International Journal of Astrobiology (2022), **21**:5 380–391
doi:10.1017/S1473550422000131

CAMBRIDGE UNIVERSITY PRESS

RESEARCH ARTICLE

Role of DNA repair pathways in the recovery of a dried, radioresistant cyanobacterium exposed to high-LET radiation: implications for the habitability of Mars

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Received: 15 September 2021; **Revised:** 22 February 2022; **Accepted:** 16 March 2022; **First published online:** 21 April 2022

Keywords: DNA damage repair, Mars habitability, high LET radiation, Chroococcidiopsis

Abstract

If life ever appeared on Mars and if it did refuge into sub-superficial environments when surface conditions turned too hostile, then it should have been periodically revived from the frozen, dormant state in order to repair the accumulated damage and reset the survival clock to zero for the next dormant phase. Thus, unravelling how long Earth dormant microorganisms can cope with high-LET radiation mimicking long-term irradiation is fundamental to get insights into the long-term resilience of a dormant microbial life in the Martian subsurface over geological timescales that might have taken advantage of periodically clement conditions that allowed the repair of the accumulated DNA damage. The exposure of dried cells of the radioresistant cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 to 2 kGy of heavy-ion radiation (Fe ions) did not significantly reduce its survival, although DNA damage was accumulated. Upon rehydration, DNA lesions were repaired as suggested by the over-expression of genes involved in the repair of double strand breaks (DSBs), oxidized bases and apurinic-apyrimidinic sites. Indeed, the monitoring of repair genes upon rehydration suggested a key role of the RecF homologous recombination in repairing DSBs. While the fact that out of the eight genes of the BER system, only one was up-regulated, suggested the absence of DNA lesions generally induced by UV radiation. In conclusion, the non-significantly reduced survival of dried *Chroococcidiopsis* exposed to 2 kGy of Fe-ion radiation further expanded our appreciation of the resilience of a putative dormant life in the Martian subsurface. Moreover, it is also relevant when searching life on Europa and Enceladus where the radiation environment might critically affect the long-term survival of dormant, frozen life forms.

Contents

Introduction

If life ever appeared on Mars, it was during the pre-Noachian period when liquid water occurred on its surface, and then, when the climatic conditions degraded, the water-rich epoch was followed by a cold and semi-arid era and then by the transition into present-day arid and cold conditions (Fairén *et al*., 2010). The opportunistic colonization of habitable environments could have occurred at any time during the Mars' history if microbial life remained viable when transported from a niche that turned non-habitable, to a habitable one (Cockell *et al*., 2012; Westall *et al*., 2013). However, if microbial life did refuge in sub-superficial environments when surface conditions turned too hostile, then it should have been periodically revived from the dormant state and have repaired the accumulated damage (Westall *et al*., 2013). Galactic cosmic rays continuously hit Mars that is unshielded by a thick atmosphere or a global magnetic field, thus severely limiting the long-term endurance of dried, frozen life forms (Kminek *et al*., 2003; Hassler *et al*., 2014). In the absence of an active repair mechanism, i.e. in the dormant state, even the radioresistant bacterium *Deinococcus radiodurans* and *Bacillus subtilis* spores would be killed in a few million years in the uppermost few meters, due to damage accumulation (Pavlov *et al*., 2002; Kminek *et al*., 2003). However, in the post-Noachian era, clement climate episodes could have allowed the revival of dormant microbial forms so that they repaired the accumulated, radiation-induced damages and reset the 'survival clock' to zero for the next dormant phase, thus extending their resilience limit over geological timescales (Hassler *et al*., 2014).

The scenario of the opportunistic colonization of protected niches on Mars gained support from the exposure of dried cells of the desert cyanobacterium *Chroococcidiopsis* to Mars-like conditions by using the ESA facility EXPOSE-R2 installed outside the International Space Station (Rabbow *et al*., 2017). In fact, dried *Chroococcidiopsis* could survive a total UV-radiation dose corresponding to a few-hour exposure on the Martian surface when cells were UV-shielded by the mixing with minerals (Billi *et al*., 2019*b*) or when bottom-layer cells were protected by the top-layer cells of the biofilm structure (Billi *et al*., 2019*a*). However, during the EXPOSE-R2 space mission, samples received a total ionizing radiation dose of 500 mGy (Dachev *et al*., 2017). Therefore, in view of the ionizing radiation dose of 76 mGy $year^{-1}$ measured at Gale Crater's surface (Hassler *et al*., 2014), the simulation of radiation doses equivalent to millions of years on Mars is extremely complicated.

New insights into Mars-relevant ionizing-radiation doses were obtained with the STARLIFE project that exposed a selection of extremophiles to X-, gamma rays and heavy ions, representing major parts of the galactic cosmic radiation (Moeller *et al*., 2017). Different types of ionizing radiation differ in their biological effectiveness depending on the linear energy transfer (LET) that represents the average energy loss of a particle per unit length of its track. Hence low-LET radiations, such as X- or gamma-rays, produce ionizations sparsely along their track, whereas high-LET radiation, such as heavy ions, induces more complex damage (Terato and Ide, 2004; Horneck *et al*., 2010).

In the context of the STARLIFE project, it was shown that dried cells of the radioresistant cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 survived up to 24 kGy of gamma radiation and 2 kGy of Fe-ion radiation, the highest high-LET dose tested (Verseux *et al*., 2017). Indeed, even though high atomic number and energy particles represent only the 1.5% of the galactic cosmic rays (Ferrari and Szuszkiewicz, 2009), they pose the ultimate limit for life (Horneck *et al*., 2010). Therefore, unravelling how life, as we know it, can cope with high-LET doses is of paramount importance. The STARLIFE project allowed for the first time the exposure of dried *Chroococcidiopsis* cells to high-LET radiation. However, the lack of the genome sequence of the investigated *Chroococcidiopsis* strain did not allow a deeper investigation of the accumulated DNA damage under high-LET radiation as well as of the repair mechanisms taking place upon rehydration. Recently, the sequencing of *Chroococcidiopsis* sp. CCMEE 029's genome allowed to unravel the role of the photoreactivation, nucleotide excision repair

https://doi.org/10.1017/S1473550422000131 Published online by Cambridge University Press

(NER) and UV damage endonuclease (UvsE)-dependent excision repair (UVER) in the recovery of dried biofilms exposed to a Mars-like UV flux (Mosca *et al.*, 2019). Similarly, the involvement of the homologous recombination RecF pathway and base excision repair (BER) was highlighted during the revival of dried cells exposed to space vacuum and cosmic ionizing radiation (Mosca *et al.*, 2021).

In the present work, we have taken advantage of the previously identified DNA repair pathways of *Chroococcidiopsis* sp. CCMEE 029 to investigate during the rehydration of dried cells exposed to high-LET radiation, the role of DNA repair mechanisms in the restoration of the induced complex DNA damage. After the exposure to 2 kGy of Fe-ion radiation, cell viability was verified using a quantitative polymerase chain reaction (qPCR) coupled with propidium monoazide (PMA) treatment, while DNA damage was determined using a PCR-stop assay. Then, an *in-silico* search for the *uvrD* gene, previously not identified, was performed. Finally, the expression of the genes involved in the RecF, BER, NER and UVER pathways was monitored by real-time quantitative polymerase chain reaction (RT-qPCR) during 1, 6 and 12 h rehydration.

Material and methods

Organism and culture conditions

Chroococcidiopsis sp. CCMEE 029 was isolated by Roseli Ocampo-Friedmann from cryptoendolithic growth in sandstone in the Negev Desert (Israel) and it is now kept at the University of Rome Tor Vergata, as part of the Culture Collection of Microorganisms from Extreme Environments (CCMEE) established by E. Imre Friedmann (Friedmann and Ocampo-Friedmann, 1995). Cultures were grown under routine conditions at 25 °C in BG-11 liquid medium (Rippka *et al.*, 1979), under a photon flux density of 40 µmol m^{-2} s^{-1} provided by fluorescent cool-white bulbs.

Cell drying, irradiation and recovery

Triplicates of dried samples were prepared by filtering on Millipore filters about 5×10^8 cells obtained from cultures in early stationary phase. Filters were air-dried overnight under a sterile hood and shipped to the Heavy Ion Medical Accelerator in Chiba (HIMAC, Gunma University Heavy Ion Medical Center, Japan) for the irradiation campaign performed in June 2018. Samples were irradiated with 198 keV μm^{-1} Fe ions with a dose rate of 12.1 Gy min^{-1} at 23 °C ± 1 °C. Dried-irradiated samples were shipped back with non-irradiated dried cells that were used as control. After the irradiation, samples were stored for one year in the dried state until the analyses were performed. For the recovery, each filter was cut in four pieces, and each piece transferred in a polystyrene falcon with 1 ml of BG-11 medium and incubated under routine conditions. Afterwards 1, 6 and 12 h recovery cells were centrifuged and pellets harvested in ice for RNA extraction (see below).

Cell viability

The viability of dried-irradiated cells was evaluated according to the PMA-qPCR assay (Nocker *et al.*, 2006). About 2.5×10^8 cells from each sample were harvested with 500 µl of PBS 1× containing 50 µM PMA (Biotium Inc., Hayward, WI, USA). After incubation for 10 min in the dark, at room temperature, samples were placed on ice and exposed to a 650 W halogen lamp for 15 min, to allow the PMA photoactivation and cross-link to DNA. Genomic DNA was extracted and amplified by qPCR as described (Baqué *et al.*, 2013). Liquid cultures and dried cells were used as control.

DNA damage evaluation

DNA was extracted from about 2.5×10^8 cells harvested from dried samples with 1 ml of PBS 1×, as described (Baqué *et al.*, 2013), and quantified with a NanoDrop Lite Spectrophotometer (Thermo

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Fisher Scientific, Waltham, MA, USA). PCR-stop assay with a 4 kbp target was performed as previously reported (Mosca *et al*., 2019). Liquid cultures and dried cells were used as control.

In-silico *search for DNA repair genes*

The sequence of *Chroococcidiopsis* sp. CCMEE 029's genome was already available and the previously unidentified *uvrD* gene (Mosca *et al*., 2019, 2021) was annotated by using BlastKOALA (Kanehisa *et al*., 2016). The sequence of the *uvrD* gene was deposited in GenBank under the accession number MT813437.

Expression of DNA repair genes

Total RNA was extracted from about 5×10^8 cells from dried, irradiated samples after 1, 6 and 12 h of rehydration by using the TRI Reagent (Sigma Aldrich, Saint Louis, MO, USA). The reverse transcription of the RNA into cDNA was performed by using the SensiFAST™ cDNA Synthesis Kit (Bioline, Meridian Life Science, Memphis, TN, USA) as previously described (Mosca *et al*., 2019). Each real-time PCR reaction was performed on StepOnePlus Real Time PCR System (Thermo Fisher Scientific) with equal volumes of cDNA synthesis mixture in 12 µl reaction volume, including 6 µl of iTaq™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 0.4 µM of forward and reverse primers (Table 1). PCR conditions were as described (Mosca *et al*., 2019) and each run was completed with a melting curve analysis to validate the specificity of amplification. Each experiment contained a no-template control and the 16S rRNA was used as housekeeping gene. Relative mRNA levels were calculated by the comparative cycle threshold (Ct) method. Values obtained for dried-irradiated cells (0-recovery) were set as 1 and values of re-hydrated, irradiated, dried cells were considered up-regulated (>1) or down-regulated (<1).

Results

Viability and evaluation of DNA damage in dried-irradiated cells

The viability of dried cells of *Chroococcidiopsis* sp. CCMEE 029 irradiated with 2 kGy of Fe-ion radiation was evaluated by using the PMA-qPCR assay (Fig. 1), that is based on fact that the cell membrane-impermeable PMA dye stains only dead cells and by binding the genomic DNA, it prevents the PCR target amplification (Nocker *et al*., 2006). Results showed a non-significant reduction of the amplified copy numbers in dried-irradiated cells compared to dried cells. While dried cells did not show any significant difference in the PCR target amplification compared to liquid cultures (Fig. 1(a)).

The PCR-stop assay with a 4 kbp target revealed an increased amount of DNA lesions in the target sequence that resulted in a progressive reduction of the amplicon intensity in dried cells and in dried-irradiated cells compared to liquid cultures (Fig. 1(b)).

Expression of RecF pathway genes during the rehydration of dried-irradiated cells

Upon rehydration, dried-irradiated cells of *Chroococcidiopsis* sp. CCMEE 029 showed a different over-expression of genes involved in the RecF DNA repair pathway (Fig. 2). The *recF* gene was up-regulated by 2.40-, 2.89- and 3.01-fold during 1, 6 and 12 h rehydration, respectively. Among the two *recJ* genes, only the *reJ1* gene was over-expressed with a 2.67-, 2.72- and 2.94-fold increase after 1, 6 and 12 h, respectively. The *recN* gene was over-expressed by 2.43-, 2.83- and 3.02-fold, at 1, 6 and 12 h, respectively. The *recQ1* and *recQ2* genes were up-regulated by about twofold after 1 h, and by 2.98- and 3.64-fold, respectively, after 6 h and by 2.81- and 3.71-fold, respectively, after 12 h. The *recO* and *recR* genes were weakly over-expressed during 1 and 6 h rehydration, while after 12 h they were up-regulated by about twofold. While the expression of the *ssb* gene was fourfold increased after 12 h rehydration (Fig. 2).

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Table 1. *List of gene and primer sequences used in the RT-qPCR analysis*

Gene	Function	Primes sequence (5′–3′)	References	Genbank accession
16S	16S ribosomal RNA	chr16S-F TACTACAATGCTACGGACAA chr16S-R CCTGCAATCTGAACTGAG	Mosca *et al.* (2019)	AF279107
alkA	DNA-3-methyladenine glycosylase II	chralkA-F CCAAGATTATCTACCTCAA chralkA-R TTTCATCGTCTATTGTTTC	Mosca *et al.* (2021)	MT829329
fpg	Formamidopyrimidine-DNA glycosylase	chrFpg-F TACAGAAGCAACTCCAATCCA chrFpg-R TAAGCAGCCAGCAAGTGA	Mosca *et al.* (2021)	MT829334
mpg	DNA-3-methyladenine glycosylase	chrmpg-F ATTGACCATAGCCTGAAT chrmpg-R TTGGACGAAGTTGATTAC	Mosca *et al.* (2021)	MT829332
mug	Uracil-DNA glycosylase	chrMug-F GAAGACGGTTCCAGATATTATTG chrMug-R GCACTGTAGAGGCTAGGA	Mosca *et al.* (2021)	MT829331
mutY	Adenine DNA glycosylase	chrmutY-F TACTATGCTCGTGCTCAT chrmutY-R ACATCTTCTAATCGGATAGGA	Mosca *et al.* (2021)	MT829330
nth	Endonuclease III	chrnth-F CAGCGAATGTGGTTCTTG chrnth-R AGTCAATCCTAAGCGGTAG	Mosca *et al.* (2021)	MT829335
recF	DNA replication and repair protein	chrRecF-F AGCGAACTCTGGTATTAG chrRecF-R ACACATCATCAAGTAATAGC	Mosca *et al.* (2021)	MT813436
recJ(1)	Single-stranded-DNA-specific exonuclease	chrRecJ_1-F GGAACAAGCACAAGTATT chrRecJ_1-R GTAACAACTCTACACTATGG	Mosca *et al.* (2021)	MT813442
recJ(2)	Single-stranded-DNA-specific exonuclease	chrRecJ_2-F GGATGGCTTAGGTCAGTT chrRecJ_2-R CCGTGTGATTGTGTTAGAC	Mosca *et al.* (2021)	MT813443
recN	DNA repair protein	chrRecN-F ATTTGTCGGAAGTATGGT chrRecN-R GATTGTTCACTGGCATTC	Mosca *et al.* (2021)	MT813438
recO	DNA repair protein	chrRecO-F CGTAAGCACAACTCAAAG chrRecO-R TAGACCTGGATAAGATTCAATA	Mosca *et al.* (2021)	MT813433
recQ(1)	ATP-dependent DNA helicase	chrRecQ_1-1F GAGATGCTTTAGTAGTGATG chrRecQ_1-1R ACGAGTGTTAATCCAGTT	Mosca *et al.* (2021)	MT813439
recQ(2)	ATP-dependent DNA helicase	chrRecQ_2-F CAAGACATTATTCAACAACTG chrRecQ_2-R TGGACTTCGTAATAGAGATT	Mosca *et al.* (2021)	MT813440
recR	Recombination protein	chrRecR-F AAGAGGTTATTCTGGCAATT chrRecR-R ATCCGTGTTACTCGTGTA	Mosca *et al.* (2021)	MT813435

ssb	Single-stranded DNA-binding protein	chrSSB-F GTAGTGTAAAGTGCCGATT chrSSB-R TCCAAAGTGAACCAGTCT	Mosca *et al*. (2021)	MT813434
udg	Uracil-DNA glycosylase	chrudg-F CATTGTAACCAGTGTCATC chrudg-R ATCGGTGCTTCTAAGTTG	Mosca *et al*. (2021)	MT829336
uvrA	Excinuclease ABC subunit A	chruvrA-F ACTTAGATGTGATTCGTTGT chruvrA-R CTACTTGCTCTGGTGTTC	Mosca *et al*. (2019)	MK135048
uvrB	Excinuclease ABC subunit B	chruvrB-F CGATTACTATCAACCAGAAG chruvrB-R CCGTAGCATATCAATCTCA	Mosca *et al*. (2019)	MK135049
uvrC	Excinuclease ABC subunit C	chruvrC-F ACGGATACAGAAGCAGAA chruvrC-R CTTGAGCAGCACATTGAA	Mosca *et al*. (2019)	MK135050
uvrD	DNA helicase II	chruvrD-F ACCAGAGCCAGATTTAGA chruvrD-R TCGTCGTATTCGTCTTCT	This study	MT813437
uvsE	UV DNA damage endonuclease	chruvsE-F TGTCCTTAGTTCTGATTCG chruvsE-R GGTAAGCCTAACAAGTCA	Mosca *et al*. (2019)	MK135047
xthA	Exodeoxyribonuclease III	chrxth-F GGTTGATGTCCTTTGTTTAC chrxth-R CGAGATAAGCGTGATAGC	Mosca *et al*. (2019)	MT829333

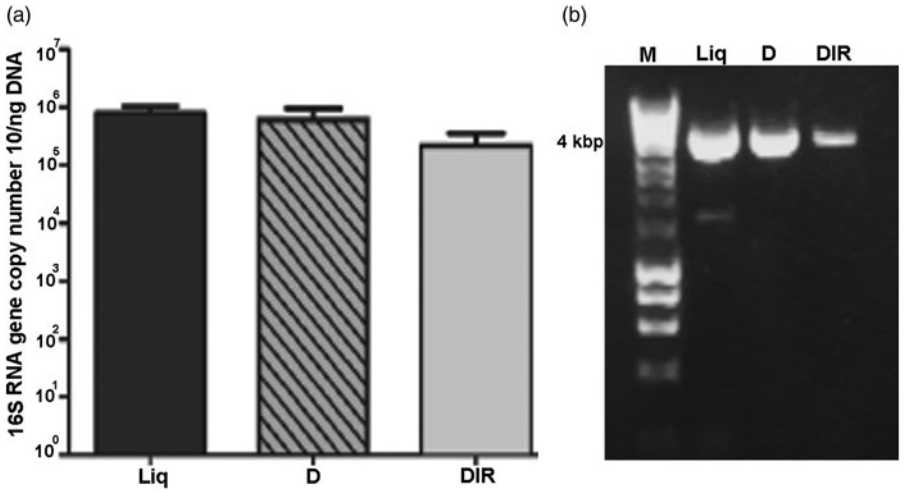

Fig. 1. *Cell viability of* Chroococcidiopsis *sp. CCMEE 029 according to PMA-qPCR assay (a). DNA damage evaluated with the PCR-stop assay (b). Liq, liquid culture; D, dried cells; DIR, dried cells irradiated with 2 kGy of Fe-ion radiation; M, hyperladder 1 kbp.*

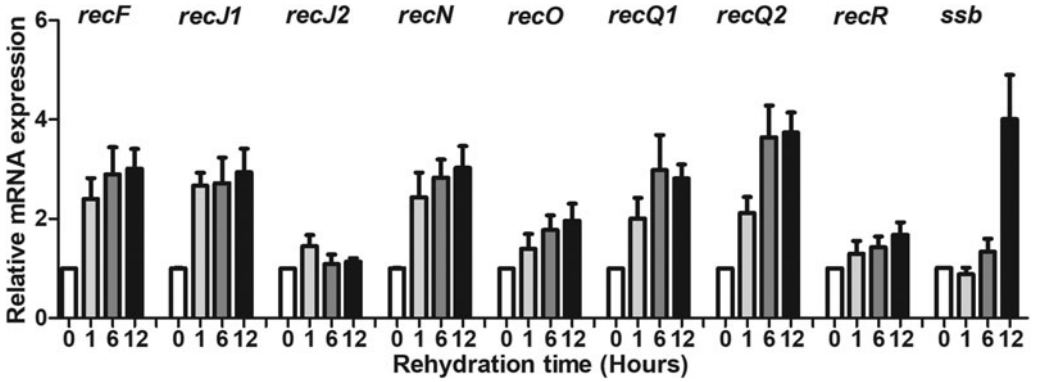

Fig. 2. *Expression of genes of the RecF repair pathway during the rehydration of dried cells of* Chroococcidiopsis *sp. CCMEE 029 exposed to 2 kGy of Fe-ion radiation. Values from non-rehydrated, irradiated cells were considered as control values and set to 1.*

Expression of BER genes during the rehydration of dried-irradiated cells

During the rehydration of dried-irradiated cells of *Chroococcidiopsis* sp. CCMEE 029, the genes involved in the BER DNA repair pathway were weakly over-expressed, exception made for the *mutY* gene that was up-regulated by 2.92-fold after 1 h, and by 6.38- and 5.91-fold after 6 and 12 h, respectively (Fig. 3). The expression of the *alkA* gene showed a 2.14-fold increase after 6 h rehydration. The *fpg* was over-expressed by 1.81-, 2.26- and 2.11-fold after 1, 6 and 12 h, respectively. The *mpg* was up-regulated by 2.53- and 2.38-fold after 6 and 12 h, respectively. While the *mug* gene was up-regulated by 2.04-, 3.15- and 3.01-fold after 1, 6 and 12 h of rehydration, respectively. There was no over-expression of the *nth* gene, while the *udg* gene was up-regulated by 2.35- and 2.03-fold after 6 and 12 h, respectively. Finally, the *xth* gene was over-expressed by 2.2-, 3.11- and 2.59-fold after 1, 6 and 12 h rehydration, respectively (Fig. 3).

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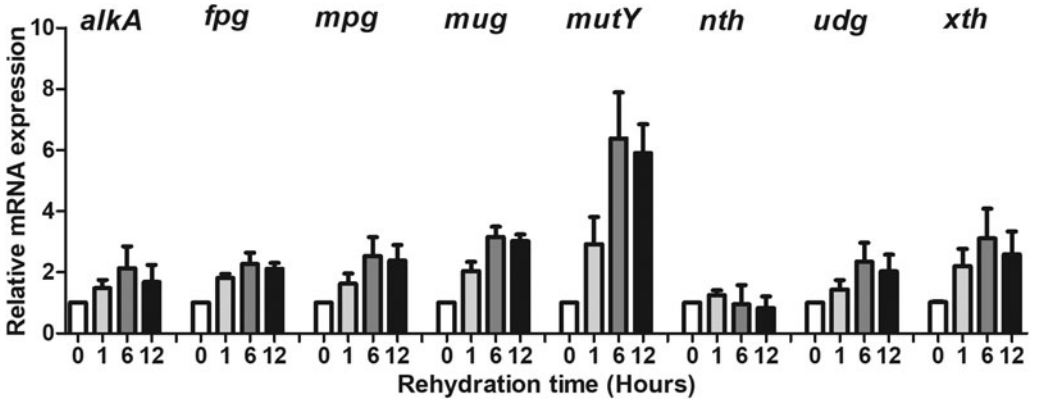

Fig. 3. *Expression of genes of the BER repair pathway during the rehydration of dried cells of* Chroococcidiopsis *sp. CCMEE 029 exposed to 2 kGy of Fe-ion radiation. Values from non-rehydrated, irradiated cells were considered as control values and set to 1.*

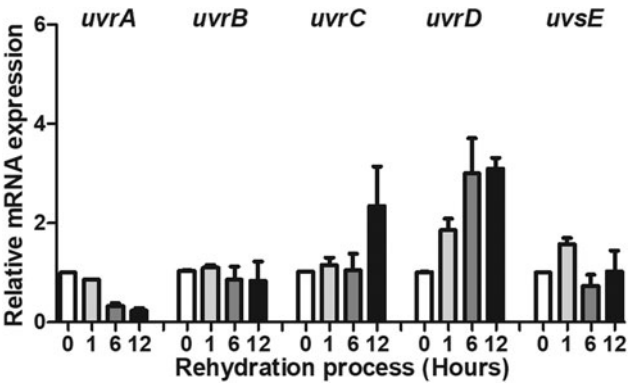

Fig. 4. *Expression of genes of the NER and UVER repair pathways during the rehydration of dried cells of* Chroococcidiopsis *sp. CCMEE 029 exposed to 2 kGy of Fe-ion radiation. Values from non-rehydrated, irradiated cells were considered as control values and set to 1.*

Expression of NER and UVER genes during the rehydration of dried-irradiated cells

Upon rehydration, dried-irradiated cells of *Chroococcidiopsis* sp. CCMEE 029 did not show any over-expression of the *uvrA* and *uvrB* genes involved in the NER repair pathway (Fig. 4). The *uvrC* gene was up-regulated by 2.34-fold only after 12 h rehydration, while the *uvrD* gene was up-regulated by 1.86-, 3.00- and 3.10-fold after 1, 6 and 12 h, respectively. Finally, the *uvsE* showed a weak over-expression only at 1 h rehydration (Fig. 4).

Table 2 shows an overview of the role of DNA repair pathways in the repair of damages induced by 2 kGy of Fe-radiation during the rehydration of *Chroococcidiopsis* sp. CCMEE 029.

Discussion

The measurement of Mars' surface radiation environment has allowed the modelling of subsurface radiation environment with paramount implications for microbial survival times (Hassler *et al.*, 2014). Any putative dormant life occurring in near-surface frozen habitats would accumulate high doses of cosmic rays over geological timescales and finally eradicated (Dartnell *et al.*, 2007). Nevertheless, during the

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Table 2. *Involvement of DNA repair pathways in the repair of damages induced by 2 kGy of Fe-radiation during the rehydration of* Chroococcidiopsis *sp. CCMEE 029*

Gene[a]	DNA repair pathway	Gene[a]	DNA repair pathway	Gene[a]	DNA repair pathway
recF	RecF	*ssb*	RecF	*udg*	BER
recJ1	RecF	*alkA*	BER	*xth*	BER
recJ2	RecF	*fpg*	BER	*uvrA*	NER
recN	RecF	*mpg*	BER	*uvrB*	NER
recO	RecF	*mug*	BER	*uvrC*	NER
recQ1	RecF	*mutY*	BER	*uvrD*	NER
recQ2	RecF	*nth*	BER	*uvrE*	UVER
recR	RecF				

RecF, RecF homologous recombination; BER, base excision repair; NER, nucleotide excision repair; UVER, UV damage endonuclease (UvsE)-dependent excision repair.
[a]Over-expressed genes are highlighted in dark grey, not over-expressed genes are highlighted in grey.

post-Noachian, brief periods of clement conditions might have occurred on timescales of several hundred thousand to a few million years era that allowed the metabolic activity recovery and repair of the radiation-induced damage (Hassler *et al*., 2014). In this scenario, the exposure of dried, radioresistant microorganisms to ionizing radiation doses mimicking long-term irradiation is fundamental to get insights into the resilience limit of putative dormant microbial life in the Martian subsurface over geological timescales.

Here the capability of repairing the DNA damage accumulated under high-LET Fe-ion radiation was investigated by irradiating dried cells of the radioresistant cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 to accelerate Fe ions up to the final dose of 2 kGy. As revealed by the PMA-qPCR assay, this exposure did not significantly affect its survivability. This result is relevant if compared to the previously reported reduced survival of dormant microorganisms exposed to Fe irradiation. For example, 500 mGy of Fe-ion radiation caused a twofold reduction of *B. subtilis* spore survival (Moeller *et al*., 2008) and a fivefold reduction in four *Bacillus* spp. from hot and cold environments (Zammuto *et al*., 2020). While 1 kGy of Fe-ion radiation reduced the viability of dried colonies of the Antarctic fungus *Cryomyces antarcticus* to 13% (Aureli *et al*., 2020).

There might be a number of possible explanations for the enhanced radiation-tolerance of dried *Chroococcidiopsis* sp. CCMEE 029. First, an efficient stabilization of dried cellular components achieved via the accumulation of sucrose and trehalose, the latter acting also as free-radical scavenger (Fagliarone *et al*., 2020). Second, an efficient DNA repair mechanism of the double strand break (DSB) taking advantage of the presence of multiple genome copies and absence of the low-fidelity non-homologous end joining system (Mosca *et al*., 2021).

It was estimated that a timescale longer than 10 million years would be required to accumulate a dose of 1 kGy of Fe-ion radiation in the Martian surface (Aureli *et al*., 2020). Therefore, the non-significant reduction in the survival of dried *Chroococcidiopsis* irradiated with 2 kGy of Fe-ion radiation further expanded our appreciation of the long-term resilience of a putative dormant microbial life in the Martian subsurface. Moreover, putative Martian microbial life forms could have evolved even a greater radiotolerance than Earth radioresistant microorganisms (Dartnell *et al*., 2007).

In the present work, the absence of a significant reduction in the survival of dried, irradiated *Chroococcidiopsis* was accompanied by DNA damage accumulation. An increased amount of DNA lesions was highlighted by PCR-stop assay with a 4 kbp target in dried, irradiated cells compared to dried cells. This is in agreement with the previously reported lack of DNA damage tested with shorter-PCR targets (Verseux *et al*., 2017), because there is a higher likelihood of encountering lesions that impair DNA polymerase progression in longer DNA sequences than in the shorter ones (Rudi *et al*., 2010).

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The monitoring of the expression of genes involved in DNA repair pathways revealed the capability of dried, irradiated *Chroococcidiopsis* to revive upon rehydration and to repair complex, accumulated DNA damage. The fact that DNA repair genes were still expressed after 12 h rehydration highlighted the time needed to repair complex DNA damage as previously reported for the repair of the severe DNA damage induced by 5 kGy of X-rays and restored after 24 h of recovery (Billi *et al*., 2000). In fact, when a high-LET radiation passes through DNA, it induces clustered damage, defined as two or more closely associated lesions consisting of DSBs along with oxidized bases and apurinic-apyrimidinic sites (Terato and Ide, 2004; Okayasu, 2012). Among clustered DNA damage, DSBs are the most severe ones, thus limiting the chance of survival (Spies and Kowalczykowski, 2005).

During the rehydration of dried-irradiated *Chroococcidiopsis*, most genes associated with the RecF pathway were over-expressed, suggesting the role of this homologous recombination pathway in the DSB repair. In particular, the over-expression of the *recN* gene was interesting since in *D. radiodurans* the RecN protein showed a cohesin-like activity and stimulated RecA-mediated recombinational repair of DNA damage (Uranga *et al*., 2017).

The bioinformatics analysis revealed the presence in *Chroococcidiopsis* sp. CCMEE 029's genome of eight genes of the BER system encoding seven nucleotide glycosylases and one endonuclease (Mosca *et al*., 2021). Such a high number of DNA glycosylases was reported for the genome of *D. radiodurans* and ruled out as important for the DNA repair efficiency (Makarova *et al*., 2001). However, upon rehydration dried-irradiated *Chroococcidiopsis* showed a weak over-expression of the BER genes, exception made for the *mutY* gene, thus suggesting a lack of DNA oxidative damage. In addition, the absence of an up-regulation of the *uvrA* and *uvrB* genes of the NER repair system suggested the lack of DNA lesions, like cyclobutane-pyrimidine dimers and 6–4 photoproducts that are generally induced by UV radiation (Rastogi *et al*., 2010). Also the *uvsE* gene was not over-expressed, thus confirming its role in repairing DNA damage due to desiccation rather than to UV radiation (Mosca *et al*., 2019) and high-LET radiation. On the other hand, the over-expression of the *uvrD* gene suggested the involvement of the encoded DNA helicase in the DSB repair as previously reported for *D. radiodurans* (Bentchikou *et al*., 2010).

In conclusion, our findings provided new insights into the resilience of radiotolerant, dried microorganisms exposed to a high-LET dose mimicking long-term exposure in the Martian subsurface. In addition, the tolerance of dried *Chroococcidiopsis* towards high-LET radiation is relevant when searching life in other astrobiology targets rather than Mars, like Europa and Enceladus, icy moon of Jupiter and Saturn, respectively, where the high radiation environments (Rettberg *et al*., 2019) might critically affect the long-term survival of dormant, frozen life forms.

Financial support. This research was supported by the Italian Space Agency (Bio-Signatures and habitable niches_Cyanobacteria - BIOSIGN_Cyano; grant 2018-15-U.0 to DB). RM was supported by the DLR grant FuE-Projekt 'ISS LIFE' (Programm RF-FuW, Teilprogramm 475). AF and RM – MEXT Grant-in-Aid for Scientific Research on Innovative Areas 'Living in Space' (Grant Numbers: 15H05935, 15K21745).

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https://doi.org/10.1017/S1473550422000131 Published online by Cambridge University Press

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https://doi.org/10.1017/S1473550422000131 Published online by Cambridge University Press