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Express Letter

Detecting pyrolysis products from bacteria on Mars

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Abstract

A pyrolysis/sublimation technique was developed to isolate volatile amine compounds from a Mars soil analogue inoculated with ~10 billion *Escherichia coli* cells. In this technique, the inoculated soil is heated to 500°C for several seconds at Martian ambient pressure and the sublimate, collected by a cold finger, then analyzed using high performance liquid chromatography. Methylamine and ethylamine, produced from glycine and alanine decarboxylation, were the most abundant amine compounds detected after pyrolysis of the cells. A heating cycle similar to that utilized in our experiment was also used to release organic compounds from the Martian soil in the 1976 Viking gas chromatography/mass spectrometry (GC/MS) pyrolysis experiment. The Viking GC/MS did not detect any organic compounds of Martian origin above a level of a few parts per billion in the Martian surface soil. Although the Viking GC/MS instruments were not specifically designed to search for the presence of living cells on Mars, our experimental results indicate that at the part per billion level, the degradation products generated from several million bacterial cells per gram of Martian soil would not have been detected.

Keywords: Mars; Viking Program; pyrolysis; *Escherichia coli*; chromatography; detection limit; amino acids

1. Introduction

The possibility that life may have arisen early in the history of Mars has been the focus of intense debate since the 1996 report of small fossilized bacteria and organic compounds in the Martian meteorite ALH84001 [1]. At present, the red planet is extremely cold and dry and the surface is exposed to intense UV solar radiation, conditions generally considered to be incompatible with life as we know it on Earth. However, there is compelling evidence that several billion years ago the climate on Mars was much more Earth-like with warmer temperatures and liquid water on the surface [2]. The emergence of primitive bacteria-like forms of life on Mars could have taken place during this 'habitable' period. The possibility that life may still persist today on Mars is of central concern with respect to Mars sample return containment issues related to potential biological hazards associated with possible extant Martian organisms being transported back to Earth [3].

One of the primary objectives of the 1976 Viking missions was to determine whether organic compounds, possibly of biological origin, were present in Martian surface soils. The Viking gas

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chromatography/mass spectrometry (GC/MS) experiments found no evidence for any organic compounds of Martian origin above a few parts per billion in the upper 10 cm of surface soil [4], suggesting the absence of a widely distributed Martian biota. It should be pointed out that the Viking GC/MS instruments were not specifically designed to search for living cells on the surface of Mars, but rather to search for the pyrolysis degradation products of organic compounds of either abiotic or biotic origin [4,5]. Recently, Benner et al. have suggested that significant amounts of non-volatile organic compounds, possibly including oxidation products of bioorganic molecules, would not have been detected in the Martian surface soils analyzed by the Viking GC/MS [6].

A recent theoretical calculation indicates that a subsurface Martian biota could be supported by an energy flux derived from the oxidation of atmospheric H_2 and CO diffusing into the Martian regolith [7]. Based on present Mars surface abundance measurements of H_2 and CO, Weiss et al. suggest that a Martian biota density of 3×10^{-8} g (dry weight) per cm^2 could be supported at a few centimeters of depth, which they claim would be below the detection limits of the Viking GC/MS. If the entire Martian biota consisted of prokaryotic cells with an average dry weight of 2.3×10^{-13} g/cell [8], we estimate that a maximum of 5×10^7 cells/cm^3 could be supported by this energy flux. Assuming an average Martian soil density of ~3 g/cm^3 [9], a maximum of 15×10^6 prokaryotic cells could be present in 1 g of Martian soil.

Based on a previous report it was estimated that there would have to be at least 10^5 microorganisms in 250 mg of Martian soil to correspond to 5 parts per million in weight in order for the Viking GC/MS to detect the presence of living cells [5]. Here we report experimental evidence that confirms that the Viking GC/MS would have been unable to detect bacterial cells present at this level.

2. Sample preparation

Palagonite, an amorphous weathered basaltic glass, was used as a Martian soil analogue material. The palagonite rock was first heated to 500°C for 3 h in order to remove any organic contaminants, then crushed and sieved through a 500–800 μm filter. The crushed palagonite was then inoculated with *Escherichia coli* bacteria using the following procedure. *E. coli* cells (strain MG1655) were grown in glass tubes with 500 mg of palagonite by shaking at 250 rpm overnight in 10 ml Luria-Bertani (LB) medium [10] at 37°C in a water bath. After overnight growth, the bacteria/palagonite suspension was centrifuged for 10 min at 5000 rpm in a Falcon plastic tube. The LB medium was then removed from the tube and the bacteria/palagonite sample was re-suspended in 1.5 ml potassium phosphate-buffered saline (KPBS), microfuged at 6000 rpm for 2 min, and the supernatant removed. The KPBS washing procedure was repeated three more times in order to remove all of the LB medium from the sample. After rinsing, the inoculated palagonite was ho-

Table 1
Amino acid results of the *E. coli* sublimation pyrolysis experiment

	Prior to heating[a] (μg/g)	After heating[b] (μg/g)
Aspartic acid	187	<0.1
Glutamic acid	310	<0.7
Serine	117	<1.0
Glycine	298	3.1
Alanine	222	7.4
Valine	102	6.5
Methylamine[c]	<0.1	8.6
Ethylamine[c]	<0.1	3.2
Total amino acids (μg/g)	1236	
# of *E. coli* (cells/g)[d]	1.1×10^{10}	

The total concentrations in parts per million (μg/g) of several protein amino acids and amine decomposition products in a crushed palagonite sample inoculated with *E. coli* cells before and after heating at 500°C for ~30 s (pressure = 5 mbar). This heating cycle is similar to the 1976 Viking pyrolysis procedure used to extract organic compounds from the Martian surface soil. All reported values were corrected using the procedural blanks.

[a]Hydrolyzed in 6 M HCl at 100°C for 24 h.

[b]Sublimed fraction collected off the cold finger.

[c]Viking GC/MS detection limit estimated to be ≥0.01 μg/g.

[d]Assuming dry total mass of amino acids in *E. coli* is 1.55×10^{-13} g/cell [8] and that the listed amino acids account for 70% of the total amino acids.

mogenized by mixing and kept cool at 4°C. A crushed palagonite control blank that had not been inoculated with *E. coli* cells was carried through the LB medium treatment and KPBS washing procedure described above.

Approximately half (250 mg) of the inoculated palagonite sample was transferred to a clean test tube and hydrolyzed in 1 ml of double-distilled 6 M HCl at 100°C for 24 h. The HCl supernatant was then removed from the palagonite, transferred to a new test tube and dried under vacuum. The dried residue was then desalted by using cation exchange resin (AG50W-X8) and analyzed by *o*-phthaldialdehyde/*N*-acetyl-L-cysteine (OPA/NAC) derivatization and high performance liquid chromatography (HPLC) separation with UV fluorescence detection [11] in order to determine the total hydrolyzable amino acid content of the cells. The palagonite control blank was carried through the entire procedure in parallel. An estimate of the total *E. coli* cell concentration in the inoculated palagonite sample was then calculated from the amino acid yield (Table 1). In addition, the total number of *E. coli* cells present was confirmed by a measurement of the total mass of a solid *E. coli* pellet (10 mg) generated by overnight growth and centrifugation of an identical volume of LB medium used to inoculate the palagonite.

3. Sublimation pyrolysis experiment

The remaining portion of the inoculated palagonite sample ($\sim$250 mg) was used for the pyrolysis experiments. To simulate the pyrolytic process used to extract organic compounds for analysis by the Viking GC/MS instrument, the inoculated palagonite rock sample and control blank were sealed separately under Martian ambient pressure (4–6 mbar air) in a quartz glass sublimation apparatus [12], and heated in a tube furnace (Lindberg/Blue M Mini-Mite) set at 1100°C. A cold finger, attached to the sublimation tube, was kept at -195°C with liquid nitrogen throughout the entire experiment. After 30 s, the apparatus was removed from the furnace and allowed to equilibrate to room temperature. According to thermocouple measurements of the temperature inside the apparatus, the palagonite sample was heated to at least 500°C for $\sim$30 s during the experiment. In the Viking pyrolysis experiments, the maximum temperature reached in each oven was 500°C for 30 s [4].

After pyrolysis/sublimation of the inoculated palagonite was carried out, a bright yellow residue coated the end of the cold finger. This colored coating was not observed after sublimation of the control blank that did not contain bacterial cells. The material on the cold finger was carefully rinsed off with double-distilled water and the water soluble amines then analyzed by the OPA/NAC derivatization and HPLC separation method.

4. HPLC results

Prior to heating, high concentrations (100–310 μg/g) of several protein amino acids, including aspartic and glutamic acids, serine, glycine, alanine and valine (Fig. 1a and Table 1), were detected in the inoculated palagonite. This is not surprising, given that amino acids are the single most abundant compounds in *E. coli* cells, comprising more than 50% of the total dry cell weight [8]. Only trace amounts (<0.1 μg/g) of methylamine and ethylamine, the α-decarboxylation products of glycine and alanine, were found. Very low levels of amino acids (<1 μg/g) were detected in the control blank after acid hydrolysis and desalting (Fig. 1c), which indicates that the amino acids recovered from the inoculated palagonite were entirely derived from the *E. coli* cells and were not associated with any remnants of the growth medium used for inoculation. Based on the amounts of the detected protein amino acids, it was calculated that the crushed palagonite sample contained about 1×10^{10} *E. coli* cells per gram (Table 1).

After heating the inoculated palagonite to 500°C for 30 s, a major fraction of the amino acids originally present in the bacterial cells had decomposed (Fig. 1b). The primary decomposition products observed were methylamine (8.6 μg/g) and ethylamine (3.2 μg/g). The control blank yielded only trace levels (<1 μg/g) of ami-

no acids and amines after sublimation (Fig. 1d). Although some glycine, alanine and valine sublimed from the inoculated palagonite, more than 98% of the amino acids present in the *E. coli* cells were destroyed by the pyrolysis procedure (Table 1).

5. Discussion

Our sublimation/pyrolysis technique coupled with HPLC was specifically designed to search for amine pyrolysis products derived from living bacterial cells and not for the organic compounds derived from long term degradation of the cells. A previous GC/MS analysis of the pyrolysis degradation products of microorganisms indicated that amides and nitriles were the most abundant compounds generated from the cells [13]. Our experiments indicate that volatile amines, especially methylamine and ethylamine, are also produced from the pyrolysis of cells. Although the HPLC analytical method used in our experiment was different than the Viking GC/MS instrument, the pyrolysis procedures were comparable. Thus, it is likely that methylamine and ethylamine would have been generated from any cells present in the Martian soil during the Viking pyrolysis procedure. The detection limits for ethylamine and methylamine, however, were not accurately determined for the Viking GC/MS instrument. A limit for amines of 0.01 μg was estimated based on the chromatographic resolution of the Viking Tenax-GC column [14]. The actual detection limit is likely to have been much higher due to the fact that volatile amine compounds would elute from the GC column during a time where the GC/MS effluent divider vented water and CO_2 in the 8000:1 split mode. Under these conditions it would be impossible to detect any methylamine or ethylamine generated from the Viking pyrolysis procedure at the 0.01 μg/g level. The Viking GC/MS, which was calibrated for nitrile-containing molecules, did not detect any acetonitrile (an oxidized form of ethylamine) above the 0.01 μg/g level in Martian surface samples [4].

Assuming a best case detectability for ethylamine in the Viking GC/MS of 0.01 μg/g, it is possible to estimate an upper limit for the number of bacterial cells in the Martian soil that would yield 0.01 μg/g of ethylamine after pyrolysis. According to the data in Table 1, ~10 billion cells/g of palagonite yielded 3.2 μg/g of ethylamine after heating at 500°C for 30 s under Martian pressure. Thus, at the detection limit of 0.01 μg/g, ethylamine from ~30 million bacterial cells could have been generated from 1 g of Martian soil during pyrolysis, and this would have likely

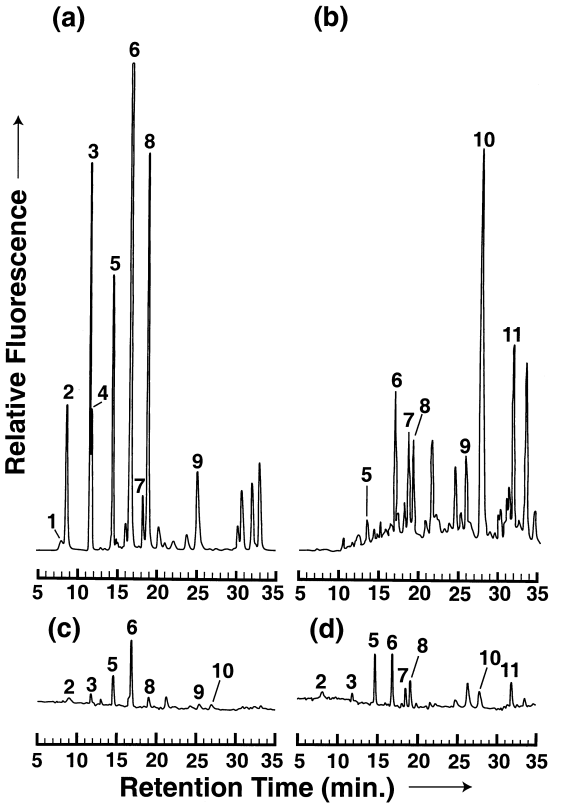

Fig. 1. The reverse-phase HPLC chromatograms of (a) the 6 M HCl hydrolyzed, desalted and (b) sublimed extracts from a crushed Mars analogue soil sample inoculated with *E. coli* cells. Also shown are chromatograms of the (c) 6 M HCl hydrolyzed, desalted and (d) sublimed extracts from palagonite without cells but treated with growth medium and carried through the entire processing procedure (procedural blanks). Peaks were identified by comparison of the retention times of an amino acid and amine standard run at the same time. Peak identifications: (1) D-aspartic acid; (2) L-aspartic acid; (3) L-glutamic acid; (4) D-glutamic acid; (5) L-serine; (6) glycine; (7) D-alanine; (8) L-alanine; (9) L-valine; (10) methylamine; and (11) ethylamine.

been missed by the Viking GC/MS instrument. Because other bacteria, as well as some blue–green algae and archaea, tend to have a lower overall dry cell weight than *E. coli* [15], the total number of prokaryotic cells that could have been present on the surface of Mars is even larger. Although the presence of a potent oxidant has generally been assumed to preclude the existence of any organisms in the soils analyzed by Viking [16], it is apparent that we cannot exclude the possibility that there could have been at least several million prokaryotic cells/g of Martian soil.

Upcoming strategies for Mars exploration will require in situ analyses by instruments that are orders of magnitude more sensitive than the Viking GC/MS in order to adequately assess whether any organic compounds, especially those that might be associated with life, are present in the Martian surface soils. One such instrument, MOD (Mars Organic Detector), which has been selected for the Mars 2005 lander payload, has a detection limit for amines that is at least 100 times greater than the Viking GC/MS instrument [17]. This means that on the order of 10^4 prokaryotic cells/g of Martian soil sample should be detectable with MOD. Future testing of the MOD prototype will be necessary to confirm these estimates.

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