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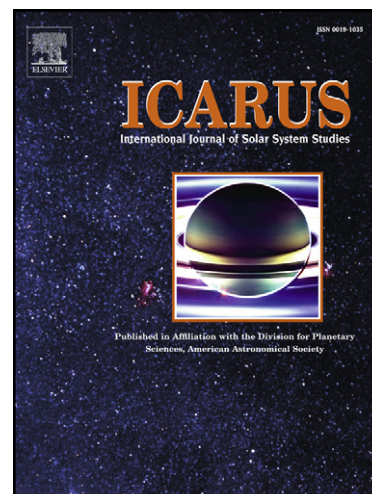
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PROTECTION OF CHEMOLITHOAUTOTROPHIC BACTERIA EXPOSED TO SIMULATED MARS ENVIRONMENTAL CONDITIONS

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Abstract

Current surface conditions (strong oxidative atmosphere, UV radiation, low temperatures and xeric conditions) on Mars are considered extremely challenging for life. The question is whether there are any features on Mars that could exert a protective effect against the sterilizing conditions detected on its surface. Potential habitability in the subsurface would increase if the overlying material played a protective role. With the aim of evaluating this possibility we studied the viability of two microorganisms under different conditions in a Mars simulation chamber. An acidophilic chemolithotroph isolated from Río Tinto belonging to the *Acidithiobacillus* genus and *Deinococcus radiodurans*, a radiation resistant microorganism, were exposed to simulated Mars conditions under the protection of a layer of ferric oxides and hydroxides, a Mars regolith analogue. Samples of these microorganisms were exposed to UV radiation in Mars atmospheric conditions at different time intervals under the protection of 2 and 5 mm layers of oxidized iron minerals. Viability was evaluated by inoculation on fresh media and characterization of their growth cultures. Here we report the survival capability of both bacteria to simulated Mars environmental conditions.

Introduction

Scientists have long speculated about the possibility of life on Mars (Klein et al., 1976; McKay, 1997). Mariner 9, a 1970s NASA mission reported Martian images with topological signatures resembling lake structures and sedimentary deposits. These scenarios suggested interesting possibilities for the development of life on Mars, but a few years later the Viking Landers reported extremely harsh physico-chemical conditions on the surface of the planet which challenged those expectations (Klein, 1978). The odds for life on Mars changed again with the new data released by the Mars Global Surveyor, launched in 1996, and from the 2004 Mars Rovers Missions. The MER Opportunity landed in Meridiani Planum where sedimentary deposits have been identified in different craters (Squyres and Knoll, 2005).

The existence of extinct or extant liquid water in Mars subsurface would increase its habitability potential. Evidence of extant water on Mars was reported by the Mars Express mission (Poulet et al., 2005). OMEGA near-infrared spectrometer reported the presence of phyllosilicates on the Mars surface. These water bearing minerals located on early Mars surfaces are solid proof of the existence of water in early Mars. Other authors have recently reported a wider diversity of phyllosilicate mineralogy using the Compact Reconnaissance Imaging Spectrometer (CRISM) on the Mars Reconnaissance Orbiter (MRO) (Mustard et al., 2008). Other evidence of a wet past of Mars come from the Opportunity rover at Meridiani Planum. The identification of hematite, goethite and sulfate rich deposits, such as jarosite (Squyres et al., 2004), gave a possible scenario for a past aqueous acidic environment on Mars.

On Earth, life works as an environmental transformer (Gómez and Amils, 2002). Chemolithotrophic microorganisms are able to transform the environments in which they develop through homeostatic process (Fernández-Remolar et al., 2008a). These homeostatic mechanisms then generate environmental modifications (physical or chemical anomalies) that could be used as biosignatures on future Mars exploration

missions (MSL or ExoMars). The presence of methane in the Mars atmosphere is an example of these chemical signatures that could be consistent with life. Some chemolithotrophic microorganisms are able to obtain energy using H_2 as the electron donor and CO_2 as an electron acceptor, producing CH_4 . Recently reported results about the presence of methane in the Martian atmosphere (Mumma et al., 2009) increased the astrobiological interest of putative protected ecosystems in the Mars subsurface. Methane has a short lifetime which means that actual identification requires active production or a reservoir large enough to maintain its presence in the atmosphere. If it is the result of biological activity this would mean extant subsurface life.

Mars and Earth might have had many similarities in their early histories. Although the subsequent evolution of both planets has been completely different, geological signatures on Mars' surface indicate the past existence of water on the red planet. Could the Martian sulfate and iron oxidized deposits detected there (Squyres and Knoll, 2005; Clark et al., 2005) be the result of acidic chemolithotrophic metabolism? Geological results from the MER rover, Opportunity, at Endurance Crater indicate a possible acidic environment in the origin of the observed geological deposits. Similar deposits are located around the Río Tinto source area as a product of chemolithotrophic metabolism of microorganisms thriving in the rich metal sulfidic deposits of the Iberian Pyritic Belt (Fernández-Remolar et al., 2005; Fernández-Remolar et al., 2008b). Further studies are required to determine whether life could take advantage of the potential protected Martian subsurface habitats.

Río Tinto is a 100km long extreme acidic environment located in the Southwestern part of the Iberian Peninsula (Amaral-Zettler et al., 2002; Gonzalez-Toril et al., 2003). The river rises in the heart of the Iberian Pyritic Belt, one of the biggest mineral sulfidic concentrations in the world. These conditions enable the establishment of an extreme iron controlled ecosystem (Amils et al., 2007). Such an extreme environment was though to be uninhabitable, but microbial ecology studies reported the presence of microorganisms belonging to the three domains of life (Amaral-Zettler et al., 2002). The system is sustained by the oxidation of iron sulfide (pyrite) by chemolithotrophic microorganisms (*Acidithiobacillus ferrooxidans*, *Leptospirillum* spp.) (López-Archilla et al., 2001; Amaral-Zettler et al., 2002; González-Toril et al., 2003, Amils et al., 2008) leading to the generation of sulfate and ferric iron, which are responsible for the two main characteristics of the river: low pH and high metal content. Due to these chemical characteristics, iron oxides and ferric sulfates, including jarosite, are abundant in the Río Tinto basin (Fernández-Remolar et al., 2003; 2005). The historical geological record of the Río Tinto area with its ancient and modern iron deposits gave this ecosystem the status of terrestrial Mars analogue, with a mineral composition very similar to some regions of Mars (Fernández-Remolar et al., 2005; Fernández-Remolar et al., 2008b). Microorganisms involved in the iron and sulfur cycles are able to obtain energy coupling oxidizing and reducing reactions to the electron transport chain (Sand et al., 2001). Some of them need oxygen for their metabolism, but this is not a strict requirement, because others, such as *Acidithiobacillus ferrooxidans*, are able to use ferric iron as an electron acceptor in anaerobic conditions to obtain energy oxidizing reduced sulfur compounds.

Acidophiles from Río Tinto are suitable for the study of habitability under simulated Mars conditions due to its capability for using iron as electron donor and the mineralogical similarity between both places. Ultraviolet doses at short wavelength are

too strong for life to develop on the surface of Mars (Schuergel et al., 2006). It is well established that some features, like ice or mineral components of rocks can efficiently protect cell systems from UV radiation (Olson and Pierson, 1986; Cleaves and Miller, 1998; García-Pitchel, 1998; Rothschild and Cockell, 1999; Phoenix et al., 2001; Bishop et al., 2006; Cockell *et al.*, 2003). Recently, it has been shown that ferric iron in solution (Gómez et al., 2007) can protect sensitive algae from UV radiation. Previous reports evaluated the effects of different extreme Martian surface conditions on terrestrial microorganisms. Some authors studied the effects of only one stress condition: ultraviolet radiation (Elasri and Miller, 1999; Schuergel, et al., 2006), heavy ions (Baltshukat et al., 1986), simulated Mars solar radiation (Tauscher, et al., 2006), extreme dryness (Dose and Gill, 1995; Kendrick and Kral, 2006) or microgravity (Sugiura et al., 1999). Other authors studied the effect of more than one stress condition: Mars atmospheric pressure and composition (Nicholson and Schuergel, 2005), space vacuum and ultraviolet irradiation (Saffary et al., 2002), or the comparative effects of several stresses on vegetative cells and spores (Díaz and Schulze-Makuch, 2006; Dose and Klein, 1996).

Mars surface dust could also play a protective role. Some images from the MERs Opportunity and Spirit on the surface of Mars have reported highly dusty conditions (Fig. 1). Both rovers are sometimes coated by dust which is mobilized by winds or dust devils. Could dust protect life against radiation? To answer this question, we report here on our studies of protection by Río Tinto Basin iron oxides and hydroxides on two model microorganisms, *Acidithiobacillus ferrooxidans*, (an acidophile isolate from Río Tinto) and *Deinococcus radiodurans* under simulated Mars surface conditions, with stressing conditions operating at the same time. *D. radiodurans* is a well-known poly-extreme resistant microorganism (Daly et al., 2004) used in this work as a reference system, and *At. ferrooxidans* is an acidophilic bacteria that could be able to develop in the geochemical conditions described in some Mars locations (Fernández-Remolar et al., 2005) with some restrictions due to iron oxidizers require oxygen, but *Acidithiobacillus ferrooxidans* is able to use ferric iron as an electron acceptor in anaerobic conditions.

Materials and Methods

Microorganisms

At. ferrooxidans isolate 3.2 was used in this study. It was isolated from Río Tinto 3.2 station by Dr. Moustafa Malki (Malki, M., 2003). *At. ferrooxidans* was grown in the conditions describe by González-Toril et al., 2006 in Mackintosh media (Mackintosh, M. E., 1978).

Deinococcus radiodurans, a poly-extremophile microorganism, from the German culture collection DSMZ, cat. n° 20539, was used as a reference system. This strain was grown in corynebacterium media (casein peptone tryptic digest 10 g, yeast extract 5 g, glucose 5 g, NaCl 5 g, distilled water 1000 ml, pH adjusted to 7.2 - 7.4). For solid media, 10 g/l of Agar were added.

The inoculated fresh media were incubated at the optimal temperature for each microorganism (30 °C for both). At different time intervals microorganisms were

filtered and cell number was determined by fluorescence microscopy after DAPI staining.

Protecting cakes preparation

A simulated protective subsurface environment was designed specifically for these experiments in the form of a two cake (one circular base and a second protective layer). Figure 2 shows the structure of the cakes. Vegetative cells from growing cultures at the stationary phase of *At. ferrooxidans* and *D. radiodurans* were harvested by centrifugation and deposited on the center of the circular base (4 mm thick). A second shielding layer (2 or 5 mm thick) was then compacted over the first one. Two sets of experiments of sample exposition to simulated Mars surface conditions (Mars atmosphere gas composition, pressure and UV irradiation) were carried out with different exposure times, 2 and 10 hours.

The material used for cake preparation was from a ferruginous deposit of Alto de la Mesa sampling station at Río Tinto, composed mainly of goethite and hematite minerals (Fernández-Remolar et al., 2005). Samples were ground in an agate mortar and pestle. Powder was sieved in order to select thin 100 μm diameter mineral grains. Powdered minerals were compressed using 6 mm diameter moulds. Material on the mould was compacted with a SPECAC hydraulic press to a final pressure of 100 At for avoiding any loss of material due to vacuum but ensuring some permeability for sample exposition to environmental stressors. Circular 4 mm thick and 6 mm diameter bases were generated for microbial exposure. Two and 5 mm thick protecting layers were deposited over the biological component. These protecting layers were compacted in the same conditions as described above. After exposure the cake was used to inoculate the corresponding fresh media. Two trials of inoculation experiments after Martian conditions exposition were done. The plotted values in figures 5 and 6 represent the mean value and error bars indicate the range between minimum and maximum value.

Planetary Atmospheres Simulation Chamber

Mars simulation experiments were performed in an especially designed environmental planetary atmospheres simulation chamber (Fig. 3) at the Centro de Astrobiología (Madrid) (Mateo-Martí et al., 2006). Mars atmospheric composition was the following: 95% CO_2 , 2.7% N_2 , 1.6% Ar and 0.6% H_2O with a pressure of 7 mbars. Temperature was set at 150 K with the intention of simulating a very restrictive Martian environment. Ultraviolet radiation source was a Deuterium lamp with a dose emission of 30 mW cm^{-2} in the wavelength range of 200-400 nm. Fig. 4 shows the radiation spectrum of the lamp used in the experiments.

Results and discussion

Two different microorganisms, the acidophilic bacteria *At. ferrooxidans* and the radiation resistant bacteria *D. radiodurans*, were used to study survival capacity in a Mars subsurface analogue environment. To simulate the subsurface environment we designed a two-layer regolith cake as a protective environment. A bacterial pellet was placed between the two layers. In order to compare the protecting effect of the layers thickness, samples were exposed to Mars surface conditions at different protective depths inside the cake, at 2 and 5 mm, of iron minerals. The cakes were exposed to

Mars extreme conditions for different periods of time (doses), 2 and 10 hours. These time periods were selected in order to obtain accumulation doses equivalents to 0.5 and 2.6 Martian Sol (Sol = Martian day; 24 h 37 min) in the worst UV scenario (location 15° S and Ls 270) (Patel et al., 2002).

When an inoculum of *At. ferrooxidans* was exposed to Mars surface conditions under the protection of either a 2 or 5 mm thick regolith protecting layer, bacterial growth was observed in all conditions after reinoculation in fresh media (fig. 5). *At. ferrooxidans* growth was severely affected by the exposure conditions but 2 mm of regolith was sufficient to protect bacteria from harsh Mars conditions (Fig. 5). Increasing the thickness of the protecting sheet lowers the deleterious effect on the bacterial growth. 5 mm regolith protection gave higher growth rates than 2 mm. When the slopes of the growth curves were compared, steeper slopes were observed with the thicker regolith protection layer (Table 1). In any case, a thickness of 2 mm of the shielding layer was enough to obtain a high survivability value (40.77 % of slope similarity with respect to the unexposed control sample). The slopes of the curves were lower when exposure to Mars conditions was longer. Bacterial survivability is lower at 2 (40.77 % and 48.50 % for 2 h and 10 h of simulated Martian conditions exposition, respectively) than at 5 mm (48.50 % and 30.05 % for 2 and 10 h) of thickness layer protection (Fig. 5). Since 1 mm thickness is enough for screening UV radiation (Fig. 4) any indirect UV effects could be noticed as previously reported by Hansen et al., (2005) and Yen et al., (2000). Thus, the observed decrease in bacterial survivability at 2 mm with respect to 5 mm depth in the soil cannot be simply explained by direct UV effect. Following data reported by Yen et al., (2000), the observed lower recovery level of bacterial viability at 2 mm could be UV-induced by the production of superoxide ions that could penetrate in the soil under a simulated Martian atmosphere. Then, UV indirect effects could go deeper in the soil than direct UV radiation goes.

Deinococcus radiodurans was much less affected than *At. ferrooxidans* to exposure to Mars conditions, which is not surprising due to its well reported stress-resistance capability. Similar growth rates (Fig. 6) were obtained from the inocula exposed during 2 and 10 hours under the protection of 2 or 5 mm thick layers of regolith. *D. radiodurans* slopes for the inocula protected under 2 and 5 mm thick layer were similar to the slope of the unexposed control inocula (Table 2). It was not possible to compare the growth curve slopes of the two bacteria due to the differences in their duplication time.

Actual Mars surface environmental conditions can be considered extreme for terrestrial microorganisms. Not only irradiation conditions but low pressure, dehydration and temperature are extreme conditions that organisms must deal with on the surface of Mars. Recent studies have reported the possible existence of water in the Mars subsurface (Malin and Edgett, 2000; Armstrong et al., 2005). Life as we know it on Earth needs water for its development. Due to the harsh oxidative conditions on the surface of Mars and the possible presence of water on the subsurface, it seems reasonable to search for life under the surface. Subsurface habitats protect against radiation. Dartnell et al., (2007) modelled the surface and subsurface radiation on Mars and demonstrated that the protective shielding depends on depth and the nature of the regolith material. Three different regolith models were used to assess radiation decay vs. depth. Their results reported 4.5 meters for wet heterogeneous regolith and 7 m in the case of the pure ice to obtain a similar protection for life than *D. radiodurans* has.

In our case, both the 2 and 5 mm thick regolith layers provided enough protection against radiation and Mars environmental conditions for bacteria to survive. These experiments clearly show that non-sporulating Gram negative microorganisms can retain viability with only a thin layer of iron minerals (fig. 7). This is the first time that a marked ability to survive Mars conditions is reported for acidophilic chemolithotrophic microorganisms. These are organisms that are able to develop in the ionic conditions detected in different Mars locations.

Real Martian surface conditions simulation is difficult due to technical limitations. Some previously published work in the field refers to simulation of one or two Martian surface conditions, but not all the Martian surface conditions at the same time (Diaz and Schulze-Makuch, 2006; Newcombe et al., 2005; Schuerger et al., 2006). Then, results comparison is difficult. Different authors have reported experiments using endospores of Gram positive bacteria (Nicholson and Schuerger, 2005; Tauscher et al., 2006). Cockell et al., 2005 reported 99 % of cell viability loss after 30 min of exposition of *Chroococcidiopsis* sp. to an 8.5 mbar 99.99 % CO₂ atmosphere. Schuerger et al., (2003) reported *Bacillus* endospores survival under Martian surface pressure using several Mars atmospheric compositions Here we reported the exposition of vegetative bacteria to several Martian surface simulation conditions (atmosphere composition, pressure, temperature, water vapour content and radiation conditions). We obtained a high percentage of survivability of vegetative bacteria in all the cases under the protection of very thin regolith layers.

The experiments reported here broaden the range of possibilities for extant life on Mars. A slim Mars regolith layer is enough to greatly reduce radiation doses and offers a shielding layer for microorganisms. Habitability increases under only a few millimetres of regolith protection. The absence of carbonates in Mars regolith allows us to speculate about the idea of a putative acidic ocean on early Mars (Fairén et al., 2004). This environment would have promoted the weathering of basalts leading to the liberation of iron and magnesium into the ocean. The oxidation of ferrous iron could drive further the acidification of the water. A similar extant model for this type of environment can be found in the Río Tinto basin. The chemical reactions in this ecosystem are promoted by chemolithotrophic bacteria leading to the acidification of the river waters. The microbial metabolic products: iron hydroxides and oxides and sulphate salts, such as jarosite, are easily located in the Río Tinto basin, and they were also identified by the MER missions on the Mars surface (Squyres et al., 2004). The resistance of *At. ferrooxidans* to extreme conditions like those existing on the Mars surface under the protection of a thin regolith layer produced by its metabolism establishes new perspectives in the possibility of a chemolithotrophic environment in a putative Mars subsurface habitat.

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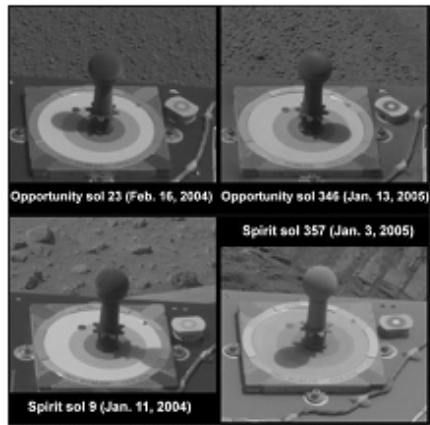
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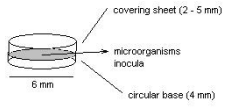
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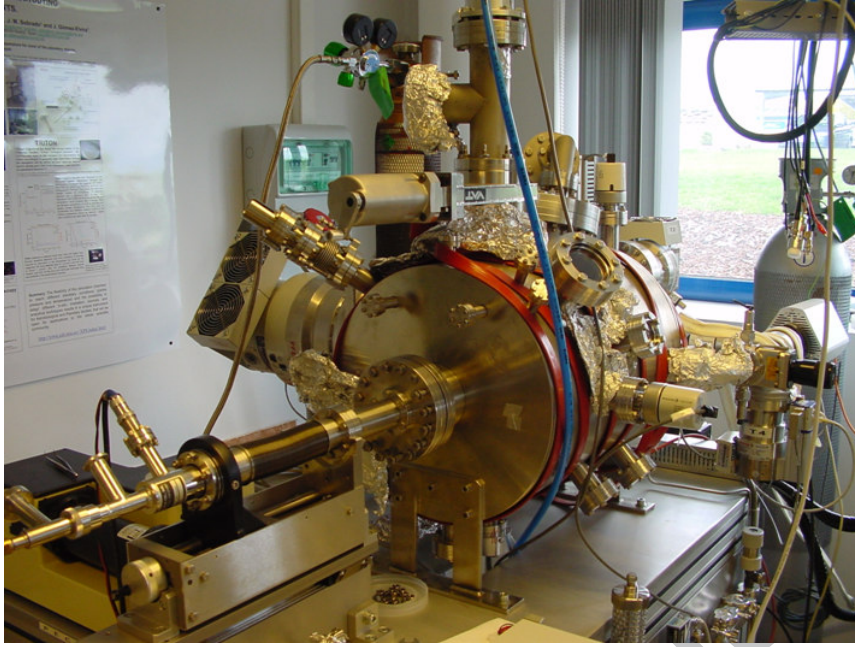
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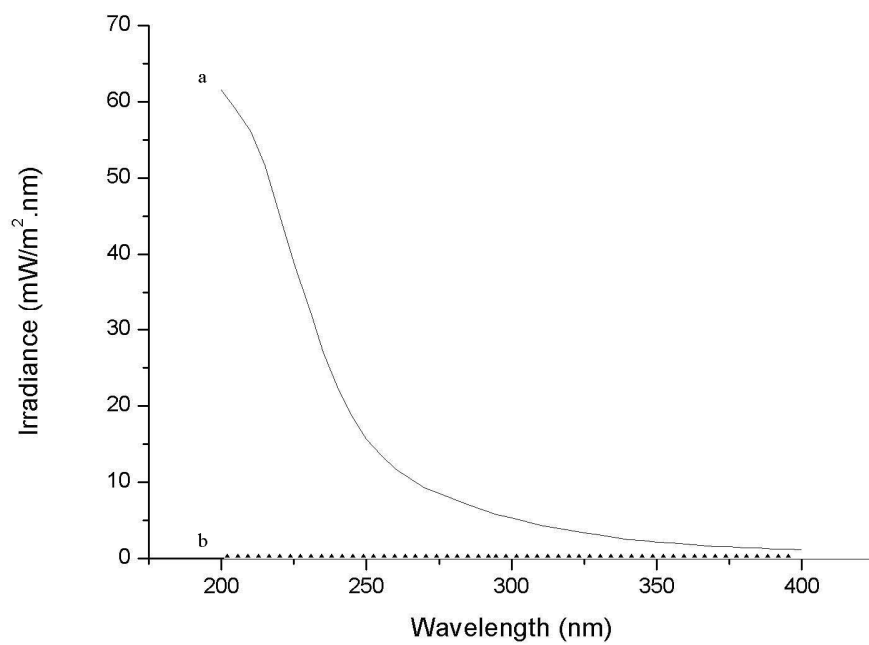
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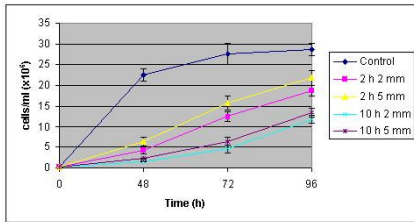
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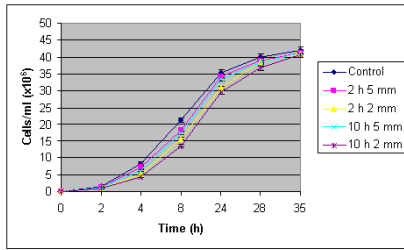
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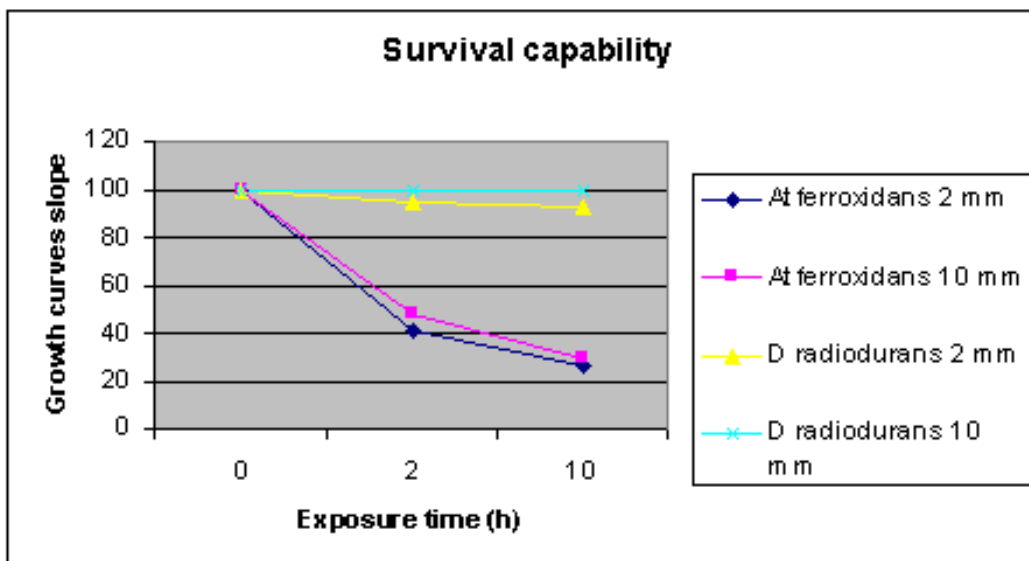
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Fig. 1: Panoramic camera's calibration target of Mars Environment Rover Spirit gradually covered by dust. The thickness of the dust layer was calculated to range between 1 to 10 micrometers. Spirit calibration target is the more dust-laden of the two rovers. As a result, a Spirit's power decrease has occurred due to the thin layer deposited on its solar panels.

(<http://marsrovers.jpl.nasa.gov/gallery/press/spirit/20050125a.html>)

Figure 2: Schematic diagram of the cakes used in the Mars environment simulation experiment. Cakes were made with Martian analogue regolith (see text for details).

Fig. 3: Photograph of the Planetary Simulation Camera used in this work.

Figure 4: a) Radiation spectrum of the deuterium lamp used in the Mars environment simulation experiment. The irradiance spectrum of the deuterium lamp is a continuum that decreases for increasing photon wavelength; it was monitored during irradiation using a spectroradiometer (Bentham DMc150FC), placed underneath the CaF₂ window. b) Radiation spectrum under 1 mm layer thickness of soil used for the protection experiments. No UV penetration is clearly observed.

Figure 5: Protective effect of regolith on *At. ferrooxidans* after exposure to Mars environmental conditions for 2 and 10 hours of exposition and under 2 mm and 4 mm protecting layer thickness. Control: not exposed. The plotted values represent the mean value and error bars indicate the range between minimum and maximum value.

Figure 6: Growth curves of *D. radiodurans* after exposure to Mars conditions during 2 and 10 hours of exposition and under 2 mm and 4 mm protecting layer thickness. Control: not exposed. The plotted values represent the mean value and error bars indicate the range between minimum and maximum value.

Fig. 7: Percentage similarities of the growth curves slopes for both microorganisms exposed to different conditions with respect to unexposed inocula. The plotted values are mean values.

Table 1: *Acidithiobacillus ferrooxidans* growth curve slopes and percentage of similarities with the control culture (not exposed to Mars conditions).

Table 2: *Deinococcus radiodurans* growth curves slope and similarities, in percentage, with the control.

Time/depth exposition	Curve's slope (x 10 ⁶)	% in comparison with the control
Control (not exposed)	0.466	100 %
2 h/ 2 mm	0.19	40.77 %
2 h/ 5 mm	0.226	48.50 %
10 h/ 2 mm	0.12	25.75 %
10 h/ 5 mm	0.14	30.04 %

Table 1: *Acidithiobacillus ferrooxidans* growth curve slopes and percentage of similarities with the control culture (not exposed to Mars conditions).

Time/depth exposition	Curve's slope ($\times 10^6$)	% in comparison with the control
Control (not exposed)	1.35	100 %
2 h/ 2 mm	1.28	94.81 %
2 h/ 5 mm	1.35	100 %
10 h/ 2 mm	1.25	92.60 %
10 h/ 5 mm	1.36	100 %

Table 2: *Deinococcus radiodurans* growth curves slope and similarities, in percentage, with the control.

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