Denitrification by *Pseudomonas aeruginosa* Under Simulated Engineered Martian Conditions

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The growth of *Pseudomonas aeruginosa* in denitrifying medium was observed for 14 days in the presence of a martian soil analog (JSC Mars-1) and elevated CO_2 levels. A four-way test was conducted comparing growth of experimental samples to growth in the presence of inert silica ("Earth soil") and normal terrestrial atmosphere. The combination of 50 mL of fluorescence-denitrification medium and 10 grams of soil additive simulated an aquatic environment, which was contained in sealed culture bottles. Nitrite assays of the media (to test for consumption during denitrification), gas sampling from the bottles to observe nitrogen production, and colony counts to quantify growth rate were all performed at 0, 7 and 14 days after inoculation. Supplemental tests performed included nitrate assays (to confirm the occurrence of denitrification) and culture fluorescence (as a non-invasive growth test). Growth and denitrification took place under all conditions, and no significant differences were observed between samples. These data indicate that the presence of simulated martian regolith and elevated CO_2 have little or no effect on the growth of or denitrification by *P. aeruginosa* at the concentrations used.

1. Introduction

The necessity of nitrogen is one of the major ecological obstacles in the terraformation of Mars [1]. Nitrogen is a very important element in Earth's biogeochemical cycle and is a necessary component of amino acids and nucleotides. Earth's primary nitrogen reservoir is its atmosphere. In contrast, nitrogen is a very small component of Mars' already thin atmosphere. However, nitrogen may be present in the martian regolith in the form of nitrates and/or nitrites formed by either abiotic or biotic processes [2]. The presence and amount of nitrogen available in the martian regolith is still a matter of some conjecture. The presence of valleys and canyons on Mars suggests the past presence of water (reviewed in [2]). As the water was lost, dissolved minerals would have been left behind as evaporites. However, there is little geographical evidence of nitrate-bearing evaporites on Mars, although it is possible that dust storms have covered such formations [3]. In addition, nitrates have been identified in martian meteorites [4, 5 and 6]. There is still not enough evidence to absolutely determine whether nitrates are indeed present in significant amounts on Mars, and this question is not likely to be resolved until the upcoming sample collection and return missions are completed. For the purposes of this paper, we assume that nitrates are present on Mars.

If the terraformation of Mars ever becomes a reality, a viable ecosystem—most likely consisting of cyanobacteria and eubacteria—would be a prerequisite for plant life [1]. Denitrifying bacteria could be used to convert nitrates to atmospheric nitrogen. Later, populations of nitrogen-fixing bacteria could be established in order to create a primitive nitrogen cycle, essential for plants and consequently beneficial to many higher life forms. Of course, before bacteria could be introduced on Mars, the atmospheric pressure and temperature would have to be increased by some physical means in order to allow the presence of liquid water.

The denitrifying bacterium, *Pseudomonas aeruginosa* was chosen for this experiment because of its commercial availability, denitrification abilities, and physiological versatility. It is commonly found in terrestrial soil, and can grow in a variety of low-nutrient conditions [7]. *P. aeruginosa* is a representative of a wide range of other denitrifying bacteria including *Pseudomonas denitrificans, Alcaligenes denitrificans,* and *Paracoccus denitrificans* [8].

Few experiments have examined the growth of bacteria in the presence of a martian soil analog. One experiment found that the growth of mixed microbial communities was slightly inhibited by analog soil [9]. Our experiment was designed to test whether the presence of the soil analog would be toxic to denitrifying bacteria, not whether the bacteria could draw sustenance from the soil itself. Therefore, a denitrifying medium that provides carbon and other nutrients was used. The bacteria were also cultured in an elevated CO_2 environment to test an additional variable that would be encountered on Mars. Because of *P. aeruginosa's* ability to anaerobically respire, we hypothesized that the combination of elevated CO_2 and martian soil analog would have little effect on growth and denitrification by this bacterium. At the end of the experiment, we observed no differences in growth or denitrification between the martian analog conditions and the controls.

2. Materials and Methods

The martian soil analog, JSC Mars-1, was obtained from NASA's Johnson Space Center. The chemical and spectral characteristics of the analog match very closely with what is currently known about the martian surface [10]. Stock cultures of *P. aeruginosa* were grown for 48 hours in 35 mL tryptic soy broth. Cultures were centrifuged at 2000 x g for 20 minutes, washed in 25 mL phosphate-buffered saline [11], centrifuged again, and resuspended in another 25 ml of the saline solution. Rinsed cultures were inoculated into 50 mL of fluorescence-denitrification (FN) medium [8] in 125 mL culture bottles containing ten grams of either sterile silica sand or sterile JSC-1 simulant. Each bottle was fitted with a rubber septum to facilitate gas addition and sampling. A total of 72 culture bottles were prepared with the following combinations of additives: 18 bottles silica (control), earth atmosphere; 18 bottles JSC Mars-1, earth atmosphere; 18 bottles silica, elevated CO_2; and finally 18 bottles of JSC Mars-1 with elevated CO_2. Half of the bottles from each group were inoculated with *P. aeruginosa*, the other half were left sterile as controls. Samples with terrestrial atmosphere were left open in a sterile laminar flow hood for 10 seconds and then sealed. Samples with simulated martian atmosphere were each injected with two full 60-cc syringes of 99.99% pure CO_2 while the caps were loosened to allow pressure equilibration. It was initially unknown exactly what CO_2 concentration this would create within the bottles, but it was later found that initial levels were at approximately 65% CO_2 with the balance composed of air. Cultures were incubated at 37°C in the dark.

Samples were taken on days 0, 7, and 14 for each bottle, with day 0 being the day of inoculation. Samples were tested for N_2, NO_3^-, NO_2^-, bacterial growth and bacterial fluorescence (a qualitative indicator of growth). N_2 was analyzed by gas chromatography-mass spectrometry by the Kennedy Space Center environmental chemistry laboratory. Nitrate and nitrite were analyzed with commercially available aquarium test kits. Bacterial growth was monitored by serial dilution and plate counts. All tests were performed in triplicate.

3. Results

All of the samples started with an initial concentration of 100 µg mL^{-1} NO_2^-, and almost all of this was consumed by day 7 in those bottles that contained *P. aeruginosa*. No significant difference in nitrite consumption between groups (whether they contained silica, martian soil analog, terrestrial atmosphere, or elevated CO_2 levels) was found. Although initial nitrate levels were not measured, it is clear that nitrates were consumed in those bottles containing *P. aeruginosa* (nitrate levels at or near zero after incubation) and not in the sterile controls (where nitrate levels remained at 20-40 ppm after 14 days). Again, these data do not show any discernible change in nitrate consumption between sample sets with different soil or atmospheric conditions.

Colony count data show growth taking place in all inoculated containers on approximately the same order of magnitude in all sample sets. There was no significant difference in growth between sample sets. All samples had on the order of 10^9 colony forming units per mL at day 14. Periodic testing revealed fluorescence in all inoculated samples, indicating growth and denitrification. The bottles containing JSC Mars-1 did not fluoresce as brightly as those with added silica; however, this may have been due to clouding of the media caused by tiny particles of the martian soil analog suspended in the medium. Sterile controls showed neither fluorescence nor colonies throughout the test period.

4. Discussion

This study showed that growth of and denitrification by *P. aeruginosa* are not prevented by the presence of martian soil analog or CO_2 at the concentrations used. Nitrite and nitrate consumption indicated that denitrification took place in all inoculated bottles, with no apparent difference caused by the presence of the martian soil analog or elevated CO_2 levels. However, since almost all of the nitrite was consumed by day 7, there may have been signifi-

cant differences in denitrification rate that were simply missed in this experiment due to the scheduling of tests.

This experiment must be considered preliminary. We have no firm idea of what the conditions on Mars will be after initial planetary engineering. We have made some very basic assumptions in order to test a single variable—the ability of a denitrifying bacterium to metabolize nitrites and nitrates in the presence of martian regolith. Clearly, much additional research is needed to determine the viability of biological cycles on an engineered Mars. As far as denitrification goes, future experiments may utilize other denitrifiers to observe variations in growth rates. Also, increasing the soil to denitrification medium ratio might amplify any effects of the soil simulant and aid in discerning whether there are any effects on bacterial growth that were hidden during this experiment. Other atmospheric compositions might also have an effect on the growth of *Pseudomonas* and other bacteria. At present, the possibilities for continued research are almost boundless.

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