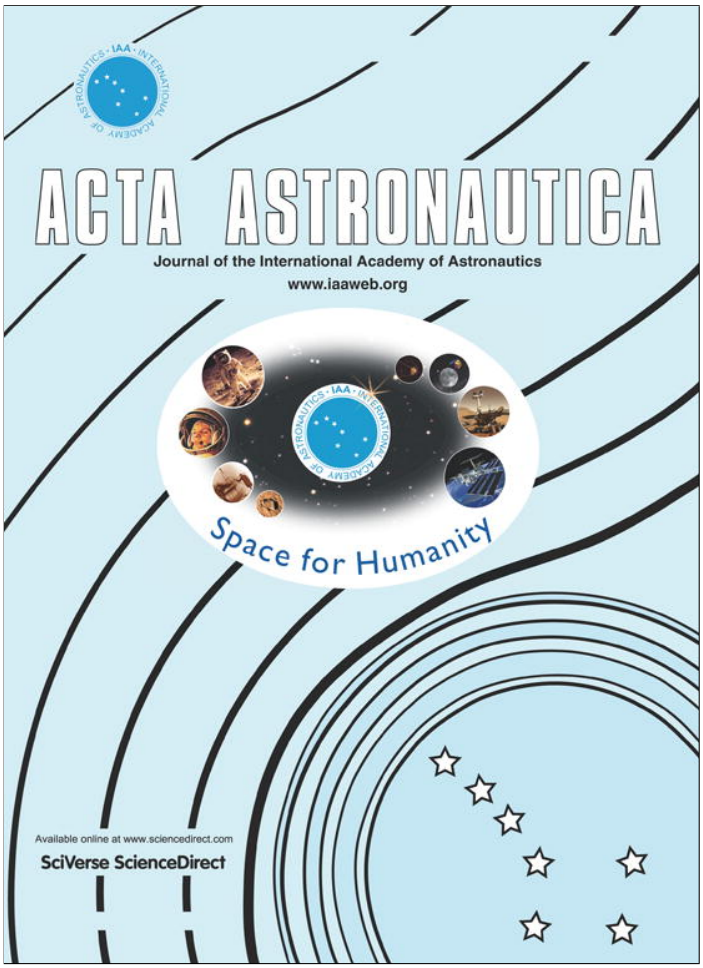

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The BOSS and BIOMEX space experiments on the EXPOSE-R2 mission: Endurance of the desert cyanobacterium *Chroococcidiopsis* under simulated space vacuum, Martian atmosphere, UVC radiation and temperature extremes.

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ABSTRACT

The proposed space experiments BOSS (Biofilm Organisms Surfing Space) and BIOMEX (BIOlogy and Mars experiment) will take place on the space exposure facility EXPOSE-R2 on the International Space Station (ISS), which is set to be launched in 2014. In BOSS the hypothesis to be tested is that microorganisms grown as biofilms, hence embedded in self-produced extracellular polymeric substances, are more tolerant to space and Martian conditions compared to their planktonic counterparts. Various microbial biofilms have been developed including those obtained from the cyanobacterium *Chroococcidiopsis* isolated from hot and cold deserts. The prime objective of BIOMEX is to evaluate to what extent biomolecules are resistant to, and can maintain their stability under, space and Mars-like conditions; therefore a variety of pigments and cell components are under investigation to establish a biosignature data base; e.g. a Raman spectral library to be used for extraterrestrial life biosignatures. The secondary objective of BIOMEX is to investigate the endurance of extremophiles, focusing on their interactions with Lunar and Martian mineral analogues. Ground-based studies are currently being carried out in the framework of EVTs (Experiment Verification Tests) by exposing selected organisms to space and Martian simulations. Results on a desert strain of *Chroococcidiopsis* obtained from the first set of EVT, e.g. space vacuum, Mars atmosphere, UVC radiation, temperature cycles and extremes, suggested that dried biofilms exhibited an enhanced survival compared to planktonic lifestyle. Moreover the protection provided by a Martian mineral analogue (S-MRS) to the sub-cellular integrities of *Chroococcidiopsis* against UVC radiation supports the endurance of this cyanobacterium under extraterrestrial conditions and its relevance in the development of life detection strategies.

1. Introduction

In the frame of the next mission of the EXPOSE facility of the European Space Agency (ESA), called EXPOSE-R2, set to be launched in April 2014, two experiments, BOSS (Biofilm Organisms Surfing Space) and BIOMEX (BIOlogy and Mars EXperiment), are planned to be exposed in Low Earth Orbit, at the exterior of the International Space Station (ISS). These two projects focus on a selection of archaea, bacteria, fungi, lichens, which are tolerant to various environmental extremes, along with their cellular components. They not only represent a follow up of

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previous astrobiological missions on EXPOSE and BIOPAN facilities and ground-based experiments [1] but also involve the exposure of new organisms and the development of novel approaches in astrobiology [2].

BOSS aims to investigate the resistance of cyanobacterial and bacterial biofilms compared to planktonic cultures. While BIOMEX will investigate selected organisms and their constituents (biomolecules like pigments and cell wall components) mixed with lunar regolith analogue rocks like anorthosite [3] and two Mars regolith analogue mixtures. Having characteristics of phyllosilicatic Mars Regolith soils (P-MRS) and sulfatic Mars regolith soils (S-MRS) the latter reflect two evolutionary epochs with environmental changes on Mars [4]. Results from BIOMEX and BOSS experiments will provide further insights into space resistance of life as we know it, also in the context of the (litho-) Panspermia and will contribute to creating a biosignature database based on spectroscopic detection of biological molecules. Indeed, a primary objective of BIOMEX is to determine the influence of interfering mineralogical features (Mars-like soils) and changes in the molecular conformation of biological substances after exposure to space and Martian-simulation on the ISS, this in order to contribute to the development of life detection strategies, such as the Raman Laser Spectrometer onboard of ExoMars [2].

BOSS and BIOMEX experiments will include desert strains of the cyanobacterium *Chroococcidiopsis*. This cyanobacterium is a photosynthetic desiccation-, radiation-tolerant prokaryote, able to thrive in extreme deserts on Earth like the Atacama desert in Chile and the Dry Valleys in Antarctica [5]. It has been selected for the next EXPOSE-R2 mission also due to its resistance to space constraints tested during the space mission EXPOSE-E and ground-based simulations [6,7]. Concerning their desiccation and radiation resistance, *Chroococcidiopsis* cells were reported to survive 15 kGy of ionizing radiation and 4 years of dry storage [8]; moreover in the dried status this cyanobacterium tolerated 10 min of a simulated Martian UV flux [9]. In the context of the previous EXPOSE-E mission [10], dried, ametabolic cells of a hot desert strain of *Chroococcidiopsis* sp., overlain by 3 mm of Antarctic sandstone, have shown resistance to simulated space and Martian conditions, including monochromatic (254 nm) and polychromatic UV (200–400 nm) exposure, Mars-like atmosphere, vacuum and temperature cycles, as expected in 1.5 year in space [7]. Remarkably, this cyanobacterium survived the real exposure in space for 548 days when augmented to an epilithic microbial community [6].

Therefore desert strains of *Chroococcidiopsis* represent a good model organism for investigating astrobiological topics. (Litho-)Panspermia, the impact-driven transfer of life between neighbor planets (see review [11]), for instance, is favoured by the proven *Chroococcidiopsis* resistance to space conditions and its capability to grow inside rocks or at the soil rock interface in its natural habitats. Moreover, its natural habitats on Earth (Atacama Desert and Dry Valleys) represent the closest analogues to a possible Martian habitat; hence the evolution paths that drove its adaptation to such conditions could help us in the definition of the possibility of life on other planets. Another field that could benefit from the study of extremophiles is space biotechnology, dealing with life-support systems for human space exploration (e.g. MELiSSA project [12]), in situ resource utilization for future Mars missions based on desiccation-, radiation-tolerant phototrophs, and the adequacy of planetary protection measures (see international regulations by COSPAR [13–16]).

In the preparation of EXPOSE-R2 space mission, selected space and Martian simulations (EVTs for Experiment Verification Tests and SVTs for Scientific Verification Tests) were scheduled to test whether the selected samples could withstand real exposure. In order to address the BIOMEX tasks *Chroococcidiopsis* cells were mixed with lunar, P-MRS and S-MRS analogues; the effects of space and Martian simulations on its cellular constituents were investigated to further understand the degradation of cyanobacterial biosignatures (e.g. photosynthetic pigments). This is valuable for the identification of detectable fluorescent break-down products [17]. In order to address BOSS tasks, dried *Chroococcidiopsis* biofilms and dried multilayer planktonic cells were compared regarding survival and sub-cellular damage.

In the present work we report the results on survival and sub-cellular integrities (genomic DNA and photosynthetic apparatus) in dried cells of the desert cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 exposed to the EVT part 1; this included the exposure to space vacuum (10^{-5} Pa), Martian atmosphere (argon 1.60%, oxygen 0.15%, nitrogen 2.70%, carbon dioxide 95.55% and ~370 ppm of water), temperature cycles (−10 °C/+45 °C), temperature extremes (+25 °C and +60 °C) and UVC radiation (up to 10.000 J/m^2). As a proof of concept PCR-based genomic fingerprinting and confocal laser scanning microscopy (CLSM) were optimized to investigate the sub-cellular integrities of cells mixed with S-MRS analogue as well as biofilms versus their planktonic counterparts. Survival of the exposed samples was also tested by assessing the colony-forming ability.

2. Material and methods

2.1. Organisms and culture conditions

Chroococcidiopsis sp. CCMEE 029 was isolated by Roseli-Ocampo Friedmann from endolithic growth in sandstone from the Negev desert, Israel and currently kept at the Department of Biology, University of Rome "Tor Vergata" as part of Culture Collection of Microorganisms from Extreme Environments (CCMEE) which was established by E. Imre Friedmann. Cyanobacterial cells were grown under routine conditions at 25 °C in BG-11 medium [18] under a photon flux density of 40 μmol/m^{2}s^1 provided by fluorescent cool-white bulbs with a 16-h/8-h light/dark cycle.

2.2. Tests facilities and exposure conditions

Ground-based simulations were performed using the Planetary and Space Simulation facilities (PSI) at the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany). Tests facilities and exposure conditions were as reported in Table 1. All tests were

Table 1
Exposure conditions during the experiment verification tests (EVTs).

EXPOSE-R2 EVT part 1 exposure experiments			
Test parameter		**Planned**	**Performed**
Vacuum		1 h	1 h, pressure $3.86 \times 10^{-3} \pm 0.12$ Pa
Vacuum		7d	7 h, pressure $8.50 \times 10^{-5} \pm 0.12$ Pa
10^{-5} Pa			
Mars atmosphere		1 h	1 h, pressure $6.08 \times 10^{2} \pm 0.12$ Pa
Mars atmosphere		7d	7d, pressure $6.00 \times 10^{2} \pm 0.12$ Pa
Temperature		50 cycles	66 cycles
−10 °C to +45 °C			2 h at −10 °C ± 1 °C; 2 h at +45 °C
Temperature max and min			
−25 °C / +60 °C 1 h each			1 h, −25 °C ± 0.5 °C; 1 h, +60 °C ± 0.5 °C
Irradiation			
254 nm	(80 μW/cm²)	0 J/m²	0 J/m²
		10 J/m²	12 s, 9.6 J/m²
		100 J/m²	2 min, 5 s, 96 J/m²
		1.000 J/m²	20 min, 50 s, 1000 J/m²
		10,000 J/m²	208 min, 20 s 10,000 J/m²

performed in triplicate, laboratory controls were kept at DLR in the dark, at RT. Biofilms were obtained by growing the cells on the top of BG-11 agarized medium [18] in Petri dishes for 2 months; then biofilms were allowed to dry by removing the parafilm from the Petri dishes; after 15 days dried biofilms were stored in the dark at room temperature (RT). For BOSS experiments plan ktonic cells were to be investigated as counterpart of biofilms grown on BG-11 agarized medium; cells obtained from liquid cultures were plated on BG-11 agar and allowed to dry overnight under sterile air, at room temperature. BIOMEX samples were obtained by mixing cells from cultures grown in liquid BG-11 medium, with S-MRS analogue and then allowed to dry. S-MRS analogue contained gabbro, olivine, quartz, hematite, goethite and gypsum as previously reported [4]. Dried samples were cut to the size of the exposure carrier, and the remaining ones stored at RT in the dark as laboratory controls. The layout of the *Chroococcidiopsis* samples in the EXPOSE-R2 is shown in Fig. 1.

2.3. PCR fingerprinting

Genomic DNA was extracted from 10 mm² in average of *Chroococcidiopsis* disk samples by using the phenol-chloroform method [19]. Briefly, after centrifugation the cells were resuspended in 250 μl of TE buffer (1 mM EDTA [pH 8.0], 10 mM Tris hydrochloride [pH 7.4]), one volume of phenol saturated with 0.1 M Tris hydrochloride (pH 7.4) and glass beads (20% [vol/vol], 0.5-mm diameter). Four 2-min cycles of heating at 65 °C and vortexing for 30 s were performed, then cell debris and glass beads were eliminated by centrifugation and the organic phase extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated with ethanol and sodium acetate (pH 4.5; 0.3 M) by overnight incubation at −20 °C; the yield was about 100 pg of DNA from 10^7 cells. DNA aliquots were normalized by suitable dilution with sterile water after reading their absorbance with an Eppendorf Biophotometer (Eppendorf, MI, Italy).

PCR-genomic fingerprinting was performed by using the primer HIP1-CA (5′–GCGATCGCCA-3′). PCR conditions were 1 cycle at 94 °C for 3 min; 30 cycles at 94 °C for 1 min, 37 °C for 30 s and 72 °C for 1 min and 1 cycle at 72 °C for 7 min. Genomic DNA extracted from one-month old liquid culture of *Chroococcidiopsis* was used as a control.

2.4. Confocal laser scanning microscopy

The presence of nucleic acids was tested by staining the cells with the cell-permeant nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, D1306), which was added at a final concentration of 5 μg/ml for 10 min, in the dark. After washing, the cells were immobilized with 1.5% agarised BG-11 medium, spotted onto slides and examined with a CLSM (Olympus Fluoview 1000 Confocal Laser Scanning System). The autofluorescence of photosynthetic pigments (phycobiliproteins and chlorophyll *a*) was investigated by exciting the cells with a 543-nm and 635-nm laser, and collecting the emission from 560 nm (or 650 nm) to 800 nm; DAPI fluorescence was acquired by using a mercury lamp (excitation 335–375, emission 440–500).

3. Results

3.1. BOSS: integrity of genomic DNA and photosynthetic pigments in Chroococcidiopsis biofilms.

The integrity of the genomic DNA of dried biofilms and planktonic counterparts exposed to UVC radiation was tested by assessing the suitability as PCR template. After UV exposure dried planktonic samples exhibited a dose-dependent reduction in the yield of the PCR amplicons, characterized by the absence of visible products after 10.000 J/m² (Fig. 2 lanes 2–5). By contrast PCR profiles of dried biofilms exposed to UVC doses up to 1.000 J/m² were virtually identical to that of

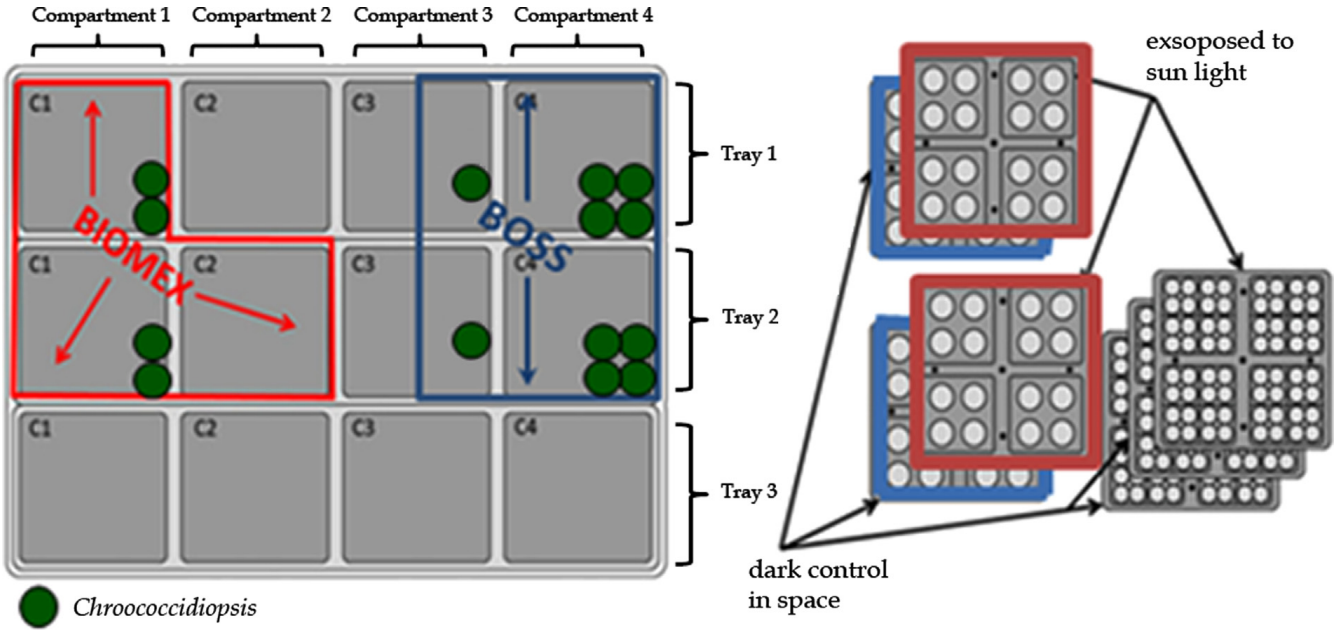

Fig. 1. Layout of *Chroococcidiopsis* samples in the EXPOSE-R2 mission.

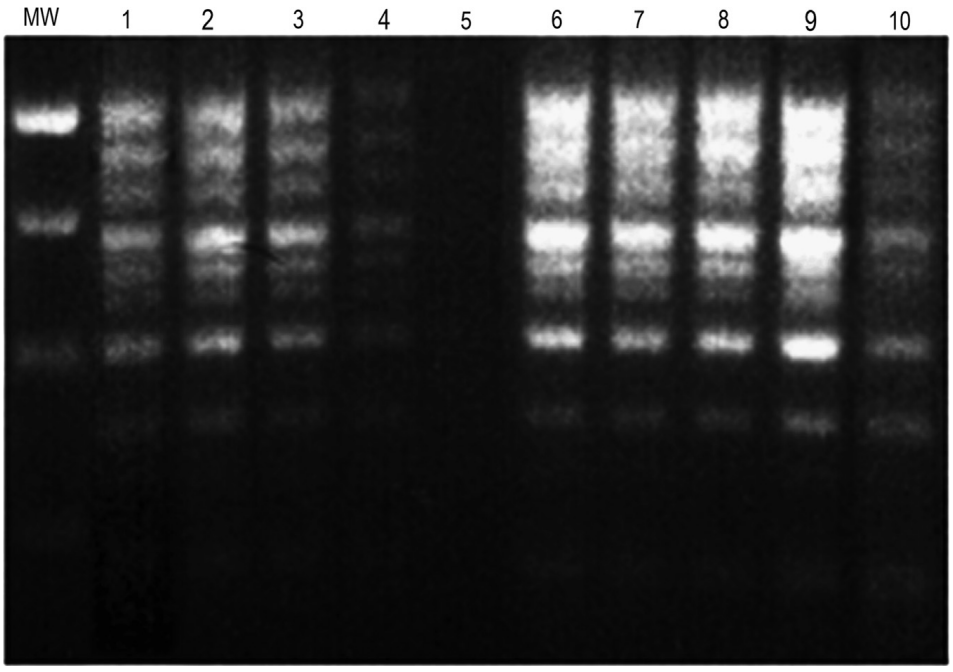

Fig. 2. Effect of UVC radiation on the genome integrity. PCR profiles of unexposed planktonic cells (lane 1), after 10, 100, 1000 and 10,000 J/m² (lanes 2–5) and of unexposed biofilm (lane 6) and of biofilms exposed to 10, 100, 1000 and 10,000 J/m² (lanes 7–10). MW = DNA ladder.

unexposed, dried control (Fig. 2 lanes 6–9); a reduced intensity of the PCR amplicons occurred only in biofilms exposed to 10.000 J/m^2 (Fig. 2 lane 10). After exposure to temperature cycles (−10 °C/+45 °C for 66 cycles), temperature extremes (−25 °C or +60 °C for one hour) band profiles of biofilms and planktonic cells were nonetheless virtually identical to that of control. The CLSM analysis of unexposed dried biofilms of *Chroococcidiopsis* highlighted a red autofluorescence throughout the whole structure; this autofluorescence was due to the presence of phycobiliproteins and chlorophyll *a* (Fig. 3a). In biofilms exposed to 10 kJ/m^2 of UVC radiation top cell layers had damaged photosynthetic pigments as suggested by the yellowish autofluorescence whereas lower cell layers maintained a red autofluorescence (Fig. 3b). Compared to dried biofilms, dried planktonic samples did not exhibit any evident morphological difference as revealed by CLSM analysis and showed after exposure to 10 kJ/m^2 of UVC radiation a comparable pigment bleaching on top cell layers (not shown).

3.2. BIOMEX: permanence of genomic DNA and photosynthetic pigments in Chroococcidiopsis mixed with S-MRS analogue

The integrity of the genomic DNA of dried *Chroococcidiopsis* cells mixed with S-MRS was affected by space

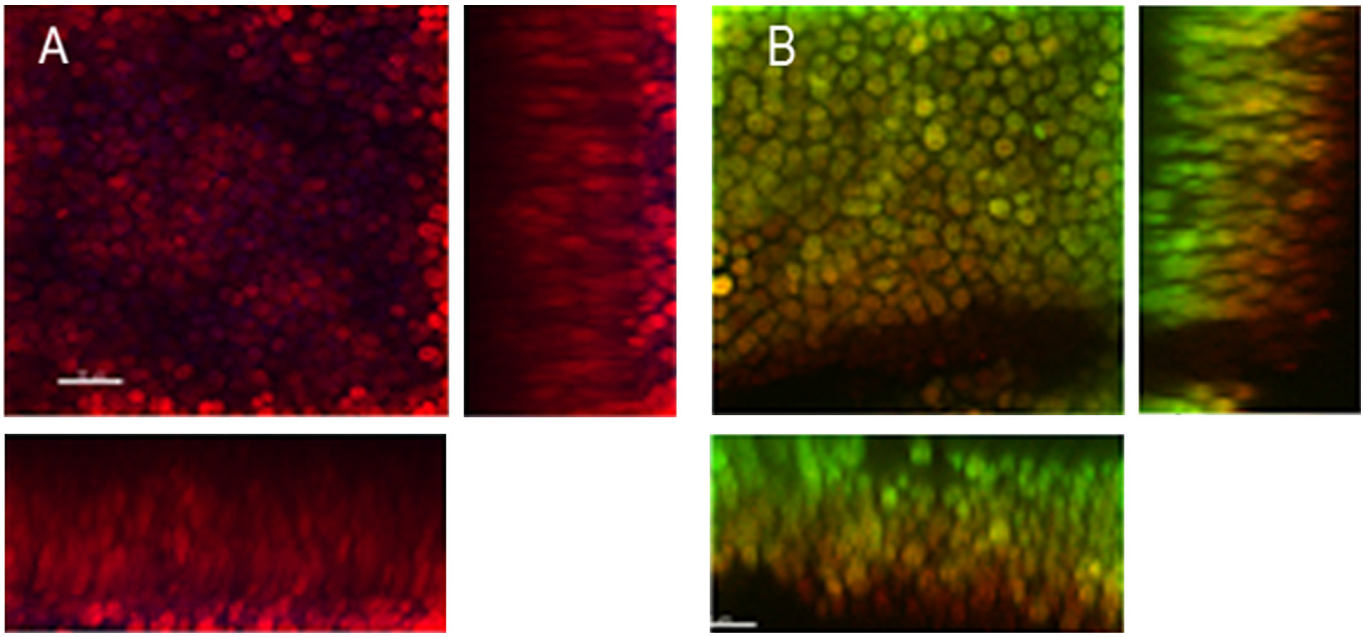

Fig. 3. CLSM image of unexposed biofilm showing the red autofluorescence of the photosynthetic pigments (A) and after 10 kJ/m² of UVC radiation causing the bleaching of top cell layers (B). Bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

vacuum as suggested when testing its suitability as PCR template (Fig. 4A and B). Compared to the PCR profile of the unexposed control (Fig. 4A, lane 1), that of cells exposed to space vacuum for 1 h or 7 days was progressively reduced in the number and intensity of the PCR amplicons (Fig. 4A, lanes 2, 3). When the genome integrity was tested in cells mixed with S-MRS and exposed to Martian atmosphere a PCR fingerprint virtually identical to that of control (Fig. 4B lane 1), was yielded after 1 h exposure (Fig. 4B lane 2), while no PCR amplicons were obtained after 7 days exposure (Fig. 4B lane 3). PCR fingerprints virtually identical to the control were also obtained after temperature cycles between −10 °C/+45 °C (Fig. 4B, lane 4) and 1 h at −25 °C or +60 °C (Fig. 4B, lanes 5–6). Cells mixed with S-MRS analogue and exposed to UVC radiation yielded PCR amplicons after each dose, although some variability in the band profiles occurred, probably as a consequence of the low homogeneity of the samples (not shown).

When *Chroococcidiopsis* cells exposed to space vacuum were stained with the cell-permeant nucleic acid stain DAPI, cells exposed to space vacuum for 1 h exhibited DAPI-stained nucleoids and red autofluorescent photosynthetic pigments (Fig. 5a). Exposure to space vacuum for 7 days resulted in a reduced autofluorescence of the photosynthetic pigments, although DAPI-stained nucleoids were visible in the cell cytoplasm (Fig. 5b).

3.3. Colony-forming ability of Chroococcidiopsis tested in BIOMEX and BOSS

The capability to undergo cell division and form colonies upon transfer into growth medium was scored for biofilms and planktonic samples and for cells mixed with S-MRS and exposed to each one of the ground-based simulations reported in Table 1.

4. Discussion

In preparation for the two space experiments BOSS and BIOMEX onboard the EXPOSE-R2 platform of the European Space Agency, ground-based simulations were performed on selected biomolecules and microorganisms, mimicking some of the conditions they will face during real exposure to space and Martian simulated environment in low Earth orbit, outside the ISS. These tests are important in order to validate the samples for the real mission and better apprehend which parameter could be more deleterious in the cocktail of the space environment.

Here we present the BOSS and BIOMEX results from simulated space vacuum (10^{-5} Pa), Mars atmosphere, temperature cycles (−10 °C/+45 °C), temperature extremes (+25 °C and +60 °C) and UVC radiation (up to 10.000 J/m^2). The resistance of dried cells of *Chroococcidiopsis* was investigated on the basis of the integrity of their genomic DNA and photosynthetic pigments. To this end, experimental approaches based on PCR-based assay and confocal microscopy techniques were optimized. Molecular approaches based on PCR-stop assays are a powerful tool for evaluating DNA damage; genomic PCR fingerprinting reveals DNA lesions by yielding altered PCR profiles [20]; this approach has already been used to study DNA damage in dried *Chroococcidiopsis* exposed to space and Martian simulations [7,21] and in hydrated cells exposed to UVC radiation [22].

In BOSS, when the PCR-stop assay was applied to dried biofilms and dried planktonic cells exposed to UVC radiation, unlike biofilms, planktonic samples did not yield any amplicons after 10.000 J/m^2. This is in line with the advantage of living as a biofilm when facing extreme conditions [23]. Microbial biofilms are among the oldest clear signs of life on Earth and might also be the first forms of life to be detected on other planets and moons of our

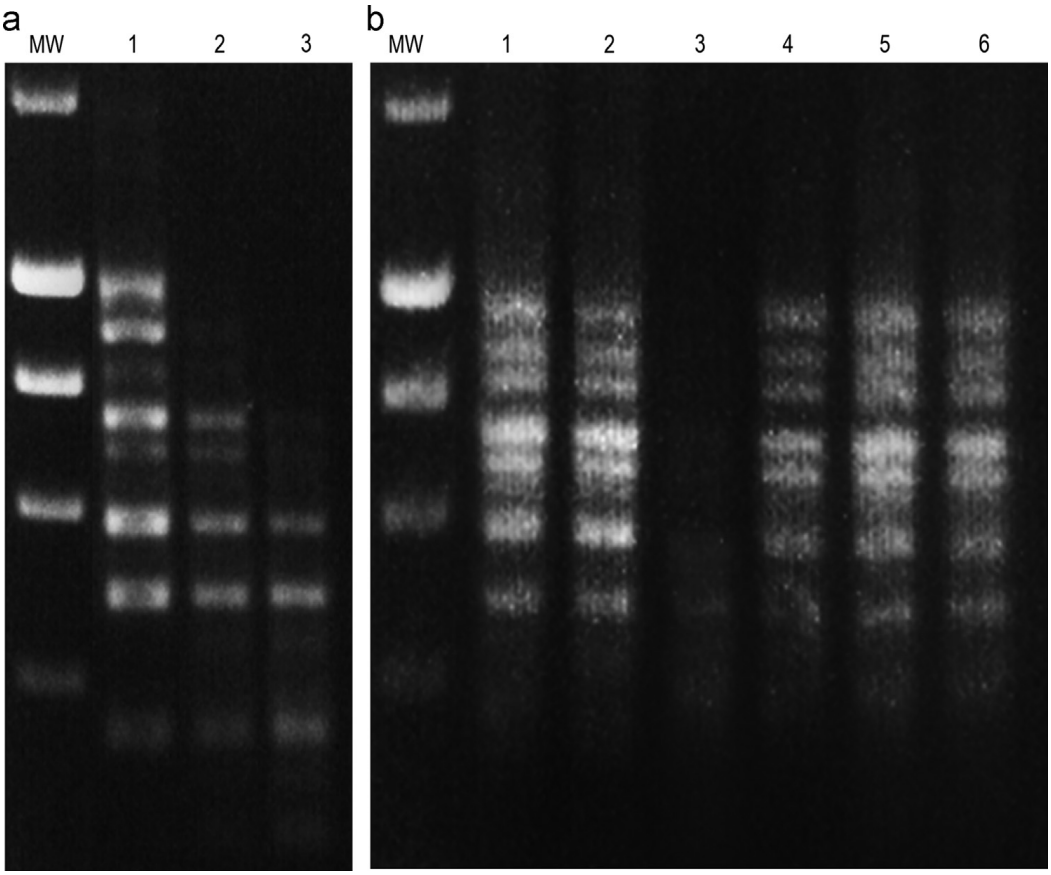

Fig. 4. PCR-fingerprint of dried cells mixed with S-MRS. (A) Unexposed sample (lane 1) and after 1 h (lane 2) and 7 days (lane 3) of space vacuum. (B) Unexposed sample (lane 1) and after exposure to Martian atmosphere for 1 h (lane 2) and 7 days (lane 3) and after 66 cycles between −10 °C/+45 °C (lane 4), 1 h at −25 °C (lane 5) and 1 h at +60 °C (lane 6). MW=DNA ladder.

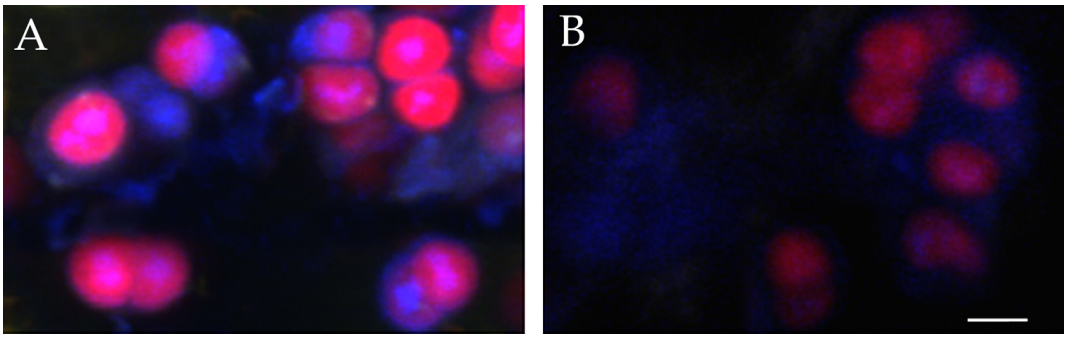

Fig. 5. CLSM images of cells mixed with S-MRS analogue exposed to space vacuum for 1 h (A) and 7 days (B) showing the red autofluorescence of the photosynthetic pigments and the white-blue DAPI-stained nucleoids. Bar=5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

solar system [23]. The CLSM analysis of biofilms exposed to 10.000 J/m² of UVC radiation revealed an extensive bleaching of the photosynthetic pigments in the upper cell layers (facing the UVC source), which provided protection to the lower layers. Such a protective effect has already been reported for multilayers of *Bacillus subtilis* spores exposed to ground-based UV sources and real space conditions [24]. Consequently, in biofilms, pigments, DNA or other cellular components could remain mostly intact in the lower layers and could serve as targets for search for life purposes. Furthermore, the presence of EPS encapsulating biofilm-forming cells might provide additional protection towards UV radiation. Ongoing studies on *Chroococcidiopsis* biofilms and their planktonic counterparts will clarify whether the production of extracellular polymeric substances is a key component in the endurance of *Chroococcidiopsis* biofilms.

For BIOMEX, the experimental procedures were optimized to minimize the effects of S-MRS analogue on PCRs; virtually identical band profiles were obtained from cells mixed or not with the Mars regolith analogue, thus suggesting that this analogue did not affect PCR (not shown). Altered PCR identified genomic DNA damage in cells exposed for 7 days to space vacuum and Mars atmosphere; DNA damage

was not revealed after exposure to temperature extremes. Despite the damage occurrence, CLSM analysis highlighted the permanence of genomic DNA in exposed cells by means of DAPI staining, as well as that of bleached but still detectable photosynthetic pigments. Although there is a need for further investigation by using RAMAN spectroscopy, the persistence of genomic DNA and photosynthetic pigments makes them promising molecular biomarkers for detecting life on Mars. This approach will allow a better selection of putative biosignatures able to withstand Martian-like or space conditions for future search for life missions [2]. The fact that DNA could be amplified by PCR in cells exposed to the highest UVC dose (10.000 J/m^2), suggested a protective role of the Mars regolith. The future investigation of *Chroococcidiopsis* mixed with P-MRS will assess eventual differences between the two regoliths in regards to cell protection under space and Martian conditions.

In neither BOSS nor BIOMEX did the investigated ground-based simulations impair the capability of the exposed cells to undergo cell division and form colonies upon rewetting. It should be also pointed out that the observed survivability was in line with previous data, thus supporting *Chroococcidiopsis*'s capability to avoid and/or limit damage to the photosynthetic apparatus and genomic DNA [7,21]; the fact that a positive colony-forming ability was scored in vacuum-exposed cells which accumulated DNA damage further highlighted this cyanobacterium's capability to repair extensive DNA damage upon rewetting. Once the planned exposures to full UV (200–400 nm) combined to space vacuum and to Martian atmosphere are carried out, a deeper insight into the survival potential of this cyanobacterium will be gained. Finally, the observed endurance complements previous results on the survivability of *Chroococcidiopsis* under ground-based simulations and exposure in low Earth orbit, in which its endurance was guaranteed by being overlain by 3 mm of sandstone and by the shielding of surface layer, respectively [6,7].

5. Conclusions

The results of the first EVT set carried out in the framework of the EXPOSE-R2 mission suggested that dried *Chroococcidiopsis* can withstand space vacuum, Mars atmosphere, UVC radiation, temperature cycles and extremes. Furthermore biofilms exhibited an enhanced survival compared to planktonic lifestyle, thus supporting the relevance of this cyanobacterium for the astrobiological tasks foreseen in the BOSS space experiment. The protection provided by S-MRS analogue to the sub-cellular integrities of *Chroococcidiopsis* against UVC radiation supports the (litho-) Panspermia theory and contributes to the development of life detection strategies as aimed at in the BIOMEX space experiment.

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