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ASTROBIOLOGY

BIOMEX Experiment: Ultrastructural Alterations, Molecular Damage and Survival of the Fungus *Cryomyces antarcticus* after the Experiment Verification Tests

Claudia Pacelli[1] · Laura Selbmann[1] · Laura Zucconi[1] · Jean-Pierre De Vera[2] · Elke Rabbow[3] · Gerda Horneck[3] · Rosa de la Torre[4] · Silvano Onofri[1]

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Abstract The search for traces of extinct or extant life in extraterrestrial environments is one of the main goals for astrobiologists; due to their ability to withstand stress producing conditions, extremophiles are perfect candidates for astrobiological studies. The BIOMEX project aims to test the ability of biomolecules and cell components to preserve their stability under space and Mars-like conditions, while at the same time investigating the survival capability of microorganisms. The experiment has been launched into space and is being exposed on the EXPOSE-R2 payload, outside of the International Space Station (ISS) over a time-span of 1.5 years. Along with a number of other extremophilic microorganisms, the Antarctic cryptoendolithic black fungus *Cryomyces antarcticus* CCFEE 515 has been included in the experiment. Before launch, dried colonies grown on Lunar and Martian regolith analogues were exposed to vacuum, irradiation and temperature cycles in ground based experiments (EVT1 and EVT2). Cultural and molecular tests revealed that the fungus survived on rock analogues under space and simulated Martian conditions, showing only slight ultra-structural and molecular damage.

Key words BIOMEX · Cryptoendolithic black fungus · DNA damage · Mars · Space simulations · Survival

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✉ Laura Selbmann
selbmann@unitus.it

[1] Department of Ecological and Biological Science (DEB), University of Tuscia, L.go dell'Università snc, 01100 Viterbo, Italy

[2] German Aerospace Center (DLR) Berlin, Institute of Planetary Research, Rutherfordstr. 2, 12489 Berlin, Germany

[3] German Aerospace Centre, Institute of Aerospace Medicine, Linder Hoehe, D 51170 Köln, Germany

[4] Department of Earth Observation, INTA – National Institute of Aerospace Technique, Madrid, Spain

Abbreviations

BIOMEX	Biology and Mars Experiment
EVT	Experimental Verification Tests
ISS	International Space Station
L	Lunar analogue
LEO	Low-Earth Orbit
LIFE	Lichen and Fungi Experiment
OS	Original Substrate or Arenaria
P-MRS	Phyllosilicatic Mars Regolith Simulant
PMA	Propidium MonoAzide
RAPD	Random amplification of polymorphic DNA
S-MRS	Sulfatic Mars Regolith Simulant
SVT	Scientific Verification Tests
TEM	Transmission Electron Microscopy

Introduction

The question whether extraterrestrial life exists has always intrigued scientists. Extremophilic and extreme-tolerant microorganisms have, as their natural niches, environments previously thought to be incompatible with active life; for this reason they are perfect models for studying the limits of habitability on Earth. The McMurdo Dry Valleys of Antarctica, Arctic regions, permafrost soils and cold deserts, for instance, are considered to be good analogues of Mars environments due to their permanently cold and dry conditions (Hansen et al. 2007). Microbes living there are pushed to the absolute limits of adaptability and represent a perfect tool for astrobiological research (Finster et al. 2007).

The resistance of these terrestrial extremophiles under both space simulation and LEO (Low Earth Orbit) has been documented (Horneck et al. 2010). Both ground based and space experiments on the International Space Station (ISS), i.e. the LIFE experiment on Expose-E (Rabbow et al. 2012), showed that some organisms, such as spores of bacteria, meristematic black fungi, and lichens, are able to survive and reactivate their metabolism after space simulations or direct exposure to space (Demets et al. 2005; de la Torre et al. 2007, de La Torre et al. 2010; de Vera et al. 2002, 2004; Horneck et al. 1994; Olsson-Francis et al. 2009; Onofri et al. 2008, 2012; Raggio et al. 2011; Sancho et al. 2007, 2008) and even simulated Martian conditions (Baqué et al. 2013; Meeßen et al. 2013a; Moeller et al. 2012; Sánchez et al. 2012).

This work is a part of BIOlogy and Mars EXperiment (BIOMEX), exposed on the EXPOSE-R2 payload (Rabbow et al. 2015), whose main goal is to detect signatures of extinct or extant life on Mars, investigating the fate of selected extreme-tolerant organisms and the stability of associated biomolecules, after exposure to actual space and simulated Mars conditions. Investigations will be based on sensitive and non-destructive approaches such as Infrared (Igisu et al. 2006, 2009) and Raman spectroscopies (Böttger et al. 2012, 2013; de Vera et al. 2012), using for comparison an international Raman library whose construction is in progress.

Another main objective of BIOMEX is to test survival in extra-terrestrial conditions of selected extreme-tolerant/extremophilic lithobionts, such as bacteria, meristematic black fungi and lichens grown on Mars and Lunar regolith analogues.

Among the selected organisms, the cryptoendolithic black fungus *Cryomyces antarcticus* CCFEE 515, from the McMurdo Dry Valleys in Antarctica, is an excellent eukaryotic model

due to its exceptional stress resistance and ability to grow inside the rock (Selbmann et al. 2005). Its survival in dried conditions after 18 months of exposure to actual space outside of the ISS, as well as to simulated Mars conditions in space, which was recently demonstrated, gave new insights to the Lithopanspermia theory (transfer of life between neighbor planets within a meteorite) (Onofri et al. 2012).

On July 24th 2014 the EXPOSE-R2 facility (Fig. 1): was launched onboard a Russian Progress cargo spacecraft (Fig. 2) from the Baikonur Cosmodrome, Kazakhstan to the ISS and mounted outside the ISS Zvezda module. The EXPOSE-R2 facility carried BIOMEX, along with the Biofilm Organisms Surfing Space (BOSS), Photochemistry on the Space Station (PSS) and an experiment from the Russian Institute of Biomedical Problems (IBMP).

This work focuses on the preparatory ground-based EVTs (Experiment Verification Tests) which include space and Martian simulations, performed on *C. antarcticus* in support of the actual space exposure.

Results give clues in searching for life in future Mars exploration missions (de Vera et al. 2012) in detecting putative biosignatures.

Material and Methods

Fungal Strain Preparation

Cryomyces antarcticus CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition 1980–81.

For the EVT tests, cell suspensions were spread on MEA (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH) in Petri dishes, mixed with Antarctic sandstone (15 g/L), Lunar and Martian analogues (1 g/L), prepared to optimize mineral/microorganisms interactions. Sandstone was the original substrate (OS) for the test fungus, Lunar analogue (L) was constituted mainly of anorthosite (Mytrokhyn et al. 2003) and two specific Martian analogues were composed of sulfatic Mars regolith (S-MRS) and phyllosilicatic Mars Regolith (P-MRS), simulating late basic Mars and early acidic Mars surface lithosphere composition, respectively (Böttger et al. 2012). Mars analogue composition

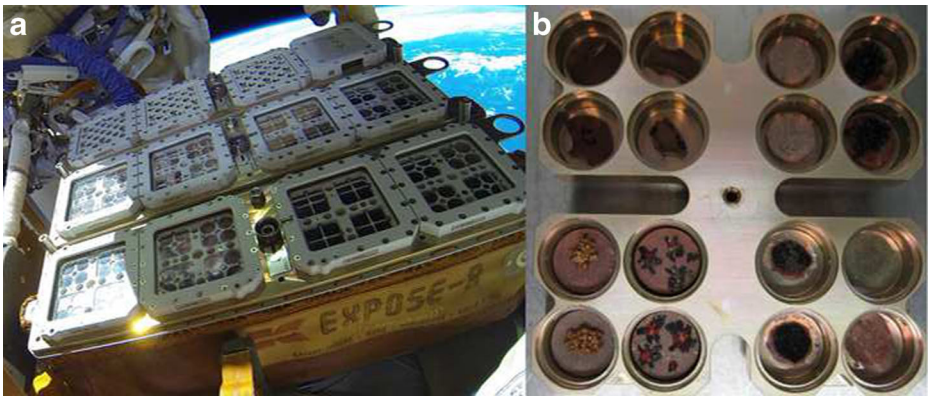


Fig. 1 **a:** EXPOSE R2 Facility mounted outside the ISS from October 22th 2014; **b:** Microorganisms integrated in the platform

Fig. 2 BIOMEX launch on July 24th 2014

was developed and produced by the Naturkundemuseum Berlin, according to the data of Mars research missions (Bibring et al. 2005; Chevrier and Mathé 2007; Poulet et al. 2005). Colonies were grown at 15 °C for 3 months. Disks, cut to fit within the wells of exposure carrier (12 mm diameter) (Fig. 1b), were drilled under sterile conditions.

Untreated samples, prepared as above and stored in the dark at room temperature, were used as controls in all the tests performed.

Tests Facilities and Exposure Conditions

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

Survival Tests

Cultivation Test

Survival of *C. antarcticus* was determined by its colony forming ability as percentages of CFU (Colony Forming Units).

For the test, three of the treated colonies were suspended in 1 mL of physiological solution (NaCl 0.9 %), and diluted to a final concentration of 3000 cells/mL; 0.1 mL of the suspension was spread on Petri dishes supplemented with MEA (5 replicates), incubated at 15 °C for 3 months and counted.

PMA Assay

The test was performed by adding the Propidium MonoAzide (PMA, Biotium, Hayward, CA) at a final concentration of 200 μM to 1–2 re-hydrated fungal colonies. PMA penetrates only

Table 1 Parameters obtained using the Planetary and Space Simulation facilities at DLR (Köln, Germany) for ground-based simulations

Test parameter	Performed
EXPOSE-R2 EVT part 1 exposure experiments	
Vacuum	1 h, pressure $3.86 \times 10^{-3} \pm 0.12$ Pa
Vacuum	7 h, pressure $8.50 \times 10^{-5} \pm 0.12$ Pa
Mars atmosphere	1 h, pressure $6.08 \times 10^{2} \pm 0.12$ Pa
Mars atmosphere	7d, pressure $6.00 \times 10^{2} \pm 0.12$ Pa
Temperature	66 cycles
min and max: -25 °C/+60 °C	2 h at −10 °C ± 1 °C; 2 h at +45 °C
min and max: -25 °C/+60 °C	1 h − 25 °C ± 0.5 °C; 1 h + 60 °C ± 0.5 °C
Irradiation	
UVC (254 nm) irradiation with Hg low pressure lamp at 80 mW/cm^2	0 J/m^2
	12 s, 9.6 J/m^2
	2 min, 5 s, 96 J/m^2
	20 min, 50 s, 1000 J/m^2
	208 min, 20 s 10,000 J/m^2
EXPOSE-R2 EVT part 2 exposure experiments	
Irradiation	28 d
Polychromatic UV irradiation (200–400 nm) with SOL2000 at 1271 Wm^{-2}	dark 0 kJ/m
	$5{,}5 \times 10^{2}$ kJ/m (7 min 12 s) (0,-1%ND Filter)
	$5{,}5 \times 10^{3}$ kJ/m (1 h 12 min) (1%ND Filter)
	$1{,}4 \times 10^{5}$ kJ/m (30 h)
	$2{,}7 \times 10^{5}$ kJ/m (60 h)
	$5{,}5 \times 10^{5}$ kJ/m (120 h))

damaged membrane cells, crosslinks to DNA after light exposure and thereby prevents Polymerase Chain Reaction (PCR).

Following DNA extraction and purification, quantitative PCR (Biorad CFX96 real time PCR detection system) was used to quantify the number of fungal Internal Transcribed Spacer (ITS) ribosomal DNA fragments present in both PMA treated and non-treated samples. Five μL of purified genomic DNA (0.1 ng/ml) were added to 12 μL of PCR cocktail containing 1X Power Sybr-Green PCR Master Mix (Applied Bios, Foster City, CA), as well as NS91 forward (5′-gtc cct gcc ctt tgt aca cac-3′) and ITS51 reverse (5′-acc ttg tta cga ctt tta ctt cct c-3′) primers, each at 5 pmol final concentration. Sterile water was added to reach the final volume of 25 μL.

These primers amplify a 203 bp product spanning the 18S/ITS1 region of rRNA encoding genes.

A standard Q-PCR cycling protocol, consisting of a denaturation step at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, was performed. Fluorescence measurements were recorded at the end of each annealing step. After forty cycles, a melt curve analysis was performed by recording changes in fluorescence as a function of raising the temperature from 60 to 90 °C in 0.5 °C per increments. All tests were performed in triplicate.

Statistical Analyses

For multiple data points, the calculation of the mean and standard deviations was performed. Statistical analyses were performed by one-way analysis of variance (Anova) and pair wise multiple comparison procedure (Tukey test), carried out using the statistical software SigmaStat 2.0 (Jandel, USA).

DNA Extraction and PCR Analyses

DNA was extracted from rehydrated colonies, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimized for fungi.

ITS and LSU amplifications were performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of each primer and 20 ng of template DNA at final volume of 25 μL. The amplification was carried out using MyCycler Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) equipped with a heated lid.

The rDNA regions were amplified as follows: for the ITS region the first denaturation step at 95 °C for 2 min was followed by denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and for the LSU region the first denaturation step at 95 °C for 3 min was followed by denaturation at 95 °C for 45 s, annealing at 52 °C for 30 s, extension at 72 °C for 3 min. The last three steps were repeated 35 times, with a last extension 72 °C for 5 min for ITS and 7 min for LSU. Primers ITS5, ITS4 (White et al. 1990), LR5 and LR7 (Vilgalys and Hester 1990) were employed to amplify ITS and LSU rDNA portions, respectively.

Random Amplification of Polymorphic DNA (RAPD) Assay

RAPD was performed using BioMix (BioLine GmbH, Luckenwalde, Germany) adding 5 pmol of the primer and 1 ng of template DNA at final volume of 25 μL. The primer used for RAPD was GGA_7 (GGA GGA GGA GGA GGA GGA GGA) (Kong et al. 2000). The conditions for amplification were: first denaturation step at 94 °C for 2 min followed by denaturation at 94 °C for 20 s, annealing at 49 °C for 60 s, extension at 72 °C for 20 s. The last three steps were repeated 40 times, with a last extension at 72 °C for 6 min.

Transmission Electron Microscopy (TEM)

Controls and UV-irradiated colonies were treated with 5 % glutaraldehyde/cacodylate sucrose buffer 0.1 M (pH 7.2) for 12 h at 4 °C, washed three times in the same buffer for 1 h each at 4 °C and fixed with 1 % OsO_4 + 0.15 % ruthenium red in 0.1 M cacodylate buffer (pH 7.2) for 3 h at 4 °C. Samples were washed in distilled water (2 times for 30 min at 4 °C), treated with 1 % uranyl acetate in distilled water for 1 h at 4 °C and washed in distilled water (2 times, 30 min at 4 °C). Samples were then dehydrated in ethanol solutions: 30 %, 50 %, 70 % (15 min each, at room temperature) and 100 % EtOH (1 h at room temperature), critical point dried and infiltrated in ethanol 100 %: LR White series with accelerator, in rotator, at 4 °C (2:1 for 3 h; 1:1 for 3 h, 1:2 overnight) and embedded in pure resin for 1 day and overnight; as final step, samples were included in pure resin in gelatinous capsule for 2 days at 48–52 °C.

Results

Cultural Tests

C. antarcticus retained the colony-forming ability after both EVT1 and EVT2 treatments. In EVT1 (Fig. 3) the fungus showed a similar trend of survival on all the substrates tested: in general, survival decreased with increased UV-irradiation doses. Yet, colonies formed even at the highest dose, 10.000 J/m^2, where percentage of survival was 5 %, 18 %, 38 % and 46 % in Lunar, Original Substrate, P-MRS and S-MRS analogues, respectively. The fungus, grown on Martian analogues, was able to survive exposure to the Martian atmosphere with no significant decrement on S-MRS with respect the control and almost 60 % of survival on P-MRS. Surprisingly, lower vitality was observed, for all substrates tested, at −25 °C than at 60 °C.

In the EVT2 treatments (Fig. 4), a progressive increase of mortality was observed with increasing of UV-irradiation doses, but 48 %, 38 % and 72 % of germination was recorded even at highest doses for colonies grown on Original Substrate, S-MRS and P-MRS, respectively. Colony-forming ability was maintained in most cases after EVT2 treatments; the only exceptions was the highest irradiation in Lunar sample.

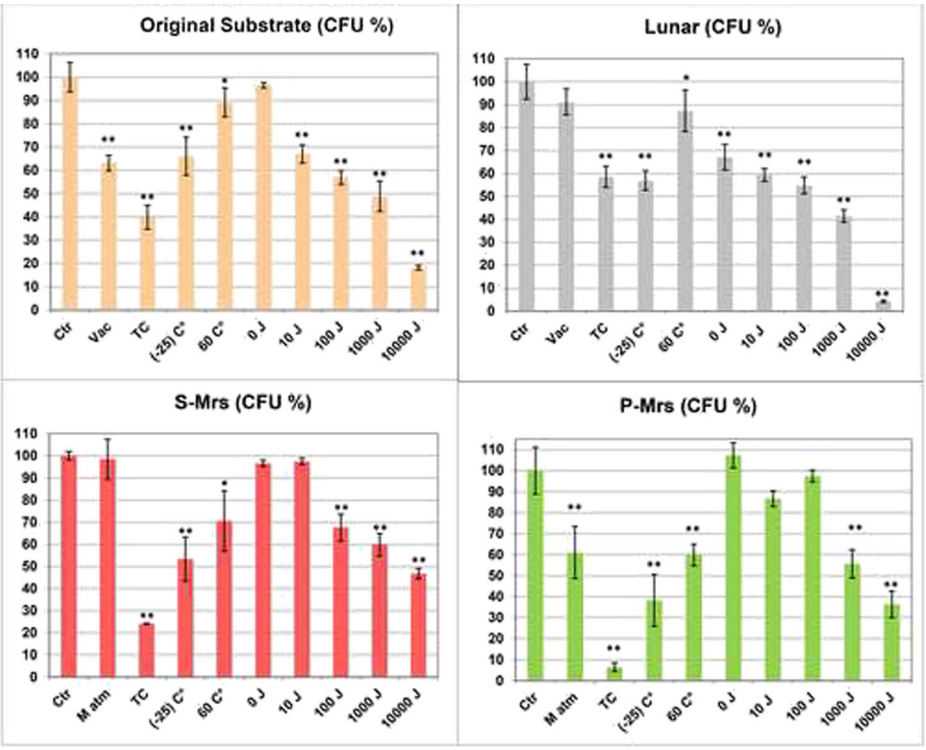


Fig. 3 Cultural test after EVT1 treatments: percentages of CFU of *C. antarcticus* on different substrates, relative to controls on the same substrate. Control (Ctr), vacuum 7 h (Vac), Martian atmosphere 7 g (M atm), temperature cycles (TC), minimum temperature (−25 °C), maximum temperature (+60 °C), irradiation at different intensities (0, 10, 100, 1000 and 10,000 J). Significant differences were calculated by Tukey test with * = $p > 0.05$. and ** = $p > 0.001$

Vitality, specifically, the integrity of the plasma membrane, was also tested through PMA assay on control and EVT2 samples treated with medium and maximum irradiation doses for each substrate (Fig. 5). This test gave a higher percentage of possible survival (measured as cells with intact membrane) compared to the results from the cultural test, even at the highest dose; for instance, no colonies were recorded under Lunar conditions but 35 % was observed with PMA assay. No significant differences between treated samples and control were obtained on P-MRS.

DNA Damage

The integrity of genomic DNA in treated samples was tested by assessing its ability to serve as a PCR template both after EVT1 and EVT2 treatments.

All the EVT2 samples were analyzed, while for EVT1, on the basis of colony forming results, only samples exposed at maximum treatments were chosen: Temperature Cycles, Vacuum 7 h, Martian atmosphere 7d, maximum dose of UV (254 nm) irradiation at 10^4J/m^2. Amplifications worked out for all the gene-lengths in EVT1 (Fig. 6a, b and c) and EVT2 (Fig. 7a, b and c) exposed samples. A reduced intensity of PCR bands was evident in panel c, with the largest gene length, in Lunar EVT1 samples exposed to 10,000 J/m^2 (Fig. 6c, Lane L 10^4) and for colonies grown on S-MRS at the highest dose of UV (>200 nm) irradiation in EVT2.

Although there was an overall decrease in band intensity, mainly for the highest molecular weight (MW) bands (about 2200 bp) the RAPD profiles were well preserved in all samples

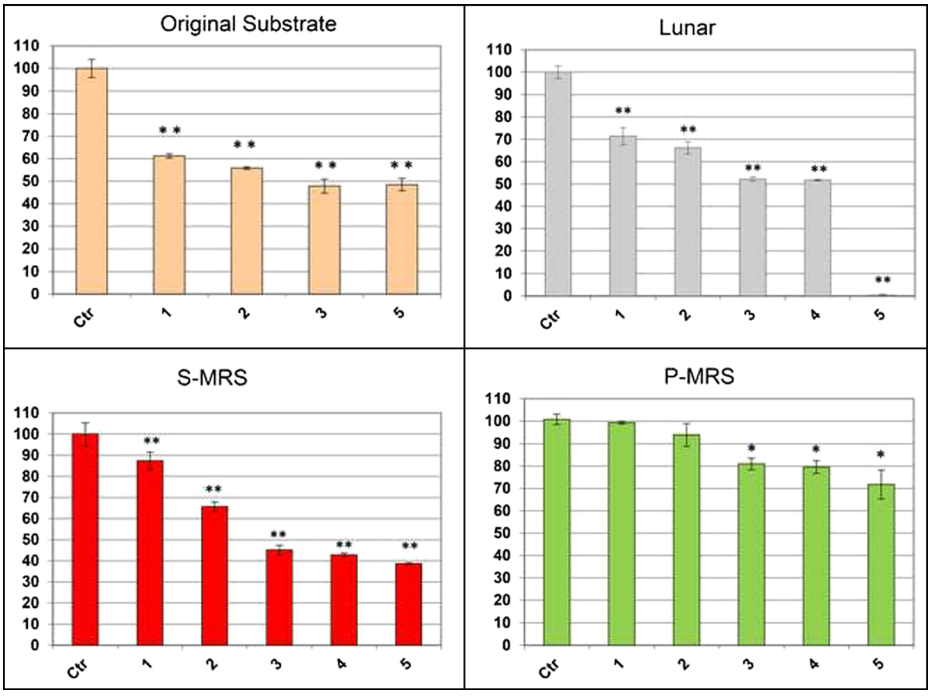


Fig. 4 Cultural test after EVT2 treatments of *C. antarcticus* grown on different substrates: CFU following exposure to UV light relative to controls on the same substrate. Control (Ctr), increasing polychromatic UV irradiation doses **1**: 1.5 × 10^3, **2**: 1.5 × 10^4, **3**: 1.5 × 10^5, **4**: 5.0 × 10^5, **5**: 8.0 × 10^5kJ/m^2. The statistical analyses were performed as Fig. 3

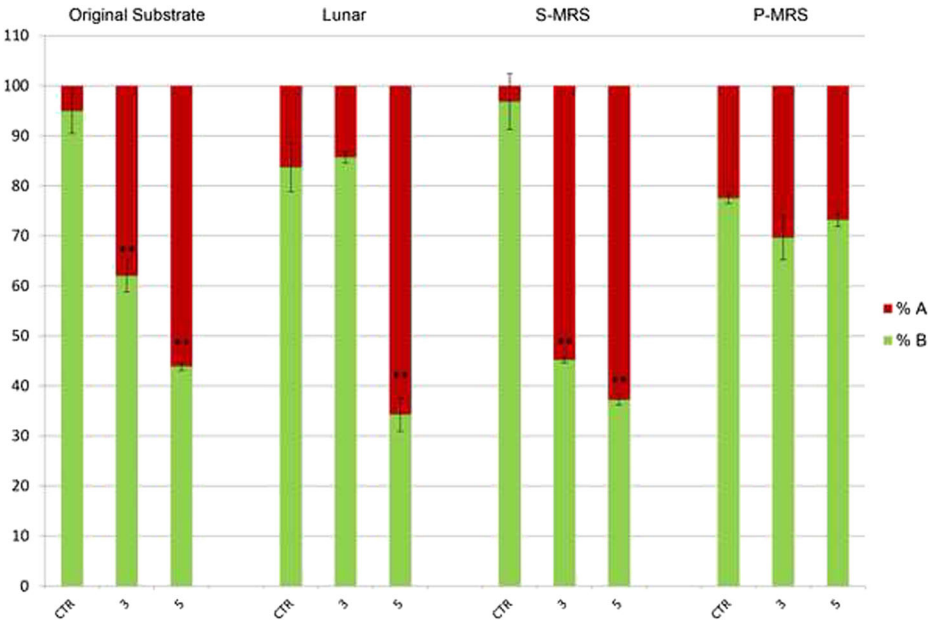


Fig. 5 Results of PMA assay coupled with qPCR after EVT2 treatments in a selection of samples (Samples correspond with those in Fig. 4): **a**: percentages of *C. antarcticus* cells with damaged membrane **b**: percentages of *C. antarcticus* cells with intact membrane. The statistical analyses were performed as in Fig. 3

both after EVT1 and EVT2 treatments (Figs. 6d and 7d), demonstrating a good preservation of the whole genomic DNA.

Ultrastructural Damage

Ultrastructural damage in *C. antarcticus* cells was observed by TEM. The samples treated at the highest irradiation dose of EVT2, for which the effect on vitality and DNA damage was evident (Fig. 8), were compared with laboratory controls.

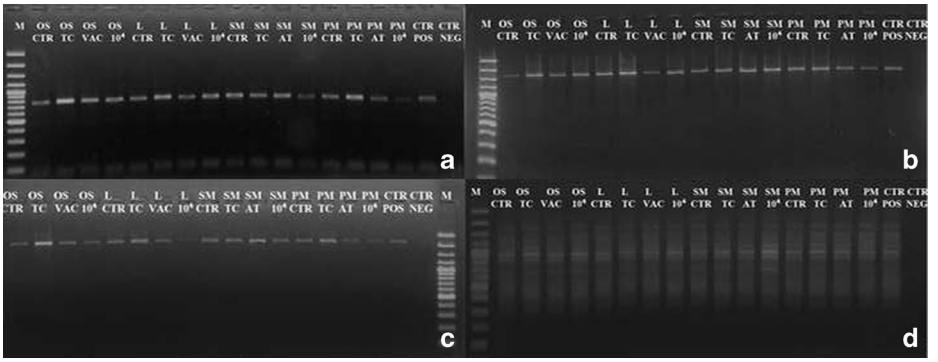


Fig. 6 Increasing detrimental effect of EVT1 treatments on the DNA integrity of genes of different lengths **a:** ITS rRNA gene (ITS5-ITS4 primers)(700 bp). **b:** LSU rRNA gene (ITS5-LR5 primers) (1600 bp). **c:** LSU rRNA gene (ITS5-LR7 primers) (2000 bp). **d:** RAPD profile of *C. antarcticus.* Treatments were as follows: Control, Thermal Cycles (TC), Vacuum (VAC) and 10^4 J/m^2, Positive PCR Control (CTRL POS), Negative PCR Control (CTRL NEG), DNA ladder (M). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS

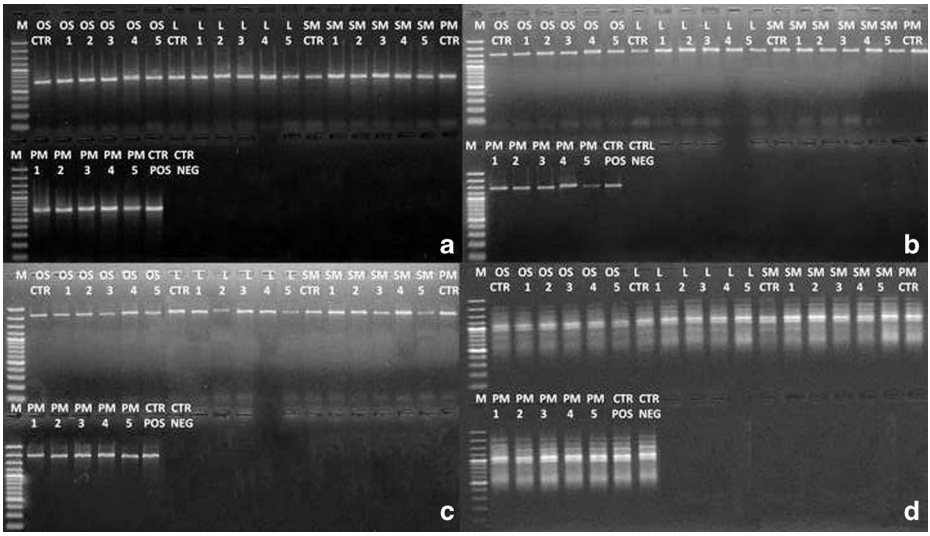


Fig. 7 Increasing detrimental effect of EVT2 treatments on the DNA integrity on genes of different lengths **a:** ITS rRNA gene (ITS5-ITS4 primers)(700 bp). **b:** LSU rRNA gene (ITS5-LR5 primers) (1600 bp). **c:** LSU rRNA gene (ITS5-LR7 primers) (2000 bp). **d:** RAPD profile of *C. antarcticus* after EVT2 treatments. Order as follows: DNA ladder (M), irradiation (sample correspondence as for Fig. 4), Control (CTR). Substrates: OS, Original Substrate; L, Lunar; SM, S-MRS; PM, P-MRS

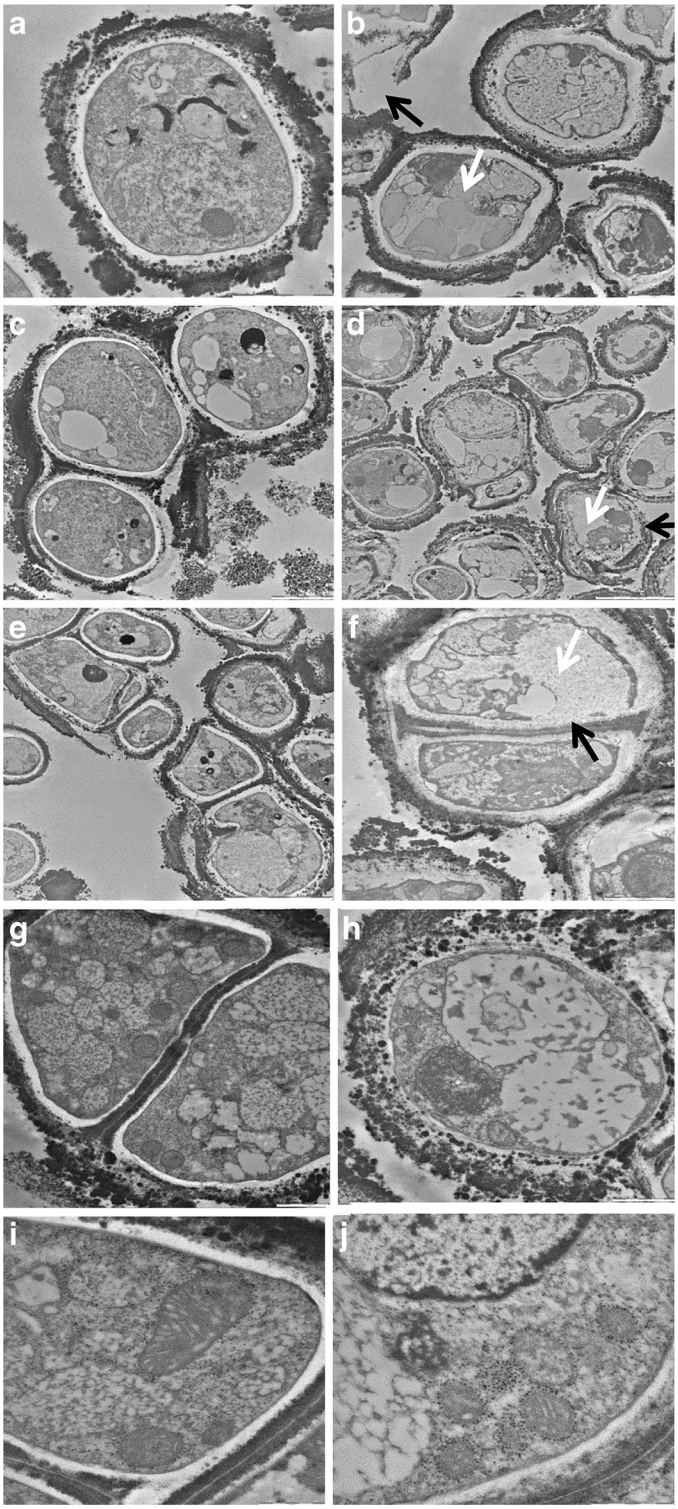
Control cells maintained mostly an intact cell membrane and a well-organized and defined cytoplasm after dehydration (Figs. 8a, c, e, g, i). By contrast, the majority of cells in the irradiated samples showed extended damage with irregular shapes, damaged cell walls, discontinuous cell membranes and a compromised organization of the cytoplasm (Fig. 8b, d, f, h, j).

Discussion

Ground-based simulations, EVT1 and EVT2, performed in the frame of the BIOMEX experiment currently on-board the EXPOSE-R2 platform fixed outside the ISS, were preliminary experiments to validate samples for the space mission. One of the aims of the project was to study dried cells of *C. antarcticus*, grown on lunar regolith analogue rocks and two Mars regolith analogue mixtures, to test survival as well as DNA and ultra-structural damage.

Although a reduced survival was observed after exposure to the most stressing parameters, *C. antarcticus* retained some colony forming ability after UV irradiation as well as after simulated treatment with other outer space stressors. These results were confirmed by TEM observations showing that, in addition to cells showing ultrastructural damage after the highest irradiation dose, a

Fig. 8 TEM micrographs. Untreated (CTR) and treated (maximum irradiation of EVT2, 8.0×10^5 kJ/m^2) microcolonies of *C. antarcticus* on sandstone (OS) **a**, **b**; Lunar (L) **c**, **d**; S-MRS **e, f**; P-MRS CTR **g**, **i** Unirradiatied **a**, **c**, **e**, **g**, **i** and irradiated **b**, **d**, **f**, **h**, **j** respectively. The structure of the cytoplasm was not conserved in many cases in the irradiated samples, the organelles are not visible (b, d, f, white arrows) and the continuity of the cell membrane (f, *black arrow*) and of the cell wall (b, d, *black arrow*) interrupted. The irradiated cells were better preserved in colonies cultivated on P-MRS where the structure of mithocondria was still well discernable (j, *white arrow*)


a
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number of cells were still in good condition (not shown), which agrees with the survival rate recorded in the colony forming assays. The main damaging factors of the EVTs were UV irradiation and temperature cycles. *C. antarcticus* survival was higher after +60 °C than after −25 °C treatment. The surprising ability of this psychrophilic fungus to tolerate very high temperatures was previously observed, when it was found to retain 100 % survival after exposure to 1 h at 90 °C (Onofri et al. 2008). Survival was high even under simulated Mars atmosphere and vacuum since more than 50 % of colonies developed in all cases. If survival was comparable in the irradiated samples on Martian analogues, values were different on the two substrata when the fungus was exposed to Martian atmosphere: no significant decrement was recorded on S-MRS with respect the control whereas here was less than 60 % of survival for P-MRS (Fig. 3). This apparently incongruent result is difficult to explain and requires additional investigations.

Most samples showed a higher percentage of apparent survival in PMA assays as compared to colony forming tests. This apparent inconsistency may be due to the coincidental preservation of cell membrane integrity in cells that have lost the ability to multiply, preventing PMA to penetrate and react with DNA; this could have led to an overestimation of vitality in some cases, i.e. Lunar sample. The membrane may be less susceptible to damage than DNA or the DNA replication enzymes under some of these conditions. Similar results were reported by Bryan et al. (2015) who also obtained higher values with the indirect XTT assay method with respect the clonogenic approach for testing survival for irradiated fungal cells of *Cryptococcus neoformans*. Data obtained in Lunar sample (8×10^5 kJ/m^2), where survival was 35 % in PMA assay compared to zero in cultural test, may be due to the loss of the ability to multiply in some cells where UV treatment caused extensive molecular damage, since UV targets more specifically the DNA rather than the membrane.

It is worth noting that the highest survival, both from cultural analysis and PMA assay, was obtained for *C. antarcticus* grown on P-MRS; the same fate was recently reported for the mycobiont of *Buellia frigida* suggesting a protective role of the substratum (Meeßen et al. 2015). The same authors observed that the highest viability was obtained when the lichen was exposed on the original rock substratum. Differently, in the frame of BIOMEX experiment, Baquè et al. (2014) reported a higher survival of the cyanobacterium *Chroococcidiopsis* when mixed with S-MRS regolith. This contrasting results led us to conclude that a possible protective role of the analogues is difficult to be sustained for now and needs to be further studied. Our results clearly demonstrate the high resistance of *C. antarcticus* to all EVT treatments, including exposure to vacuum, simulated Mars atmosphere, and different doses of monochromatic (254 nm) and polychromatic (>200 nm) UV radiation.

Further studies on *C. antarcticus* in the last ground tests (Scientific Verification Test, SVT) are still in progress and will clarify the real role of substrates in the protection; it was suggested that survival in space could benefit from the shielding provided by melanin. Melanin is a biological macromolecule mainly known for its protective role against UV, extreme temperatures, desiccation and osmotic stress (Sterflinger 2006; Plemenitaš et al. 2008). The high tolerance to UV-B exposure of single cells of black fungi has been reported by Onofri et al. (2007). It is known that melanin strongly absorbs UVB, UVA, and PAR, thereby protecting fungi and lichens against those stressors (Nybakken et al. 2004; Meeßen et al. 2013b). Moreover, it was observed that melanized fungal spores, in addition to resistance to UV radiation, even resist γ-ray and X-ray treatment better than melanin-deficient ones (Bell and Wheeler 1986) suggesting a role for this pigment in radioprotection of fungi (Henson et al. 1999; Dadachova et al. 2007). In this study, the presence of either Martian and Lunar analogues did not affect molecular analyses since genomic DNA was successfully extracted and amplified even from samples that had lost the ability to form colonies. In agreement with what is reported in the

literature, PCR band intensity decreased mainly in the highest molecular weight fragments in single-gene PCR (Atienzar et al. 2002). However, most of the amplicons were still obtained even at highest doses of UV-irradiation and RAPD profiles were well preserved in all samples. This surprisingly high DNA resistance to UV-irradiation, if protected by screening pigments, the outer cell envelop or a dust layer, suggests DNA as a possible biosignature candidate in future exploration missions (Lyon et al. 2010). Of course its resistance to UV-radiation needs to be proved over much longer timescales.

It is worth noting that ancient DNA was actually recovered on Earth from samples between 400 thousand and 1.5 million years old (Sankaranarayanan et al. 2014); it was also postulated that present Mars conditions (in terms of dryness and low temperatures) may even preserve ancient DNA much better than Earth conditions (Sephton 2010) with a theoretical preservation of a 100 bp fragment of DNA along a timescale of 3.4×10^9 years at −50 °C and 3×10^{21} years at −110 °C at the Martian polar ice caps (Willerslev et al. 2004). Moreover, some specific conditions could improve the long-term preservation of ancient DNA in halite crystals, permafrost, amber depositions and marine sediments (Panieri et al. 2010). Of course, some other damaging factors, such as ionizing radiation (Hassler et al. 2014) and oxidative environments (Yen et al. 2000; Hecht et al. 2009) on Mars, must be taken into consideration; but our simulation experiments suggest that we may have a good chance to reveal the presence of DNA, in present, in putative extraterrestrial samples, by using some low-specificity based approach such as random primers (as for RAPD) or non-specific staining such as orange acridine or non-toxic ones such as gel red and sytox green.

Conclusion

C. antarcticus is an astonishingly resistant fungus, able to withstand even long term exposure to actual Space conditions (Onofri et al. 2012); the present study proved that the fungus survives space simulated stressors even when grown on extraterrestrial rock analogues. The awareness that a terrestrial microbe may survive extraterrestrial conditions is an important clue in searching for life on other planets, above all on Mars. Many efforts are now devoted to the definition of proper biosignatures to detect whether life was ever present in an extraterrestrial sample from a putatively habitable region. Our results show that genomic DNA can be successfully extracted even in the presence of Martian or Lunar analogues; the ease of isolation and detection are key characteristics for a suitable biosignature, and optimizing the extraction is an important challenge in detecting biomarkers (Aerts et al. 2014). PCR was also successful and amplifications were obtained for most of the genes even at a length of up to 2000 bp; this was regardless of the treatments, revealing a high DNA persistence. Further analyses on samples treated in more stressing ground based experiments (SVT) or exposed to actual space conditions in the frame of the BIOMEX experiment, will provide further information on the detectability of this molecule and its suitability as biomarker in future exploration missions. Studies are in progress to define additional biomolecules to be used as good biomarkers using non-destructive approaches as Raman and Infrared spectroscopies, according to the instruments available on ExoMars.

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