MICROBIAL LIFE IN MARTIAN REGOLITH SIMULANT JSC MARS-1.

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Introduction: JSC Mars-1 is a Martian regolith simulant developed to support research, instrument development, and education. This study measures the simulant’s concentration of cellular material and quantifies and identifies a subset of the microbial population.

Sample Description: Martian regolith simulant JSC Mars-1 is the <1 mm fraction of weathered volcanic ash from Pu‘u Nene, a cinder cone on the Island of Hawaii. This material was chosen based on its spectral similarity to Martian bright regions, extensive previous characterization, and availability in suitable quantity [1].

JSC Mars-1 was obtained from the surficially altered tephra of Pu‘u Nene, located at 1,850 m elevation on the south flank of Mauna Kea volcano. An overlying soil horizon 30-40 cm in thickness was first removed from a 180 m² area on the steep flank of Pu‘u Nene. Altered ash was then collected from a 40-60 cm thick zone of underlying material. This material was hand excavated to minimize contamination from the soil above and the unaltered glassy tephra below. The material was then partially field-dried and passed through stainless steel sieves to separate the <1 mm fraction. It was further dried in a Hilo warehouse using solar and propane heaters at temperatures below 80°C. The dried ash was again passed through 1 mm sieves. It was finally sealed in plastic buckets for shipment and storage [2]. Samples for the current experiments were randomly selected from buckets which had been sealed for one to two years.

Analyses and Results:

Cellular Material: Lipopolysaccharides, along with beta glucans and peptidoglycans, form the cell walls of most microorganisms. Their presence in a sample provides very strong evidence of life, without reference to a particular species.

A highly sensitive assay of cell wall components has been developed, based on the powerful immune response of the horseshoe crab, Limulus polyphemus. Results of the limulus amebocyte lysate (LAL) test [3] are reported as EU (Endotoxin Unit = 10⁻¹⁰ g) per mass of sample.

An LAL assay of JSC Mars-1 yielded a concentration of 4.1 EU/mg of simulant, equivalent to 4.1x10⁻⁷ g/g. For comparison, a spherical bacterium with a diameter of 1 µm has a mass of approximately 5x10⁻¹³ g. The LAL assay is thus equivalent to approximately one million microbial cells per gram of simulant.

Culturable Microbes: Splits of JSC Mars-1 weighing 250 mg each were diluted in 5 ml of sterile water and placed in culture media. Five commercially available media (Table 1) were selected for their compatibility with a wide range of fungi and bacteria. Triplicate cultures were prepared for each split. All cultures were incubated for seven days under aerobic conditions in darkness at a temperature of 27°C.

The cultures were examined by eye and with a low power binocular microscope to determine the number of distinct isolates. Fungal and bacterial colonies were counted for each culture and converted to estimates of colony-forming units, i.e. culturable, dividing cells, per gram of simulant (CFU/g).

Table 1. Fungi and bacteria plate counts

<table>
<thead>
<tr>
<th>Media</th>
<th>Fungi Isolates</th>
<th>Bacteria Isolates</th>
<th>CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDX</td>
<td>5   100 ± 40</td>
<td>6</td>
<td>3100 ± 700</td>
</tr>
<tr>
<td>R2A</td>
<td>0   0</td>
<td>7</td>
<td>27000 ± 6000</td>
</tr>
<tr>
<td>NA</td>
<td>0   0</td>
<td>3</td>
<td>18000 ± 4000</td>
</tr>
<tr>
<td>EMB</td>
<td>2   60 ± 35</td>
<td>10</td>
<td>8900 ± 1800</td>
</tr>
<tr>
<td>ACT</td>
<td>0   0</td>
<td>10</td>
<td>24000 ± 7000</td>
</tr>
</tbody>
</table>

Media: SDX (Sabouraud’s dextrose agar for fungi), R2A (minimal agar media for non copiotrophic heterotrophs), NA (nutrient agar for bacterial copiotrophs), EMB (agar for the identification of coliforms), ACT (Actinomycete isolation agar) [4]

Mean and uncertainty (± 1σ) in CFU/g calculated from triplicate plate counts.
Identification: Frozen isolates from approximately 25% of the culture plates were replated onto Tryptic Soy Agar to enable easier isolation of single colonies. All preparations were carried out in a laminar flow cabinet with approved aseptic technique.

If, during visual identification of the plate, there appeared to be bacterial growth of only one type (a pure culture), a Gram stain was undertaken and the results observed under a light microscope. After Gram staining, identification of the cultures was undertaken using a bioMerieux VITEK system, an industry standard for automated bacterial identification. The VITEK system uses small amounts of bacteria in solution with sterile water, which are inoculated onto test cards chosen on the basis of Gram stain results.

The microbes that have been classified to date include eight Bacillus species: B. thuringiensis, B. subtilis, B. pulmus, B. sphaericus, B. megaterium, B. cereus, B. licheniformis, and B. alvei. Also present were several species of Corynebacterium and Actinomyces, including a member of the genus Streptomycetes, which could not be further identified by the VITEK.

Identification of fungal isolates was performed based on morphology, as observed under an optical microscope. The fungi include Aspergillus flavus and species of Penicillium, Fusarium and Hyphomycete.

Discussion: JSC Mars-1 is altered volcanic ash, taken directly beneath a vegetated soil horizon. The ash thus contains a variety of microbes typically found in soil. During preparation the ash was dried at <80°C. This temperature may have killed large numbers of heat-sensitive microbes but was not sufficient to sterilize the sample. Storage at ambient temperatures in sealed plastic buckets for several years also proved insufficient to destroy many soil microbes.

The concentration of cell wall components in one sample of simulant is on the order of 1 ppm, roughly equivalent to one million cells per gram. In typical surface soils the population of natural bacteria can approach \(10^8\) and even \(10^9\) cells per gram [5].

Most of the microbial species in the environment, including soils, have never been cultured. Our experiments included five culture media but only one set of conditions: air, darkness, 27°C, seven days. As a result, only a fraction of the bacteria in the simulant were successfully cultured.

Depending on the medium used, as many as five distinct, culturable isolates of fungi and ten isolates of bacteria were recognized. The mean concentration of all culturable fungal species in the simulant ranged from zero to 100 CFU/g, depending on the medium. Bacterial concentrations ranged from approximately 3100 to 27,000 CFU/g. Thus, these experiments cultured a few per cent of the total population of cells in the simulant.

The bacteria identified by VITEK represent only a subset of the culturable species. One fourth of the culture plates were selected for VITEK identification. In addition, our method selected for pure cultures of bacteria which had survived frozen storage.

The microbes identified in JSC Mars-1 are typical of many soils. Bacillus species and Corynebacterium are some of the most commonly found bacteria in the environment. They are also prevalent on human skin [6]. The fungi found in the simulant are also widespread in soil [7]. No unusual species of bacteria or fungus were identified in this study.

Implications: JSC Mars-1 was prepared to support a variety of research and instrument development concerning the planet Mars, as well as for classroom instruction and student projects. The current study has implications for both types of use.

A wide range of experiments could potentially benefit from the microbial content of JSC Mars-1. These include life detection technologies for Mars landers, studies of biological cross-contamination on spacecraft, and determination of sample sterilization methods. Other measurements, including stable isotope and volatile release profiles, could be altered in unexpected ways by microbial activity.

A biologically-active, Mars-like soil is a useful teaching and learning resource. JSC Mars-1 contains enough organic material to support limited plant growth experiments. The simulant can be used to demonstrate the classic life-detection methods employed on the Viking landers. It can also be used to compare the effectiveness of sterilization techniques.

This simulant, like any other terrestrial soil, is host to large numbers of microbes comprising scores or perhaps hundreds of species. JSC Mars-1 should be treated with the same care as any unknown material, but with the realization that humans have learned to survive and thrive in a dirty, microbe-filled world.