A Desert Cyanobacterium under Simulated Mars-like Conditions in Low Earth Orbit: Implications for the Habitability of Mars

Daniela Billi,1 Cyprien Verseux,1 Claudia Fagliarone,1 Alessandro Napoli,1 Mickael Baqué,2 and Jean-Pierre de Vera2

Abstract

In the ESA space experiment BIOMEX (BIOlogy and Mars EXperiment), dried Chroococcidiopsis cells were exposed to Mars-like conditions during the EXPOSE-R2 mission on the International Space Station. The samples were exposed to UV radiation for 469 days and to a Mars-like atmosphere for 722 days, approaching the conditions that could be faced on the surface of Mars. Once back on Earth, cell survival was tested by growth-dependent assays, while confocal laser scanning microscopy and PCR-based assay were used to analyze the accumulated damage in photosynthetic pigments (chlorophyll a and phycobiliproteins) and genomic DNA, respectively. Survival occurred only for dried cells (4–5 cell layers thick) mixed with the martian soil simulants P-MRS (phylosilicatic martian regolith simulant) and S-MRS (sulfatic martian regolith simulant), and viability was only maintained for a few hours after space exposure to a total UV (wavelength from 200 to 400 nm) radiation dose of 492 MJ/m² (attenuated by 0.1% neutral density filters) and 0.5 Gy of ionizing radiation. These results have implications for the hypothesis that, during Mars’s climatic history, desiccation- and radiation-tolerant life-forms could have survived in habitable niches and protected niches while transported. Key Words: Cyanobacteria—Mars—Extreme environments—Habitability—Low Earth orbit.

1. Introduction

The question as to whether Mars ever harbored life remains open; since NASA’s life-detection experiments carried out with the Viking landers nearly 40 years ago, no other mission has been dedicated to the search for life (Levin, 2015). The Viking results were inconclusive, in large part due to our lack of knowledge of the martian surface, making it impossible either to detect life or to distinguish it from physicochemical reactions (Schulze-Makuch et al., 2015). In 2020, the ESA ExoMars rover mission, a new astrobiology mission, will be carried out to search for extant life on Mars. This mission, however, will also be equipped with a drill that can collect material at depth as great as 2 m (Vago et al., 2017). Hence, it is possible the ESA ExoMars rover mission could also detect extant life if it exists on Mars within drilled surface sediments.

Since the Viking missions, our understanding of the martian surface and limits for life on Earth has improved considerably. Many environments on Earth that were once believed to be lifeless have been discovered to harbor microorganisms with unusual protection and adaptation mechanisms that allow them to cope with physicochemical conditions lethal to most organisms (Harrison et al., 2013). However, although the Viking mission failed to detect life on Mars, the discovery of the endolithic communities in the Dry Valleys in Antarctica, previously considered too dry for life (Friedmann and Ocampo, 1976), holds out hope for the existence of life on Mars in similar environments (Friedmann and Ocampo-Friedmann, 1984).

A key feature of lithic communities is that the physical properties of the substrate guarantee the maintenance of habitable niches with an extended period of liquid water availability and access to moisture after a rewetting event (Davila et al., 2010). The study of evaporitic deposits and permafrost, respectively, in the hyperarid core of the Atacama Desert in Chile and in the Dry Valleys in Antarctica offers a new understanding of the liquid water availability and water exchange between surface and atmosphere. For example, in the Atacama Desert, halite evaporites provide liquid water to endolithic photosynthetic and heterotrophic microorganisms due to the deliquescence of salt at certain
relative humidity levels (Davila et al., 2008, 2013, 2015). By contrast, in the Antarctic Dry Valleys, the formation of dew or rime has been reported to have reactivated the metabolism of endolithic cyanobacteria (Büdel et al., 2008).

Antarctic Dry Valleys and hot deserts are considered Mars analogs of key relevance for planetary field analog studies (Martins et al., 2017). In particular, they bear similarities with the last step of the presumed three main climatic stages of Mars, this beginning with a water-rich period, followed by a cold and semiarid one, and ending with the present-day arid and cold environment (Faiéren et al., 2010). In deserts with varying degrees of dryness, lichens are often dominated by members of the genus Chroococcidiopsis (Bahl et al., 2011; Stuavleta et al., 2012; Wierzchos et al., 2013). In view of their remarkable tolerance against desiccation and radiation (Faglioni et al., 2017; Verseux et al., 2017), these cyanobacteria are good candidates for exploring microbial endurance under simulated Mars-like conditions and hence contribute to a better appreciation of the habitability of Mars (Billi et al., 2017; Billi, 2018).

By considering Mars as habitable from the point of view of life proliferation and survival, it was postulated that microbial life could have colonized habitats with trapped water, subsequently entering a survival state and eventually dying out when conditions remained unfavorable to proliferation (Westall et al., 2013). The possibility of microbial life in environmental niches on Mars depends on the interaction between atmosphere moisture and soil components. It was reported that phyllosilicatic martian regolith simulants (P-MRS) and sulfatic martian regolith simulant (S-MRS) that mimic the (supposed) wetter early and dryer late Mars soil, respectively, differ in their hydration and dehydration properties, losing (upon heating to 873 K) about 10 and 5 wt % of water, respectively (Jänchen et al., 2014). It has also been shown that, for at least a few weeks under Mars-like conditions in laboratory simulations, lichens containing symbiotic microorganisms and cryptoendolithic cyanobacteria remain viable and perform photosynthesis in the presence of available liquid water (de Vera et al., 2010, 2014a, 2014b).

Indeed, planetary laboratory simulations and space experiments expand our knowledge of the boundaries of life on Earth as well as of the potential for habitability of other worlds by improving our ability to identify environments that could support life (Horneck et al., 2016).

In the BIOlogy and Mars Experiment (BIOMEX) experiment selected for the EXPOSE-R2 space mission, Chroococcidiopsis sp. CCME 029 and other extremophiles were exposed to space and Mars-like conditions in low Earth orbit (LEO) mixed with P-MRS and S-MRS, simulating minerals formed during the Noachian and Hesperian/Amazonian epochs, respectively (de Vera et al., 2012). The aim was to investigate the resistance of extremophiles and stability/degradation of their macromolecules when mixed with lunar and martian regolith simulants (de Vera et al., 2012). Both the martian regolith simulants contain gabbro, olivine, quartz, and hematite. P-MRS resembles igneous rocks and also contains montmorillonite, kaolinite, siderite, and hydromagnesite (the carbonates hypothesized to have formed on Mars due to precipitation or interaction between a primitive CO2-rich atmosphere and basaltic subsurface rocks). S-MRS is an analog of a more acidic environment with sulfate deposits and, in addition to gabbro, olivine, quartz, and hematite, it contains goethite and gypsum (Böttger et al., 2012).

The microorganism used in this study was Chroococcidiopsis strain CCME 029 collected originally from the Negev Desert, Israel, that was preferred to CCME 123 from a coastal desert in Chile, and CCME 134, collected from the Dry Valleys in Antarctica, all clustering in a desert lineage of desiccation- and radiation-tolerant Chroococcidiopsis isolates (Faglioni et al., 2017). Strain CCME 029 was selected for this study (e.g., instead of CCME 123 and CCME 134) for the following reasons: (i) after exposure to space and Mars simulations in the dried state and overlain by 3 mm of Antarctic sandstone, CCME 134 did not survive (Billi et al., 2011); (ii) when ground-based simulations were performed before the EXPOSE-R2 mission, CCME 123 proved to be less tolerant than CCME 057, CCME 064, and CCME 029, all clustering in the above-mentioned desert lineage and used in the EXPOSE-R2 Biofilm Organisms Surfing Space (BOSS) experiment (Billi et al., 2019); (iii) CCME 029 was successfully exposed in LEO during the EXPOSE-E and EXPOSE-R space missions (Cockell et al., 2011; Bryce et al., 2015); and (iv) only a few positions were available in the EXPOSE-R2 facility for Chroococcidiopsis samples to be included in the BIOMEX experiment.

Here, we report on the survival and integrity of subcellular components of thin layers of dried cells of Chroococcidiopsis sp. CCME 029 that were mixed with martian regolith simulants after exposure in LEO to conditions simulating those on Mars. P-MRS and S-MRS’s protective role for survival of this cyanobacterium was investigated by growth-dependent assays, while the integrity of the photosynthetic pigments and of the genomic DNA was assessed by laser scanning microscopy and PCR-based assay.

2. Material and Methods

2.1. Organisms and sample preparation

Chroococcidiopsis sp. CCME 029 (hereafter Chroococcidiopsis) was isolated by Roseli Ocampo-Friedmann from cryptoendolithic growth in sandstone in the Negev Desert (Israel). This isolate is part of the Culture Collection of Microorganisms from Extreme Environments (CCMEE), established by E. Imre Friedmann, that is currently maintained at the University of Rome Tor Vergata. Before preparing dried samples, Chroococcidiopsis was grown under routine conditions at 25°C, in BG-11 medium, under a photon flux density of 40 μmol m⁻² s⁻¹ provided by fluorescent cool-white bulbs with a 16/8 h light/dark cycle.

Dried samples were obtained after resuspending cell pellets obtained from cultures in the exponential growth phase to a density of 5 × 10⁶ cells/mL, and aging 400μL aliquots mixed with 0.2 g of martian simulants P-MRS or S-MRS (see de Vera et al., 2019) onto 1.5% agarized BG-11 medium in 100 mm diameter Petri dishes in order to have 1 × 10⁶ cells/mg of mineral mixtures. To achieve a comparable and random distribution of the mineral phases in S-MRS and P-MRS, aliquots of the martian regolith simulants were taken from a small flask immediately after vigorous shaking just before mixing with the cells and prior to plating. Samples were immediately air-dried under a laminar flow hood for 2 days, in the dark, and then 12 mm diameter
disks were cut. This sample preparation was validated during the vibrational tests performed at the Microgravity User Support Center in Cologne to ensure sample integrity and stability during the spaceflight. Disks were stored in the dark at room temperature until exposure (ground reference and flight samples) or throughout the mission (laboratory controls). Control samples (cells not mixed with minerals) were prepared by plating 40 μL aliquots of liquid cultures concentrated to $5 \times 10^{10}$ cells/mL onto 1.5% agarized BG-11 medium that were then immediately dried as described above. The use of the higher volume of cell-mineral mixture was necessary to achieve a comparable sample thickness in the experimental samples with the martian regolith simulants and the controls.

2.2. Exposure in low Earth orbit

Dried, ametabolic *Chroococcidiopsis* cells mixed with P-MRS (or S-MRS) were accommodated in compartment 1 of a two-layer stacked sample carrier of Tray 2 in the EXPOSE-R2 flight hardware (Fig. 1A). The dried samples of *Chroococcidiopsis* cells without minerals, accommodated in compartment 4 of Tray 2 (Fig. 1A), were also part of the BOSS experiment (Billi *et al.*, 2019) that was designed to investigate the resistance of dried biofilm and planktonic samples that had been grown in liquid cultures.

For the simulation of a Mars-like UV spectrum with wavelength $>200$ nm, Tray 2 was covered with quartz windows and a long-pass cutoff filter of approximately 50% transmission at 216 nm. Samples were further covered with 0.1% neutral density (ND) filters, attenuating solar UV radiation by approximately 3 orders of magnitude (Rabbow *et al.*, 2017). In this two-layer stacked conformation, top samples were exposed to solar UV radiation, while bottom samples in the lower carrier were completely shaded, providing the in-flight dark controls. After closure, nitrogen in Tray 2 was exchanged with a Mars-like atmosphere (980 Pa of a gas mixture composed of 95.55% CO$_2$, 2.70% N$_2$, 1.60% Ar, 0.15% O$_2$, and ca. 370 ppm H$_2$O).

The EXPOSE-R2 hardware containing the samples was launched to the International Space Station (ISS) on 23 July 2014, on board the Progress 56 cargo spacecraft. After 26 days on board the ISS, it was installed on the Russian Svezda module and remained in the dark for 9 weeks, then exposed to solar UV for 469 days. Samples were brought back inside the ISS, where they remained for 136 days before being sent back to Earth on board the Soyuz 45S capsule on 18 June 2016. The sample racks were opened 5 days after landing, leading to a repressurization of the sample compartments and ending the exposure to a Mars-like atmosphere after 722 days. *Chroococcidiopsis* samples were then sent back to the University of Rome Tor Vergata for analysis after about 900 days from the integration of the mission.

During the EXPOSE-R2 space mission, samples in Tray 2 were exposed under Mars-like conditions (Mars-like UV spectrum and atmosphere) to a total UV dose (200–400 nm) of about 492 MJ/m$^2$ (Rabbow *et al.*, 2017) attenuated to $4.92 \times 10^2$ kJ/m and 0.5 Gy of ionizing radiation (Dachev *et al.*, 2017).

2.3. Ground references and laboratory controls

A simulation of the flight mission, the Mission Ground Reference (MGR), was performed at DLR’s Microgravity User Support Center in Cologne (Rabbow *et al.*, 2016).

![FIG. 1. EXPOSE-R2 flight hardware with *Chroococcidiopsis* sp. 029 sample distribution in Tray 2 with simulated Mars-like conditions. (A) Compartment 1 contained dried cells mixed with P-MRS or S-MRS (BIOMEX experiment), and compartment 4 dried cells not mixed with minerals (BOSS experiment). For each sample, one disk was in the top (t) and one in the bottom (b) position of the two-layer stacked sample carrier. In the top-layer carrier, cells were exposed to a Mars-like UV flux and Mars-like atmosphere; in the bottom-layer carrier (dark in-flight control) only to Mars-like atmosphere. (B) Total UV dose received during the flight and MGR. (C) Visual inspection of the samples after exposure in LEO or MGR; the reddish spots are due to glue used for the sample integration. Photo credits: Roscosmos, ESA.](image-url)
Duplicates of the flight samples were kept in flight-similar hardware, which was exposed to conditions mimicking the flight mission environment, based on available data and within the limits of the simulation facilities. No temperature data was available for the dark outgassing period, as data acquisition systems were turned off to avoid heating of the samples under the cover. During the simulation of this period and until data was available, MGR trays were kept at 15°C. Afterward, MGR conditions lagged approximately 2 months behind the flight mission conditions. More information on MGR parameters can be found in Rabbow et al. (2017).

2.4. Cell viability tests

Cell viability of dried samples was assessed by using the colony-forming unit (cfu) assay. Cells were removed by gentle resuspension with 50 μL of BG-11, from fragments (about 4 mm²) of dried disks prepared before the launch by platting cells and minerals on the top of agarized medium. The cell density was measured with a counting chamber, and 10⁶ cells were diluted and plated onto agarized BG11 and incubated under routine growth conditions for 6 months. From those samples that did not yield colonies, about 2×10⁷ cells (from about 25 mm² fragments) were inoculated, under sterile conditions, in 2 mL of BG11 medium in 15 mL sterile Falcon tubes and incubated under routine conditions. After 3 months, cell densities were measured at 730 nm (OD 730 nm) with a spectrophotometer.

2.5. Genomic PCR fingerprinting

Sample fragments (about 2 mm²) not used for inoculation were resuspended in sterile double distilled water (50 μL). Cells were pelleted (10,000g for 5 min), washed twice and resuspended in 20 μL of sterile double distilled water. DNA was extracted by performing 3 cycles of freeze-thawing (-80°C for 10 min and 60°C for 1 min) followed by 10 min boiling, as previously optimized (Baqué et al., 2016). After centrifugation (10,000g for 10 min), DNA concentrations were determined using the Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Invitrogen). Then 25 μL PCR mixtures were prepared with 12.5 μL of Taq polymerase Master Mix (MyTaq TM Red Mix, Bioline), genomic DNA (50 ng), and 2 μM of HIP1-CA 5′-GCGATCGC-CA-3′ primer derived from the Highly Iterated Palindrome (HIP1) sequence (Robinson et al., 1995) by adding 2 nucleotides at the 3′ end (Smith et al., 1998). The following PCR program was used: 1 cycle at 94°C for 3 min, 30 cycles at 94°C for 30 s, 37°C for 30 s and 72°C for 1 min, and 1 cycle at 72°C for 7 min, as previously optimized for Chroococcidiopsis sp. CCME029 (Billi 2009).

Polymerase chain reactions were loaded onto 1.5% agarose gel containing 0.5 μg/mL ethidium bromide, subjected to electrophoresis for about 1 h at 90V, and then visualized with a trans-illuminator.

2.6. Confocal laser scanning microscopy

Sample fragments (about 2 mm² collected from the initial dried disks) were placed directly between two cover glasses in the presence of a drop of water and observed with a confocal laser scanning microscope (CLSM; Olympus Fluoview 1000 Confocal Laser Scanning System). Images were acquired using a 60× objective and processed with the Imaris v. 6.1.0 software (Bitplane AG Zürich, Switzerland) for XYZ projections. The autofluorescence of photosynthetic pigments (phycobiliproteins and chlorophyll a) was revealed by successively exciting the samples with a 543 nm laser and a 635 nm laser and collecting the emitted fluorescence in the 555–609 and 655–755 nm channels, respectively. The reflective signal of minerals was recorded between 490 and 510 nm after excitation with a 488 nm laser.

Three-dimensional images were obtained after scanning optical sections throughout the samples, moving along the z axis with a step size of 0.52 μm. Sample thickness was determined by multiplying the step size (0.52 μm) by the number of steps required to focus through the sample along the z axis. To accommodate sample heterogeneity, at least six locations per sample were analyzed.

Spectral analysis (CLSM-scan) was performed with a 543 nm laser operating at 0.54 mW with emission collected from 555 to 800 nm. The mean fluorescence intensity was measured on n≥15 cells from three different areas. Curve plotting and normalization were performed using the GraphPad Prism program (GraphPad Software, San Diego, CA).

The presence of bacterial contamination was assessed by staining small fragments (about 2 mm²) of dried samples with the cell-permeant nucleic acid stain 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes, D1306) added at a final concentration of 5 μg/mL for 10 min, in the dark. Images were taken by exciting DAPI-stained cells with a 405 nm laser and collecting the emission between 430 and 470 nm.

3. Results

3.1. Visual inspection of cells mixed versus not mixed with martian regolith simulant after exposure to Mars-like conditions

After the space mission (about 900 days from launch to sample return to the lab), dried samples of Chroococcidiopsis, mixed either with P-MRS or S-MRS or not mixed with minerals, were removed from Tray 2 of the flight hardware, from the top- and bottom-layer carriers of compartment 1 and 4, respectively (Fig. 1A). Calculations provided by RedShift showed that during the EXPOSE-R2 mission each sample was exposed to a different UV dose depending on its position within a given compartment (Fig. 1B). CCME029 cells mixed with P-MRS (position 2-1-t-12) and mixed with S-MRS (position 2-1-t-16) received a UV + PAR dose of 2.70×10⁵ kJ/m² and 2.48×10⁵ kJ/m², respectively, while cells not mixed with minerals (position 2-4-t-15) received a UV + PAR dose of 4.45×10⁵ kJ/m² (see de Vera et al., 2019), corresponding to 2.19×10⁵ kJ/m², 1.94×10⁵ kJ/m², and 3.19×10⁵ kJ/m² of UV radiation (200–400 nm), respectively (Fig. 1B). During MGR, each compartment was exposed to the mean UV radiation (200–400 nm) fluence as transmitted during the space mission (Rabbow et al., 2017), but it was not possible to reproduce a different dose for each different position within a given compartment of the flight hardware (Fig. 1B).

The visual inspection of the dried samples not mixed with minerals from the top-layer carrier revealed a yellowish-
green coloration, which differed noticeably from the blue-green samples from the bottom-layer carriers (Fig. 1C). No differences occurred among samples not mixed with minerals from the bottom-layer carrier and the laboratory control.

Dried samples mixed with S-MRS or P-MRS had a reddish or brownish coloration, respectively, due to the presence of MRS, which made impossible the detection of cell bleaching (Fig. 1B). No differences were noticed among samples de-integrated from the flight or MGR hardware (Fig. 1B).

3.2. Cells mixed with P-MRS or S-MRS survive a simulated Mars-like UV flux

After exposure to Mars-like conditions in LEO and in MGR, *Chroococcidiopsis* survival was evaluated by plating about 10⁶ cells (Table 1).

Colonies were scored only from flight samples exposed in the dark (i.e., bottom-layer carrier), either mixed with P-MRS or S-MRS, or not mixed with minerals. Dried cells mixed or not mixed with minerals were stored under laboratory conditions and recovered after about 900 days of desiccation, corresponding to the entire space mission length. No colonies were obtained from any flight sample exposed to simulated Mars-like UV flux (i.e., top-layer carrier) and MGR samples (i.e., top- and bottom-layer carriers).

For samples that did not yield any colonies, survival was further investigated by inoculating 2 x 10⁷ cells into liquid BG-11 medium (Table 1). After 3 months, a reduced growth compared to control was observed for flight samples mixed with P-MRS or S-MRS and exposed to simulated Mars-like UV flux (i.e., top-layer carrier), but not for cells not mixed with minerals. For the MGR samples, a reduced growth was scored for samples mixed with P-MRS or S-MRS, exposed to a simulated Mars-like UV flux or exposed in the dark (i.e., top- and bottom-layer carriers). No growth was observed for cells not mixed with mineral after MGR (Table 1).

### Table 1. Viability of Dried *Chroococcidiopsis* Mixed or Not Mixed with Martian Regolith Simulants and Exposed to Mars-like Conditions in LEO (Flight), Mission Ground Reference (MGR), and under Laboratory Conditions (Lab Control)

<table>
<thead>
<tr>
<th>Dried cells</th>
<th>Lab control % cfu</th>
<th>Flight (Mars-like)</th>
<th>MGR (Mars-like)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top</td>
<td>Bottom</td>
<td>Top</td>
</tr>
<tr>
<td>no-MRS</td>
<td>100%</td>
<td>0%</td>
<td>0.03%</td>
</tr>
<tr>
<td>P-MRS</td>
<td>100%</td>
<td>0%</td>
<td>0.04%</td>
</tr>
<tr>
<td>S-MRS</td>
<td>100%</td>
<td>0%</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dried cells</th>
<th>Lab control % OD₇₃₀nm</th>
<th>Flight (Mars-like)</th>
<th>MGR (Mars-like)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top</td>
<td>Bottom</td>
<td>Top</td>
</tr>
<tr>
<td>no-MRS</td>
<td>100%</td>
<td>0%</td>
<td>n.d.</td>
</tr>
<tr>
<td>P-MRS</td>
<td>100%</td>
<td>47%</td>
<td>n.d.</td>
</tr>
<tr>
<td>S-MRS</td>
<td>100%</td>
<td>42%</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Viability was determined according to the colony-forming unit (cfu) assay by using 10⁶ cells (A). When no colonies occurred, viability was determined by measuring the optical density at 730 nm (OD₇₃₀nm) of cultures obtained from 2 x 10⁷ cells after 3 months of incubation (B). No-MRS: dried cells unmixed with minerals; P-MRS: dried cells mixed with P-MRS; S-MRS: dried cells mixed with S-MRS. Top: exposed in the top-layer carrier of a two-layer stack. Bottom: exposed in the bottom-layer carrier of a two-layer stack. n.d.: not determined.

3.3. Cells mixed with P-MRS or S-MRS are protected from DNA damage under a simulated Mars-like UV flux

Genomic DNA damage in exposed *Chroococcidiopsis* cells was assessed by evaluating DNA suitability as a template in a PCR-based assay, as compared to the amplificability of the genomic DNA from dried cells maintained under laboratory conditions (Fig. 2). The resulting genomic fingerprints obtained with a primer derived from the cyanobacterial HIP1 sequence showed a nearly complete absence of PCR amplicons in dried cells not mixed with minerals and exposed in LEO to simulated Mars-like UV flux (i.e., top-layer carrier) but not in those exposed in the dark (i.e., bottom-layer carrier). In dried cells exposed to the simulated Mars-like UV flux, the presence of P-MRS or S-MRS minerals resulted in the disappearance only of the longer amplicons and in a slight reduction of the other PCR band intensity, while samples exposed in the dark yielded PCR band profiles practically identical to unexposed, dried control cells (Fig. 2). A similar protective role of P-MRS and S-MRS on the DNA integrity was observed in dried cells exposed to MGR (Fig. 2).

3.4. Reduced photosynthetic pigment fluorescence in cells mixed with P-MRS or S-MRS after exposure to simulated Mars-like UV flux

When observed under CLSM, *Chroococcidiopsis* cells not mixed with minerals appeared as cell layers of about 15–30 µm with an intense photosynthetic pigment autofluorescence (Fig. 3A). The staining of nucleoids with the cell-permanent DAPI fluorochrome did not reveal any bacterial cells among cyanobacteria (Fig. 3B). The mixing of *Chroococcidiopsis* cells with S-MRS or P-MRS resulted in 4–5 cell-layers of about 15–30 µm in thickness, although cells and minerals were heterogeneously mixed (Fig. 3C, 3D).
After the space mission, the photosynthetic pigment autofluorescence of the flight samples and the laboratory controls was analyzed with a CLSM (Fig. 4). A comparison of the autofluorescence of the photosynthetic pigments of the laboratory control sample of dried cells mixed with P-MRS (Fig. 4A) or S-MRS (Fig. 4D) and the flight samples mixed with P-MRS (Fig. 4B) or S-MRS (Fig. 4E) that were exposed to Mars-like UV-flux conditions (i.e., top-layer carrier) in LEO (Fig. 4B, 4E) showed a demonstrable reduction in autofluorescence in the flight samples. Such a reduction did not

![FIG. 2. A representative image of DNA damage in Chroococcidiopsis as revealed by genomic PCR fingerprints. Lab: dried cells stored under laboratory conditions; Flight: simulated Mars-like conditions in LEO; MGR: mission-ground simulations; t: top-layer carrier; b: bottom-layer carrier; No-MRS: dried cells not mixed with minerals; P-MRS: dried cells mixed with P-MRS; S-MRS: dried cells mixed with S-MRS; MW: DNA ladder (lane 1 Hyperladder 100 bp, lane 15 Hyperladder 1 kbp, Bioline).](image)

![FIG. 3. CLSM images of dried Chroococcidiopsis cells. (A) Cells showing photosynthetic pigment autofluorescence; (B) DAPI-stained nucleoids; scale bar=20 μm. Cells mixed with S-MRS (C), P-MRS (D), mineral reflectance in white; scale bar=40 μm. Projections in xz- (below) and yz- (right side).](image)
occur in dried cells mixed with P-MRS or S-MRS that were exposed to Mars-like UV-flux conditions in the dark (i.e., the bottom-later carrier) in LEO (Fig. 4C, 4F). A similar result was obtained for MGR samples (not shown).

The effects of exposure conditions on the emission spectrum of photosynthetic pigments were evaluated by performing CLSM-lscan analysis (Fig. 5). A typical spectrum of hydrated cells (liquid culture), obtained with a 543 nm excitation laser and by collecting the emission from 555 to 800 nm, showed a peak at 648 nm due to the phycobiliprotein fluorescence, which was used as a reference point. Dried cells not mixed with MRS and stored under laboratory conditions for about 900 days showed a reduced emission spectrum. No further reduction occurred in dried cells mixed with P-MRS or S-MRS and stored under laboratory conditions for about 900 days. Such reduction was less pronounced in dried cells (about 70% of hydrated cells) mixed with P-MRS and exposed to martian simulation in the bottom-layer carrier during the space mission (P-MRS bottom flight) and MGR (P-MRS bottom MGR). The exposure to a simulated Mars-like UV flux (top-layer carrier) resulted in a peak emission intensity that was reduced to about 20% of hydrated cells for the flight samples (P-MRS top flight) and to about 15% for the MGR samples (P-MRS top MGR). Among dried cells mixed with P-MRS and exposed to a simulated Mars-like UV flux, areas with a less reduced emission peak (about 35% of hydrated cells) were observed (P-MRS top 2 flight).

The emission intensity peak of dried cells mixed with S-MRS stored in the laboratory (S-MRS control) was reduced to about 45% of hydrated cells (Fig. 5B). However, opposite to P-MRS, a comparable reduction occurred in cells exposed to simulation Mars-like conditions in the bottom-layer carrier in flight samples (S-MRS bottom flight) while a reduction to about 70% of hydrated cells occurred in cells exposed in the dark during the MGR (S-MRS bottom MGR). The exposure to a simulated Mars-like UV flux (top-layer carrier) resulted in a peak emission intensity being reduced to about 10% of hydrated cells in both flight and MGR samples.

4. Discussion

To discern whether Mars is habitable and whether we can find traces of extinct or extant life, it is essential to expose extremophiles isolated from Mars-relevant environments to Mars-like conditions. In this context, one of the aims of the BIOMEX experiment was to investigate the survival of extremophiles mixed with Mars-analog regoliths (P-MRS or S-MRS) after exposure to simulated Mars-like conditions in LEO (de Vera et al., 2012). On the basis of the Science Verification Tests (SVT) performed prior to the mission on this cyanobacterium mixed with P-MRS or S-MRS, the exposure to 570 MJ/m² of UV radiation (200–400 nm)—a dose expected after 1 year exposure in LEO—led to the lack of survivors (Baqué et al., 2016). For the real mission, it was therefore decided to attenuate the solar UV radiation with 0.1% ND filter as previously decided for the EXPOSE-R mission (Demets et al., 2015; Rabbow et al., 2015). The use of such filters also made it possible to accurately simulate the radiation that reaches micronsiches known to be a common habitat of the endolithic cyanobacteria.

During the EXPOSE-R2 space mission, dried cell-layers of the desert cyanobacterium Chroococcidiopsis sp. CCME
029, mixed with P-MRS or S-MRS, or not mixed with minerals, were subjected in LEO to Mars-like conditions (simulated Mars-like UV flux and Mars-like atmosphere). Cells in the top-layer carrier were exposed to the direct solar UV irradiation for 469 days in addition to a Mars-like atmosphere, while those placed in the bottom-layer carrier were only exposed to the Mars-like atmosphere for 722 days (Rabbow et al., 2017). While in LEO, dried Chroococcidiopsis cells mixed with P-MRS or S-MRS or not mixed with minerals were exposed to 2.19 × 10^2 kJ/m², 1.94 × 10^2 kJ/m², and 3.19 × 10^2 kJ/m² of UV radiation (200–400 nm), whereas during the MGR cells mixed or not mixed with regoliths were exposed to 4.37 × 10^2 kJ/m² and 5.05 × 10^2 kJ/m², respectively.

Overall, the MGR results suggest that mixing cells with martian regolith simulants plating them to a thin layer with a

**FIG. 5.** CLSM-l scan with a 543 nm laser at 0.54 mW of photosynthetic pigments in dried Chroococcidiopsis cells mixed with P-MRS (A) or S-MRS (B) after exposure to Mars-like conditions in LEO (flight samples) or in MGR. Emission spectra of cells from liquid cultures and dried cells mixed with P-MRS or S-MRS or unmixed with minerals stored under laboratory conditions (P-MRS control, S-MRS control, and no-MRS control). Data points represent normalized fluorescence intensity at 653 nm ± standard error for n ≥ 15 cells as a function of emission wavelength.
thickness of about 15–30 µm (corresponding to 4–5 cell layers) provided sufficient UV shielding to guarantee their survival after exposure to $4.37 \times 10^5$ kJ/m² of UV radiation (200–400 nm). The same conclusion cannot be drawn for samples exposed for 469 days in LEO because dried cells mixed with P-MRS or S-MRS in compartment 1 of Tray 2 experienced almost half the dose of that received by samples not mixed with the regolith simulants in compartment 4 of Tray 2. Dried cells not mixed with minerals did not survive the exposure in LEO to $3.19 \times 10^2$ kJ/m² of UV radiation (200–400 nm).

Our results also confirm that UV irradiation is the major damaging factor in space, due to the DNA damage caused by its absorption of UVB and UVC from the solar spectrum (Horneck et al., 2010). Indeed, thin-cell layers of Chroococcidiopsis in the absence of minerals did not recover upon rehydration. Consistently, dried Chroococcidiopsis cells not mixed with minerals and exposed to a simulated Mars-like UV-flux failed to yield PCR amplicons in the PCR-based assay—in agreement with results obtained during the SVT (Baqué et al., 2016); this suggests that the accumulated DNA damage exceeded the repair capability of the cells upon rehydration. The genomic DNA from cells mixed with minerals and exposed to Mars-like UV-flux DNA proved to be suitable for PCR fingerprinting, although it experienced some damage, as suggested by the reduced band intensities of PCR fingerprints. It should also be noted that DNA oxidized nucleotides do not significantly affect the PCR amplification process (Furda et al., 2014). The exploitation of the PCR fingerprinting based on the HIP1-derived primer was possible thanks to its occurrence in the genome of Chroococcidiopsis sp. CCMEE 029 for 4333 times, a value in the range of the periodicity reported for the cyanobacterial genomes sequenced so far (Xu et al., 2018).

When comparing the survivors scored among cells mixed with P-MRS or S-MRS and exposed to space versus MGR with the lack of survivors reported after exposure to 570 MJ/m² of UV radiation (with or without the 0.1% ND), it must be pointed out that during the SVT the recovery was determined by colony-forming ability by plating up to $10^9$ cfu (Baqué et al., 2016). In the present work, when colonies were not scored after plating $10^6$ cells, $2 \times 10^7$ cells were inoculated into liquid BG-11. Increasing the cell number was impossible due to the reduced sample availability. The scored growth for dried cells mixed with P-MRS or S-MRS exposed in the top-layer mixture (Baqué et al., 2016). During the SVT, exposure of the dried cells mixed with S-MRS or P-MRS to 570 MJ/m² of UV radiation (200–400 nm) resulted in a reduction in the photosynthetic pigment autofluorescence of about 0.001%, as determined on Luria-Bertani agarized medium. DAPI staining of Chroococcidiopsis biofilms did not reveal bacteria, thus suggesting that they did not account for the scored survival. In addition, no bacterial colonies were scored after plating onto Luria-Bertani medium aliquots of cyanobacterial cultures obtained from samples exposed in the top-layer carrier to Mars-like conditions in LEO and MGR (not shown).

After 900 days of storage (from launch to sample return), control dried cells mixed with S-MRS or P-MRS or not mixed with minerals showed no differences in pigment fluorescence. The exposure to a Mars-like UV flux led to a reduction in the emission spectra of dried cells mixed with P-MRS or S-MRS, the reduction being slightly greater in samples exposed to MGR, likely due to the higher UV dose. Dried cells mixed with P-MRS or S-MRS and stored in the air-dried state under laboratory conditions for about 900 days showed a greater reduction in the photosynthetic pigment fluorescence compared to samples exposed in the bottom-layer to Mars-like conditions in space and ground-based simulations. This was likely because cells accumulated oxidative damage during the prolonged dried storage under laboratory conditions that did not occur under a gas mixture consisting of 95.55% CO₂. A reduction in the photosynthetic pigment fluorescence was not observed in dried cells stored under laboratory conditions during the SVT (Baqué et al., 2016), likely due to the shorter storage period (89 days) as compared to the EXPOSE-R2 mission.

A comparison of the CLSM-λ scan of the martian regolith simulant/cell mixtures and their ground-based controls showed that unlike the S-MRS samples, dried cells that were mixed with P-MRS and exposed to Mars-like UV flux exhibited areas with an emission spectrum reduced only to 35% of that of the control. Better protected zones were also observed in samples of dried cells that were mixed with P-MRS and exposed to the SVT, where the fluorescence was reduced only to 50% of that of hydrated cells; this finding was ascribed to the thinner grain-size of P-MRS (compared to S-MRS), which might have led to thicker areas with enhanced shielding due to preparation of the cells/mineral mixture (Baqué et al., 2016). During the SVT, exposure of the dried cells mixed with S-MRS or P-MRS to 570 MJ/m² of UV radiation (200–400 nm) resulted in a reduction in the photosynthetic pigment autofluorescence of 3.5% and 16.7% that of the control, respectively. The increased protective role of P-MRS might have been due to the fact that it contained a large fraction of clay minerals (notably montmorillonite, which accounts for 45% of the dry weight), which are considered to be good matrices for organic matter preservation in that they adsorb water molecules that might otherwise oxidize organic molecules and strongly absorb organic material (see, e.g., Hedges and Keil, 1995; Farmer and Des Marais, 1999).

5. Conclusion

Ultraviolet radiation is known to be the most harmful factor to microorganisms in space (Horneck et al., 2010).
Based on the model of Cockell et al. (2000), the UV dose received by the dried thin cell-layers in LEO, $3.19 \times 10^2 \text{kJ/m}^2$, corresponded to approximately 4 h of an average UV flux of Mars at the equator at the vernal equinox. Though dried monolayers of the *Chroococcidiopsis* strain used in this study have previously been reported to have withstood up to $15 \text{kJ/m}^2$ of a Mars-like UV flux (Cockell et al., 2005), survivors could not be cultivated from dried cell-layers of *Chroococcidiopsis* that were exposed during the MGR to a simulated Mars-like UV-flux of $5.05 \times 10^2 \text{kJ/m}^2$ of UV radiation (200–400 nm). In this experiment, survival of the *Chroococcidiopsis* strain occurred only with those cells that were mixed with martian regolith simulant and plated as thin layers (about 15–30 $\mu$m, corresponding to 4–5 cell layers). Though the martian regolith simulants provided shielding against the UV radiation, the role of a high intracellular manganese concentration in the resistance of the investigated desert cyanobacterium, as reported for UVC-resistant bacteria (Paulino-Lima et al., 2016), in synergy with other enzymatic and non-enzymatic antioxidant systems, remains to be investigated (Faglialone et al., 2017). Our finding suggests that a putative microbial life-form at least as resistant to desiccation and radiation as the investigated desert cyanobacterium could withstand some exposure to UV on the martian surface.

It is unlikely that cell viability was affected in our experiments by the exposure to 0.5 Gy of ionizing radiation, the total ionizing radiation dose of the EXPOSE-R2 mission (Dachev et al., 2017). Based on the dose of 76 mGy/year measured by the Curiosity rover at the surface in Gale Crater (Hassler et al., 2013), the ionizing radiation dose experienced during the EXPOSE-R2 mission corresponds to no more than a half decade of exposure on Mars; therefore, it would not represent a limiting factor for short-term survival when compared to the limitations posed by martian UV irradiation. Our findings support the hypothesis that opportunistic colonization of protected niches on Mars, such as in fissures, cracks, and microcaves in rocks or soil, could have enabled life to remain viable while being transported to a new habitat (Westall et al., 2013).

Our study also demonstrated the capability of dried *Chroococcidiopsis* to repair, upon rehydration, damage that accumulated during the EXPOSE-R2 space mission. Such findings support the use of this cyanobacterium in future astrobiology experiments carried out on cells rehydrated in orbit after exposure to space conditions. This endeavor will allow for evaluation of cyanobacterial adaptation to extraterrestrial environments and a better understanding of the limits and adaptation potential of life as we (now) know it (Cottin et al., 2017).

**Acknowledgments**

This research was supported by the Italian Space Agency (ASI grant 051-R.0 to D.B.) and the German Helmholtz Association through the Helmholtz-Alliance “Planetary Evolution and Life”. We thank Dr. Elke Rabbow (DLR Cologne) for conducting the ground-based simulations, Dr. René Demets (ESA) for supporting EXPOSE experiments, and Dr. Elena Romano, Centre of Advanced Microscopy “P.B. Albertano”, University of Rome Tor Vergata, for her skillful assistance in using the CLSM. M.B. acknowledges support from the Alexander von Humboldt Foundation.

**References**


---

**Address correspondence to:**

Daniela Billi

University of Rome Tor Vergata

Department of Biology

Via della Ricerca Scientifica

Rome 00133

Italy

E-mail: billi@uniroma2.it

Submitted 13 December 2017

Accepted 4 January 2019

---

**Abbreviations Used**

BIOMEX = BIOlogy and Mars EXperiment

BOSS = Biofilm Organisms Surfacing Space

CCMEE = Culture Collection of Microorganisms from Extreme Environments

CLSM = confocal laser scanning microscope

DAPI = 4′,6-diamidino-2-phenylindole

HIP1 = Highly Iterated Palindrome

ISS = International Space Station

LEO = low Earth orbit

MGR = Mission Ground Reference

ND = neutral density

P-MRS = phyllosilicatic martian regolith simulant

P-MRS = sulfatic martian regolith simulant

SVT = Science Verification Tests