Psychrophilic Microorganisms from Areas Associated with the Viking Spacecraft

TERRY L. FOSTER* AND LUTHER WINANS, JR.

Department of Biology, Hardin-Simmons University, Abilene, Texas 79601

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Microorganisms capable of growth at 7 C were enumerated and isolated from soil samples from the manufacture and assembly areas of the Viking spacecraft. Populations ranging from 4.2 \times 10^8 to 7.7 \times 10^9/g of soil were isolated from the 15 soil samples examined. Temperature requirements were determined, and those growing at 3 C, but not at 32 C, were designated as obligate psychrophiles in this investigation. Populations of soil bacteria, including aerobic sporeformers, ranging from 1.5 \times 10^8 to 9.8 \times 10^9/g were capable of growth at 3 C, but not at 32 C. Bacterial isolates were identified to major generic groups. No psychrophilic sporeformers were isolated from soil from the manufacture area, but psychrophilic sporeformers ranged from 0 to 6.1 \times 10^9/g from soil from the assembly area.

One purpose of the United States planetary quarantine policy is to determine guidelines for the prevention of contamination of Mars with terrestrial microorganisms which might grow in the Martian environment (6). For this reason, it is essential that various groups of microorganisms associated with planetary vehicles destined for Mars be studied in this respect. A great deal of previous research has been conducted on organisms isolated from the manufacture and assembly areas of interplanetary spacecraft, but most of these studies dealt primarily with mesophilic bacteria and the heat resistance of mesophilic sporeformers. The Viking spacecraft will be decontaminated by means of dry heat (13). The standard procedures for the microbiological assay of such spacecraft environments call for the exclusive use of 32 C as the incubation temperature (11). This procedure limits the assay to detection of mesophilic organisms, which make up the majority of microorganisms found on spacecraft surfaces (15).

Although it is generally agreed that psychrophiles may not be the most heat resistant of the microorganisms, they should not be excluded from investigations related to planetary quarantine, because these may include organisms with the physiological characteristics to grow in the hostile environment of Mars (7). Also, it is known that some Bacillus spp. and Clostridium spp. can grow at low temperatures (9, 17), and sporeformers are the more heat-resistant microorganisms.

It is recognized that there are numerous definitions of psychrophilic organisms, including those based on optimum growth temperature and those based on possible growth ranges (8). This present study is not intended to debate the definition of psychrophiles but to demonstrate that the present incubation temperature of 32 C used in the microbial monitoring of spacecraft environments might be excluding populations of microorganisms of significance to planetary quarantine. The Viking spacecraft is scheduled to be launched to Mars in 1975; consequently, the primary objective of this investigation was to determine the presence and concentration of psychrophilic microorganisms in various environmental areas associated with the spacecraft.

MATERIALS AND METHODS

Selection of samples. Soil samples were obtained from three sites where the Viking spacecraft is manufactured (Denver, Colo., M), and 12 sites where the spacecraft is housed in preparation for launching (Cape Canaveral, C). Surface soil samples no deeper than 6 inches (ca. 15 cm) were taken from areas around main entrances through which dust contamination might enter the buildings.

Isolation of microorganisms. Immediately after being returned to the laboratory, each sample was thoroughly mixed, and 10-g portions of each were decimally diluted in 1.0% peptone to a final dilution of 10^{-4} prior to plating. The first bottle (90 ml) in each dilution series contained glass beads, the bottle was sonicated for better dispersal of soil particles (14). Subsequent dilution tubes (9 ml) were mixed on a Vortex mixer to assure thorough mixing. Amounts of 0.1 ml were transferred to the surface of Trypticase soy agar (Baltimore Biological Laboratories [BBL]) and Mycophil agar (BBL), pH 4.0, and spread with glass spreading rods.
Because all isolations included incubation of samples at 7 C, media used throughout this investigation were stored at 7 C for at least 24 h prior to use. To prevent possible damage to psychrotolerant organisms by addition of molten agar, the spread-plate technique was employed in all counts. All manipulations were performed in a laminar flow cabinet (Enviro-MiniBench, model MBO-48, Albuquerque, N.M.).

Duplicate plates were prepared for aerobic, anaerobic, and fungal counts. The plates were placed in the 7 C incubator (Freams model 805) immediately after inoculation, and only the anaerobic plates were allowed to reach room temperature during the manipulations. The anaerobic plates were recriled after inoculation, placed in Brewer Anaerobic jars with GasPaks and anaerobic indicators (BBL), and plated in the 7 C incubator as soon as anaerobic conditions were achieved as shown by the indicator.

A freshly inoculated Trypticase soy agar slant of Alcaligenes fecalis (NASA standard test organism, Center for Disease Control, Phoenix, Ariz.) was placed in each anaerobe jar as a biological indicator of anaerobiosis. In no case did this control organism grow in the anaerobe systems. All incubators were monitored with maximum-minimum registering thermometers (Taylor model 5458), which were checked daily. Slight increases in temperature occurred only during times when samples were being added to or removed from the incubators.

Temperature studies. Colonies from plates having 30 to 300 colonies after 14 days of incubation were transferred to Trypticase soy agar slants for incubation at 3 C (10 to 14 days), 24 C (3 to 5 days), and 32 C (48 h). After growth had occurred, the results were recorded, and organisms showing growth at 3 C, but not at 32 C, were classified as psychrophilic, according to the definition used in this investigation.

Identification of isolates. All isolates from the temperature studies were examined individually by staining and biochemical testing. From these results, the temperature studies, and colonial characteristics, the organisms were identified to major generic groups. The isolates were stained for their Gram reaction, tested for motility by phase-contrast microscopy, tested for oxygen requirements, and subjected to numerous biochemical tests (4). Organisms thought to be sporeformers were grown on AK-2 sporulating agar (BBL) at either 7 C (10 to 14 days) or 24 C (2 to 3 days). These were then stained to demonstrate production of spores. Micrococcales were identified according to the method of Baird-Parker (1). The gram-positive rods were identified as sporeformers (Bacillus) or nonsporeformers. On the basis of the tests performed, the latter group was designated as the Corynebacterium-Brevisbacterium group. The gram-negative rods were placed into one of two groups. The nonpigmented ones were placed into the Alcaligenes-Acinetobacter group and the pigmented ones into the Flavobacterium-Cytophaga group, although the taxonomic relationship of this group is still uncertain (16).

The fungi were identified to genus according to the methods of Barnett and Hunter (2) and Barron (3), and the yeasts were identified following the method of Lodder (10). This was performed with the assistance of John Brandsberg, Center for Disease Control, Kansas City, Kansas.

RESULTS

Population studies. Viable counts of microorganisms growing at 7 C from soils from the manufacture and assembly areas of the Viking spacecraft are presented in Table 1. Viable counts from the manufacture area are approximately 2 logs higher than those from the assembly area. In all but one sample (C-4) the aerobic bacterial counts were the highest, with sample C-4 containing a higher population of anaerobes. In 10 of the 15 samples the anaerobic counts were higher than the fungal counts.

Temperature studies. One of the means of grouping the various isolates for identification included their ability to grow at 3 C (10 to 14 days), 24 C (3 to 5 days), and 32 C (48 h). Organisms showing growth at 3 C, but not at 32 C, in the designated time are defined as psychrophiles. Many of these did show growth at 24 C (Table 2). Since many investigators prefer a more rigid definition of psychrophiles, results in Table 2 also show the percentage of organisms that grew at 3 C but not at the other two temperatures. Even though the total population is higher in the samples from Denver, the percentage of psychrophiles is higher in the samples from Cape Canaveral. This is espe-

| Sample | Bacteria | Fungi |
|--------|---------|-------|
|        | Aerobic | Anaerobic |        |
| M-1    | $1.7 \times 10^6$ | $2.7 \times 10^4$ | $1.3 \times 10^4$ |
| M-2    | $3.5 \times 10^5$ | $4.4 \times 10^4$ | $4.5 \times 10^5$ |
| M-3    | $7.7 \times 10^6$ | $3.0 \times 10^3$ | $1.4 \times 10^4$ |
| C-1    | $4.8 \times 10^4$ | $9.6 \times 10^5$ | $2.8 \times 10^4$ |
| C-2    | $5.1 \times 10^4$ | $2.8 \times 10^4$ | $2.8 \times 10^5$ |
| C-3    | $1.3 \times 10^4$ | $2.1 \times 10^3$ | $1.4 \times 10^4$ |
| C-4    | $6.7 \times 10^3$ | $2.4 \times 10^4$ | $5.3 \times 10^4$ |
| C-5    | $2.3 \times 10^4$ | $3.9 \times 10^3$ | $4.1 \times 10^4$ |
| C-6    | $9.4 \times 10^4$ | $4.1 \times 10^3$ | $3.7 \times 10^5$ |
| C-7    | $1.3 \times 10^4$ | $1.7 \times 10^3$ | $4.1 \times 10^4$ |
| C-8    | $6.5 \times 10^5$ | $2.5 \times 10^3$ | $3.9 \times 10^5$ |
| C-9    | $4.2 \times 10^4$ | $4.5 \times 10^3$ | $9.9 \times 10^5$ |
| C-10   | $1.9 \times 10^4$ | $2.2 \times 10^3$ | $3.3 \times 10^5$ |
| C-11   | $6.1 \times 10^4$ | $1.9 \times 10^3$ | $3.8 \times 10^5$ |
| C-12   | $1.4 \times 10^5$ | $6.9 \times 10^3$ | $2.3 \times 10^5$ |

* Results are given in colony-forming units per gram of soil.

* Based on duplicate plates.
TABLE 2. Percentage of aerobic isolates that grew at 3 C but not at 32 C and were isolated from the manufacture (M) and assembly (C) areas of the Viking spacecraft

| Sample | Growth at 3 C, but not at 24 or 32 C | Growth at 3 and 24 C, but not at 32 C |
|--------|-------------------------------------|-------------------------------------|
| M-1    | 2                                   | 24                                  |
| M-2    | 2                                   | 6                                   |
| M-3    | 4                                   | 9                                   |
| C-1    | 10                                  | 26                                  |
| C-2    | 16                                  | 12                                  |
| C-3    | 7                                   | 10                                  |
| C-4    | 7                                   | 11                                  |
| C-5    | 8                                   | 12                                  |
| C-6    | 7                                   | 12                                  |
| C-7    | 10                                  | 46                                  |
| C-8    | 2                                   | 25                                  |
| C-9    | 10                                  | 14                                  |
| C-10   | 28                                  |                                     |
| C-11   | 2                                   |                                     |
| C-12   | 2                                   |                                     |

* Given in percentage of aerobic count from Table 1.

especially true in the group that includes organisms growing at 3 C but not at 24 or 32 C. The results given for percentage of obligate psychrophiles from these soil samples may appear high, but it must be remembered that they are based upon counts in which the organisms were originally isolated at 7 C.

Identification. Anaerobic isolates from the manufacture area were stained and found to consist of approximately 70 to 80% sporeformers and 20 to 30% gram-positive non-sporeforming rods. Samples from Cape Canaveral yielded almost 100% sporeformers. During further testing, it was determined that these were facultative aerobes belonging to the genus Bacillus. It was also determined that many of the aerobic isolates were facultative. From these results, it was decided to exclude the anaerobes from further investigation because obligate anaerobes were not demonstrated.

The fungi were indentified to genus, and these results are presented in Table 3. Molds were not identified to species. The yeasts from the manufacture area were all determined to be Cryptococcus albidus. Those from Cape Canaveral were identified as either C. albidus, Cryptococcus laurentii, Rhodotorula rubra, or Rhodotorula minuta.

The aerobic bacteria were identified to major generic groups including Micrococcus, Bacillus, Corynebacterium-Brevibacterium, Alcaligenes-Acinetobacter, and Flavobacterium-Cytophaga. Different isolates within each major group were recognized, and representatives of these have been lyophilized. The results of these determinations are presented in Table 4. 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 Corynebacterium-Brevibacterium 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 Canaveral contain primarily members of the genus Bacillus and the Corynebacterium-Brevibacterium group.

Percentages given in Table 4 are calculated from the aerobic counts of Table 1. The actual number of Bacillus per gram of soil is similar when the manufacture area samples are compared to the Canaveral samples. Even though the percentage of Bacillus in the aerobic population of the manufacture area samples is quite low, M-3 contained the largest population of aerobic sporeformers.

TABLE 3. Mean percentage of fungi isolated at 7 C from the manufacture (three samples) and assembly (12 samples) areas of the Viking spacecraft

| Genus         | Manufacture area | Assembly area |
|---------------|------------------|---------------|
| Alternaria    | 3                | 8             |
| Aspergillus   | 6                |               |
| Chrysosporium | 23               | 9             |
| Cladosporium  | 2                | 4             |
| Cryptococcus  | 33               | 8             |
| Fusarium      | 5                |               |
| Geniculosporium |                | 7             |
| Penicillium   | 16               | 29            |
| Rhodotorula   | 1                | 5             |
| Rhizoctonia   | 1                |               |
| Ulocladium    | 16               | 24            |

TABLE 4. Mean percentage of the major groups of microorganisms isolated at 7 C from soil samples from the manufacture (three samples) and assembly (12 samples) areas of the Viking spacecraft*

| Generic groups | Manufacture area | Assembly area |
|----------------|------------------|---------------|
| Bacillus spp.  | 1                | 31            |
| Corynebacterium-Brevibacterium | 57 | 41 |
| Alcaligenes-Acinetobacter | 8 | 10 |
| Flavobacterium-Cytophaga | 3 | 4 |
| Micrococcus    |                   |               |
| Subgroup 1     | 9                | 3             |
| Subgroup 7     | 2                | 7             |
| Subgroup 8     | 16               |               |
| Yeasts and molds | 1            | 3             |
| Unable to subculture | 3 | 1 |

* Percentage of aerobic count from Table 1.
Distribution of obligate psychrophiles. From the results of the temperature studies and the distribution of major groups of isolates, the percentage of psychrophiles (growth at 3°C, but not at 32°C) within each group was determined (Table 5). The majority of psychrophiles from the manufacture area belong primarily to the Corynebacterium-Brevibacterium group or to Micrococcus subgroup 8. Samples from Cape Canaveral contained a more diverse population, but the majority of obligate psychrophiles from these samples was also either gram-positive rods or gram-positive cocci. No psychrophilic Bacillus were isolated from the Denver samples, but 10 of the 12 samples from Cape Canaveral contained members of this genus which grow at 3°C but not at 32°C. Means for the three sample sites of the manufacture area show that approximately 16% of the aerobic cultures isolated at 7°C are obligate psychrophiles and that none of these are sporeformers. Means for the 12 sample sites from the assembly area show that approximately 21% of the cultures isolated at 7°C are obligate psychrophiles and approximately 6% are psychrophilic sporeformers. Based upon these studies, this would give approximately $2.1 \times 10^5$ psychrophilic sporeformers/g of Cape Canaveral soil.

**DISCUSSION**

Attempts to prevent the contamination of interplanetary spacecraft intended to enter the atmosphere of Mars have at least a twofold purpose. One is to prevent the contamination of the Martian surface with terrestrial organisms which might alter the state of the planet; the other is to assure that terrestrial contaminants do not interfere with the life detection experiments on the spacecraft (6). It is accepted that the conditions on the Martian surface are not such that mesophilic organisms will be exposed to a temperature approaching 32°C (12), and the Viking Lander Biological Instrument will be maintained at a temperature of approximately 15°C (H. P. Klein, personal communication).

Standard NASA procedures for the microbial monitoring of spacecraft specifies incubation at the single temperature of 32°C (11). The majority of contaminants found in the spacecraft are mesophilic organisms (15), but the use of this single temperature could possibly exclude organisms better adapted to grow in the cold environment of Mars or in the Viking Lander Biological Instrument. This present investigation has demonstrated the presence of relatively large soil populations of organisms which would not be detected by the present microbial monitoring procedures for spacecraft environments. This estimate might be low because of the use of a single isolation temperature of 7°C.

Of the populations just described, the sporeforming rods are probably the most important group, because the Viking spacecraft will be subjected to dry-heat decontamination prior to launch. The soil samples from Denver showed no psychrophilic sporeformers, whereas the samples from Cape Canaveral showed an average of $2.1 \times 10^5$ psychrophilic sporeformers/g of soil. These results demonstrate the presence of a population that may have been excluded by present monitoring procedures.

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