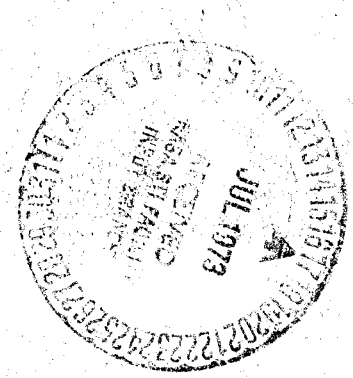
July, 1973

Report No. 2
January 1, 1973 – June 30, 1973

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

Submitted by

Terry L. Foster
Department of Biology



Hardin-Simmons University
Abilene, Texas

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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. 2 of Planetary Quarantine Activities
January 1, 1973 - June 30, 1973

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July, 1973

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

Presented at the

Semi-Annual NASA Spacecraft Sterilization
Technology Seminar

The Denver Hilton Hotel, Denver, Colorado
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FOREWORD

This second 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, and covers the period from January 1, 1973 - June 30, 1973. The relationship with the NASA Planetary Quarantine Program has been most stimulating to the Division of Science at H-SU, and it is hoped that this project will be a significant contribution to the activities of NASA in its present and future planetary exploration programs.

This report includes a more detailed investigation of the types of psychrophilic organisms isolated from soil samples from areas associated with the Viking spacecraft. Results are presented which show the distribution of the major generic groups of organisms isolated at low temperature from these areas and the percentage of these which are obligate psychrophiles. The methods employed for subjecting samples to some of the environmental conditions suggested for Mars are described in detail, and results of subjecting the original soil samples to these conditions are presented. The report also includes a description of work currently in progress and future work planned for the remainder of the grant period. Emphasis in all areas is toward application of these results to the objectives of the planetary quarantine program.

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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

The importance of psychrophilic organisms from areas associated with the Viking spacecraft was presented in the first report of this project (H-SU Preliminary Report - January, 1973). That report described the procedures used for original isolation of these organisms at 7 C and showed an early survey of the percentage of the various samples which would not grow at 32 C. It is felt that this population may be of significance to planetary quarantine because organisms not growing at 32 C might be excluded from microbial monitoring of the Viking spacecraft. A description of procedures, media, and a preliminary population survey was presented in the first report and will not be presented here.

This second report presents a more detailed investigation of the psychrophilic populations, and the isolates have been placed into major generic groups. The percentages of each group which are obligate psychrophiles are given, and it has been determined that the most important organisms for further investigations are the psychrophilic, facultative aerobes. The majority of organisms originally isolated under anaerobic conditions were determined to be facultative aerobes.

Upon completion of the population survey of the various samples, procedures were devised for subjecting organisms to some of the

environmental conditions suggested for Mars. Although there are still many factors of the Martian environment which are debatable, the conditions used here are based upon some of the more recent reports about the conditions of the Martian environment. It is felt that such a study is of importance because it utilizes organisms which were isolated directly from areas associated with the Viking spacecraft and includes isolates which are capable of growth at lower temperatures. The procedures used to establish these conditions are described in this report, and results of the first of these investigations are presented. Similar investigations are currently in progress and will be reported at a later time.

Procedure

Soil samples were selected from the manufacture and assembly areas of the Viking spacecraft and treated as described in the first report of this project (H-SU Preliminary Report - January, 1973). These samples sites are presented in Table 1.

Growth Temperature Studies

Plates showing countable (30-300 colonies) results after 14 days incubation were selected, and all colonies were transferred from these to TSA slants for incubation at 3 C (10-14 days), 24 C (3-5 days), and 32 C (48 hrs). Plates with higher counts were examined for low populations of organisms which did not appear on the countable plates. After growth had occurred, the results were recorded and organisms showing growth at 3 C, but not at 32 C, were classified as psychrophilic. The organisms were then reincubated for 7-10 more days for better growth and pigment production.

Table 1. Sample Sites

CODE	SOURCE
	From the Manufacture Area in Denver, Colorado
M-1	Outside high bay area on cooling tower side
M-2	West side of high bay area*
M-3	Back of high bay area
	From the Assembly Area at Cape Kennedy
K-1	Bldg. M7-1469 East of low bay door on north side of bldg.
K-2	Bldg. M7-1469 West of low bay door on north side of bldg.
K-3	Bldg. M7-1469 Directly in front on low bay door
K-4	Bldg. M7-1469 East of high bay door on south side of bldg.
K-5	Bldg. M7-1469 West of high bay door on south side of bldg.
K-6	Bldg. M7-1469 Directly in front of high bay door
K-7	Bldg. AO Directly in front of high bay (west side) Dark sand
K-8	Bldg. AO Directly in front of high bay (west side) Light sand
K-9	Bldg. AO From the curb directly in front of high bay
K-10	Bldg. AO Main personnel entrance on east side of bldg.
K-11	Bldg. AO From vacuum units inside bldg.
K-12	Bldg. AE Outside main entrance to the clean room

*From roadbed of fill dirt - not native soil

Identification of Isolates

Due to the large number of isolates and because of the problems associated with identification of psychrophilic, soil microorganisms, identification procedures consisted of placing these organisms into various generic groups. The isolates were gram-stained, tested for motility of phase-contrast microscopy, and subjected to selected biochemical tests including Hugh-Leifson's test (glucose), Kovac's oxidase test, nitrate reduction, citrate utilization, starch hydrolysis, gelatin liquefaction, and lactose, sucrose, and glucose fermentation. Although all of these tests are not included in the identification scheme, they were useful in differentiating strains within the various groups. Organisms thought to be sporeformers were grown on AK-2 Sporulating Agar (BBL) at either 7 C (10-14 days) or 24 C (2-3 days). These were then spore-stained to demonstrate production of spores.

From these results, the organisms were placed into major groups according to the scheme presented in Fig. 1. As can be seen, members of the Micrococcaceae were identified according to the method of Baird-Parker, and no Streptococci were isolated. The Corynebacterium-Brevibacterium group may also include members of the genera Arthrobacterium, Microbacterium, and Cellulomonas, but attempts were not made to differentiate these. The Alcaligenes-Acinetobacter group may also contain members of other closely-related genera such as Achromobacterium, but according to J. M. Shewan (personal communication), this genus is being absorbed into the Alcaligenes one. The Acinetobacter genus is included in this group because of inconsistencies in

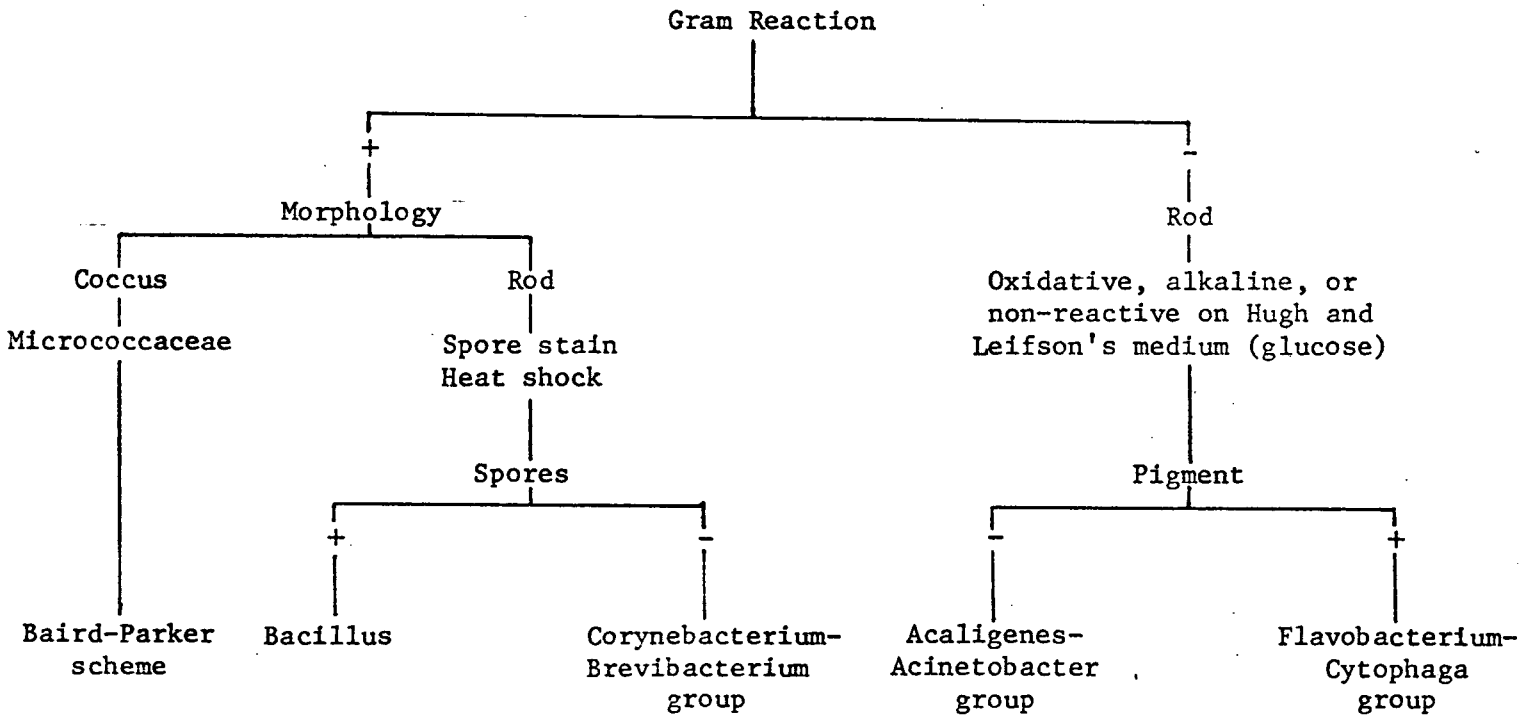

Fig. 1. Diagnostic key for grouping of isolates

the results of the oxidase and motility tests. As a general rule, organisms of this group were oxidase negative and non-motile. The taxonomic relationship of the Flavobacterium-Cytophaga group and related organisms is still under debate; therefore, they have been placed into a single group in this scheme.

The fungi were identified to genus according to the methods of Barnett and Hunter and Barron, and the yeasts were identified following the methods of Lodder. This was performed with the assistance of Dr. John Brandsberg, Center for Disease Control, Kansas City, Kansas.

All aerobic isolates were subjected to anaerobic conditions to determine which ones were facultative anaerobes. Since evidence indicates that only a trace amount of oxygen is present in the Martian atmosphere, the most likely organisms to grow in this environment will be the anaerobes or facultative anaerobes. The anaerobic isolates were subjected to aerobic conditions to determine if they were obligate or facultative anaerobes.

Since two mechanisms of contamination of the spacecraft are inadequate terminal sterilization and recontamination after sterilization, it is likely that the contaminating flora, if it is present, will consist primarily of sporeforming organisms. For this reason, all isolates were cultured on AK-2 Sporulating Agar (BBL) and spore-stained in an attempt to demonstrate spore formation. These were then washed and suspended in phosphate buffer (pH 7.0), heat-shocked at 80 C for 15 min, and plated on TSA plates to demonstrate survival.

Representatives of all isolates were lyophilized for future

reference by washing a 48 hr TSA culture with 2 ml of 10% skim milk, placing in a Virtis Vac Vial, quick-freezing in a dry ice-acetone mixture, and drying on a Virtis Unitrap lyophilizer (Virtis Co., Inc., Gardiner, New York).

Artificial Martian Environment

The experimental environment was established by a modification of the method described by A. A. Imshenetsky at the COSPAR meeting in Madrid in 1972. These conditions consisted of an atmosphere of 80% CO_2 and 20% Argon at a pressure of 6-7 mb (4.4-5.2 mm Hg). The moisture level was either excess (1.0 ml of water per gm of soil) or 10% (0.1 ml water per gm of soil) unless otherwise stated. The soil mixture was 1.0 gm of either native soil (from the original soil samples) or a 40:40:10:5:5 mixture of felsite:limonite:garden soil:peat:volcanic rock, unless otherwise stated. The temperature cycle used represents a day-night cycle at the Martian equator and consisted of 16 hrs at -65 C and transferred to +25 C for 8 hrs. Ultraviolet irradiation was not used as a condition of this artificial Martian environment because it has been demonstrated that bacteria deposited in the soil will be shielded by the soil particles. Since the organisms must be shielded to withstand the intense bombardment of ultraviolet radiation, light was not considered as a necessary factor in this study.

These experimental conditions were established by attaching the tubes to a sterile manifold and evacuating to 6-7 mb (4.4-5.2 mm Hg). The vacuum pressure was accomplished with a Welch Duo-Seal pump (Model 1400) and regulated with a Cartesian Manostat (Model 9). To assure proper calibration of the manostat, the pressure was also monitored with a McLeod gauge (Model 10-224-b). A mercury vapor

trap was placed between the gauges and the manifold to prevent mercury contamination of the samples. When the desired pressure was achieved, the tubes were flushed with a gas mixture containing 80% CO_2 and 20% Argon (Paul Carroll Oxygen Supply, Abilene, Texas). A Matheson gauge (Model 49) was used between the gas cylinder and manifold to reduce the gas pressure before it entered the tubes, and the gas mixture was sterilized by passing it through a Sartorius membrane filter (pore size 0.20 um) before it entered the manifold. This evacuation-flushing cycle was performed three times to assure that the atmosphere in the tubes had been replaced with the 80% CO_2-20% Argon mixture. The tubes were then heat-sealed under this atmosphere at 6-7 mb pressure. The tubes (16 x 150) were fitted with a one-hole, double 0 stopper containing a 6-8 in length of ¼ in, medium-wall, Pyrex glass tubing. This was attached to the manifold, subjected to the artificial environment, and sealed at the ¼ in tubing. The stoppers were secured with parafilm to assure continued maintenance of conditions in the tubes. All tubes were examined for vacuum prior to counting, and all showed evidence of vacuum conditions. The general arrangement of the system used to establish these conditions is seen in Fig. 2.

Sealed tubes to be tested in the artificial environment were then placed in an ultra-low temperature incubator (Revco Model ULT-1175B) at -65 C for 16 hrs, then transferred to + 24 C for 8 hrs (Freas Model 805). This daily freeze-thaw cycle approximates the day-night conditions at the Martian equator and was maintained throughout the duration of the experiment.

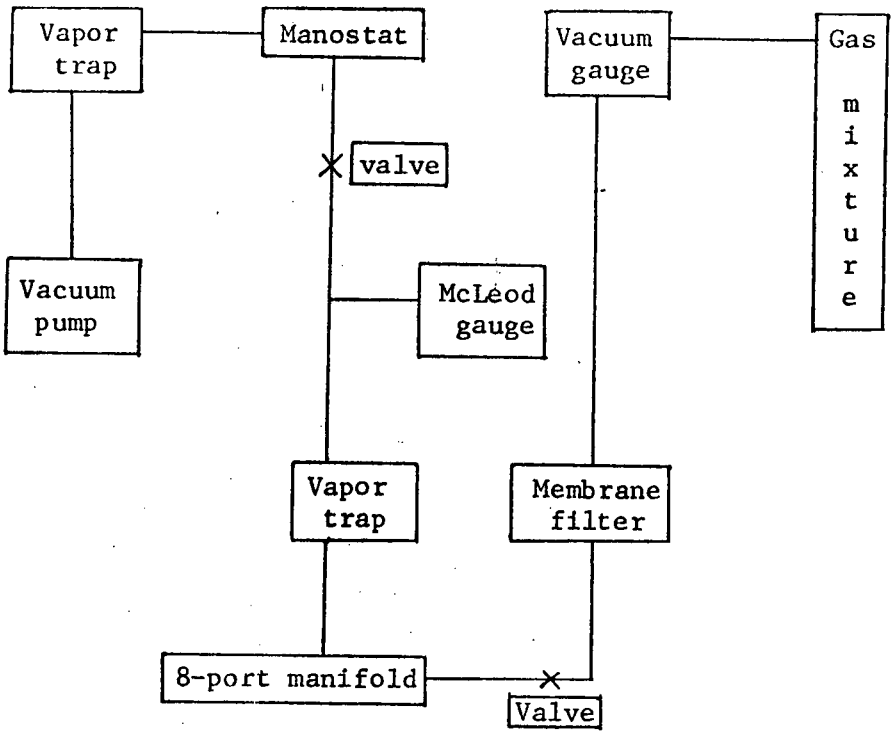

Fig. 2. Apparatus for establishing Martian atmosphere

The -65 C temperature was monitored on a recording thermometer, and the freezer provided very reliable stability and rapid recovery. The temperature rose about 5-10 C when the samples were removed from or placed into the freezer, and the recovery time to -65 C was less than 30 min. Otherwise, the temperature fluctuated no more than -65 ± 2 C. The 24 C temperature was registered on a maximum-minimum registering thermometer which was checked daily and showed $+24\pm2$ C even when samples were being transferred.

Preparation of Tubes for Experimental Environment

In preparing the tubes to be used in the artificial Martian environment, two different techniques were used. The slide method was prepared by cutting standard microscope slides (25 x 75 mm) in half, longitudinally, and coating them with agar containing the desired nutrients. One set was designed to provide sufficient nutrients and consisted of TSA-coated slides; the other was designed to contain limited nutrients and was composed of an extract of a 40:40:10:5:5 mixture of felsite:limonite:garden soil:peat:volcanic rock. This medium was prepared by adding 100 gms of the soil mixture to 100 ml of distilled water, boiling for 2 hrs, and centrifuging at 5,000 rpm for 15 min. The supernatant was added to an equal volume of melted 3% water agar, examined for contaminating microbial cells, and sterilized at 121 C for 20 min. This represents an experimental Martian soil of predominantly felsite and limonite with limited organic nutrients. All manipulations requiring aseptic technique were performed in a laminar flow cabinet.

The sterile agar-coated slides were then dried by placing them into petri dishes containing absorptive discs in the lids. These were

placed in a dry heat oven at 45 C overnight. The time required for the slides to show no weight loss was determined, and it was found that overnight at 45 C was sufficient. This lack of weight loss was an indication of dryness. After drying, the slides were inoculated with 0.01 ml of the desired test organism diluted to give approximately 50 - 100 organisms in a microscopic field under oil immersion. The inoculated slides were then placed into individual, sterile, 16 x 150 test tubes containing a saturated aqueous solution of K_2SO_4. This procedure provides a moisture concentration of approximately 4%. The tubes were then placed onto the sterile manifold and subjected to the artificial Martian atmosphere described previously.

Duplicate slides of each sample were removed immediately after sealing and examined under oil-immersion phase microscopy (1000x) using a Swift Phase Master (Model M-970) with Plan, achromatic objectives.Polaroid micrographs were made of these slides and were labeled day 0 to be used as a reference for comparison of growth. Duplicate slides were removed at day 14, examined as before, and micrographs were compared to day 0 micrographs. Evidence of growth was considered positive upon the formation of microcolonies.

The other system, the tube method, was prepared by adding one gram of various soil mixtures to the tubes and inoculating with known populations of microorganisms. This system is advantageous because the desired nutrients and moisture levels can be more easily regulated and quantitative determinations of population changes can be made.

The inoculated tubes were then subjected to the Martian environment as described previously. Counts were performed by adding 9 ml of

1.0% peptone solution to the tubes after incubation for the required time in the Martian environment. This was then diluted using ten-fold dilutions and plated in duplicate for counting. Counts were performed after 0, 1, 2, 7, 14, and 21 days incubation in the freeze-thaw cycle. Samples were removed from the -65 C incubator and held at 25 C for at least 5-6 hrs before counts were performed.

The first set of experimental samples to be subjected to the Martian environment in the tube method included the original fifteen soil samples. One gram of each was placed into separate tubes, 1.0 ml of sterile water was added to provide excess water. The tubes were sealed as described and subjected to the freeze-thaw cycle for the designated time intervals. The first counts were performed immediately upon sealing to give a count at day 0. In all series of tubes where water was added, the volume of peptone added to the tube was reduced according to the volume of water which was added to assure a 1:10 dilution in the tube. No nutrients were added to this series other than what was present in the original soil sample, and counts were performed on the designated days. Plates were also incubated anaerobically, and all counts were performed in duplicate. All plates in this series were incubated at 7 C for 14 days prior to counting.

Another series of tubes were prepared using the original soil samples as described previously except that the samples were heat-shocked at 80 C for 15 min to destroy germinating cells. The resulting soil population consisted of spores and was then subjected to the artificial environment. Population increases in this series of tubes was considered a result of sporulation and growth of the sporeforming organisms.

Use of the original soil samples to study population changes has certain disadvantages as related to parameters of this investigation, and, for this reason, investigations were performed on various pure cultures of bacteria isolated from the samples at 7 C. These isolates were grouped on the basis of various characteristics and then tested to determine their temperature and oxygen requirements. After this was accomplished, representative isolates from each group were selected for testing in the two experimental systems. The predominant groups of organisms isolated from these samples are presented in the results, and the representatives of each group selected for testing are given in Table 2. The codes used indicate the source (M = manufacture area, K = Cape Kennedy samples), the sample number, and the colony number of the isolated organism. These organisms represent a cross-section of those present in the soil samples with major emphasis of investigation being placed on the sporeforming organisms. Those organisms selected for testing have been shown to be facultatively anaerobic, and they grow at 3 C, but not at 32 C.

Preparation of Inoculum

The organisms to be subjected to the artificial environment were cultured on AK Sporulating Agar for 48 hrs at 24 C or 10 days at 7 C (if they did not grow at 24 C). The slants were washed with phosphate buffer (pH 7.0), the suspension transferred to a sterile tube and centrifuged; and the sample was washed three times with phosphate buffer. The organisms were then diluted to a concentration of approximately 10^6 cells/ml and 0.1 ml was transferred to the soil mixture in the tubes (16 x 150) or 0.01 ml to the agar-coated slides.

Table 2. Isolates subjected to the artificial Martian environment

BACILLUS		
Off-white		Tan
K-1- 6	K- 3- 89	K- 3- 64
K-1- 8	K- 4-118	K- 3-110
K-2-21	K- 7-140	K- 8- 42
K-2-38	K-10- 99	K-12- 59
K-3-27	K-11- 38	
K-3-44		

CORYNEBACTER-BREVIBACTER GROUP	
Off-white	Pigmented
K-6-79	K-11-1
K-7-20	

ALCALIGENES-ACINETOBACTER GROUP	FLAVOBACTER-CYTOPHAGA GROUP
K-5-34	K-1-32

MICROCOCCUS - 7	MICROCOCCUS - 8
K- 1-40	M-1-2
K-11-84	M-3-6

In this part of the experiment, the soil consisted of 1 gm of the solid mixture described in the preparation of the soil extract agar slides. All tubes were then subjected to the artificial Martian atmosphere, sealed, and immediately placed into the -65 C incubator to begin the required freeze-thaw cyclization. Most plated organisms were incubated aerobically at 24 C for 48-72 hrs to give more rapid results, but those not growing at 24 C were incubated at 7 C for 10-14 days.

Controls

In all of these investigations, control tubes included identical treatments as the experimental tubes with one set of control tubes being incubated under the Martian environment at 24 C, and one set being incubated anaerobically at 24 C. Counts were performed on these tubes at day 0 and day 7.

Results

Results of total counts isolated at 7 C in 10-14 days were presented in our first report, but are included for reference in this report in Table 3.

Identification

The anaerobic isolates were stained and found to consist of approximately 70-80% sporeformers and 20-30% gram positive rods in the samples from the manufacture area. Samples from Cape Kennedy yielded almost 100% sporeformers. During further testing, it was determined that nearly all of these were facultative aerobes. It was also determined that many of the aerobic isolates were facultative aerobes. Since it was felt that representatives of the anaerobic population would be selected from the aerobic isolates,

Table 3. Number of organisms isolated from each sample*
(% of total)

SAMPLE	TOTAL	AEROBIC	ANAEROBIC	FUNGI
M-1	1.74×10^{6}	1.7×10^{6} (97.7)	2.7×10^{4} (1.6)	1.3×10^{4} (0.7)
M-2	3.94×10^{5}	3.5×10^{5} (88.8)	4.4×10^{4} (11.1)	4.5×10^{2} (0.1)
M-3	9.10×10^{6}	7.7×10^{6} (84.6)	3.0×10^{3} (0.03)	1.4×10^{6} (15.4)
K-1	6.04×10^{4}	4.8×10^{4} (79.5)	9.6×10^{3} (15.9)	2.8×10^{3} (4.6)
K-2	8.18×10^{4}	5.1×10^{4} (62.3)	2.8×10^{4} (34.2)	2.8×10^{3} (3.5)
K-3	1.60×10^{4}	1.3×10^{4} (78.1)	2.1×10^{3} (13.1)	1.4×10^{3} (8.8)
K-4	3.60×10^{4}	6.7×10^{3} (18.6)	2.4×10^{4} (66.7)	5.3×10^{3} (14.7)
K-5	2.73×10^{4}	2.3×10^{4} (84.2)	3.9×10^{3} (14.3)	4.1×10^{2} (1.5)
K-6	1.02×10^{5}	9.4×10^{4} (92.2)	4.1×10^{3} (4.1)	3.7×10^{3} (3.7)
K-7	1.89×10^{4}	1.3×10^{4} (69.3)	1.7×10^{3} (9.0)	4.1×10^{3} (21.7)
K-8	7.14×10^{3}	6.5×10^{3} (91.0)	2.5×10^{2} (3.5)	3.9×10^{2} (5.5)
K-9	5.64×10^{3}	4.2×10^{3} (74.0)	4.5×10^{2} (8.0)	9.9×10^{2} (18.0)
K-10	2.45×10^{4}	1.9×10^{4} (77.6)	2.2×10^{3} (9.0)	3.3×10^{3} (13.4)
K-11	8.38×10^{3}	6.1×10^{3} (73.0)	1.9×10^{3} (23.0)	3.8×10^{2} (4.0)
K-12	1.47×10^{5}	1.4×10^{5} (95.1)	6.9×10^{3} (4.7)	2.3×10^{2} (0.2)

*Cells/gm of soil

it was decided to exclude the anaerobes from further investigation.

The fungi were identified to genus, and these results are presented in Table 4. The molds were not identified to species, but it was observed that most genera showed only one or two different species, with the exception of Penicillium. The yeasts from the manufacture area were all determined to be Crytococcus albidus. Those from Cape Kennedy were identified as either C. albidus, C. laurentii, Rhodotorula rubra, or R. minuta.

The aerobic bacteria were identified to major generic groups including Micrococcus, Bacillus, Corynebacterium-Brevibacterium (C-B), Alcaligenes-Acinetobacter (A-A), and Flavobacterium-Cytophaga (F-C). Since these isolates included such a diverse group and due to the large numbers of isolates involved, speciation was not attempted. Various strains within each major group were noted, and representatives of these have been lyophilized. The results of these determinations are presented in Table 5. The fungi isolated on these plates were not identified, but were included in the counts.

Samples from the manufacture area appear to contain primarily members of the C-B group and members of the genus Micrococcus, especially subgroup 8. Of interest is the fact that these samples contain only low percentages of aerobic sporeformers. In contrast to this, samples from Cape Kennedy contain primarily members of the genus Bacillus and the C-B group, with the exceptions of K-5 which contains a high percentage of the A-A group and K-9 which contains a high percentage of Micrococcus subgroup 7.

Table 5 includes the number of organisms in each group per gram of soil from the various samples. One interesting result demonstrated

Table 4. Types of fungi isolated
(Given in % of fungal count from Table 3)

Manufacture Area

	Alt*	Cep	Chr	Cla	Cry	Fus	Pen	Rho	Rhi	Ulo
M-1	4.6	-	16.9	4.6	36.9	9.2	-	4.6	3.1	20.0
M-2	-	0.9	39.8	-	0.9	-	40.7	-	-	17.6
M-3	5.0	-	12.5	-	60.0	5.0	7.5	-	-	10.0

Cape Kennedy Area

	Alt	Asp	Chr	Cla	Cry	Gen	Pen	Rho	Tub	Ulo
K-1	-	-	-	10.0	-	-	90.0	-	-	-
K-2	8.1	-	-	31.6	2.9	44.9	8.8	3.7	-	-
K-3	8.7	-	42.8	-	2.2	12.3	29.0	5.1	-	-
K-4	2.8	-	0.9	-	11.0	-	4.6	-	-	80.7
K-5	-	7.4	1.1	-	4.6	-	7.5	3.5	-	75.9
K-6	41.6	1.4	1.4	-	27.8	2.8	15.3	6.9	-	2.8
K-7	-	-	-	-	-	-	97.6	-	-	2.4
K-8	5.2	-	-	2.6	-	-	-	-	5.1	87.1
K-9	17.4	5.0	7.2	-	31.1	-	2.9	18.8	-	-
K-10	-	18.2	18.2	-	3.0	18.2	36.4	-	-	6.1
K-11	6.6	27.9	8.2	-	6.6	-	26.2	21.3	-	3.3
K-12	-	-	27.0	-	5.4	-	28.4	5.4	-	33.8

*Alt = Alternaria
Asp = Aspergillus
Cep = Cephalosporium
Chr = Chrysosporium
Cla = Cladosporium
Cry = Cryptococcus
Gen = Geniculosporium
Fus = Fusarium
Pen = Penicillium
Rho = Rhodotorula
Rhi = Rhizoctonia
Tub = Tubercularia
Ulo = Ulocladium

Table 5. Types of organisms isolated from aerobic TSA plates*
(% of aerobic count)

	Bac.	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	No growth on subculture
M-1	-	$6.1x10^5$	$2.4x10^5$	$6.8x10^4$	$2.7x10^5$	$6.8x10^4$	$3.7x10^5$	-	$6.8x10^4$
	-	(36.0)	(14.0)	(4.0)	(16.0)	(4.0)	(22.0)	-	(4.0)
M-2	$7.0x10^3$	$2.3x10^5$	$3.5x10^4$	-	$3.5x10^4$	$7.0x10^3$	$2.1x10^4$	-	$1.4x10^4$
	(2.0)	(66.0)	(10.0)	-	(10.0)	(2.0)	(6.0)	-	(4.0)
M-3	$1.4x10^5$	$5.4x10^6$	-	$4.2x10^5$	-	$6.9x10^4$	$1.6x10^6$	$1.4x10^5$	-
	(1.8)	(69.8)	-	(5.5)	-	(0.9)	(20.2)	(1.8)	-
K-1	$1.9x10^4$	$1.3x10^4$	$9.6x10^2$	$1.9x10^3$	-	$1.9x10^3$	-	$8.6x10^3$	$1.9x10^3$
	(40.0)	(28.0)	(2.0)	(4.0)	-	(4.0)	-	(18.0)	(4.0)
K-2	$1.9x10^4$	$2.4x10^4$	$3.1x10^3$	$2.0x10^3$	-	-	$1.0x10^3$	$1.0x10^3$	-
	(38.0)	(48.0)	(6.0)	(4.0)	-	-	(2.0)	(2.0)	-
K-3	$6.5x10^3$	$4.6x10^3$	$9.1x10^1$	$1.2x10^3$	-	$3.9x10^2$	-	-	$2.9x10^2$
	(50.1)	(35.1)	(0.7)	(8.9)	-	(3.0)	-	-	(2.2)
K-4	$2.6x10^3$	$2.7x10^3$	$3.6x10^2$	$2.6x10^2$	-	$1.0x10^2$	-	$2.6x10^2$	$2.0x10^2$
	(39.1)	(43.5)	(5.3)	(3.8)	-	(1.5)	-	(3.8)	(3.0)
K-5	$3.5x10^3$	$1.2x10^3$	$1.6x10^4$	-	-	$2.3x10^3$	-	-	-
	(15.0)	(5.0)	(70.0)	-	-	(10.0)	-	-	-
K-6	$1.2x10^4$	$3.8x10^4$	-	$2.2x10^3$	$3.1x10^4$	$1.1x10^4$	-	-	-
	(12.5)	(40.9)	-	(2.3)	(33.0)	(11.3)	-	-	-
K-7	$1.4x10^3$	$1.1x10^4$	$9.1x10^1$	-	-	$3.8x10^2$	-	-	-
	(10.7)	(85.7)	(0.7)	-	-	(2.9)	-	-	-
K-8	$1.3x10^3$	$2.9x10^3$	$1.7x10^3$	$5.5x10^2$	$9.1x10^1$	-	-	-	-
	(19.6)	(45.1)	(25.5)	(8.4)	(0.4)	-	-	-	-
K-9	$3.5x10^2$	$1.1x10^3$	-	$3.5x10^2$	-	$2.1x10^3$	-	$8.8x10^1$	$1.8x10^2$
	(8.4)	(27.0)	-	(8.3)	-	(50.0)	-	(2.1)	(4.2)
K-10	$1.4x10^4$	$4.1x10^3$	$9.5x10^1$	$9.5x10^1$	-	$2.1x10^2$	$2.1x10^2$	$9.5x10^1$	-
	(74.8)	(21.6)	(0.5)	(0.5)	-	(1.1)	(1.1)	(0.5)	-
K-11	$3.1x10^3$	$1.7x10^3$	$1.9x10^2$	$3.8x10^2$	-	$4.7x10^2$	$9.8x10^1$	$1.9x10^2$	-
	(50.7)	(27.6)	(3.1)	(6.2)	-	(7.7)	(1.6)	(3.1)	-
K-12	$2.8x10^4$	$1.2x10^5$	$9.8x10^2$	$5.6x10^3$	-	-	-	-	-
	(12.0)	(83.3)	(0.7)	(4.0)	-	-	-	-	-

*Cells/gm of soil

in this table is the fact that the actual number of Bacilli per gram of soil is very close when the Denver samples are compared to the Kennedy samples. Even though the percentage of Bacilli in the aerobic population of the Denver samples is quite low, M-3 contains the largest population of aerobic sporeformers. Another point of interest from this table is the fact that the majority of these isolates are gram positive organisms. This seems to support a more recent concept that psychrotolerant organisms do not have to be predominantly gram negative rods.

Distribution of Obligate Psychrophiles

Based upon the temperature studies, the percent and number of obligate psychrophiles within each group were determined and are presented in Table 6. The majority of psychrophiles from the manufacture area belong primarily to the C-B group or to Micrococcus subgroup 8. Of the organisms isolated from this area, only 2.0% proved to be gram negative rods. Samples from Cape Kennedy showed a more diverse distribution, but the majority of obligate psychrophiles from these samples were also either gram positive rods or cocci. Sample K-1 contained a large portion (18%) of psychrophilic fungi in its population isolated aerobically on TSA, and samples K-8 and K-4 had a fairly high population of gram negative rods (16.9% and 8.3%, respectively). No obligately psychrophilic Bacilli were isolated from the Denver samples, but 10 of the 12 samples from Cape Kennedy contained members of this genus which grew at 3 C, but not at 32 C. The counts for the Kennedy samples were quite low, ranging from 150 cells/gm of soil (K-4) to 6,100 cells/gm of soil (K-2).

Table 6. Types of aerobic isolates which are obligate psychrophiles*
(% of aerobic count)

	Bac.	C-B	A-A	F-C	Mic. 1	Mic. 7	Mic. 8	Yeasts &Molds	Total
M-1	-#	1.7×10^5 (10.0)	0	3.4×10^4 (2.0)	1.0×10^5 (6.0)	0	1.4×10^5 (8.0)	-	4.4×10^5 (26.0)
M-2	0@	2.1×10^4 (6.0)	0	-	0	0	7.0×10^3 (2.0)	-	2.8×10^4 (8.0)
M-3	0	6.3×10^5 (8.2)	-	0	-	0	3.5×10^5 (4.6)	0	9.8×10^5 (12.8)
K-1	2.9×10^3 (6.0)	1.9×10^3 (4.0)	9.6×10^2 (2.0)	9.6×10^2 (2.0)	-	1.9×10^3 (4.0)	-	8.6×10^3 (18.0)	1.7×10^4 (36.0)
K-2	6.1×10^3 (12.0)	4.1×10^3 (8.0)	2.0×10^3 (4.0)	2.0×10^3 (4.0)	-	-	0	0	1.4×10^4 (28.0)
K-3	9.6×10^2 (7.4)	1.8×10^2 (1.4)	0	0	-	9.1×10^1 (0.7)	-	-	1.2×10^3 (9.5)
K-4	1.5×10^2 (2.3)	0	0	0	-	0	-	0	1.5×10^2 (2.3)
K-5	0	0	1.9×10^3 (8.3)	-	-	0	-	-	1.9×10^3 (8.3)
K-6	3.2×10^3 (3.4)	9.6×10^3 (10.2)	-	0	2.2×10^3 (2.3)	2.2×10^3 (2.3)	-	-	1.7×10^4 (18.2)
K-7	3.7×10^2 (2.9)	2.1×10^3 (16.4)	0	-	-	0	-	-	2.5×10^3 (19.3)
K-8	5.5×10^2 (8.5)	1.7×10^3 (26.8)	1.1×10^3 (16.9)	2.7×10^2 (4.2)	0	-	-	-	3.6×10^3 (56.4)
K-9	0	3.5×10^2 (8.3)	-	8.8×10^1 (2.1)	-	6.1×10^2 (14.6)	-	8.8×10^1 (2.1)	1.1×10^3 (27.1)
K-10	4.2×10^3 (22.1)	2.1×10^2 (1.1)	0	0	-	0	0	9.5×10^1 (0.5)	4.5×10^3 (23.7)
K-11	7.0×10^2 (11.5)	4.2×10^2 (6.9)	0	2.3×10^2 (3.8)	-	2.3×10^2 (3.8)	9.8×10^1 (1.6)	0	1.7×10^3 (27.6)
K-12	9.8×10^2 (0.7)	9.8×10^2 (0.7)	9.8×10^2 (0.7)	0	-	-	-	-	2.9×10^2 (2.1)

*Cells/gm of soil
@This type was isolated originally, but none were psychrophilic.
#This type was not isolated originally.

Means for Sample Areas

Means for the three sample sites of the manufacture area and the twelve sites from the assembly area are presented in Table 7. This table provides a representation of the aerobic population distribution from the two major areas associated with the Viking spacecraft. It indicates that approximately 14.8% (4.9×10^5 cells/gm soil) of the aerobic population originally isolated at 7 C from the manufacture area consists of obligately psychrophilic organisms. Of this population, 14.5% consists of gram positive rods or cocci, none of which are sporeformers. It also indicates that approximately 15.9% (5.6×10^3 cells/gm soil) of the population from the Kennedy samples consists of obligate psychrophiles. Of this population approximately 4.7% (1.7×10^3 cells/gm soil) are aerobic sporeformers. The remainder of this population includes gram positive rods and cocci, fungi, and smaller populations of gram negative rods.

Response of Original Soil Samples to the Artificial Martian Environment

The results of the response of the original soil samples to the artificial Martian environment are presented in Figs. 3, 4, and 5. Counts were performed on days 0, 1, 2, 7, 14, and 21, and incubation for counting was performed at 7 C. This temperature is in keeping with the overall objectives to determine the effects of populations growing at low temperatures instead of the standard NASA temperature of 32 C. Due to the problems associated with counting soil populations, evidence of decreased or increased populations is indicated only by changes in excess of approximately one log. Data points not shown on the graphs were lost due to uncountable plates, and these were regarded as no increase in the population.

Table 7. Means for all samples from the two sample areas (Based on original aerobic TSA counts)

	Manufacture Area (M)		Assembly Area (K)	
	Mean No.* (Mean %)	Mean No.Psy. (Mean % Psy.)	Mean No. (Mean %)	Mean No.Psy. (Mean % Psy.)
Bac.	4.9×10^4 (1.5)	- -	9.3×10^3 (26.1)	1.7×10^3 (4.7)
B-C	2.1×10^6 (63.9)	2.7×10^5 (8.4)	1.8×10^4 (50.0)	1.8×10^3 (5.1)
A-A	9.1×10^4 (2.8)	- -	2.0×10^3 (5.6)	5.7×10^2 (1.6)
F-C	1.6×10^5 (5.0)	1.1×10^4 (0.3)	1.2×10^3 (3.4)	2.8×10^2 (0.8)
Mic.1	1.0×10^5 (3.1)	3.4×10^4 (1.0)	2.6×10^3 (7.3)	1.8×10^2 (0.5)
Mic.7	4.8×10^4 (1.5)	- -	1.5×10^3 (4.3)	4.2×10^2 (1.2)
Mic.8	6.5×10^5 (20.0)	1.7×10^5 (5.1)	1.1×10^2 (0.3)	- -
Fungi	4.6×10^4 (1.4)	- -	8.2×10^2 (2.4)	7.1×10^2 (2.0)
No growth on sub-culture	2.7×10^4 (0.8)	- -	2.1×10^2 (0.6)	- -
TOTALS	3.3×10^6 (100.0)	4.9×10^5 (14.8)	3.5×10^4 (100.0)	5.6×10^3 (15.9)

*Cells per gm of soil

Fig. 3 contains the results of the three samples from the manufacture area. As can be seen, after 21 days these organisms were dying at a rapid rate. Samples M-1 and M-2 experienced a rapid decrease after the first day in this environment, recovered on day 2, but decreased steadily thereafter. Sample M-3 showed a slower but sustained death curve and showed no indication of recovery.

The results from the original soil samples from Cape Kennedy are presented in Figs. 4 and 5. Sample K-9 has been omitted because it showed a very low population at the beginning of the experiment. The figures show that these samples demonstrate almost identical responses during the first two days. This includes rapid death after one day, followed by rapid recovery on the second day, except for samples K-6 and K-12 which showed no significant recovery. The various samples responded differently after the first two days, but at least 7 of the 10 samples for which data were complete demonstrated definitive growth in the environment after 14 to 21 days. Samples K-1, K-2, K-5, K-7, and K-10 appear to be increasing in population after 21 days. Samples K-3 and K-4 showed definite growth after 14 days, but began dying rapidly during the next seven days. Results of samples K-8 and K-11 are inconclusive because of the loss of two data points for each. Since these points were unavailable for analysis, it was assumed that there was no increase in their populations. From the response of the other samples it can be estimated that sample K-8 did not grow in the Martian environment, and that sample K-11 may have continued to increase at least until the fourteenth day. It is apparent from these results that the soil samples from areas associated with the Viking spacecraft do contain populations which can grow under the

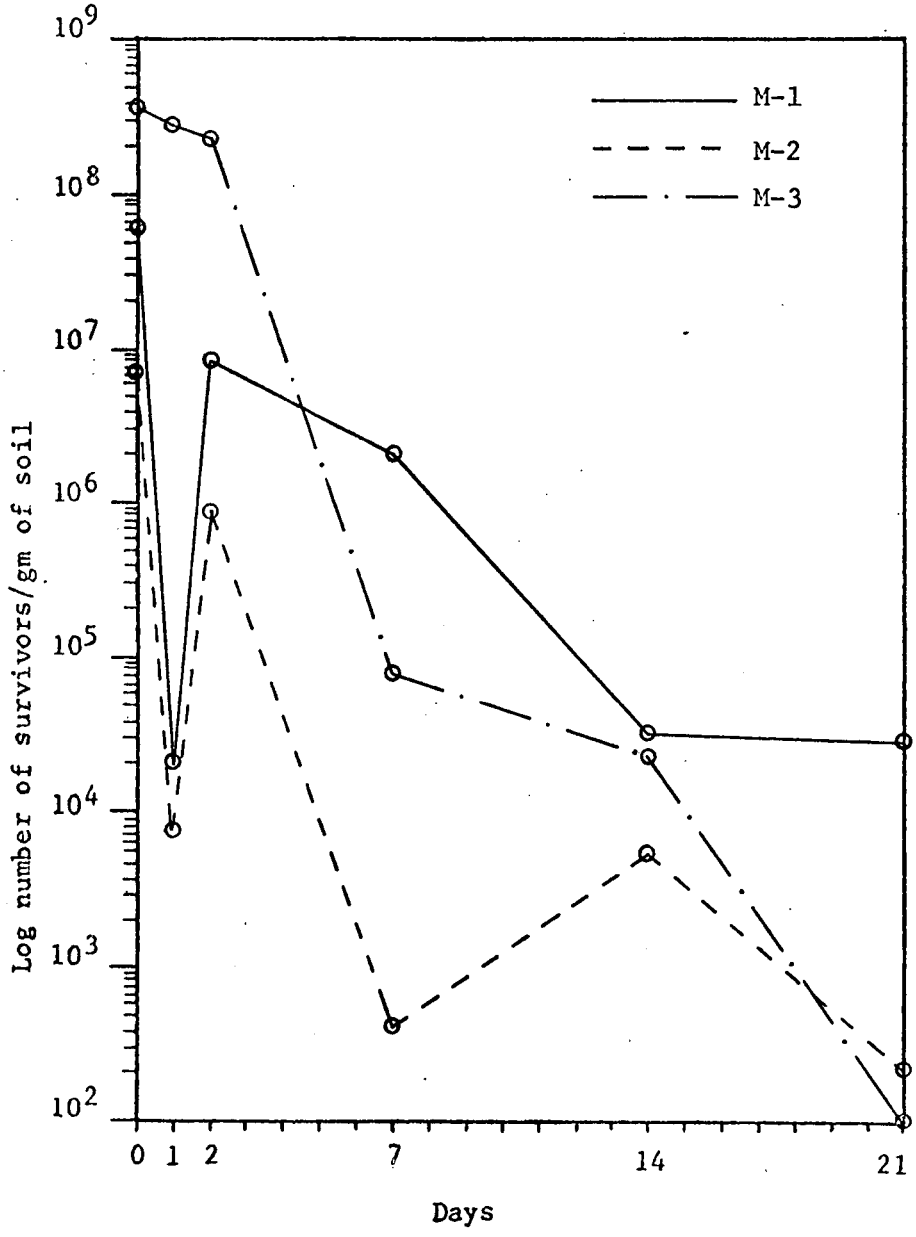

Fig. 3. Effect of experimental environment on original soil samples from manufacture area

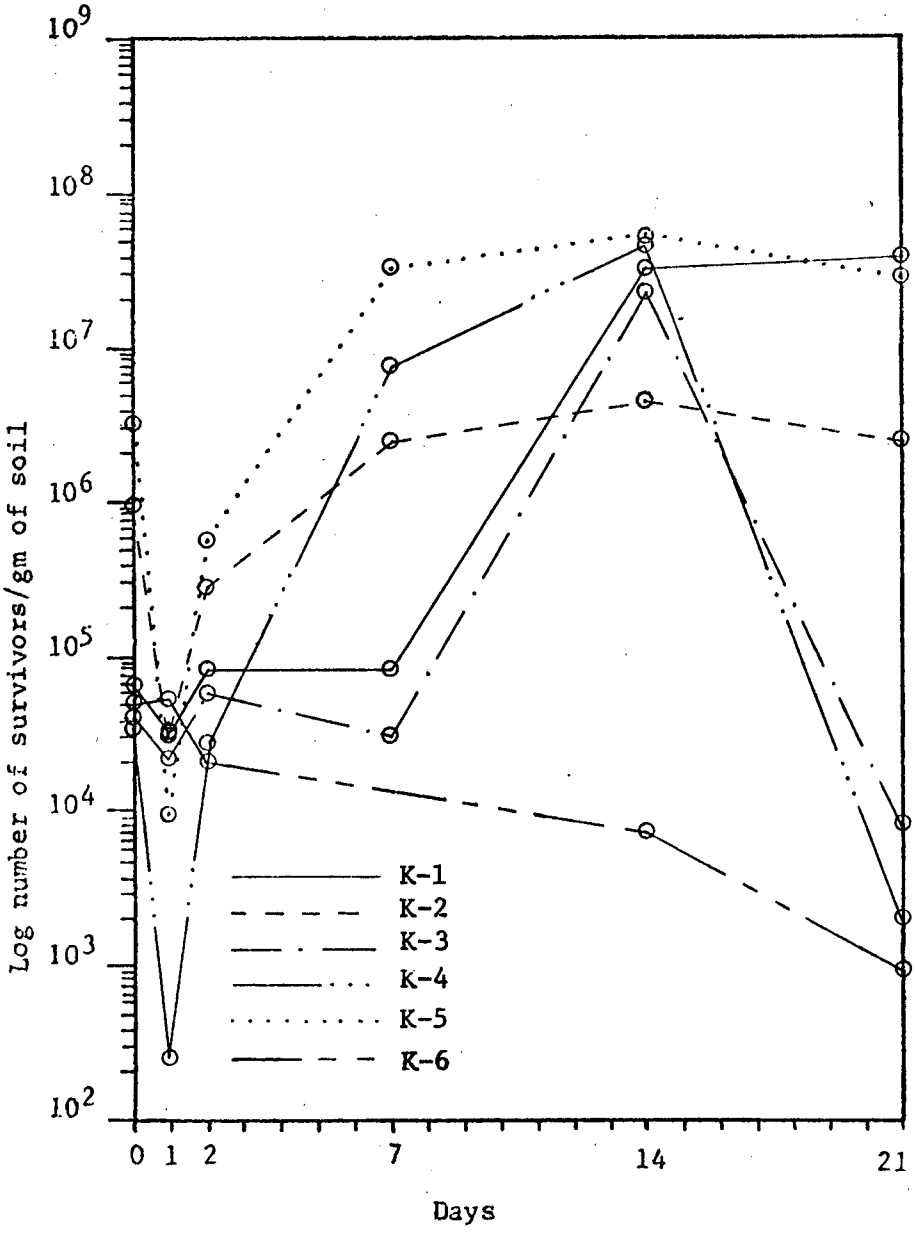

Fig. 4. Effect of experimental environment on original soil samples from Cape Kennedy (K1-K6)

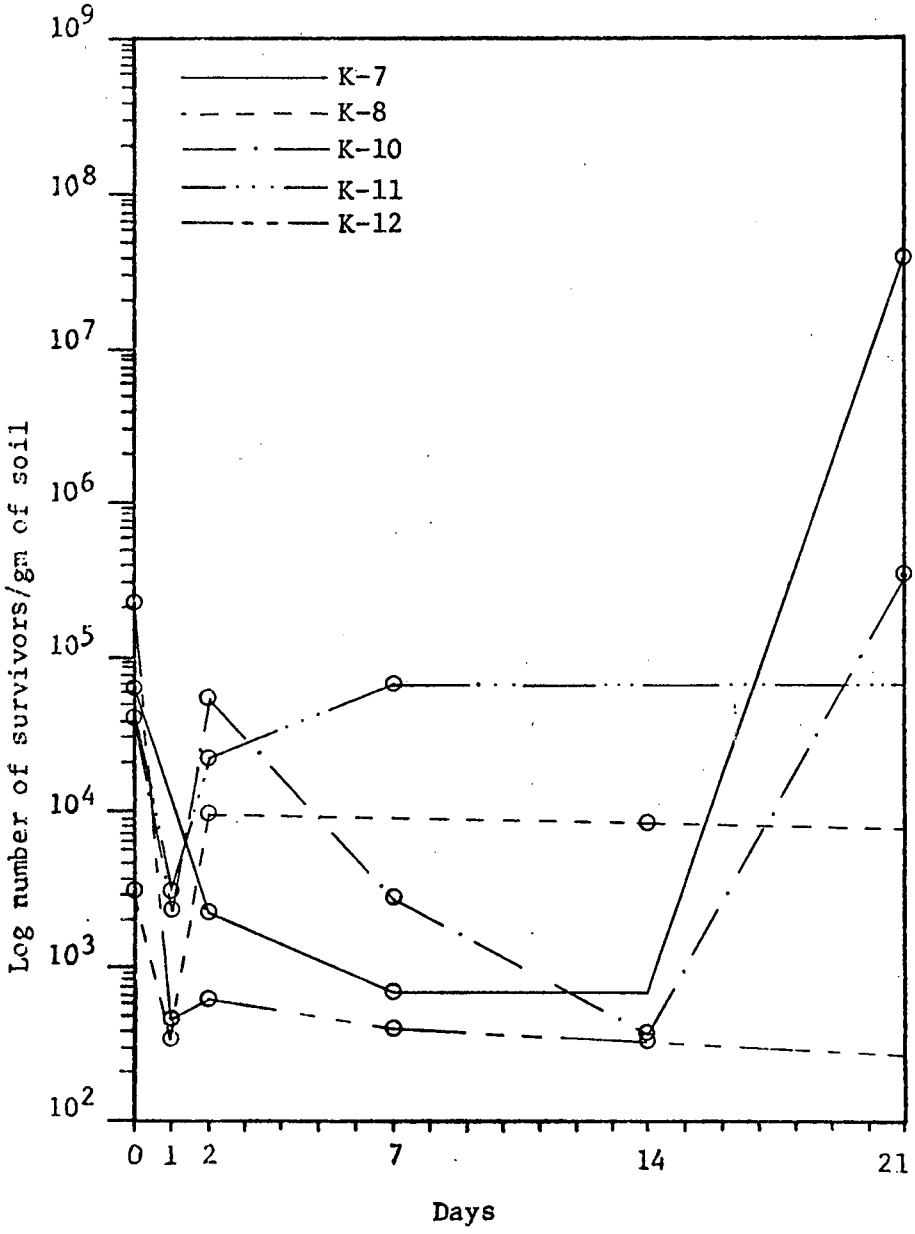

Fig. 5. Effect of experimental environment on original soil samples from Cape Kennedy (K7-K12)

conditions of this artificial Martian environment.

Response of Heat-Shocked Original Soil Samples to the Artificial Martian Environment

The original soil samples were heat-shocked at 80 C for 15 min and subjected to the experimental conditions of this investigation. The heat-shock procedure was employed to kill vegetative cells and to induce spore formation in the samples. These were then treated in the same manner as the non-heat-shocked samples. The results from these procedures on the samples from the manufacture area are presented in Fig. 6. As can be seen, there is no decline in the populations on the first day, as seen in the previous experiment, and all three samples showed a definite increase in the population after 21 days incubation. Samples M-1 and M-3 increased their populations by 3 logs after only two days, and they maintained this high population. The data point at day 7 for M-3 may be an artifact, but at any rate, the sample demonstrated good growth under these conditions. Sample M-2 shows a much slower increase in the population, reaching a one log increase only after 14 days.

Results of the Cape Kennedy samples are quite similar to those for the Denver samples and are presented in Figs. 7 and 8. Samples K-9 and K-11 have been omitted because their counts were very low at the onset of this part of the investigation. Only one of these samples (K-4) showed a decrease of one log on the first day, the others showing either little or no loss, or an increase on day 1. On the second day, 6 of the 10 samples tested showed increases of greater than one log, with four of these (K-4, K-6, K-10, and K-12) showing increases of two logs or greater. All samples except K-8 showed increases of at least

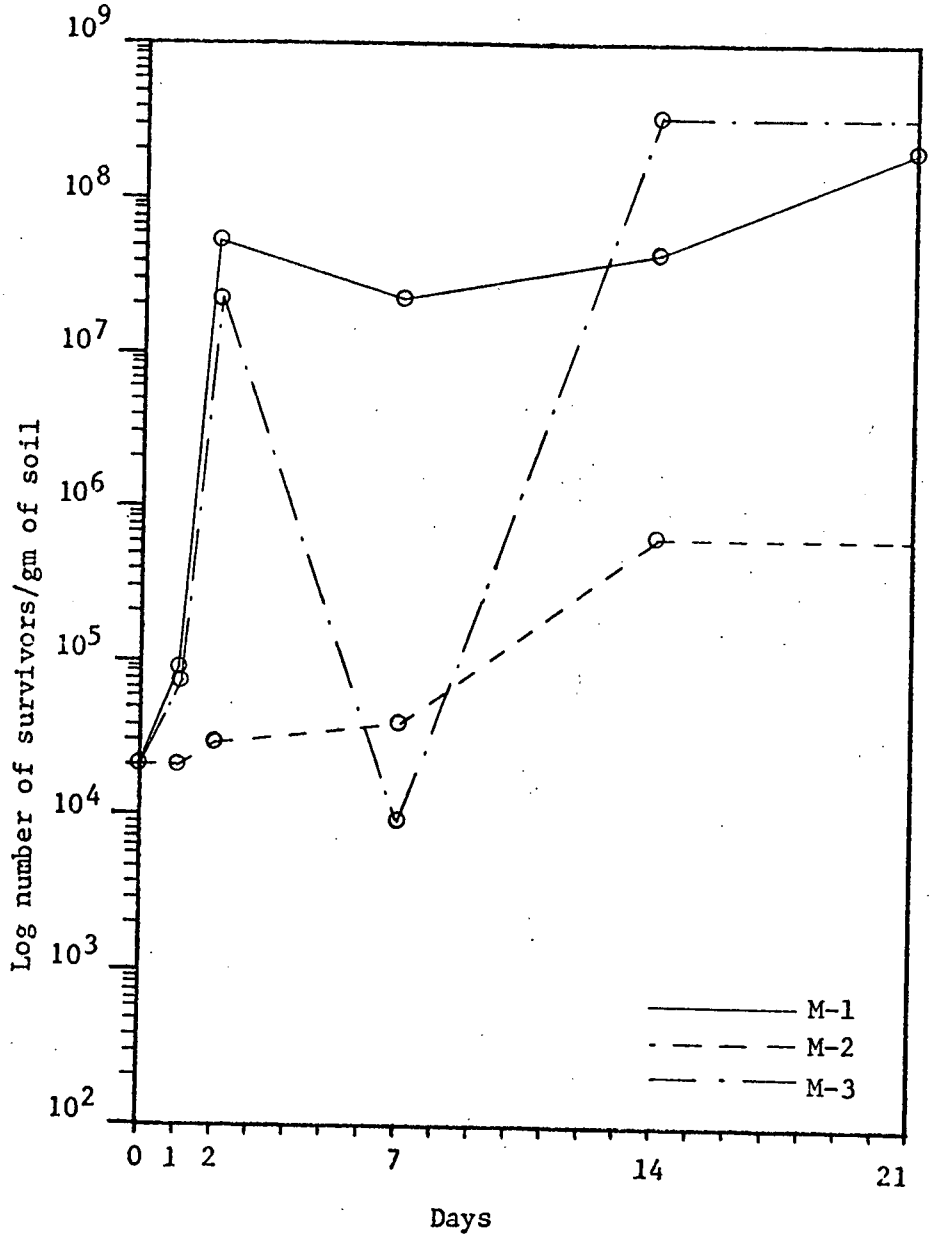

Fig. 6. Effect of experimental environment on heat-shocked soil samples from the manufacture area

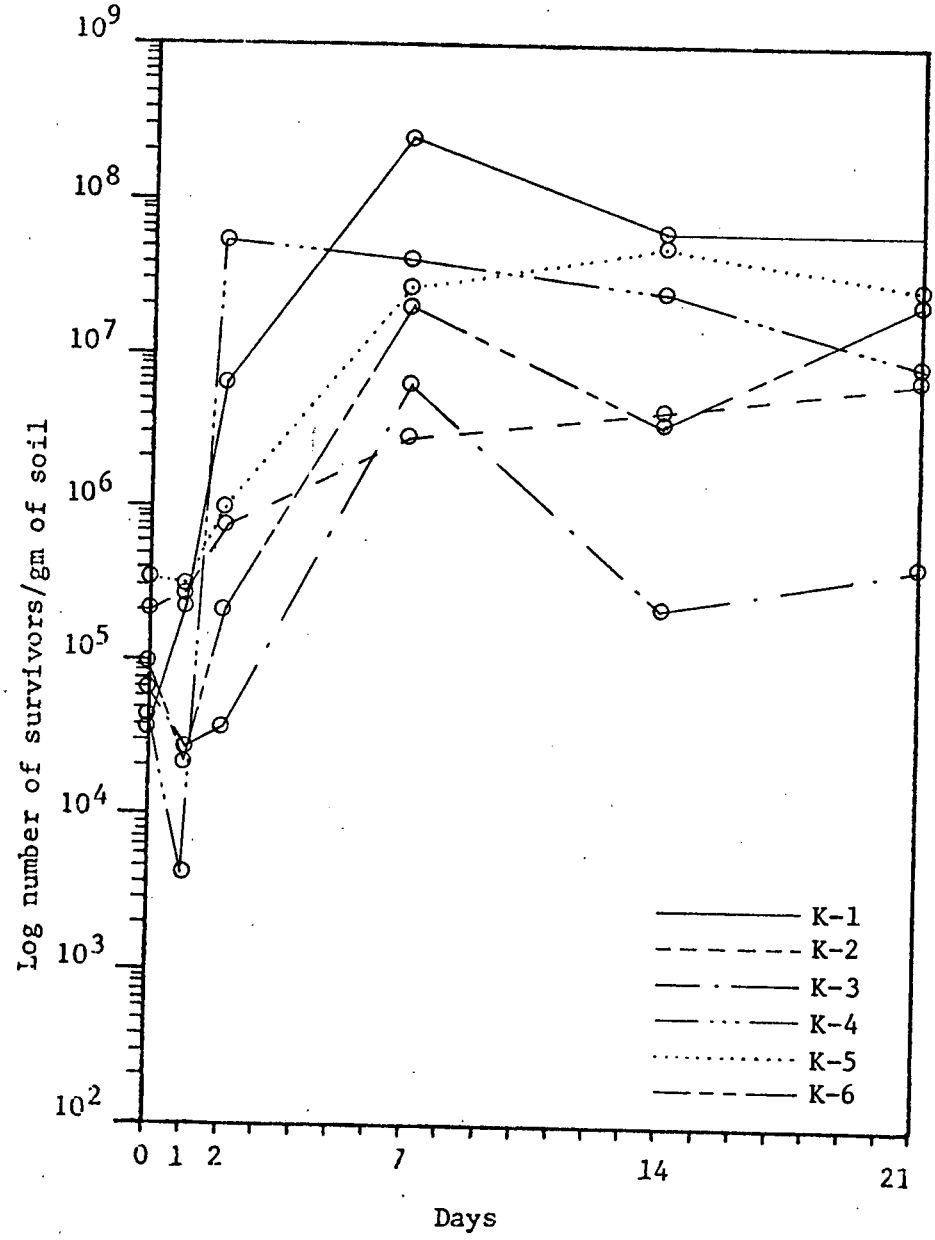

Fig. 7. Effect of experimental environment on heat-shocked soil samples from Cape Kennedy (K1-K6)

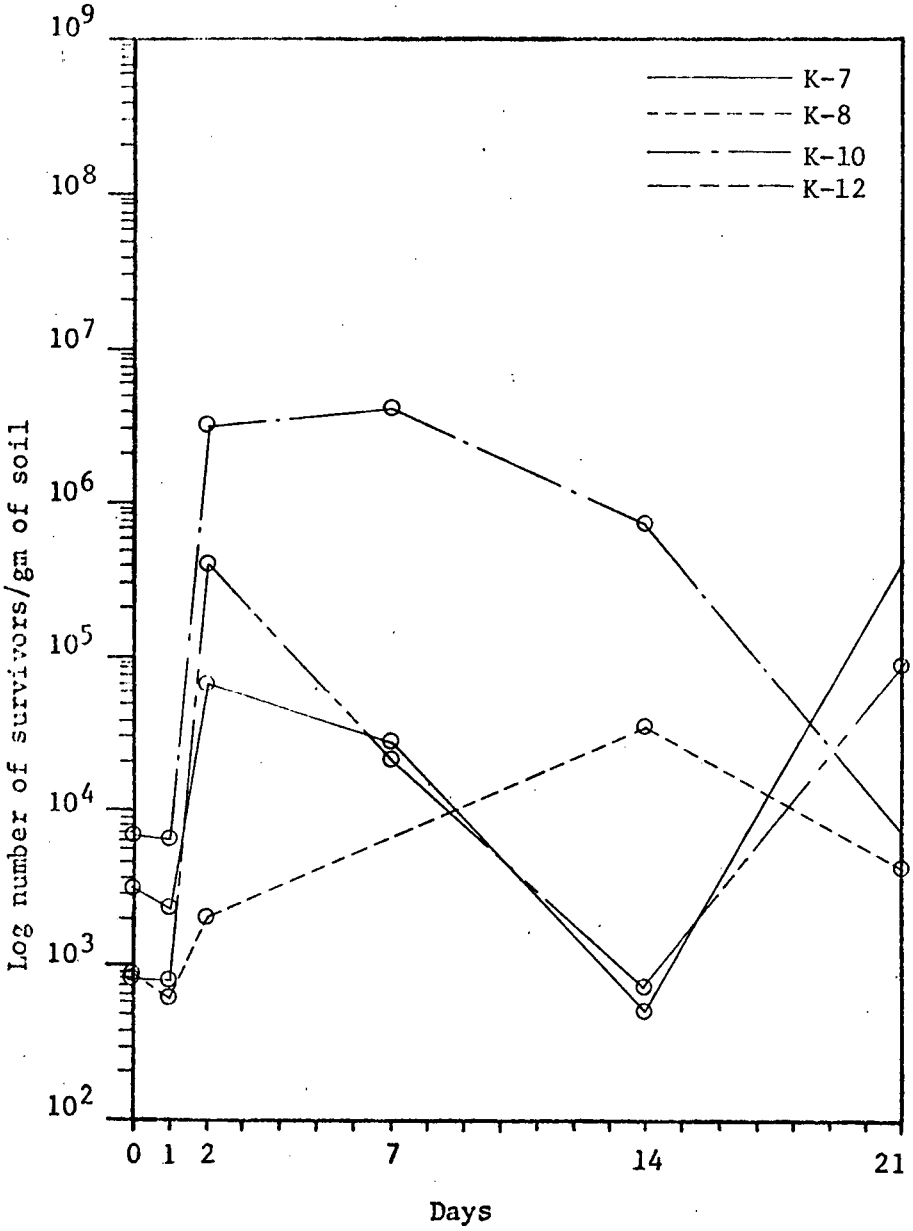

Fig. 8. Effect of experimental environment on heat-shocked soil samples from Cape Kennedy (K7-K12)

one log during the entire incubation period, and only sample K-10 dropped significantly after high populations had been established. Eight of these 10 heat-shocked samples appeared to be maintaining or increasing their populations after 21 days in the experimental Martian environment.

Response of Isolated Pure Cultures to the Experimental Martian Environment

Representative isolates were selected from the major groups of organisms and subjected to the artificial Martian environment in pure culture. The conditions for testing these organisms were the same as those for the original soil samples except that these were inoculated into an experimental Martian soil instead of being tested in their native soil. Since the sporeformers are of primary interest, 15 of the 24 isolates tested belonged to this group, while 3 belonged to the Corynebacterium-Brevibacterium group, one to the Alcaligenes-Acinetobacter group, one to the Flavobacterium-Cytophaga group, and four were pigmented Micrococci. Results from these studies over 21 days showed that there was almost no change in the populations. The curve for organism K-1-6 (Bacillus) in Fig. 9 is exemplary of nearly all of the isolates tested in pure culture. As is shown, the population did not change by one log in either direction. The other three organisms presented in this figure show the most drastic changes that occurred of the 24 isolates examined, and these are indeed not very drastic. Isolates K-2-38 and K-12-59 belong to the genus Bacillus, and K-5-34 belongs to the Alcaligenes-Acinetobacter group. Again, none of these showed a final change of more than one log. Sample K-2-38 did decrease and recover, but its final population exhibited no evidence of growth.

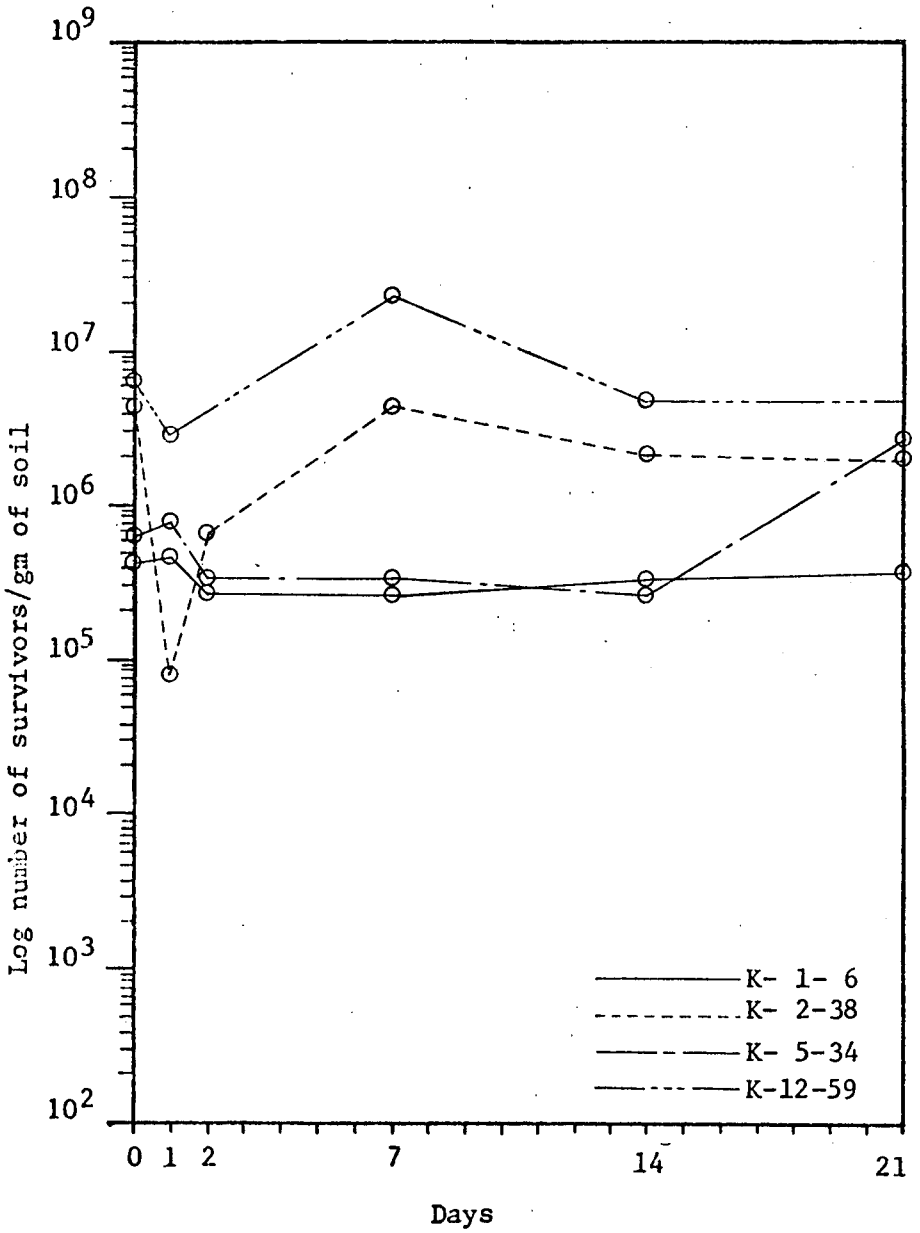

Fig. 9. Effect of experimental environment on selected pure culture isolates from Cape Kennedy soil samples

From these studies, it appears that the organisms could not grow under the conditions of this investigation, although the conditions were not lethal to the isolates. This lack of change in populations in the simulated Martian soil could be an effect of available nutrients. This aspect was investigated and is discussed later.

Response of Pure Cultures to the Artificial Martian Environment in the Slide Technique

The slide technique was used in this investigation to determine if it offered any significant advantages over the tube method in investigations such as this. This procedure was only recently set up, and the results are not completed at the time of this report. They will be reported at a later date.

Discussion and Conclusion

From the results it can be seen that the soil samples from areas associated with the Viking spacecraft do contain populations of organisms not capable of growth at 32 C on primary isolation. The samples from the manufacture areas contain as much as 15% of their populations which are obligate psychrophiles, none of which were shown to be sporeformers. Approximately 16% of the soil populations from Cape Kennedy contain obligate psychrophiles, of which about 5% are sporeformers. In other words, it appears that approximately 1.7×10^3 sporeformers per gm of soil and 4.9×10^5 total organisms per gm of soil are not capable of growth at 32 C on primary isolation. It is felt that these results are actually low because of the single isolation temperature of 7 C. If other primary isolation temperatures were used, additional organisms might be found which cannot grow at 32 C.

The investigations to demonstrate growth of microorganisms under the conditions of this experiment have shown that these soil samples, especially those from Cape Kennedy, do contain populations capable of growth under these experimental conditions. Some interesting results are seen when the results of the non-heat-shocked original samples are compared to the results of the heat-shocked samples. This is seen by comparing Figs. 3 and 6, Figs. 4 and 7, and Figs. 5 and 8. Upon comparing the curves of the samples from the manufacture area, it can be seen that heat-shocking appears to have induced the organisms to grow in the artificial Martian environment. The three non-heat-shocked samples showed no growth, while definite growth can be seen with the heat-shocked samples. Comparison of results from the Cape Kennedy samples are not as obvious as those of the Denver samples because most of the former demonstrated growth whether or not they were heat-shocked. The most obvious features seen by these comparisons are the lack of decreased populations after the first day and the very high increases which follow. These features are also seen in the Denver samples. In most cases, the inocula of the various samples in these two experiments were about the same, or even lower in the heat-shocked experiment; yet this latter population showed a substantially larger increase in population than the non-heat-shocked samples.

By making these comparisons, it appears that heat-shocking renders the population more able to grow in the artificial Martian environment. In the samples from the manufacture area it appears that the populations are so injured by the initial subjection to the Martian environment that they cannot recover. On the other hand, heat-shocking seems to induce a hardiness in at least a portion of

the population (the sporeformers) that enables them to survive the initial shock of the artificial environment and then grow abundantly under these conditions. Similar results are demonstrated in the Kennedy samples. The drop in population on the first day is appreciably decreased in the heat-shocked samples, and the resulting overall increase in the population is higher than in the non-heat-shocked samples. Also, more samples in the heat-shocked group showed continued increases after 21 days.

Even though it appears that heat-shock induces organisms to grow in the artificial environment, it may be possible that heat-shocking is sublethally injuring the populations and that some of the organisms then recover after a few days in the environment. If this is true, then counts at days 0, 1, and possibly 2 would appear lower than they actually were and subsequent apparent growth would be due to the recovery of these injured organisms. If this is the mechanism involved, then what appears to be very rapid growth may actually be only slow or no growth. At any rate, the point of major interest is that these soil samples do contain populations which appear to grow in the artificial Martian environment.

As seen in Fig. 9 pure cultures subjected to the experimental environment demonstrated no growth. The main difference between these and the original soil samples which showed growth is the difference in nutrients available. The original samples were maintained in their native soils while the pure cultures were inoculated into the artificial Martian soil. In order to better understand the effect of other parameters on growth in this environment, moisture and nutrient studies are being performed and will be reported at a later date.

Plans for Future Work

Several parts of this project are being investigated currently, but results are not complete at the time of this report. All experiments dealing with growth of microorganisms under the conditions of this project include incubation in the experimental environment for 60 days. These are being counted at present, but were not ready for reporting at this time. Since the pure cultures did not respond to the growth conditions of this experiment, it was decided to determine the cause of the difference in their results and the results of the original soil samples. For this reason, additional experiments based on the effects of moisture and nutrients are being performed. The slide technique which was described in the procedures may offer some advantages in investigations such as this, but this part of the project was only recently started, and results are not yet available.