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Exposure to low Earth orbit of an extreme-tolerant cyanobacterium as a contribution to lunar astrobiology activities

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Research Article

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Abstract

By investigating the survival and the biomarker detectability of a rock-inhabiting cyanobacterium, *Chroococcidiopsis* sp. CCMEE 029, the BIOMEX space experiment might contribute to a future exploitation of the Moon as a test-bed for key astrobiology tasks such as the testing of life-detection technologies and the study of life in space. Post-flight analyses demonstrated that the mixing of dried cells with sandstone and a lunar regolith simulant provided protection against space UV radiation. During the space exposure, dried cells not mixed with minerals were killed by 2.05×10^2 kJ m^{-2} of UV radiation, while cells mixed with sandstone or lunar regolith survived 1.59×10^2 and 1.79×10^2 kJ m^{-2}, respectively. No differences in survival occurred among cells mixed and not mixed with minerals and exposed to space conditions in the dark; this finding suggests that space vacuum and 0.5 Gy of ionizing radiation did not impair the cells' presence in space. The genomic DNA of dead cells was severely damaged but still detectable with PCR amplification of a short target, thus suggesting that short sequences should be targeted in a PCR-based approach when searching for traces of life. The enhanced stability of genomic DNA of dried cells mixed with minerals and exposed to space indicates that DNA might still be detectable after prolonged periods, possibly up to millions of years in microbes shielded by minerals. Overall, the BIOMEX results contribute to future experiments regarding the exposure of cells and their biomarkers to deep space conditions in order to further test the lithopanspermia hypothesis, the biomarker stability and the microbial endurance, with implications for planetary protection and to determine if the Moon has been contaminated during past human missions.

Introduction

Fifty years after Apollo 11, the Earth's natural satellite, the Moon, is again the target for manned space programs, namely with the ESA's Moon Village concept (http://exploration.esa.int/moon/59374-overview/), the NASA's Artemis program (https://www.nasa.gov/artemis/) and the Gateway, an outpost orbiting around the Moon, endorsed by the International Space Station partners (https://www.nasa.gov/feature/multilateral-coordination-board-joint-statement).

Although the Moon is hostile to life (Schuerger *et al.*, 2019), nevertheless it is relevant to astrobiology since it may hold key information on how life developed on Earth. Its surface, lacking recent geological and erosional activities, could have preserved the organics delivered to it and to Earth during the late heavy bombardment period (Gronstal *et al.*, 2007; Crawford *et al.*, 2012). An exogenous origin of prebiotic organics delivered by carbonaceous chondrites, comets and interstellar dust particles, all of which might have provided the first building blocks, has been suggested (for reviews see Bernstein, 2006; Kitadai and Maruyama, 2018). Although a terrestrial origin of organics was also proposed according to the Miller–Urey synthesis in the primordial atmosphere and/or through the serpentinization process in hydrothermal vents (for a review see Bernstein, 2006). In addition, considering the large amount (about 200 kg km^{-2}) of terrestrial ejecta that reached the lunar surface 3.8–3.9 Gyr ago (Koeberl, 2003), a possible Earth-to-Moon transfer of microbial life inside rocks might have occurred. Indeed, the transport of microorganisms from one celestial body to another aboard planetary ejecta, resulting from impacts of asteroids and comets, has been proposed for the Earth–Mars system (Mileikowsky *et al.*, 2000). Such a phenomenon, known as lithopanspermia, is constrained by three events: (i) the launch of microbe-bearing rocks from a donor planet into space; (ii) the transit through space to a recipient planet and (iii) the entry into a recipient planet (for review see Nicholson, 2009). Compared to the Earth–Mars system, the transfer of rock-inhabiting microbes form the Earth to the Moon could have been easier, given the lower distance of the Moon. Moreover, the chances of surviving the landing on the Moon

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might have been increased by a reduced impact velocity due to the presence of a lunar atmosphere, which appeared about 3.5 Ga ago, with a lifetime of about 70 million years (Needham and Kring, 2017). In the context of the Earth-to-Moon lithopanspermia, relevant implications depend on the possibility of the Moon having once been habitable (Schulze-Makuch and Crawford, 2018).

The Moon's surface with its high doses of ultraviolet irradiation, temperature extremes, low pressure and high levels of ionizing radiation (Schuerger *et al.*, 2019) has a remarkable relevance when testing life-detection instrumentations (Carpenter *et al.*, 2012; de Vera *et al.*, 2012). Currently, ground-based simulations and exposure in low Earth orbit (LEO) are used to understand how biomarkers are affected by extra-terrestrial conditions and contribute to future life detection missions (de Vera *et al.*, 2019*a*).

A first Moon-analogue experiment was performed as part of the BIOlogy and Mars EXperiment (BIOMEX) project carried out by using the ESA EXPOSE-R2 platform installed outside the ISS in LEO from July 2014 to June 2016 (de Vera *et al.*, 2019*b*). Among the extremophiles selected for the BIOMEX space experiment, there was the rock-inhabiting cyanobacterium *Chroococcidiopsis* sp. CCMEE 029, isolated from sandstone in the Negev Desert. Cyanobacteria belonging to this genus survive in extremely hot and cold deserts, where they take refuge inside porous rocks or colonize soil–rock interfaces (Billi, 2018). In particular, it was demonstrated that under laboratory conditions, *Chroococcidiopsis* sp. CCMEE 029 tolerates at least 4 years of air-drying (Billi, 2009; Fagliarone *et al.*, 2017), up to 12 kGy of γ-radiation and at least 2 kGy of Fe ions (Verseux *et al.*, 2017).

The objective of the BIOMEX experiment was to investigate the endurance of extremophiles and stability of biomolecules under two simulated environments: under a Mars-like atmosphere (in contact or not with Mars regolith analogues), and under space conditions (contact with natural substrata or lunar regolith analogue). The former environment has already provided significant data concerning the habitability of Mars and the likelihood of Earth–Mars lithopanspermia (Billi *et al.*, 2019; de Vera *et al.*, 2019*b*). The latter environment will contribute to evaluating the effects of the lunar regolith on space-exposed biosignatures in order to optimize life detection technologies to be tested on the Moon by avoiding potential pitfalls (de Vera *et al.*, 2012; Fox and Strasdeit, 2017).

Here we report on the survival of dried *Chroococcidiopsis* cells mixed with sandstone or a lunar regolith analogue after exposure to space conditions in LEO for 672 days as well as simulated conditions on Earth during the mission ground reference (MGR). The protection provided by mixing the cells with minerals was determined by evaluating the survival threshold while the detectability of a biomarker, namely DNA, was evaluated by using polymerase chain reaction (PCR)-stop assays.

Material and methods

Organisms and sample preparation

Chroococcidiopsis sp. CCMEE 029 (hereafter *Chroococcidiopsis*) was isolated by Roseli Ocampo-Friedmann from cryptoendolithic growth in sandstone in the Negev Desert (Israel) and now maintained at the University of Rome Tor Vergata, as part of the Culture Collection of Microorganisms from Extreme Environments (CCMEE) established by E. Imre Friedmann. Liquid cultures were grown under routine conditions at 25 °C, in BG-11 medium (Rippka *et al.*, 1979), 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.

In order to have 1×10^7 cells per mg of mineral mixtures, 400 µl of 5×10^9 cells per ml were mixed with: (i) 0.2 g of a lunar regolith analogue (about 100–1000 µm grain size and mainly composed of anorthosite; for details see de Vera *et al.*, 2019*b*); and (ii) 0.2 g of a red Antarctic sandstone (kindly provided by Silvano Onofri, Tuscia University, Italy) that was crushed to about 250 µm grain size. The mixing of the cells with minerals was performed on the top of 1.5% agarized BG-11 medium within a 70 mm diameter surface of a 100 mm-diameter Petri dish. After plating, samples were air-dried under a laminar flow hood for 2 days, in the dark and then cut in 12 mm-diameter disks. Disks were stored in the dark at room temperature until exposure (MGR and flight samples) or throughout the mission (laboratory controls). Controls were prepared by plating 40 µl aliquots of 5×10^{10} cells per ml onto agarized BG-11 medium without adding minerals.

Exposure in low Earth orbit and mission ground reference

In the EXPOSE-R2 flight hardware, dried cells mixed with lunar analogue or smashed sandstone were accommodated in the compartment 1 of a two-layer stacked sample carrier (Tray 1) while control, dried samples not mixed with minerals (part of the BOSS experiment) were accommodated in the compartment 4 of Tray 1 (Fig. 1(a)).

Tray 1 was covered with MgF_2 windows for exposure to space UV radiation ($\lambda > 120$ nm) under space vacuum. 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. Both layers were subjected to ionizing radiation exposure from solar energetic particles, galactic cosmic rays and trapped within the radiation belts, estimated to have reached approximately 0.5 Gy of absorbed dose during the EXPOSE-R2 mission (Dachev *et al.*, 2017). *Chroococcidiopsis* samples were sent back to the University of Rome Tor Vergata for analysis after about 900 days from the integration of the mission.

A simulation of the flight mission, the MGR, was performed at DLR's Microgravity User Support Center in Cologne (Rabbow *et al.*, 2016) by using duplicates of the flight samples that were kept in a flight-similar hardware exposed to conditions simulating the flight mission, based on transmitted data and the capabilities of the simulation facilities (Rabbow *et al.*, 2017). Control samples were stored in the dark at room temperature throughout the mission (laboratory control).

Cell viability

Cell viability of each dried sample was assessed by inoculating about 2×10^7 cells (from about 25 mm^2 fragments) into 2 ml of BG11 medium in a 15 ml sterile Falcon tube. After 3 months of growth under routine growth conditions, cell densities were inferred from optical density measurements at 730 nm (OD 730 nm) with a spectrophotometer.

Evaluation of genomic DNA integrity by PCR-stop assays

Sample fragments (about 2 mm^2) were resuspended in 50 µl of sterile bidistilled water and the removed cells washed twice and resuspended in 20 µl of sterile bidistilled water. Genomic DNA was extracted by performing three cycles of freeze–thawing (−80 °C for 10 min and 60 °C for 1 min) followed by 10 min boiling. After centrifugation,

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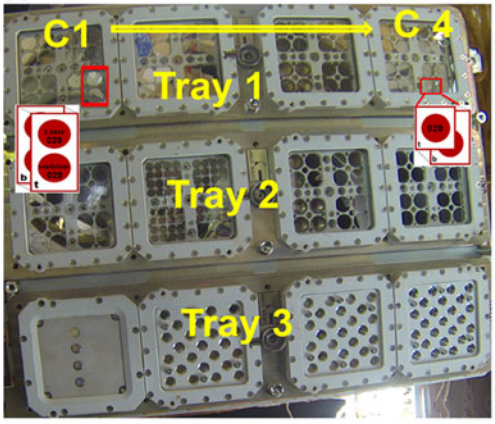
(a)



(b)

Carrier position	Flight space: Tray 1 Compartment 1 Sample 12 029 Lunar	Flight space: Tray 1 Compartment 1 Sample 16 029 Sandstone	Flight space: Tray 1 Compartment 4 Sample 15 029 no-mineral	MGR space: Tray 1 Compartment 1 Sample 12 029 Lunar	MGR space: Tray 1 Compartment 1 Sample 16 029 Sandstone	MGR space: Tray 1 Compartment 4 Sample 15 029 no-Mineral
Top						
Bottom						
Lab control						

(c)

Solar irradiation	Flight (space): Tray 1 Compartment 1 Sample 12 029 Lunar	Flight (space): Tray 1 Compartment 1 Sample 16 029 Sandstone	Flight (space): Tray 1 Compartment 4 Sample 15 029 no-mineral	MGR (space): Tray 1 Compartment 1 Sample 12 029 Lunar	MGR (space): Tray 1 Compartment 1 Sample 16 029 Sandstone	MGR (space): Tray 1 Compartment 4 Sample 15 029 no-mineral
UV + PAR	3.0×10^3 kJ/m^2	2.77×10^3 kJ/m^2	4.26×10^3 kJ/m^2	n.d.	n.d.	n.d.
$UV_{200-400\ nm}$	1.79×10^2 kJ/m^2	1.59×10^2 kJ/m^2	2.05×10^2 kJ/m^2	4.39×10^2 kJ/m^2	4.39×10^2 kJ/m^2	5.05×10^2 kJ/m^2

PAR = photosynthetically active radiation
n.d. = not determined

Fig. 1. EXPOSE-R2 flight hardware with *Chroococcidiopsis* sp. CCMEE 029 distribution. Tray 1 compartment 1 with dried cells mixed with sandstone or lunar regolith simulant, and compartment 4 with dried cells not mixed with minerals; sample in the top-layer (t) and in the bottom-layer (b) carrier (a). Visual inspection of samples exposed in space and MGR; reddish spots are due to the glue used for the sample integration (b). Total UV dose received during the flight and MGR (c). Photo credits: Roscosmos, ESA. MGR: mission ground reference.

DNA concentrations were determined using the Qubit dsDNA HS Assay Kit and a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). The following PCR-stop assays were used.

Single-gene PCR

A 1027-bp fragment of the 16S rRNA gene was amplified by using the primers CYA-359F (5′-GGGGAATTTTCCGCAATGG-3′) and CRev (5′-ACGGGCGGTGTGTAC-3′), 0.5 µM each, in 12 µl PCR reaction mixtures containing genomic DNA (6 ng) and 6 µl of MyTaq™ Red Mix (Bioline). PCR conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 3 min; and 7 min at 72 °C.

A 500-bp fragment of the 16S rRNA gene was amplified using the primers 029-16SF2 (5′-GGAGTGCGGTAGGGGTAGAG-3′) and 029-16SR2 (5′-CTAGAGTGCCCAACTTAATGCT-3′), 0.5 µM each, in 12 µl PCR reaction mixtures containing genomic DNA (15 ng) and 6 µl of MyTaqTM Red Mix (Bioline). PCR conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 45 °C for 30 s and 72 °C for 30 s; and 7 min at 72 °C.

Genome PCR fingerprinting

PCR reaction mixtures of 25 µl were prepared using 12.5 µl of Taq polymerase Master Mix (MyTaq TM Red Mix, Bioline), genomic DNA (50 ng) and 2 µM of HIP1-CA primer (5′-GCGATC GC-CA-3′) derived from the Highly Iterated Palindrome (HIP1) sequence. The following PCR program was used: 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 72 °C for 7 min as previously optimized for *Chroococcidiopsis* sp. CCMEE 029 (Billi, 2009).

Results

Visual inspection of cells mixed and not mixed with lunar regolith analogue or sandstone after space exposure

After the EXPOSE-R2 space mission, samples of *Chroococcidiopsis* sp. CCMEE 029 were removed from the top- and bottom-layer carriers of Tray 1 (Fig. 1(a)) and from the hardware used for the MGR. Visual inspection of the dried samples not mixed with minerals from the top-layer carrier of the hardware exposed in LEO and in the MGR revealed a yellowish-green colouration, due to pigments bleaching, as opposed to the blue-green samples from the bottom-layer carriers (Fig. 1(b)). For the top-layer carrier of the flight and ground hardware, cells mixed with lunar regolith analogue or sandstone showed a slight yellowish-green colouration compared to green samples from the bottom-layer carrier (Fig. 1(b)). Laboratory controls retained a blue-green colouration (Fig. 1(b)).

Calculations provided by RedShift showed that during the space mission, each sample was exposed to a different UV dose depending on its position within the compartment (Fig. 1(c)). *Chroococcidiopsis* cells mixed with lunar regolith (position 1-1-t-12) and mixed with sandstone (position 1-1-t-16) received a UV + PAR dose of 3.0×10^3 kJ m^{-2} and 2.77×10^3 kJ m^{-2}, respectively. While cells not mixed with minerals (position 2-4-t-15) received a UV + PAR dose of 4.26×10^3 kJ m^{-2}, corresponding to 1.79×10^2, 1.59×10^2 and 2.05×10^2 kJ m^{-2} of UV radiation (200–400 nm), respectively (Fig. 1(c)). During the MGR, each compartment was exposed to the mean UV radiation (200–400 nm) fluency, as transmitted during the space mission (Rabbow *et al.*, 2017) (Fig. 1(c)).

Dried cells survived space conditions when mixed with lunar regolith analogue or sandstone

Post-flight analyses were performed on *Chroococcidiopsis* samples after storage in the dried state for about 2.5 years due to the time required for sample preparation, integration into the flight hardware, EXPOSE-R2 space mission (696 days from launch to landing) and distribution to the various laboratories after retrieval (about 900 days from launch to sample return to the laboratory).

The viability of dried *Chroococcidiopsis* cells exposed to space conditions in LEO or in MGR was evaluated by determining the culture densities ($OD_{730\ nm}$) 3 months after the inoculum of about 2×10^7 cells into liquid BG11 medium (Table 1).

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Table 1. Viability of dried *Chroococcidiopsis* mixed or not mixed with Lunar regolith simulant and sandstone and exposed to space conditions (Flight), Mission Ground Reference (MGR) and under laboratory conditions (Laboratory control). Viability was determined by measuring the increase in optical density at 730 nm (OD_{730nm}) of liquid cultures obtained from 2×10^7 cells after 3 months of growth (B). Top: exposed in the top-layer carrier. Bottom: exposed in the bottom-layer carrier

		Flight (space)		MGR (space)	
Dried cells	Laboratory control % $OD_{730\ nm}$	Top	Bottom	Top	Bottom
No-mineral	100%	0%	80%	0%	78%
Lunar	100%	38%	78%	19%	79%
Sandstone	100%	51%	77%	28%	76%

After exposure in LEO to UV irradiation, space vacuum and cosmic radiation (i.e. top-layer carrier), only cells mixed with lunar regolith analogue or sandstone recovered in the liquid medium. The recorded OD_{730nm} values for cultures obtained from cells mixed with lunar regolith analogue or sandstone were reduced to 38 and 51%, respectively, of the value obtained for dried cells stored in the laboratory throughout the space mission (laboratory control).

Similarly, after the MGR, in which dried cells were exposed to UV irradiation and space vacuum, only cells mixed with minerals underwent cell division after transfer into liquid growth medium, although they reached OD_{730nm} values of 19 and 28% compared to dried cells stored in the laboratory (Table 1). Dried cells mixed or not mixed with minerals and exposed in the dark to space vacuum and cosmic radiation (i.e. bottom-layer carrier) or exposed during the MGR in the dark to simulated space vacuum showed OD_{730nm} values ranging from 80 to 76% of the OD_{730nm} of laboratory control (Table 1).

Intact genomic DNA in cells mixed with lunar regolith analogue after space exposure

The effect of space exposure on the genome integrity was evaluated by assessing its suitability as a PCR template.

When the amplification of a 1027-bp fragment of the 16S rRNA gene was attempted on the genomic DNA of dried cells exposed in LEO to UV irradiation, space vacuum and cosmic radiation (i.e. top-layer carrier), no amplicons were obtained from cells not mixed with minerals (Fig. 2, lane 3). Cells mixed with lunar regolith analogue did not yield a PCR amplicon (Fig. 2, lane 8) in contrast to cells mixed with sandstone that yielded a very faint PCR band (Fig. 2, lane 13). An intense PCR band was obtained from laboratory control, i.e. dried cells not mixed with minerals and stored in the dark throughout the space mission (Fig. 2 lane 2), as well as from dried, laboratory control cells mixed with lunar regolith analogue (Fig. 2, lane 5) or with sandstone (Fig. 2, lane 10). When the amplification of the 1027-bp fragment was carried out on dried cells exposed in the dark to space vacuum and ionizing radiation (e.g. bottom-layer carrier), cells mixed or not with minerals yielded intense PCR amplicons (Fig. 2, lanes 4, 9, 14).

During the MGR, the exposure of dried cells mixed with minerals to simulated space UV radiation and vacuum resulted in PCR amplicons of reduced intensity (Fig. 2, lanes 6, 11) compared to bands obtained from cells exposed in the dark (Fig. 2, lane 7, 12).

When the amplification of a shorter 500-bp fragment of the 16S rRNA gene was attempted by using genomic DNA from dried cells exposed to LEO conditions in the top-layer carrier position, amplicons were obtained from cells not mixed with minerals (Fig. 3 lane 3), mixed with lunar regolith analogue (Fig. 3 lane 8) or mixed with sandstone (Fig. 3 lane 13). A similar result was obtained for cells from the top-layer carrier position of the MGR (Fig. 3 lanes 6, 11) as well as from the bottom-layer carrier

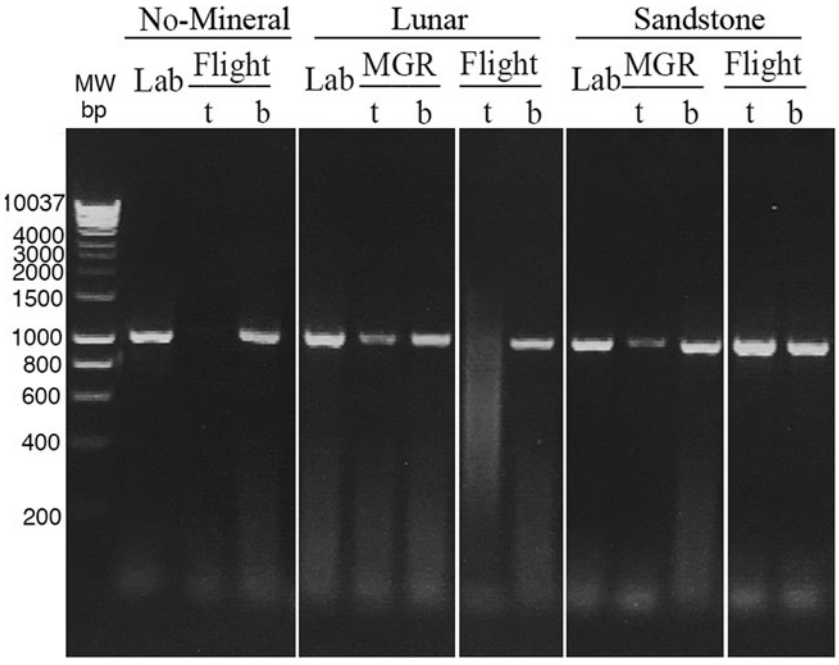

Fig. 2. Evaluation of DNA integrity by PCR amplification of a 1027-bp gene target. Laboratory: dried cells stored under laboratory conditions; Flight: space conditions in LEO; MGR: mission ground reference; t: top-layer carrier; b: bottom-layer carrier; No-mineral: dried cells not mixed with minerals; Lunar: dried cells mixed with lunar regolith simulant; Sandstone: dried cells mixed with natural substratum; MW: DNA ladder (Hyperladder 1 kbp, Bioline).

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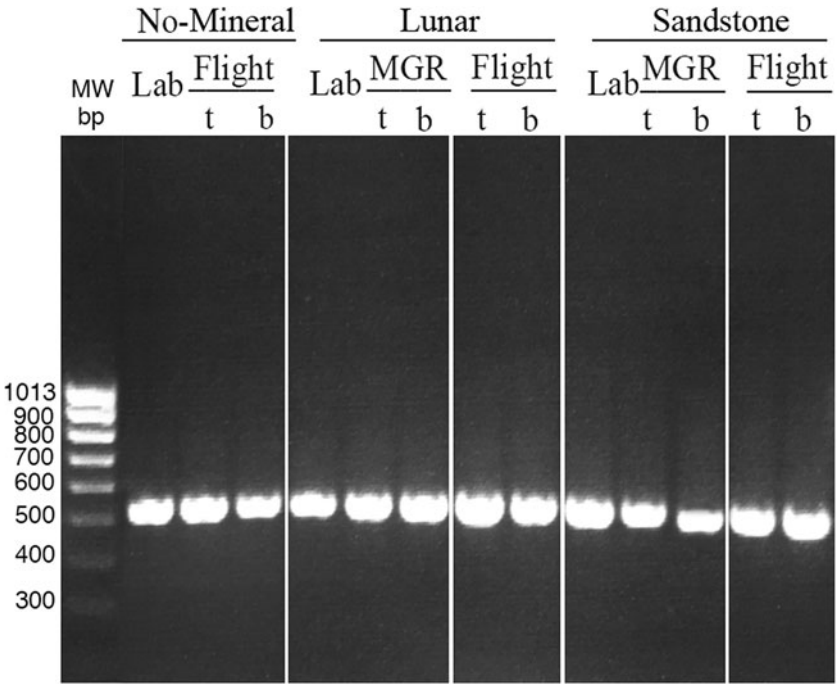

Fig. 3. Evaluation of DNA integrity by PCR amplification of a 500-bp gene target. Laboratory: dried cells stored under laboratory conditions; Flight: space conditions in LEO; MGR: mission ground reference; t: top-layer carrier; b: bottom-layer carrier; No-mineral: dried cells not mixed with minerals; Lunar: dried cells mixed with lunar regolith simulant; Sandstone: dried cells mixed with natural substratum; MW: DNA ladder (Hyperladder 100 bp, Bioline).

position (Fig. 3 lanes 7, 12). A 500-bp band was obtained from laboratory controls, including cells not mixed with minerals (Fig. 3, lane 2,) and cells mixed with lunar regolith analogue (Fig. 3, lane 5) or sandstone (Fig. 3, lane 10).

When the DNA integrity was evaluated by performing genome PCR fingerprinting, after exposure in LEO (i.e. top-layer carrier), dried cells not mixed with minerals yielded no amplicons (Fig. 4 lane 3), while a band profile was obtained from laboratory control, i.e. dried cells not mixed with minerals and stored in the dark throughout the space mission (Fig. 4, lane 2). Dried cells mixed with minerals form the top-layer carrier yielded band profiles with a slight reduction in the intensity of the low-size bands and lacking the higher-size bands (Fig. 4 lanes 8, 13). While the band profile of dried cells exposed in LEO in the dark (e.g. bottom-layer carrier) was virtually identical to that of laboratory controls, i.e. cells mixed with lunar regolith analogue (Fig. 4, lane 5) and with sandstone (Fig. 4, lane 10).

Discussion

By investigating among other topics, the survival and the biomarker detectability of the rock-inhabiting cyanobacterium,

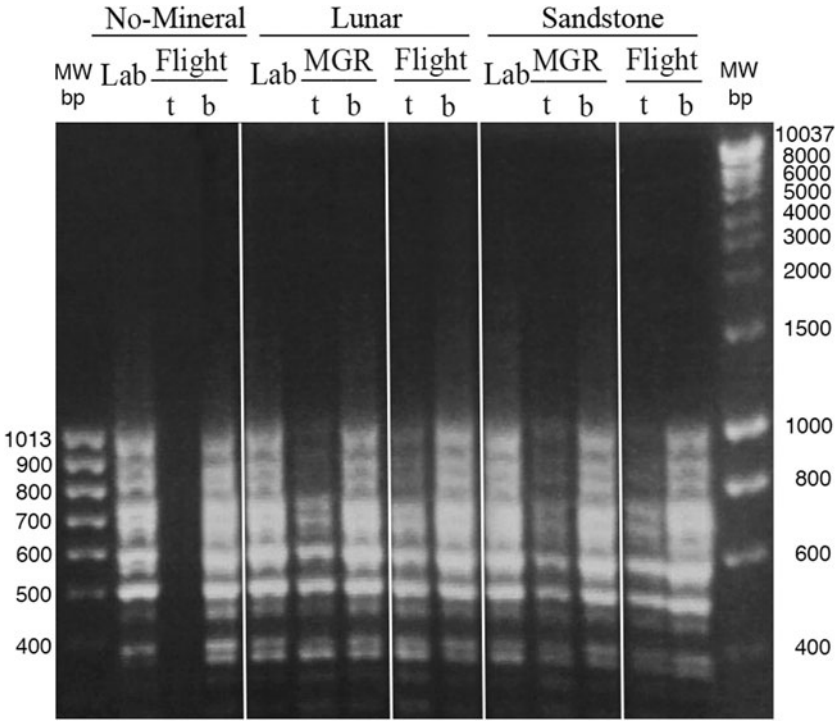

Fig. 4. Evaluation of DNA integrity by genome PCR fingerprinting. Laboratory: dried cells stored under laboratory conditions; Flight: space conditions in LEO; MGR: mission ground reference; t: top-layer carrier; b: bottom-layer carrier; No-mineral: dried cells not mixed with minerals; Lunar: dried cells mixed with lunar regolith simulant; Sandstone: dried cells mixed with natural substratum; MW: DNA ladder (Hyperladder 100 bp, Bioline).

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Chroococcidiopsis sp. CCMEE 029, the BIOMEX space experiment might contribute to a future exploitation of the Moon as a test-bed for key astrobiology tasks such as the testing of life-detection technologies and the study of life in space.

During the EXPOSE-R2 space mission, the shielding against UV radiation provided by minerals led to the survival of dried *Chroococcidiopsis* cells exposed to solar UV radiation (in top-layer carrier). In compartment 1 of Tray 1, a few cells mixed with sandstone or lunar regolith survived 1.59×10^2 and 1.79×10^2 kJm^{-2} of UV radiation, respectively, while in compartment 4 of Tray 1, cells not mixed with minerals were eradicated by 2.05×10^2 kJ m^{-2} of UV radiation (Fig. 1). The differences in the UV doses within the EXPOSE-R2 hardware were due to the ISS shadowing (Rabbow *et al.*, 2017). In addition, solar UV radiation was attenuated with 0.1% ND filters as suggested by the ground-based simulations performed before the space mission. In fact, dried *Chroococcidiopsis* cells mixed with lunar regolith analogue survived 1.5×10^3 kJ m^{-2} of UV radiation, but not 1.5×10^4 kJ m^{-2} and were killed by a UV dose of 5×10^2 kJ m^{-2} when combined with space vacuum (Baqué *et al.*, 2014).

During the MGR, samples were exposed to UV doses higher than their counterparts in space due to the fact that each compartment was exposed to a mean UV radiation fluency, as transmitted during the space mission, combined with simulated space vacuum (Rabbow *et al.*, 2017). Hence in compartment 4 of Tray 1, cells not mixed with minerals were killed by 5.05×10^2 kJ m^{-2} of UV radiation, whereas in compartment 1 of Tray 1, cells mixed with sandstone or lunar regolith survived 4.39×10^2 kJ m^{-2} of UV radiation. Overall, the scored survival matched the threshold UV dose of 5×10^2 kJ m^{-2}, combined with space vacuum, as reported during the ground-based simulations (Baqué *et al.*, 2014).

The mixing of *Chroococcidiopsis* cells in the proportion of 1×10^7 cells per mg of minerals yielded survivors after exposure to 1.79×10^2 kJ m^{-2} of UV radiation received during the space mission, and to 4.39×10^2 kJ m^{-2} during the MGR (see below). These results are in line with the observation that mineral grains act as a shield against UV radiation for cells located beneath them (Horneck *et al.*, 2001). Indeed, the SURVIVAL experiment performed during the BIOPAN space flights demonstrated that unprotected layers of bacterial spores were completely or nearly completely inactivated, whereas an increased survival occurred when 2.5×10^9 spores per ml were mixed with 100 mg ml^{-1} of powdered sandstone or 10 mg mL^{-1} of powdered meteorite (Horneck *et al.*, 2001).

Although the EXPOSE-R2 space mission exposure conditions exceeded the survival potential of *Chroococcidiopsis* cells unshielded by minerals, insights were provided into the detectability of traces of DNA-based life forms. In fact, the deeply damaged genomic DNA of *Chroococcidiopsis* cells killed by space UV radiation was still detectable by PCR by using a 500-bp target; by contrast, the PCR amplification of 1027-bp target and genome fingerprinting failed. Indeed, lesions block the DNA polymerase progression on the template, resulting in decreased/absent amplicons (Kumar *et al.*, 2004). Moreover, compared to the amplification of short targets, longer ones are impaired, due to the higher likelihood of encountering DNA lesions (Rudi *et al.*, 2010).

On the other hand, the mixing of dried *Chroococcidiopsis* cells with sandstone increased the detectability of genomic DNA after space exposure, leading to successful PCR amplifications of both short and long targets (500 and 1027-bp gene fragments) and genome PCR fingerprinting. The lack of the 1027-bp amplification in cells mixed with lunar regolith analogue and exposed in LEO was a puzzling result, possibly due to the occurrence in this DNA sequence of lesions, such as cross-links with proteins or carbohydrates which severely impair DNA polymerase progression (Shirkey *et al.*, 2003). Indeed, positive amplifications were obtained from cells mixed with lunar regolith analogue and exposed in LEO when attempting not only the 500-bp amplification but also the genome PCR fingerprinting.

The detectability of extensively damaged DNA by means of short-target PCR amplification could be relevant when searching for traces of DNA-based life, for instance due to microbial transfer via lithopanspermia. By taking advantage of miniaturization technologies, such as the Oxford Nanopore's MinION (Loman and Watson, 2015), a PCR-based approach has been proposed in order to detect possible remains of life on Mars (Carr *et al.*, 2013). At the same time, short-target PCR amplification could detect traces of dead microbes present on the external surfaces of spacecraft landed on the Moon (Schuerger *et al.*, 2019).

Due to the low ionizing radiation rates occurring in LEO, the EXPOSE-R2 space mission came to no conclusion regarding DNA detectability after prolonged exposure on the Moon. Nevertheless, it was previously demonstrated that the genomic DNA of dried *Chroococcidiopsis* cells exposed to 113 kGy of γ radiation was still suitable for amplifying a 1027-bp target and for yielding genome PCR fingerprints, although with altered band profiles (Verseux *et al.*, 2017). Cells mixed with minerals and exposed to high ionizing radiation doses are expected to show an increased DNA stability. Indeed, in the cyanobacterium *Nostoc* sp. CCCryo 231-06, it was reported that carotenoids were detectable after exposure to up to 56 kGy, while cells mixed with Martian mineral analogues showed totally preserved carotenoids after exposure to 117 kGy (Baqué *et al.*, 2018). Therefore, considering that the lunar surface is annually exposed to doses ranging from around 100 mGy to 1 Gy (depending on solar activity and solar particle events) of cosmic rays (Reitz *et al.*, 2012), DNA might be detectable after hundreds of thousands to millions of years and, potentially, even after longer periods in cells mixed with minerals. Hence a DNA-based approach might be applied in searching for life in other astrobiology-relevant targets: however, its success depends on a shared ancestry hypothesis, this being less and less likely as one proceeds deeper into the Solar System (Johnson *et al.*, 2018).

In addition, by investigating the survival of cells mixed with regoliths and exposed in LEO, the BIOMEX space experiment has contributed to testing the likelihood of the lithopanspermia hypothesis (de Vera *et al.*, 2012; de Vera *et al.*, 2019*b*). Space exposure is one of the major constraints to the interplanetary transport of microbes inside rocks, as well as the ejection from one celestial object and entry into another one (Horneck *et al.*, 2001). Solar UV radiation is immediately lethal due to its high energy efficiently absorbed by biological macromolecules; however, it is relatively easily shielded against (Nicholson *et al.*, 2005). It was previously reported that covering dried cells of *Chroococcidiopsis* CCMEE 123 (from coastal desert, Chile) with 3 mm of smashed sandstone ensured its survival under 1.5×10^5 kJ m^{-2} of UV radiation (Billi *et al.*, 2011).

Another factor limiting microbial survival in space is vacuum (Horneck *et al.*, 1994). During the EXPOSE-R2 space mission, dried *Chroococcidiopsis* cells exposed in the bottom-layer carrier experienced a vacuum ranging from 1.33×10^{-3} to 1.33×10^{-4} Pa and combined with a total dose of ionizing radiation of about 0.5 Gy (Dachev *et al.*, 2017). During the MGR, samples

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in the bottom-layer carrier were exposed to 1×10^{-5}–6×10^{-5} Pa, although in the absence of ionizing radiation (Rabbow *et al.*, 2017). However, the survival of dried cells exposed in the dark in LEO (i.e. vacuum and ionizing radiation) did not differ from that of cells exposed in the dark during the MGR (i.e. only vacuum). The cyanobacterial endurance after 672 days of space vacuum might be ascribable to its desiccation tolerance (Fagliarone *et al.*, 2017). Such a capability is likely due to the accumulation of disaccharides (Billi *et al.*, 2017), which may stabilize subcellular structures and macromolecules during the extreme dehydration induced by vacuum (Nicholson *et al.*, 2000). Indeed bacterial spores embedded in chemical protectants, e.g. disaccharides, survived 6 years under space vacuum during the Long Duration Exposure Facility experiment (Horneck *et al.*, 1994).

The absence of an evident reduction in the survival of *Chroococcidiopsis* exposed to 0.5 Gy of ionizing radiation was something expected in view of its capability to cope with 12 kGy of γ radiation and at least 2 kGy of Fe ions (Verseux *et al.*, 2017). Indeed, an enhanced desiccation and radiation tolerance would be needed when microbes are ejected into space in order to face the lethal effects of cosmic and solar radiation combined with space vacuum (Nicholson, 2009). Therefore, even if galactic cosmic rays and solar particle events are limiting factors for microbial survival in space (Ferrari and Szuszkiewicz, 2009), one could hypothesize a prolonged survival of this cyanobacterium when protected by rocky materials.

Ejection from a donor planet and entry into a recipient one also limit the interplanetary transfer of microbes inside rocks (Horneck *et al.*, 1994). It has been demonstrated that *Chroococcidiopsis* survives simulated impact ejections with shock pressures up to 10 GPa (Horneck *et al.*, 2008). Yet during the STONE space experiment, *Chroococcidiopsis* cells inoculated 5 mm depth into gneiss rocks were killed during the re-entry process of the Foton-M capsule (Cockell *et al.*, 2007). Therefore, it was suggested that atmospheric transit may prevent the dispersal of endolithic phototrophs because the re-entry heat ablates and heats the rocks to temperatures that exceed the upper temperature limit for life (Cockell *et al.*, 2007). Nevertheless, it has been hypothesized that an enhanced survival during atmospheric transit could occur in the case of chasmoendoliths inhabiting deep fractures or deeply buried within rocks, both being possibilities in nature under peculiar situations (Cockell, 2008). Indeed cyanobacteria related to endolithic and hypolithic representatives of genus *Calothrix*, *Chroococcidiopsis* and *Microcoleus* were recently detected in deep subsurface rock samples (Puente-Sánchez *et al.*, 2018).

In conclusion, BIOMEX results contribute to the future exploitation of the Moon as a test-bed to unravel key questions dealing with the origin of life on Earth, the limits and adaptation of life beyond Earth, the testing of life-detection technologies and the development of biological life-support systems (Cockell, 2010). Indeed the Moon could be used as a natural laboratory for unravelling microbial behaviour over several generations (Cockell, 2010), thus contributing to their use in biological life support systems (Horneck *et al.*, 2010). Future experiments regarding prolonged exposure of cells and their biomarkers to a severe ionizing environment will further test the likelihood of the lithopanspermia hypothesis, but they will also allow a deeper understanding of biomarker stability. Additionally, this research has implications for planetary protection and determining if the Moon has been contaminated during past human missions.

In this context, the use of future platforms on the Moon's surface or orbiting around it, e.g. the Gateway, will require the real-time monitoring of microbial activities, possibly by using gene expression reporters such as the green fluorescent protein, a method already applied to *Chroococcidiopsis* sp. CCMEE 029 (Billi, 2010, 2012).

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