prepared with salt concentrations from 1% to 10% at 1% increments. These were inoculated with a standard suspension of the heat-survivors, incubated at 24°C for 4 days, and turbidity was again determined with the Spec 20 at 660 nm. From these data, the optimum salt concentration was determined for each isolate.

6. At the request of Dr. Hall, an experiment was designed to determine the effect of storage of the spores of the 33 heat-survivors under vacuum for short periods of time. This task was only recently begun, and because of difficulty in culturing some of these organisms for preparation of the spore suspensions, only 21 of the 33 isolates have been subjected to the vacuum conditions, and these for periods of only 14-26 days. Results of the other 12 and results of extended storage will be reported later.

Spore suspensions were prepared according to standard procedures, washed in phosphate buffer, washed in 95% ethanol, and finally stored in 95% ethanol. Spore titers of these suspensions were performed, and the suspensions were diluted to give approximately 10^5 - 10^6 spores/ml. Sterile 1.0 ml plastic cups were inoculated with 0.1 ml of the spore suspensions and allowed to dry under laminar-flow for 12 hrs. The cups were then placed into Virtis Bulk Vac Bottles (Virtis #BCB-84CA),

and half of these bottles were subjected to a vacuum of 10^{-2} Torr before sealing, while the other half were sealed at atmospheric pressure. Three bottles of each were placed at -65°C, 20°C, and 55°C. Uninoculated controls received identical treatment and were incubated with the test cups.

At various time intervals, samples will be removed, transferred to separate bottles of 1% Tween 80, insonated (cups turned downward or toward the power source), diluted in 1% peptone, and plated in duplicate in molten supplemented TSA. At the time of this report, counts have been performed after 14-26 days of incubation of the cups.

Results

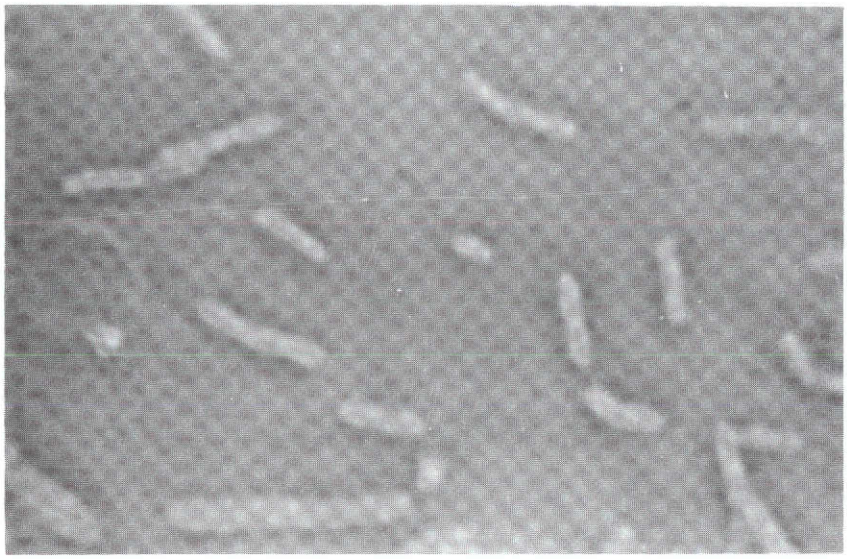
Response of Original Soil Samples to the Experimental Martian Environment

As described in H-SU Report No. 2, changes in populations in the soil samples incubated in the experimental Martian environment were considered significant only if there was a change of at least one log. Results of incubation after 60 days in this environment demonstrated that some of the populations continued to increase while others declined to below a countable level (less than 10^2 in this study). In the three non-heat-shocked samples from Denver (manufacture area), samples M-1 and M-2 showed a continuing decline in population from the count at 21 days, while sample M-3 showed an increase of more than one log over the 21-day count. All three heat-shocked samples showed a decline in populations when incubation was extended to 60 days.

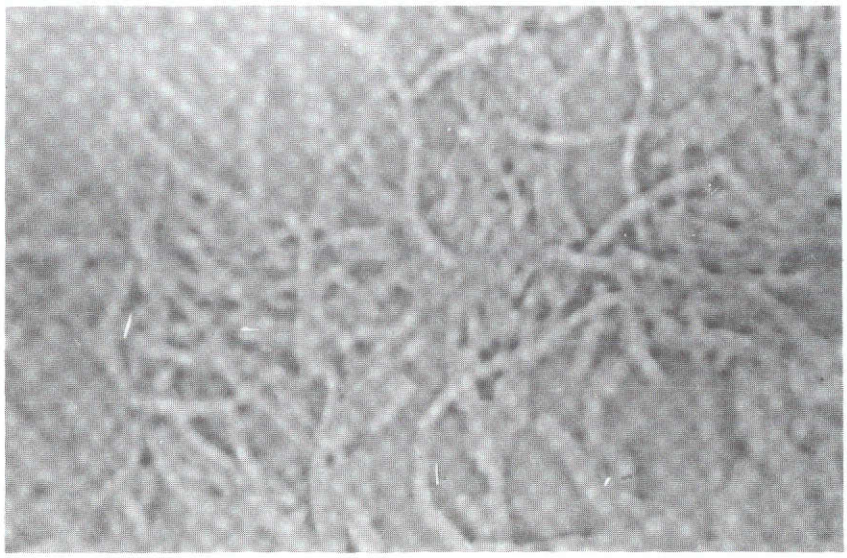
The samples from Cape Kennedy demonstrated a variable response to incubation in this environment after 60 days. Of the non-heat-shocked samples, populations in K-2, K-6, K-7, K-8, K-10, K-11, and K-12 (7 of 11 samples) showed a decline; K-1 showed no change; and samples K-3 and K-4 were the only two of eleven samples which demonstrated an increase from 21 to 60 days. Of the heat-shocked samples, populations in K-1, K-5, K-6, and K-10 showed a decrease; K-2, K-3, K-4, and K-12 showed no change; and only samples K-7 and K-8 showed an increase of greater than one log from 21 to 60 days incubation.

Slide Culture Technique

Examples of results of this part of the investigation are presented in Figure 1A and Figure 1B (page 13). Figure A is an example of the



(A)



(B)

Fig. 1. Phase contrast micrographs (1000X) of psychrophilic isolates from Cape Kennedy after 0 days incubation (A) and 10 days incubation (B) on agar-coated slides in the experimental Martian environment.

appearance of isolated cells on the agar-coated slides at Day 0. All suspensions were diluted to give a similar appearance, with the cells scattered and in low density, immediately after inoculation. Micrographs were made of duplicate slides of all 24 pure cultures on both TSA and artificial Martian soil extract (AMS) agar-coated slides. This was done to assure that none of the inoculations produced overcrowding of cells, which might later lead to misinterpretation of results.

Figure B is an example of a micrograph after ten days incubation in the experimental Martian environment. Again, micrographs were taken of duplicate slides of all 24 isolates on both types of agar-coated slides. These micrographs were compared to those prepared at Day 0; and, as can be seen by a comparison of the two figures, it appears that microcolonies have been formed on the slide. This formation of microcolonies is considered evidence of growth according to the method described by Imshenetsky.

Of the 24 pure cultures examined by this procedure, all showed the formation of microcolonies on both TSA and AMS agar-coated slides. Results of this technique are qualitative only, but the microcolonies on TSA slides were generally more dense than those on the AMS slides. Of these 24 pure cultures examined in this part of the investigation, fifteen were sporeforming rods.

Response of Selected Microorganisms to Different Moisture and Nutrient Levels in the Experimental Martian Environment

The effect of moisture and nutrients on the growth of microorganisms in the simulated Martian environment was determined by subjecting a sporeformer (K-1-6) and a non-sporeformer (K-11-1) to various nutrient and moisture conditions. The results of this

investigation are presented in Figures 2, 3, and 4 (pages 16, 17, and 18) and indicate that both factors have a definite effect on ability of the organisms to grow. It is apparent from Figures 3 and 5 that the sporeformer is better able to survive more harsh conditions than the non-sporeformer because the latter decreases more drastically in population. This survival ability is no doubt attributed to the formation of spores.

The effect of moisture on growth of these isolates is seen in these three figures. In all soil mixtures, only those organisms with excess water (1.0 ml/gm soil) were capable of growth. At lower moisture levels the sporeformer was able to survive, but the non-sporeformer showed decreasing populations. That nutrients affect growth is seen by comparing the growth of the sporeformer on the three different media. As can be seen, it was unable to grow in the simulated Martian soil, it grew slowly after 7 days in the soil enriched with 1% peptone, but it grew rapidly after 2 days in the soil enriched with 1% TSB and yeast extract. In contrast to this, the non-sporeformer showed growth at all nutrient levels if excess water was available.

These figures also demonstrate that even if the test organisms are going to grow, they do not do so within the first two days. This could be due to the organisms' being transferred from a more favorable environment to the shock of the artificial Martian environment. After they become accustomed to this new environment, they can then grow if moisture and nutrients are available.

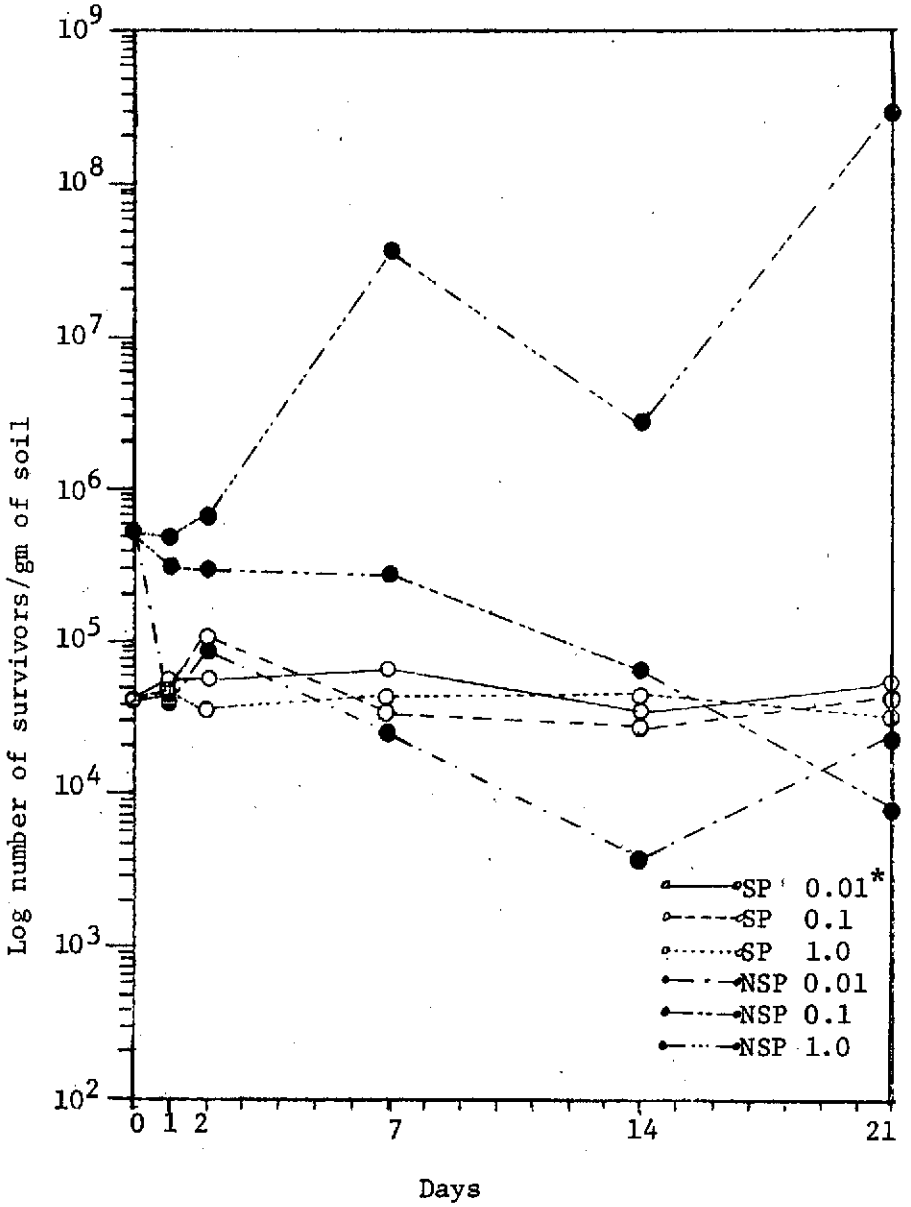


Fig. 2. Effect of the simulated Martian environment on the growth of selected psychrophilic microorganisms in artificial Martian soil at different moisture levels

*SP = sporeformer
NSP= non-sporeformer
ml H_2O/gm soil

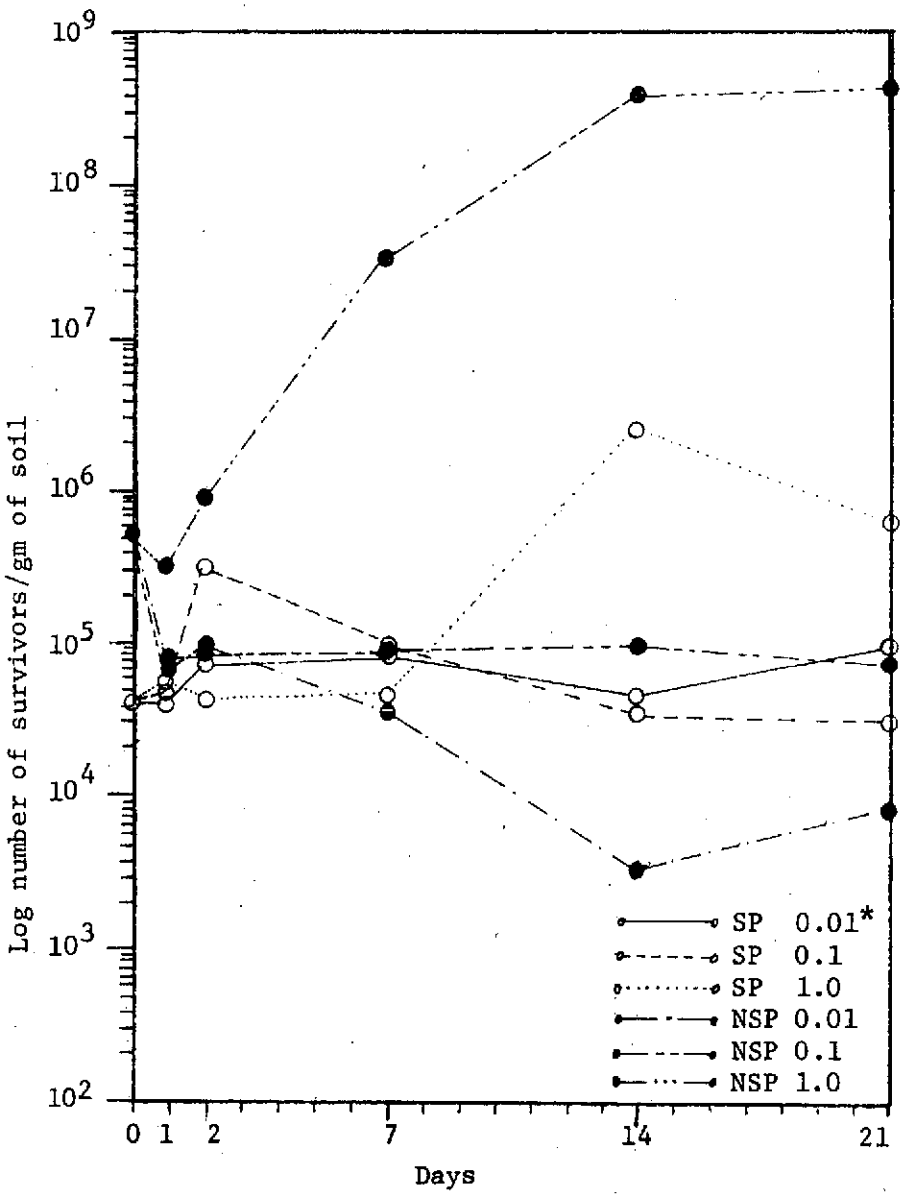


Fig. 3. Effect of the simulated Martian environment on the growth of selected psychrophilic microorganisms in artificial Martian soil plus 1% peptone at different moisture levels

*SP = sporeformer
NSP= non-sporeformer
ml H_2O/gm soil

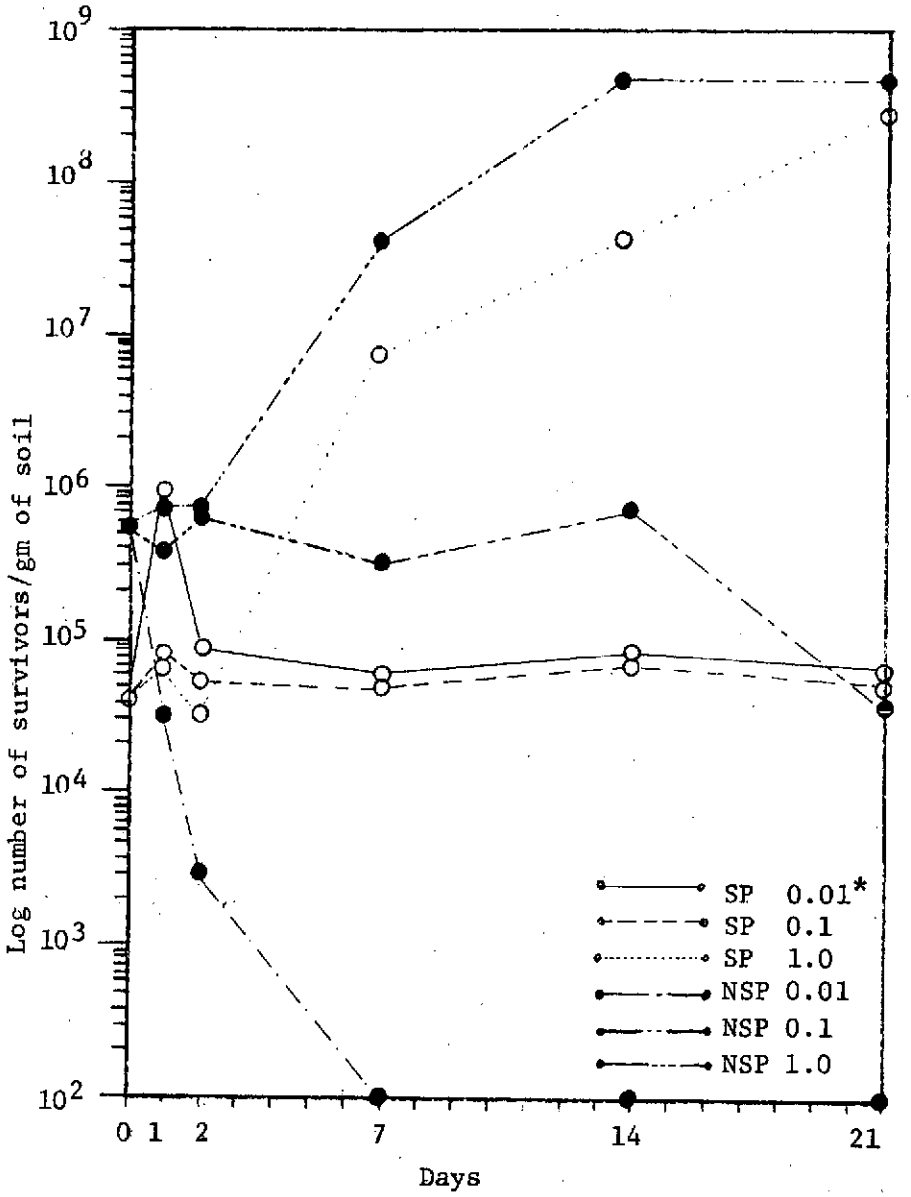


Fig. 4. Effect of the simulated Martian environment on the growth of selected psychrophilic microorganisms in artificial Martian soil plus 1% TSB and yeast extract at different moisture levels

*SP = sporeformer
NSP= non-sporeformer
ml H_2O/gm soil

Psychrophilic (7°C) Isolates from Cape Kennedy Teflon Ribbon Experiments

Of the ten isolates from the N_o determinations (3 from the non-heat-shocked samples and 7 from the heat-shocked samples) performed by Mr. Puleo, all were capable of growth at 32°C. Only two of these (18AH and 6BH) were capable of growth at 0°C in 14 days. One of the (6B) was a yeast; one (19A) was a non-sporeforming rod; and the remainder were sporeformers. After preparation of ethanol spore suspensions, four of these (6BH, 10AH, 18AH, and 18BH) were taken to the University of Minnesota for dry-heat studies.

Psychrophilic Counts From Cape Kennedy Soil Samples

Populations of organisms from various Cape Kennedy soil samples are presented in Table 3.

Table 3. Bacterial counts from Cape Kennedy soil samples incubated at various low temperatures (CFU/gm soil)

Sample	10°C	7°C	0°C	-5°C
A	2.5×10^5	3.0×10^3	0	0
B	1.4×10^5	6.7×10^2	0	0
C	1.4×10^5	0	0	0
D	7.9×10^5	3.9×10^4	4.8×10^3	4.6×10^2
E	8.0×10^5	7.3×10^3	1.0×10^3	1.0×10^2
F	3.0×10^4	1.9×10^4	6.3×10^3	2.1×10^3
G	4.9×10^7	5.1×10^6	1.1×10^4	2.0×10^3
Mean	7.3×10^6	7.4×10^5	3.3×10^3	1.2×10^3

As can be seen the counts generally decrease as the temperature decreases. The -5°C results are being repeated because the temperature differential was not as close as was desired. The work is being repeated with a more reliable incubator (Freas 815) which will be used in future studies.

Countable plates from the 0°C incubation temperature were selected for further study, thus samples A, B, and C are not included in the remainder of the investigation. The colonies from samples D through G were inoculated onto TSA slants and incubated at 0°, 3°, 7°, 24°, and 32°C. The results demonstrated that all isolates were capable of growth at the three low temperatures in at least 14 days, but several were not capable of growth at 32°C in 48-72 hrs. These results are seen in Table 4.

Table 4. Population of 0°C soil isolates not capable of growth at 32°C

Sample	% of Total Count	CFU/gm Soil
D	24	1.2×10^3
E	0	0
F	15	1.5×10^2
G	12	1.3×10^3

These counts represent those organisms in Cape Kennedy soil which have the ability to grow at 0°C, but not at 32°C.

Attempts to Count Bacteria at Sub-zero Temperatures

Table 5 shows the preliminary results of attempts to isolate organisms at sub-zero temperatures. Incubation times were 14 days at 7°C and 3°C, 21 days at 0°C, 40 days at -5°C, and 60 days at -10°C.

Table 5. Counts of Cape Kennedy soil samples at various low temperatures on different media

Incubation Temperature		Medium				
		TSA	TSA + 3% Agar	TSA + 5% Glycerol	TSA + 7.5% Glycerol	TSA + Membrane Filter
7°C	NHS*	9.9×10^4	1.1×10^5	5.1×10^4	3.3×10^4	1.3×10^5
	HS	1.8×10^4	3.2×10^4	1.8×10^4	1.4×10^4	+
3°C	NHS	2.6×10^5	7.6×10^4	5.3×10^4	4.0×10^4	1.4×10^6
	HS	6.9×10^4	3.6×10^4	4.5×10^4	3.9×10^4	2.5×10^2
0°C	NHS	3.1×10^3	2.0×10^4	2.9×10^3	3.0×10^3	+
	HS	9.9×10^3	1.9×10^4	3.5×10^3	1.7×10^3	+
-5°C	NHS	0	0	0	0	0
	HS	0	0	5.2×10^3	0	0
-10°C	NHS					
	HS	0	11	0	0	0
		0	3	2	0	0

*NHS=Non-heat-shocked +=Unreadable
HS=Heat-shocked

At 7°C and 3°C all plates were in good condition; at 0°C, all media had ice cracks except 3% agar which appeared to be in good condition; at -5°C, all media were frozen and in poor condition; and at -10°C, all media were frozen and impossible to read. In these investigations, it was observed that colonies on media containing glycerol were considerably smaller and difficult to observe. Even though total counts on TSA and TSA + glycerol showed slight variation, it is suspected that the glycerol has a slight inhibitory effect on colony formation.

The plates with the membrane filters were disappointing because of colonies running together. Most of the growth was around the periphery of the filters, and counts were not possible when the filters were on frozen medium.

Heat Survivors from Cape Kennedy Teflon Ribbon Experiments

The variability of biochemical activity of 33 isolates, which survived the 113°C dry-heat cycle at Cape Kennedy, after several subcultures on supplemented TSA is demonstrated in Table 6. These survivors were identified at Cape Kennedy and later at Hardin-Simmons, and Table 6 shows differences in results of these identifications. These were incubated at 32°C for 2 to 4 days before reactions were recorded.

Table 6. Results of identification procedures performed at Cape Kennedy and Hardin-Simmons on heat survivors from Cape Kennedy teflon ribbon experiments

Isolate Number		Starch	Casein	Mannitol	VP	Citrate	Nitrate	Anaerobic Growth	Tyrosine	Phenylalanine	Identification
	*A	+	+	-	-	-	+	-	-	-	B. firmus
1-12	B	-	-	-	-	-	+	-	+	-	Atypical
	A	-	-	-	-	-	-	-	-	-	Atypical
1-29	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	-	-	-	-	-	-	-	B. lentus
2-18	B	+	+	+	-	-	-	+	-	-	B. circulans
	A	-	-	-	-	-	+	-	-	-	Atypical
4-6	B	-	-	+	-	-	+	-	-	-	Atypical
	A	-	-	-	-	-	-	-	-	-	Atypical
5-19	B	+	+	+	-	-	+	+	+	-	B. laterosporus
	A	-	+	-	-	-	+	-	-	-	B. brevis
6-11	B	-	+	+	-	-	+	+	-	-	Atypical
	A	+	-	-	-	-	-	-	-	-	B. lentus
6-12	B	-	-	-	-	-	-	+	-	-	Atypical
	A	+	+	-	-	-	-	-	-	-	Atypical
6-25	B	-	+	+	-	+	-	-	-	-	B. pumilus

Table 6, continued

Isolate Number		Starch	Casein	Mannitol	VP	Citrate	Nitrate	Anaerobic Growth	Tyrosine	Phenylalanine	Identification
	A	+	-	-	-	-	-	-	-	-	B. lentus
6-28	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	-	-	-	-	-	-	-	B. lentus
6-32	B	+	-	+	-	-	-	-	-	-	B. lentus
	A	+	-	-	+	-	+	+	-	-	B. coagulans
7-11	B	-	-	-	+	-	+	+	-	-	B. coagulans
	A	-	+	-	-	-	-	-	+	-	B. brevis
8-14	B	-	+	+	-	-	-	-	+	-	B. brevis
	A	+	+	-	-	-	+	-	-	-	B. firmus
8-25	B	-	-	-	-	-	+	+	-	-	B. macerans
	A	+	+	-	-	-	+	-	-	-	B. firmus
8-28	B	-	+	-	-	-	+	+	-	-	Atypical
	A	+	+	+	-	-	-	-	-	-	B. lentus
9-12	B	+	-	+	-	-	-	-	-	-	B. lentus
	A	+	-	-	-	-	-	-	-	-	B. lentus
9-13	B	-	-	-	-	-	-	+	-	-	Atypical
	A	+	+	-	-	-	+	-	-	-	B. firmus
10-2	B	-	-	-	-	-	+	+	-	-	B. macerans
	A	-	-	-	-	-	-	-	-	-	Atypical
10-17	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	+	-	-	-	-	-	-	B. lentus
10-20	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	-	-	-	+	-	-	+	Atypical
10-30	B	-	-	-	-	-	+	-	-	-	Atypical
	A	+	-	-	-	-	-	-	-	-	B. lentus
13-20	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	-	-	-	-	-	-	-	B. lentus
16-16	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	-	+	-	-	+	-	-	-	B. macerans
16-23	B	+	-	+	-	-	+	-	-	-	B. macerans
	A	+	-	-	-	-	+	-	-	+	Atypical
18-10	B	-	-	-	-	-	+	+	-	-	B. macerans
	A	+	-	-	-	-	-	-	-	-	B. lentus
18-16	B	+	-	+	-	-	-	-	-	-	B. lentus
	A	+	-	-	-	-	-	-	-	-	B. lentus
18-24	B	+	-	-	-	-	-	-	-	-	B. lentus
	A	+	-	-	-	-	-	-	-	-	B. lentus
18-29c	B	+	+	+	-	-	-	-	-	-	B. lentus
	A	+	-	-	-	-	-	-	-	-	B. lentus
18-31	B	-	-	-	-	-	-	-	-	-	Atypical
	A	+	+	-	-	-	+	-	-	-	B. firmus
20-27	B	-	+	-	-	-	+	-	-	-	B. brevis

Table 6, continued

Isolate Number		Starch	Casein	Mannitol	VP	Citrate	Nitrate	Anaerobic Growth	Tyrosine	Phenylalanine	Identification
	A	ND									ND
30-6	B	-	+	-	-	-	+	+	-	-	Atypical
	A	ND									ND
32-15	B	-	-	-	-	-	+	+	-	-	B. macerans
	A	ND									ND
33-22	B	-	+	-	-	-	+	+	-	-	Atypical
	A	ND									ND
34-11	B	-	-	-	-	-	+	-	-	-	Atypical

*A=Cape Kennedy
B=Hardin-Simmons
ND=Not done

The reactions of the heat-survivors from the Cape Kennedy teflon ribbon experiment on various carbohydrates are presented in Table 7 (page 25). These carbohydrates were added to Phenol Red Broth Base, inoculated in duplicate with a standard suspension of the organisms, and incubated 4 days at 24°C. These results demonstrate that these isolates are generally not very reactive on carbohydrates. They grew well on all media except xylose where 18 of the 32 cultures did not grow.

Table 8 (page 26) contains the results of various environmental conditions for the Cape Kennedy heat survivors. Results of anaerobic growth in the Brewer Anaerobe Jar are given as positive or negative after incubation at 24°C for 21 days. Aerobic controls inoculated onto supplemented TSA slants at the same time demonstrated these to be viable. Results of pH, salt concentration, and decreasing available nutrients

Table 7. Reactions of Cape Kennedy heat survivors on various carbohydrates

Isolate No.	Arabinose	Dextrose	Dulcitol	Galactose	Inulin	Inositol	Lactose	Maltose	Mannose	Raffinose	Rhamnose	Sorbitol	Sucrose	Xylose
1-12	K	A	K	K	K	K	K	A	A	K	K	K	A	K
1-24	-	K	K	K	K	K	K	K	-	K	K	-	K	*
2-18	K	K	K	K	K	K	K	K	K	K	K	K	-	K
4-6	-	-	K	K	K	K	K	A	A	K	K	K	K	K
5-19	K	K	K	K	K	K	K	K	K	K	K	K	K	K
6-11	-	A	K	K	K	K	K	-	-	K	K	K	A	K
6-12	-	A	-	-	-	-	-	A	A	-	-	-	-	-
6-25	K	A	K	K	K	K	K	K	K	K	K	K	A	K
6-28	K	A	K	K	K	K	K	A	A	K	K	K	A	K
6-32	-	A	K	K	K	K	K	A	A	K	K	K	A	*
7-11	K	A	K	K	K	K	K	-	K	K	K	K	-	*
8-25	K	A	K	-	K	A	K	A	A	K	K	K	A	*
8-28	K	A	K	-	K	A	K	A	A	K	K	K	A	*
9-12	A	A	-	A	-	-	-	A	A	-	-	-	A	K
9-13	K	-	K	K	K	K	K	-	K	K	K	K	A	K
10-2	K	A	K	K	K	K	K	A	A	K	K	K	A	*
10-17	K	A	K	K	K	K	K	K	K	K	K	K	-	*
10-20	-	A	K	K	K	K	K	A	K	K	K	K	A	K
10-30	K	A	K	K	K	-	K	A	A	K	K	K	A	*
13-20	K	A	K	K	K	K	K	A	-	K	K	K	A	K
16-16	-	A	K	-	K	K	K	A	-	K	K	K	A	*
16-23	K	-	K	A	K	K	K	-	K	K	K	K	K	*
18-10	K	A	K	-	K	A	K	A	A	K	K	K	A	*
18-16	-	A	K	K	K	K	K	A	K	K	A	A	A	-
18-29	K	-	K	-	K	K	K	-	A	K	K	K	K	*
18-29c	K	-	K	A	K	K	K	A	A	K	K	K	K	*
18-31	K	A	K	-	K	K	K	A	A	K	K	K	A	K
20-27	K	A	K	-	K	K	K	A	A	K	K	K	A	*
30-6	K	A	K	K	K	K	K	A	A	K	K	K	A	*
32-15	K	A	K	-	K	-	K	A	A	K	K	K	A	*
33-22	K	A	K	K	K	K	K	A	A	K	K	K	A	*
34-11	K	A	K	K	K	-	K	A	A	K	K	K	A	*

*=No growth
A=Acid
K=Alkaline
-=No change

Table 8. Results of various environmental conditions for heat survivors from the Cape Kennedy teflon ribbon experiments

Isolate No.	Anaerobic Growth	pH Range	pH Optimum	Salt Conc.(%) Range	Salt Conc.(%) Optimum	Final Nutrient Dilution Showing Good Growth*
1-12	+	6.0-9.5	7.5	1-10	5	10^{-9}
1-24	-	5.5-8.5	6.0	1-2	1	10^{-4}
2-18	+	5.0-9.5	7.0	1-10	7	10^{-9}
4-6	+	6.5-9.5	8.5	1-8	2	10^{-8}
5-19	+	5.0-9.5	6.0	1-10	9	10^{-6}
6-11	+	5.0-9.5	7.0	1-10	3	10^{-4}
6-12	+	6.5-9.0	7.5	1-4	2	10^{-4}
6-25	+	5.0-9.5	5.0	1-10	5	10^{-7}
6-28	-	5.5-8.0	7.0	1-3	2	10^{-5}
6-32	+	7.0-9.5	8.5	1-5	2	10^{-5}
7-11	-	ND		ND	ND	ND
8-14	-	ND		ND	ND	ND
8-25	-	5.5-9.5	7.0	1-5	2	10^{-2}
8-28	+	6.5-9.5	7.5	1-5	1	10^{-2}
9-12	+	5.5-8.5	6.0	1-3	2	10^{-3}
9-13	+	6.0-8.5	6.0	1-2	1	10^{-4}
10-2	+	6.5-9.0	8.5	1-4	2	10^{-6}
10-17	-	ND		ND	ND	ND
10-20	+	6.5-8.5	7.0	1-2	1	10^{-6}
10-30	+	6.5-9.5	8.5	1-5	1	10^{-6}
13-20	+	5.5-8.5	6.0	1-2	1	10^{-5}
16-16	-	ND		ND	ND	ND
16-23	+	6.5-9.0	8.0	1-3	3	10^{-1}
18-10	+	6.5-9.5	8.5	1-5	1	10^{-2}
18-16	+	5.5-9.5	7.5	1-10	2	10^{-9}
18-29	-	ND		ND	ND	ND
18-29c	-	ND		ND	ND	ND
18-31	+	5.5-8.5	6.0	1	1	10^{-4}
20-27	+	6.5-9.5	7.5	1-5	3	10^{-4}
30-6	+	6.5-9.5	8.5	1-5	2	10^{-3}
32-15	+	6.5-9.5	7.5	1-5	2	10^{-3}
33-22	+	6.5-9.5	8.5	1-5	2	10^{-2}
34-11	+	6.5-9.5	7.5	1-5	1	10^{-3}

*Good growth is considered when the turbidity yields less than 90% transmittance

ND - not done

were determined by recording the turbidity (Spec 20) of broth cultures after 2 and 4 days incubation at 24°C.

Survival of the spores of the Cape Kennedy heat survivors under vacuum and at various temperatures is presented in Table 9 (page 28). This experiment has only recently been started, and complete results will be ready later. Some of the spore suspensions contained populations which were too low to be reliable and were not used in the present investigation. These are being prepared again and will be used in the next run. At the time that this experiment was being performed, our vacuum system was inaccessable; therefore, a vacuum of only 10^{-2} Torr could be used. During the next run of this experiment, a vacuum of 10^{-7} to 10^{-9} Torr should be attainable.

Table 9. Counts of spores stored under atmospheric pressure and 10^{-2}Torr at different temperatures ($\times 10^{6}$)

Isolate No.	Orig. Spore Counts *	Atmospheric Pressure			10^{-2} Torr		
		-65°C (26 days)	20°C (24 days)	50°C (28 days)	-65°C (16 days)	20°C (10 days)	50°C (24 days)
1-12	14.00	3.10	8.0	3.30	4.60	1.00	3.60
2-18	1.30	250.00	230.0	28.00	49.00	37.00	33.00
4-6	1.10	13.00	4.0	1.30	0.70	0.80	0.70
5-19	1.60	170.00	180.0	78.00	1.00	30.00	41.00
6-11	1.60	71.00	76.0	37.00	12.00	9.40	16.00
6-12	0.20	0.84	2.3	0.10	0.04	0.30	0.09
6-25	2.60	370.00	40.0	160.00	39.00	9.20	47.00
6-32	64.00	11.00	11.0	7.90	7.40	7.10	4.00
7-11	470.00	9.30	11.0	3.30	2.50	3.90	2.60
8-14	8.30	12.00	7.7	1.90	1.20	5.10	4.30
8-28	9.80	6.80	5.3	5.10	7.60	3.80	1.00
9-12	240.00	10.00	6.9	1.90	3.30	0.90	6.60
10-30	0.20	1.20	1.0	0.40	1.00	1.00	0.90
13-20	96.00	22.00	7.3	1.50	8.20	7.60	5.50
16-23	53.00	1.30	4.2	1.20	3.20	1.00	2.00
18-16	110.00	5.10	4.6	0.45	3.40	2.00	1.50
18-29c	30.00	1.10	4.1	1.30	8.10	4.20	3.60
20-27	0.50	0.86	0.8	0.52	0.20	0.09	0.50
30-6	55.00	4.60	1.8	0.36	1.20	1.30	1.10
32-15	2.40	7.50	11.0	6.00	10.00	4.20	3.50
33-22	0.07	0.56	0.5	0.43	0.30	0.03	0.20

*CFU/ml.

Discussion and Future Work

Response of Original Soil Samples to the Experimental Martian Environment

The response of the various soil samples to the experimental Martian environment after 60 days incubation was rather varied and unpredictable. An attempt was made to correlate these results to the types of organisms present in the original soil samples (H-SU Report No. 2), but no consistent conclusion could be drawn. Because the responses were so varied and appeared to follow no consistent pattern, it is felt that the prolonged freeze-thaw cycle (-65°C for 16 hrs. and 24°C for 8 hrs. for 60 days) may have disrupted the integrity of the experimental conditions within the sealed tubes. For this reason, a more reliable closure system has been developed and will be used in all future experiments involving the experimental Martian environment with the freeze-thaw cycle.

Slide Culture Technique

In interpreting the results of this experiment, it should be remembered that the 24 pure cultures used were also inoculated into one gram of the artificial soil mixture, with excess water, and subjected to the experimental environment. The results as presented in Report No. 2 indicated that these organisms neither increased nor decreased in population after 21 days incubation. Upon extending this incubation to 60 days, at least 8 of these showed decreased populations, with the remainder being unchanged.

On the other hand, results of the slide culture technique indicate that all 24 isolates can produce microcolonies on TSA and AMS agar-coated slides after incubation of 10 days in the experimental Martian

environment. This apparent conflict of results of the tube method and the slide culture method indicates that the slide method deserves continued study. It provides a rapid means of demonstration of growth in a simulated Martian environment by the formation of microcolonies on agar-coated slides. Because no counts are involved, this technique requires considerably less equipment and time, and the incubation time is shortened. On the other hand, population dynamics cannot be determined. All that can be stated is an affirmative or negative answer to the question of growth, but nothing can be said of how the population behaved at different stages of incubation. In addition, it cannot be definitively stated that there was a net increase in the population. The cells could be dying as fast as growing with no net gain in the total population. When comparing the results of the pure culture studies, it can be seen that the tube method showed no change in the population of the majority of the samples tested, but the slide technique would be reported as growth in the experimental environment. This investigation indicates that the slide technique is a rapid, simplified method in answering the question of growth in a simulated environment, but it does not lend itself to studies of population dynamics, nor can it be used to determine if the organisms are actually alive after a given incubation period. It can be concluded from a comparison of the tube and slide methods that growth of the pure cultures was so slight that it could not be detected by the tube method, but it could be seen by the formation of microcolonies in the slide method. This indicates that the slide method may be a more sensitive method for demonstration of growth in experimental environments.

Because this technique may indeed provide a reliable and sensitive method for demonstration of microbial growth under experimental conditions, continued investigations are planned to improve this technique. A new slide arrangement is being examined which will allow the deposition of at least 8 different cultures per slide, and incubation times between one and ten days will be studied to determine the earliest period at which growth can be confirmed. This will again be performed in conjunction with the tube method so that population dynamics can be investigated.

Response of Selected Microorganisms to Different Moisture and Nutrient Levels in the Experimental Martian Environment

The results of this investigation demonstrate that the non-sporeformer appears to be influenced primarily by the amount of moisture in the environment as is seen by its lack of growth or loss of population at a moisture concentration of 10% or less. On the other hand, after 7 days in the experimental environment it grows very well in all media with excess moisture, increasing its population by approximately two logs. The sporeformer appears more able to survive the adverse conditions, and when placed into an environment of excess moisture and nutrients can grow very well. The growth of the sporeformer is influenced more by the amount of nutrients than is the non-sporeformer. This is shown by a marked response of the Bacillus in the soil extract enriched with 1% TSB plus yeast extract, slight growth response in the peptone, and none in the experimental Martian soil. In contrast, the non-sporeformer grew well in all three nutrient levels if excess water was available.

It is recognized that all three moisture levels used in this experiment are higher than those expected on Mars. The apparatus used to establish the experimental Martian environment has just recently been redesigned and future experiments will be performed with moisture levels considerably lower than those used in this investigation.

Psychrophilic Counts from Cape Kennedy Soil Samples

As seen in Table 3, the lower the temperature of incubation, the lower the count, which is as expected. The decrease from 10°C to 7°C causes a reduction in population of an average of one log, while the decrease from 7°C to 0°C causes an average reduction of over 2 logs. In this report, the -5°C will not be considered because of fluctuations in the incubator temperature.

Temperature studies on the 0°C isolates demonstrated that only a small percentage of these are not capable of growth at 32°C, and their populations are quite small (0, 150, 1200, 1300 CFU/gm of soil). These procedures are to be continued on additional soil samples, and attempts will be made to determine which low temperature of incubation will yield the consistently highest percentage of obligate psychrophiles.

Attempts to Count Bacteria at Sub-zero Temperatures

This was a preliminary attempt to evaluate some simple procedures for counting colonies at sub-zero temperatures. From these results, it appears that this technique will require considerable additional study, and a more sensitive method of counting should be investigated because of the extremely small size of colonies at these low temperatures. No attempt will be made to expand this work at present, but if the need is justified, it may be continued at a future date.

Heat Survivors from Cape Kennedy Teflon Ribbon Experiments

As can be seen from the results of the identification of Bacillus isolates from Cape Kennedy, these organisms exhibit deviations in biochemical activity following subculture. There is a possibility of variations in individual interpretation of some of the reactions, but the degree of variability of the two sets of results probably cannot be explained so easily. In order to more completely and reliably investigate these deviations in biochemical reactions, we have recently received a duplicate set of these cultures from Cape Kennedy and will investigate their biochemical activity following different methods of preservation.

This new set of isolates will be compared to the original set to observe any variations of biochemical activity, and they will also be subjected to some of the conditions described in this report.

Upon completion of detailed characterization of the heat-survivors from Cape Kennedy, it will probably be easier to group these isolates. By performing such tests, additional distinguishing characteristics might be added to or replace some of the biochemical tests in the identification scheme used at Cape Kennedy. It is hoped that such a detailed investigation might eventually result in a diagnostic scheme which is not so influenced by biochemical deviations of the isolates. If this cannot be done, procedures will need to be standardized concerning time of testing, number of transfers prior to testing, and so forth, so that identifications can be consistent from one lab to another.

Since these organisms have survived the proposed dry-heat cycle of the Viking spacecraft, they will be of special interest to planetary

quarantine because these may be the types that will most likely be contaminants of the spacecraft. For this reason, a complete characterization of these isolates will be of value in attempts to predict their role in the possibility of contamination of Mars. These isolates will also be subjected to the artificial Martian environment to determine how they respond.

One area of interest associated with these organisms is their ability to survive vacuum conditions. From the early results of these investigations at low pressure, it appears that they will be capable of survival under vacuum at temperatures between $-65^{\circ}C$ and $50^{\circ}C$. The apparent increase in some of these populations can be explained by the clumping of cells. Some of the original spore suspensions were observed to have clumps even after insonation, and it is felt that these were disrupted upon the second insonation when counts were performed. These experiments will continue for longer time periods; they will be repeated at a vacuum of 10^{-7} to 10^{-9} Torr; and they will be repeated under a nitrogen atmosphere.