Services Provided in Support of the Planetary Quarantine Requirements of the National Aeronautics and Space Administration under Contract W-13,062

Report No. 29
January - March 1970

Applied Microbiology and
Planetary Quarantine Section
Phoenix Laboratories
Ecological Investigations Program
National Communicable Disease Center
Public Health Service
U.S. Department of Health, Education, and Welfare
Phoenix, Arizona

Contributors:

Biophysics Unit	Experimental Microbiology Unit	Spacecraft Bioassay Unit
N. Petersen	W. Bond	J. Puleo
J. Marshall	L. Carson	G. Oxborrow
	N. Crase	N. Fields

Report submitted by:

Martin S. Favero, Ph.D., Chief
Applied Microbiology and
Planetary Quarantine Section

Report reviewed and forwarded by:

James E. Maynard, M.D., Chief
Ecological Investigations Program
Phoenix Laboratories

April 1970

1. A cooperative study with the University of Minnesota to determine the effect of test systems and personnel on D_{125C} values for two spore crops of Bacillus subtilis var. niger was partially completed. The study consisted of three phases. Phase I was completed during the week of January 12, 1970, at which time simultaneous on-site tests were conducted by both groups in Phoenix. Phase II consisted of the same tests conducted by both groups in Minnesota during the week of January 26, 1970. Phase III involves simultaneous testing by each group in their respective laboratories and has not been completed to date. All data obtained thus far has been analyzed by the computer and statistical services of the University of Minnesota.

A brief outline of major procedural differences is as follows:

I. Spores.

A. Minnesota: B. subtilis var. niger sporulated on TAM Sporulation Agar and stored in 95% ethanol at 4 C.

B. Phoenix: B. subtilis var. niger sporulated in SSM-10 liquid medium (Report No. 25) and stored in 95% ethanol at 4 C.

II. Inoculation and conditioning prior to heating.

A. Minnesota: Stainless steel strips (1/2" x 1/2") were inoculated with the suspensions using a 0.02 ml Eppendorf Microliter Pipette. Strips were then conditioned overnight in a laminar cross flow room (35% RH and 25 C) and were protected from direct exposure to the air flow.

B. Phoenix: Stainless steel strips (1/2" x 1/2") were inoculated using a 0.025 ml dropper pipette (Report No. 25) and conditioned overnight in a vacuum (26" hg) over activated silica gel.

III. Heating systems.

A. Minnesota: Rectangular copper boats each holding 5 strips were heated at 125 C on a hot plate device in the laminar crossflow room (35% RH, 25 C). The hot plate was protected from the airflow and the temperature was monitored with a recording thermometer.

B. Phoenix: The strips were suspended in a forced air oven at 125 C (Report No. 25) located in a laminar downflow room (42± 2% RH, 72± 2 F).

IV. Recovery of spores.

Teams in both locations plated appropriate dilutions with TSA. Colony counts were made after 48 hr. incubation at 32 C.

In both locations, trial runs were conducted during the first day to establish appropriate dilutions for each heating interval. Subsequently, each team performed one test on each spore crop for two consecutive days. During the first test in Phoenix, a member of one team counted a complete set of plates. The counting marks were then erased, and the set was passed to a member of the other team for counting. Differences in individual counting were found to be statistically significant. However, the differences in resultant D_{125C} values could not be considered as significant in a microbiological sense (i.e., 42.6 min. vs. 48.9, 45.6 vs. 45.0 for Phoenix spores and 25.2 vs. 25.1, 22.6 vs. 22.8 for Minnesota spores). Counting differences arose primarily from individual assessments of coalescing growth on the bottoms of many plates. For the remaining tests at both locations, D_{125C} values obtained by both teams showed close correlation with each spore crop and heating system.

When D_{125C} values of the two spore crops were compared, the Phoenix crop grown in SSM-10 liquid medium had higher values in both test systems than did the Minnesota crop grown on TAM Sporulation Agar. In Phoenix, the Phoenix crop (P) D_{125C} values by both teams averaged 39.3 min. while the Minnesota crop (M) averaged 27.5 min. In Minnesota, P averaged 21.9 min. and M averaged 14.3 min.

Perhaps the most striking observation resulting from the tests was that, when considering both locations, both teams, and both spore crops, the D_{125C} values obtained on the second day of testing were consistently and significantly lower than those values obtained on the first days. This phenomenon has yet to be completely resolved. In Minnesota, indications are that the variable positioning of the copper boats on the hot plate may have accounted for the differences. In Phoenix, however, the positioning of strips in the forced air oven remained consistent over the two days of testing. Further investigation of this phenomenon and the results of Phase III will be reported later.

Thus far, the results of this study are encouraging. Indications are that several laboratories performing dry heat inactivation tests will be able to "calibrate" or relate their D_{125C} values by using a common protocol and a standard spore crop.

2. Based on the same data from the studies described above a comparison of D values obtained using the Minnesota method of analysis was made with D values obtained using the Phoenix method of analysis. The Minnesota method utilizes a computerized least squares regression analysis which applies rules for accepting or rejecting certain plate counts and which ignores N_o values because of commonplace non-linearity of data during the first time period of heating. The Phoenix method involves hand calculation of a similar least squares regression analysis with rules for accepting or rejecting certain plate counts. Also, N_o values are always used. If inspection of the data suggests a polyphasic survivor curve (i.e., "shoulder" or "tailing") D values are calculated for each

linear portion of the curve (Report Nos. 21 and 27). Table 1 presents the comparison of D_{125C} values obtained from the data of the interlaboratory cooperative study using the two methods of analysis. It appeared that the D_{125C} values obtained by the Phoenix method of analysis were more consistent than those obtained using the Minnesota technique. Basically, however, the same general trends and conclusions did not change.

3. The reproducibility of the multiple replicate unit testing (FN-MPN) system used for the frequency distribution of D_{125C} values in Report No. 26 was examined. Two of the Mariner '69 spore isolates from the frequency distribution and the standard B. subtilis var. niger (BGSSM-10) were tested on two consecutive days, and the results are shown in Table 2. The reproducibility of the system was found to be acceptable with the coefficient of variation being less than 10%. Future tests will examine the relationship between D_{125C} values obtained by the FN-MPN system and those obtained by plate counting.

4. The study to estimate concentration of buried microbial contamination in electronic components (Report No. 28) was continued. Ten percent of the tubes containing fractured components in TSB and demonstrating no growth after 30 days of incubation at 32 C were inoculated with B. subtilis var. niger and again incubated at 32 C. After 24 hours all 21 inoculated tubes showed heavy growth indicating an absence of inhibitory materials.

   The fractured components in group A were aseptically removed from the tubes containing TSB, each half resistor was broken in two, four quarter pieces were placed in each of 40 tubes containing TSB and the tubes were incubated at 32 C. During this procedure it was noted that two of the original tubes containing fractured components in TSB contained significant amounts of bottom sediment. These tubes had initially been considered positive for growth after 24 hours of incubation but attempts at subculturing were unsuccessful. This lead to the conclusion that the turbidity was due to a non-viable precipitate. However, microscopic examination of the sediment made at the time when the component pieces were removed from the tubes indicated the presence of large numbers of gram positive rods. Attempts were again made to subculture the sediment using TSA, TSB, fluid Thioglycollate medium and cooked meat medium incubated aerobically and anaerobically at 32 C and 37 C. No growth was detected in any of these media.

   The component pieces recovered from the tubes containing the sediment were aseptically washed several times in sterile buffered distilled water to remove cells that might be adhering to the surfaces and were then aseptically placed in tubes of TSB and incubated at 32 C for 48 hours. Although no obvious turbidity was observed in the tubes a sediment was noted and microscopic examination of this sediment revealed large numbers of cells similar to those observed in the original tubes. Attempts to subculture these cells were unsuccessful. Work will continue with these components and attempts will be made to recover the bacteria in a viable state and identify them. None of the 40 tubes containing the refractured components of this type were positive for growth.

5. A total of 2,037 bacterial colonies were isolated and identified from the Apollo 12 spacecraft. The 38 different species or groups detected are shown in Table 3. Four different culture media (Trypticase Soy Agar [TSA], Blood Agar, MacConkey's Agar and Mycophil Agar) were employed in these studies. Table 4 shows the types of aerobic mesophilic microorganisms isolated from the different spacecraft components using TSA. The types of microorganisms on components of Apollo 12 were similar to those on Apollo 10 and 11 (Report No. 27). A comparison of the microbial types isolated on TSA and Blood Agar is shown in Table 5. Preliminary examination of these data indicate that no distinct qualitative or quantitative advantages were gained by the use of Blood Agar. Similar results were obtained when the data from Apollo 10 and 11 were evaluated (Report No. 27).

A comparison of the microorganisms detected on Apollo 10, 11 and 12 spacecraft is shown in Table 6. The vast majority of microorganisms detected on all the spacecraft were those considered to be indigenous to human hair, skin and respiratory tract. The occurrence of microorganisms associated with soil and dust in the environment were comparatively low (Table 7). These qualitative profiles were remarkably similar for all three Apollo spacecraft.

Generic identification of molds isolated from Apollo 12 is shown in Table 8. A total of 11 genera were detected on the four culture media employed. Six genera were detected on TSA, nine on Mycophil, two on MacConkey's and one on Blood Agar. Four genera were isolated only on Mycophil Agar, one only on TSA and one only on MacConkey's agar.

A comparison of the numbers and types of microorganisms detected on Apollo 10, 11 and 12 spacecraft is shown in Table 9. The quantitative data for all three spacecraft are similar.

The 2,037 bacterial colonies isolated and identified from Apollo 12 spacecraft were lyophilized and stored for future reference.

All quantitative and qualitative data obtained from Apollo 12 mission were stored on a CDC 3100 computer at Cape Kennedy. Computer printouts were compiled and sent to the Planetary Quarantine Officer.

Studies were initiated on the Apollo 13 spacecraft, and results will be reported next quarter.

The identification schemes for microorganisms isolated from spacecraft were revised and will be completed during the next quarter. Work on the development and evaluation of a computer identification system in conjunction with the Sandia Corporation is in progress.

In accordance with a request from the Planetary Quarantine Officer, approximately 500 cultures isolated from the primates associated with

Bio-Satellite Mission (Primate Mission Flight D) were received from the Ames Research Center. The cultures will be identified and the results reported next quarter.

The Sandia modified air sampler (Royco and Anderson air sampler combination) has been received and field studies have been initiated.

TABLE 1. COMPARISON OF D_{125C} VALUES BASED ON PHOENIX AND MINNESOTA METHODS OF ANALYZING INTERLABORATORY COOPERATIVE STUDY DATA.

Spore Crop	Experimenting Team	Counting Team	D_{125C} Value in Minutes	
			Phoenix Analysis	Minnesota Analysis
P[1]	P	P	48.4	42.6
P	P	M	48.7	48.9
P	M	P	47.8	45.6
P	M	M	48.2	45.0
M[2]	P	P	20.2	25.2
M	P	M	20.1	25.1
M	M	P	19.4	22.6
M	M	M	19.5	22.8

[1] Phoenix

[2] Minnesota

TABLE 2. D_{125C} VALUES OBTAINED BY THE FN-MPN TECHNIQUE.

Spore Crop	D_{125C} Values (Min)	Mean	Standard Deviation	Coefficient Variation
M-4-5[1]	< 5.0, 6.0, 6.0, 6.3	5.8	0.5	9%
M-4-45[1]	17.0, 16.2, 13.7, 16.2	15.8	1.2	8%
BGSSM-10[2]	33.8, 37.1, 35.5, 31.4	34.4	2.1	6%

[1] Mariner '69 spore isolate (Report No. 26).

[2] B. subtilis var. niger sporulated with SSM-10 liquid medium (Report No. 25).

TABLE 3. TYPES OF MICROORGANISMS FOUND ON APOLLO 12 SPACECRAFT.

Staphylococcus spp.	*Corynebacterium*-*Brevibacterium* Group
Group I	
Group II	*Alcaligenes* spp.
Group III	
Group IV	*Flavobacterium* spp.
Group V	
Group VI	Actinomycetes
Micrococcus spp.	Streptomycetes
Group 1	
Group 2	Yeasts
Group 3	
Group 4	Molds
Group 5	
Group 6	Atypical *Micrococcus* spp.
Group 7	
	Atypical *Bacillus* spp.
Bacillus spp.	
B. alvei	
B. badius	
B. brevis	
B. cereus	
B. circulans	
B. coagulans	
B. firmus	
B. laterosporus	
B. lentus	
B. licheniformis	
B. megaterium	
B. pantothenticus	
B. polymyxa	
B. pulvifaciens	
B. sphaericus	
B. subtilis	

TABLE 4. TYPES OF MICROORGANISMS DETECTED ON APOLLO 12 SPACECRAFT USING TRYPTICASE SOY AGAR.

TYPE	CSM-108 %	LAI-6 %	LAE-6 %	LDE-6 %	SLA-12 %	IU-12 %	S-4B-12 %	All Components of Spacecraft %
Staphylococcus spp.								
Group II	25.2	6.1	21.4	20.1		4.8	1.7	14.3
Group III	4.5	0.7	0.7	3.5		1.6		2.0
Group IV	14.5	16.7	6.9	4.2	20.0	1.6	6.8	12.0
Group V	8.6	5.2	4.8	6.9		6.3	1.7	6.1
Group VI	11.0	14.4	5.5	7.6		1.6		10.1
Micrococcus spp.								
Group 1	8.3	10.6	0.7	2.8	40.0	4.8	3.4	7.2
Group 2	6.5	3.6	1.4	4.2		1.6		3.8
Group 3	3.1	5.4	2.8			1.6	3.4	3.5
Group 4		0.2						0.1
Group 5	1.0	0.4				4.8	3.4	0.9.
Group 6						1.6	1.7	0.2
Group 7	11.0	20.5	24.8	9.0		20.6	22.0	17.2
Bacillus spp.								
B. alvei						1.6		0.1
B. badius			0.7			4.8		0.3
B. brevis							1.7	0.1
B. cereus	0.3							0.1
B. circulans			2.1	4.2			1.7	0.9

TABLE 4. continuation

TYPE	CSM-108 %	LAI-6 %	LAE-6 %	LDE-6 %	SLA-12 %	IU-12 %	S-4B-12 %	All Components of Spacecraft %
Bacillus spp.								
B. coagulans		0.4					3.4	0.3
B. firmus	0.3	0.2	2.1					0.4
B. laterosporus							1.7	0.1
B. lentus		1.1	0.7				1.7	0.6
B. licheniformis				0.7				0.1
B. megaterium			2.1					0.3
B. pantothenticus	0.3	0.4		0.7		1.6	1.7	0.5
B. pulvifaciens	0.7	0.2						0.3
B. sphaericus	0.3	0.4				3.2		0.4
Corynebacterium-Brevibacterium group	1.7	4.5	13.1	28.5		17.5	23.7	9.6
Achromobacter spp.		0.2						
Alcaligenes spp.	0.3							
Flavobacterium spp.			0.7			1.6	5.1	0.4
Actinomycetes			1.4				1.7	0.3
Streptomycetes			0.7					0.1
Yeasts	0.3	0.9					1.7	0.5
Molds		0.2	1.4	0.7		9.5	5.1	1.1
Atypical Micrococcus spp.	1.0	1.8		2.1		7.9	1.7	1.7
Atypical Bacillus spp.							1.7	0.1
No growth on subculture	0.7	5.6	6.2	4.7	40.0	1.6	3.4	4.2
Number isolated	290	444	145	144	5	63	59	1150

TABLE 5. A COMPARISON OF THE TYPES AND LEVELS OF AEROBIC MESOPHILIC MICROORGANISMS DETECTED ON THE APOLLO 12 SPACECRAFT USING TRYPTICASE SOY AGAR AND BLOOD AGAR.

	TSA %	Blood Agar %
Staphylococcus spp.		
Group II	14.3	15.0
Group III	2.0	0.9
Group IV	12.0	8.6
Group V	6.1	12.3
Group VI	10.1	9.1
Micrococcus spp.		
Group 1	7.2	7.3
Group 2	3.8	3.6
Group 3	3.5	2.3
Group 4	0.1	
Group 5	0.9	0.9
Group 6	0.2	0.5
Group 7	17.2	19.1
Bacillus spp.		
B. alvei	0.1	
B. badius	0.3	0.5
B. brevis	0.1	
B. cereus	0.1	0.5
B. circulans	0.9	0.5
B. coagulans	0.3	
B. firmus	0.4	0.9
B. laterosporus	0.1	
B. lentus	0.6	0.5
B. licheniformis	0.1	
B. megaterium	0.3	
B. pantothenticus	0.5	1.4
B. pulvifaciens	0.3	
B. sphaericus	0.4	
Corynebacterium-*Brevibacterium* Group	9.6	9.1
Alcaligenes spp.	0.2	
Flavobacterium spp.	0.4	0.5
Actinomycetes	0.3	

TABLE 5. continuation

	TSA %	Blood Agar %
Streptomycetes	0.1	
Yeasts	0.5	0.9
Molds	1.1	0.5
Atypical *Micrococcus* spp.	1.7	0.9
Atypical *Bacillus* spp.	0.1	
No growth on subculture	4.2	4.5
Number isolated	1150	220

TABLE 6. GENERAL TYPES OF MICROORGANISMS DETECTED ON THE APOLLO 10, 11 AND 12 SPACECRAFT USING TRYPTICASE SOY AGAR.

Type	SPACECRAFT					
	Apollo 10		Apollo 11		Apollo 12	
	Number	Percent	Number	Percent	Number	Percent
Indigenous to humans	1067	96.2	1109	94.8	1080	93.8
Indigenous to dust and soil	42	3.8	61	5.2	70	6.1

TABLE 7. A COMPARISON OF TYPES OF MICROORGANISMS ASSOCIATED WITH THE COMPONENT PARTS OF APOLLO 10, 11 AND 12 SPACECRAFT USING TRYPTICASE SOY AGAR.

Source	Apollo 10		Apollo 11		Apollo 12	
	Human Type	Environmental Type	Human Type	Environmental Type	Human Type	Environmental Type
	Percent		Percent		Percent	
CMI	97.6	2.4	99.3	0.7	97.9	2.1
LAI	99.5	0.5	98.0	2.0	96.8	3.2
LAE	90.7	9.3	92.6	7.4	88.9	11.1
LDE	97.5	2.5	91.7	8.3	93.7	6.3
SLA	82.4	17.6	25.0	75.0	100.0	0.0
I.U.	88.9	11.1	84.4	15.6	79.4	20.6
S-4B	87.5	12.5	76.2	23.8	79.7	20.3

TABLE 8. MOLDS DETECTED ON APOLLO 12 SPACECRAFT IN RELATION TO RECOVERY MEDIA.

Genus	Number of Isolates			
	Trypticase Soy Agar	Blood Agar	MacConkey's Agar	Mycophil Agar
Alternaria	1	0	0	1
Aspergillus	7	1	1	4
Aureobasidium pullulans	0	0	0	1
Bipolaris	2	0	0	1
Cephalosporium	0	0	0	1
Curvularia	2	0	0	3
Fusarium	0	0	0	2
Nigrospora	0	0	0	1
Penicillium	1	0	0	5
Phoma	1	0	0	0
Scopulariopsis	0	0	1	0

TABLE 9. COMPARISON OF THE NUMBERS AND TYPES OF MICROORGANISMS DETECTED ON THREE APOLLO SPACECRAFT.

	Apollo 10	Apollo 11	Apollo 12
Staphylococcus spp.			
Group I	142	28	1
Group II	260	355	334
Group III	39	6	29
Group IV	131	242	229
Group V	163	215	155
Group VI	134	154	217
Micrococcus spp.			
Group 1	142	207	161
Group 2	85	103	82
Group 3	68	36	77
Group 4	0	0	1
Group 5	20	26	20
Group 6	0	0	3
Group 7	94	104	283
Group 8	4	1	0
Streptococci-Enterococcus Group	2	0	0
Streptococci-Viridans Group	15	5	0
Streptococci-Pyogenic Group	3	0	0
Bacillus spp.			
B. alvei	0	0	2
B. badius	0	3	5
B. brevis	2	1	1
B. cereus	2	4	7
B. circulans	15	3	27
B. coagulans	8	4	7
B. firmus	5	3	9
B. laterosporus	1	1	1
B. lentus	8	4	8
B. licheniformis	6	7	3
B. megaterium	3	0	0
B. pantothenticus	1	13	11
B. polymyxa	4	19	4
B. pulvifaciens	3	0	5
B. pumilus	1	13	0
B. sphaericus	1	2	5
B. subtilis	0	0	6

TABLE 9. continuation

	Apollo 10	Apollo 11	Apollo 12
Corynebacterium-Brevibacterium Group	160	183	172
Achromobacter spp.	0	2	0
Aerobacter spp.	7	0	0
Alcaligenes spp.	3	9	2
Flavobacterium spp.	1	1	8
Pseudomonas spp.	1	0	0
Actinomycetes	2	7	3
Streptomycetes	0	0	1
Yeasts	5	8	13
Molds	37	34	36
Atypical Micrococcus spp.	9	57	28
Atypical Bacillus spp.	1	3	2
No growth on subculture	409	178	75
Number isolated	1991	2041	2037