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Biomarker Preservation and Survivability Under Extreme Dryness and Mars-Like UV Flux of a Desert Cyanobacterium Capable of Trehalose and Sucrose Accumulation

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Unraveling how long life can persist under extreme dryness and what kind of environmental extremes can be faced by dried microorganisms is relevant to understand Mars habitability and to search for life on planets with transient liquid water availability. Because trehalose and sucrose stabilize dried anhydrobiotes, an *in silico* survey of the genome of the desert cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 was performed to identify pathways for trehalose and sucrose biosynthesis. The expression of the identified genes was induced in response to desiccation, and trehalose and sucrose accumulation was detected in dried cells. This adaptation strategy enabled viability and biomarker permanence under extreme dryness and Mars-like UV flux. *Chroococcidiopsis* survivors were scored in 7-year dried biofilms mixed with phyllosilicatic Mars regolith simulant and exposed to 5.5×10^3 kJ/m^2 of a Mars-like UV flux. No survivors occurred after exposure to 5.5×10^5 kJ/m^2 although, in dead cells, photosynthetic pigments, and nucleic acids, both DNA and RNA, were still detectable. This suggests that dried biofilms mixed with phyllosilicatic Martian regolith simulant are suitable candidates to identify biosignatures embedded in planetary analog minerals as planned in the future BioSignatures and habitable Niches (BioSigN) space mission to be performed outside the International Space Station.

Keywords: biosignatures, anhydrobiosis, Mars simulation, desert cyanobacteria, life detection

INTRODUCTION

Unraveling how long life can persist under extreme dryness and which environmental extremes can be faced in the dried state is relevant for long-term models of Mars habitability (Davila and Schulze-Makuch, 2016) and for searching for life on planets with transient availability of liquid water (Schulze-Makuch et al., 2017; Wilhelm et al., 2018). Moreover, according to the so called geogenetic latency hypothesis, subsurface microbes could survive planetary surface extinction and be re-exposed to the surface via geological processes, when conditions allow water to flow (Boston et al., 2019).

Indeed dryness is one of the main life-threatening factors; nevertheless, upon desiccation, a small group of taxonomically diverse organisms enter a reversible metabolic dormancy, a phenomenon known as anhydrobiosis (Crowe et al., 1992). Anhydrobiotes survive water removal as specialized structures, such as bacterial spores, cyanobacterial akinetes, and certain crustacean cysts and insect larvae, and a few among cyanobacteria, lichens, fungi, rotifers, nematodes, and tardigrades enter anhydrobiosis in the vegetative state (Alpert, 2006).

Hot and cold deserts are relevant Martian field analogs due to their aridity, temperatures, and/or geological features (Martins et al., 2017) and are considered of great interest to appreciate biosignature preservation under conditions similar to those of Mars (Aerts et al., 2020). So, if life ever existed on Mars' surface, it must have faced the presumed three main climatic stages of Mars: this beginning with a water-rich period, followed by a cold and semiarid one, and ending with the present-day arid and cold environment (Fairén et al., 2010). Because everything we know about biology we have learned from life on Earth (McKay, 2010), desiccation-tolerant microorganisms might be the best-case biologic scenario for understanding the habitability of Mars (Wilhelm et al., 2018) and identifying protective biomolecules to be used as a biomarker database (Jorge-Villar and Edwards, 2013).

Desert cyanobacteria of the genus *Chroococcidiopsis*, being metabolically active for a few hours per year, provide some evidence of their anhydrobiotic potential (Friedmann et al., 1993; Warren-Rhodes et al., 2006). Under laboratory conditions, they recovered after 4 and 7 years of air-dried storage (Billi, 2009; Fagliarone et al., 2017; Mosca et al., 2019) although new evidence will be provided by the 500-Year Microbiology Experiment aimed to investigate the desiccation longevity of dried *Chroococcidiopsis* and *Bacillus subtilis* spores, sealed in glass vials with silica gel beads (Cockell, 2015; Ulrich et al., 2018).

Insights into the environmental extremes that desert strains of *Chroococcidiopsis* can face have been revealed by challenging dried cells with the exposure to Mars simulations, either in the laboratory or in space. Under Mars laboratory simulations, dried biofilms survived 1.5×10^3 kJ/m^2 of a Mars-like UV flux followed by 7 years of air-dried storage (Mosca et al., 2019). During the EXPOSE-R2 space mission, dried samples survived 18-month exposure to Mars simulations in low Earth orbit outside the International Space Station (Rabbow et al., 2017). Under these conditions, samples face extreme dryness induced by a space vacuum and Mars simulated pressure combined with solar UV radiation and cosmic ionizing radiation (de Vera et al., 2019). In the Biofilm Organisms Surfing Space (BOSS) space experiment, within dried biofilms, top cell layers shielded bottom cell layers against UV radiation (Billi et al., 2019a). Whereas in the BIOlogy and Mars EXperiment (BIOMEX) space experiment, dried cells were shielded by mixing with Martian regolith simulants (Billi et al., 2019b).

In the present work, by taking advantage of the BIOMEX and BOSS results, biofilms of *Chroococcidiopsis* sp. CCMEE 029 were obtained by growing cells mixed with phyllosilicatic Martian regolith simulant (P-MRS) and then exposed to Mars laboratory simulations. The hypothesis is that, within the biofilm structure, the mixing of cells with minerals guarantees better UV radiation shielding. Indeed, the identification of suitable biosignatures embedded in planetary analog minerals is one the aims of the future BioSignatures and habitable Niches (BioSigN) space experiment to be performed outside the International Space Station (de Vera and The Life Detection Group of BIOMEX/BIOSIGN, 2019).

No doubt, unraveling the biochemical mechanisms that stabilize dried *Chroococcidiopsis* cells will help identify key molecules for dryness adaptation that might enable survival and/or biomarker permanence under Mars-like conditions. In this scenario, a crucial role should be played by trehalose and sucrose, two non-reducing sugars accumulated by anhydrobiotes, which replace water molecules, prevent membrane phase transition, and allow cytoplasmic vitrification (Sun and Leopold, 1997; Crowe et al., 1998). Indeed vitrification is supposed to underlie *Chroococcidiopsis* survival after exposure to subfreezing temperatures in salt solutions as inferred for Europa's icy surface (Cosciotti et al., 2019). Trehalose is also proposed to act as free-radical scavenger, thus avoiding oxidative damage (Benaroudj et al., 2001). Indeed, in a radioresistant fungus, the overexpression of a trehalose–synthase gene yields an increased trehalose accumulation and enhanced resistance to gamma irradiation, UV light, and heavy metal ions (Liu et al., 2017). Notably, glucose addition to *Bacillus subtilis* spores increased survival after 6-year exposure to a space vacuum inside the NASA Long Duration Exposure Facility (Horneck et al., 1994).

Here an *in silico* analysis of the genome of *Chroococcidiopsis* sp. CCMEE 029 was performed in order to identify genes involved in the following trehalose and sucrose biosynthesis: (i) the TreY/TreZ pathway catalyzing the transglycosylation of maltodextrins into maltooligosyl trehalose and trehalose hydrolytic release; (ii) the TreS pathway transforming maltose into trehalose; (iii) the TPS/TPP pathway catalyzing the formation of trehalose-6-phosphate and its dephosphorylation into trehalose; (iv) the SPS/SPP pathway with a sucrose–phosphate synthase and sucrose–phosphate phosphatase; and (v) the SUS pathway catalyzing the reversible transfer of a glucosyl moiety between fructose and a nucleoside diphosphate (for a review, see Avonce et al., 2006; Kolman et al., 2015).

The role of the identified genes in the desiccation tolerance of *Chroococcidiopsis* was evaluated by monitoring their expression by real-time quantitative PCR (RT-qPCR) in 10- and 60-min dried cells. Trehalose and sucrose content was also determined in dried cells. After a prolonged air-dried storage (7 years), dried biofilms mixed with P-MRS and exposed to Mars laboratory simulations were investigated for viability and biomarker permanence by mean of SYTOX-Green staining and RT-qPCR, respectively.

MATERIALS AND METHODS

Organism, Culture Conditions, and Desiccation

The cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 from the Negev Desert (Israel) is maintained at the Department of Biology as part of the Culture Collection of Microorganisms

from Extreme Environments (CCMEE) established by E. Imre Friedmann and Roseli Ocampo-Friedmann. The strain was grown under routine conditions at 25°C in liquid BG-11 medium under a photon flux density of 40 μmol/m^2/s provided by fluorescent cool-white bulbs.

Biofilms were obtained by plating about 2 × 10^8 cells mixed with 0.2 g of a Phyllosilicatic Mars Regolith Simulant (P-MRS; see Baqué et al., 2016, for regolith composition) on top of BG-11 agarized medium in Petri dishes sealed with Parafilm and allowed to grow for 2 months.

Desiccation was performed as follows: (i) over silica gel: liquid-culture aliquots were immobilized on 0.2-μm polycarbonate filters (Millipore) and stored for 10 and 60 min in a glass vacuum desiccator with anhydrous silica gel at room temperature (RT) in the dark; (ii) through air-drying: liquid-culture aliquots were dried under a laminar flow hood for 24 h and stored at RT in the dark. Biofilms were allowed to air-dry for 15 days under routine conditions by removing the Parafilm from the Petri dishes (Billi et al., 2019a).

Mars Laboratory Simulations

Dried biofilms mixed with minerals were accommodated in the DLR 16-well aluminum sample carriers at the Planetary and Space Simulation facilities (Microgravity User Support Center, DLR Cologne, Germany) and exposed to Mars simulations between September 2013 and December 2013 as planned in the context of the EXPOSE-R2 space mission (de Vera et al., 2019). The top layer of the carrier samples were exposed in triplicate to 5.5 × 10^3 kJ/m^2, 1.4 × 10^5 kJ/m^2, 2.7 × 10^5 kJ/m^2, and 5.5 × 10^5 kJ/m^2 with a SOL200 lamp (1271.2 W/m^2 200–400 nm) for 1 h 12 min, 30 h, 60 h, and 120 h under Earth's atmosphere, and in the bottom layer, samples were kept in the dark (see **Table 3**, de Vera et al., 2019). In another simulation, samples were exposed in the top layer of the carrier in triplicate to 5.7 × 10^5 kJ/m^2 with a SOL200 lamp (1,271 W/m^2 200–400 nm) for 98 h 73 min under a CO_2 Mars-like atmosphere (at 1 kPa), and in the bottom-layer carrier, samples were kept in the dark under a Mars-like atmosphere (see **Table 4**, de Vera et al., 2019). After the Mars simulations, dried samples were kept in the dark at RT for about 7 years.

Identification of Genes Encoding Trehalose and Sucrose Biosynthetic Enzymes

The genome of *Chroococcidiopsis* sp. CCMEE 029 was sequenced by using Illumina/Solexa technology (CD Genomics, NY, USA), gene annotation was performed by using the PROKKA v.1.11 software (Seemann, 2014), and the interface was provided by Galaxy-based framework Orione (Cuccuru et al., 2014). Genes encoding trehalose and sucrose biosynthetic enzymes were identified by a BlastN (Nucleotide Query Searching a Nucleotide Database) search for nucleotide sequences of sequenced cyanobacterial orthologs as previously reported for DNA repair genes (Mosca et al., 2019).

RNA Extraction and RT-qPCR

Total RNA was extracted from each sample by using 1 ml of TRI Reagent® (Merck) and treatment with RQ1 RNase-Free DNase I (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Then, 1 μg of total RNA for each sample was retrotranscribed to single-strand cDNA by using the SensiFAST™ cDNA Synthesis Kit (Bioline Meridian Life Science, Memphis, TN, USA). Real-time reactions were performed in 12 μl, including 1 μg of cDNA template, 6 μl of iTaq™ universal SYBR® Green supermix (BioRad Laboratories, Hercules, CA, USA), and 400 nM of the appropriate primer (**Table 1**). Primer specificity was confirmed by melting curve analysis. 16S rRNA (GenBank accession number AF279107) was used as a reference gene (Pinto et al., 2012). PCR cycling conditions were performed in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) as follows: a cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min, followed by a ramp from 60 to 95°C for the melting curve stage.

TABLE 1 | Primers used for RT-qPCR.

Gene	PCR primers	Sequence (5′-3′)	PCR product size (bp)
16S	chr16S-F	TACTACAATGCTACGGACAA	83
	chr16S-R	CCTGCAATCTGAACTGAG	
sps	chrsps-F	TCGGATTGAGGATATTGT	91
	chrsps-R	ATAGTACATTGGCAGGAT	
spp	chrspp-F	CAGCAGACCATCATTACT	81
	chrspp-R	ATTCCAAGAAGCCGAAAT	
susB	chrsusB-F	TTCCTCAACCGTTACCTATG	91
	chrsusB-R	CGTATTGCCGTCCTTGTA	
susA	chrsusA-F	CAAGAGTGCTGCCTTACA	82
	chrsusA-R	TTCGTTGTCGTCTGATTCT	
treY	chrtreY-F	TAATGCGGCTACAACAAT	87
	chrtreY-R	AGGATAGAAGGCGGTTAT	
treZ	chrtreZ-F	TTCTTCATCGTTGGAGTAA	128
	chrtreZ-R	CTTAGATTCAGCAGAGTCT	
treS1_long	chrtreS1-F	GAACTCCATATTGCTCTG	96
	chrtreS1-R	GGCGTATTGGTAGATAGA	
treS2_short	chrtreS2-F	CAGAGCAAGGATGAGATT	93
	chrtreS2-R	CCAGCGAAGGATATTACC	

(i) Gene expression in response to desiccation. Real-time reactions were performed by using total RNA extracted from 10- and 60-min dried cells and liquid cultures. Relative mRNA levels were calculated by the comparative cycle threshold (Ct) method. Primer specificity was confirmed by melting curve analysis. 16S rRNA (GenBank accession number AF279107) was used as a reference gene (Pinto et al., 2012). Values obtained for liquid control were set as one, and values of dried cells were considered to be upregulated (>1) or downregulated (<1). For each gene target, $n \geq 3$ qPCR reactions were conducted, each one in duplicate.

(ii) Permanence of 16S rRNA and mRNA in dried biofilms exposed to Mars laboratory simulations. RT-qPCR was performed by using total RNA extracted from 7-year-dried biofilms mixed with P-MRS and exposed to Mars

TABLE 2 | Genes encoding trehalose biosynthetic enzymes in *Chroococcidiopsis* sp. CCMEE 029.

Gene	KEGG enzyme	Gene length (nt)	Genbank accession number
treY	EC 5.4.99.15 Maltooligosyl trehalose synthase	2,793	MT078991
treZ	EC 3.2.1.141 maltooligosyl trehalose trehalohydrolase	1,827	MT078990
treS1 long	EC 5.4.99.16 maltose alpha-D-glucosyltransferase	3,393	MT078993
treS2 short	EC 5.4.99.16 maltose alpha-D-glucosyltransferase	1,641	MT078994

TABLE 3 | Genes encoding sucrose biosynthetic enzymes in *Chroococcidiopsis* sp. CCMEE 029.

Gene	KEGG enzyme	Gene length (nt)	Genbank accession number
sps	EC 2.4.1.14 Sucrose-phosphate synthase	1,281	MT078996
spp	EC 3.1.3.24 Sucrose-phosphate phosphatase	747	MT078989
susB	EC 2.4.1.13 Sucrose synthase	2,412	MT078992
susA	EC 2.4.1.13 Sucrose synthase	2,421	MT078995

laboratory simulations. Then, cycle threshold (Ct) values were acquired and used for statistical analyses. For the gene target, $n \geq 3$ qPCR reactions were conducted, each one in duplicate.

HPLC Analysis

The quantitative analysis of trehalose and sucrose content was performed by reverse phase ion pair (RPIP)-HPLC48 on a Luna 5lC18 reversed phase column (150 × 4.6 mm) (Phenomenex, Torrance, CA, USA) in acetonitrile (8.5%) and tetra-n-butylammonium hydrogen sulfate (1.2 mM; Fluka, St. Louis, MO, USA) and by applying a stepwise gradient of 0.2 M NaCl from 1 to 62%. The HPLC system (Dionex, Sunnyvale, CA, USA) was run at a flow rate of 1.1 ml/min and the fluorescent labeling reaction was performed by post-column addition of 2-cyanoacetamide (0.25%; Sigma) and NaOH (0.25%) at a flow rate of 0.35 ml/min by a PC10 post-column pneumatic delivery package (Dionex, Sunnyvale, CA, USA). Signals were quantified by comparison with known amounts of standard disaccharides analyzed in parallel runs. Data were processed by using ThermoScientific Chromeleon Chromatography Data System (CDS) software.

Cell Viability

Cell viability was assessed by staining with the cell-impermeant nucleic acid dye SYTOX Green (Molecular Probes, Inc., Eugene, OR, US) at a final concentration of 50 μM for 5 min in the dark as previously reported (Billi, 2009). Images were taken with an Olympus FV1000 confocal laser-scanning microscope (CLSM) by exciting the cells with a 488-nm laser and collecting the emissions between 510 and 530 nm. Photosynthetic pigment autofluorescence was excited with a 635-nm laser and emissions collected from the 645–800 nm emission range.

Statistical Analysis

Experiments were carried out with at least three independent replicates, data are shown with standard deviation and significance assessed by using Student's *t*-test.

RESULTS

Genes Encoding Trehalose Biosynthetic Enzymes

The *in silico* analysis identified in *Chroococcidiopsis* sp. CCMEE 029's genome genes for trehalose biosynthesis according to TreY/TreZ and TreS pathways (**Table 2**). The *treY* gene contained a 2,793-bp open reading frame predicted to encode a maltooligosyl trehalose synthase (TreY) and showing the highest similarity (BlastN output: query cover 99%, e-value 0.0, and total score 2,267) with the ortholog of *Microcoleus* sp. PCC 7113 (GenBank: CP003630.1:c4333883-4331094). The t*reZ* gene contained an open reading frame of 1,827-bp potentially encoding a maltooligosyl trehalose trehalohydrolase (TreZ) and sharing the highest similarity (BlastN output: query cover 99%, e-value 0.0, and total score 2,547) with the ortholog of *Microcoleus* sp. PCC 7113 (GenBank: CP003630.1:c4339755-4337926).

Two *treS* genes encoding a maltose alpha-D-glucosyltransferase (TreS) were identified in *Chroococcidiopsis* sp. CCMEE 029's genome (**Table 2**). The *treS1* had a 3393-bp open reading frame predicted to encode a long TreS and shared the highest similarity (BlastN output: query cover 99%, e-value 0.0, and total score 2,859) with the ortholog of *Microcoleus* sp. PCC 7113 (GenBank: CP003630). Although the *treS2* contained a 1,641-bp open reading frame predicted to encode a short TreS, it showed the highest similarity (BlastN output: query cover 100%, e-value 0.0, and total score 1,657) with the ortholog of *Scytonema* sp. NIES-4073 (GenBank: AP018268.1). The *in silico* analysis did not identify any gene involved in the TPS/TPP pathway for trehalose biosynthesis.

Genes Encoding Sucrose Biosynthetic Enzymes

The *in silico* analysis of the genome of *Chroococcidiopsis* sp. CCMEE 029 identified genes for the sucrose synthesis according to SPS/SPP and SUS pathways (**Table 3**). The *sps* gene had a 1,281-bp open reading frame predicted to encode a sucrose–phosphate synthase (SPS) and showing the highest similarity (BlastN output: query cover 97%, e-value 0.0, and total score 135) with the gene of *Chroococcidiopsis thermalis* PCC 7203 encoding a glycosyltransferase (GeneBnk AF87753.1). The *spp* gene contained a 747-bp open reading frame encoding a

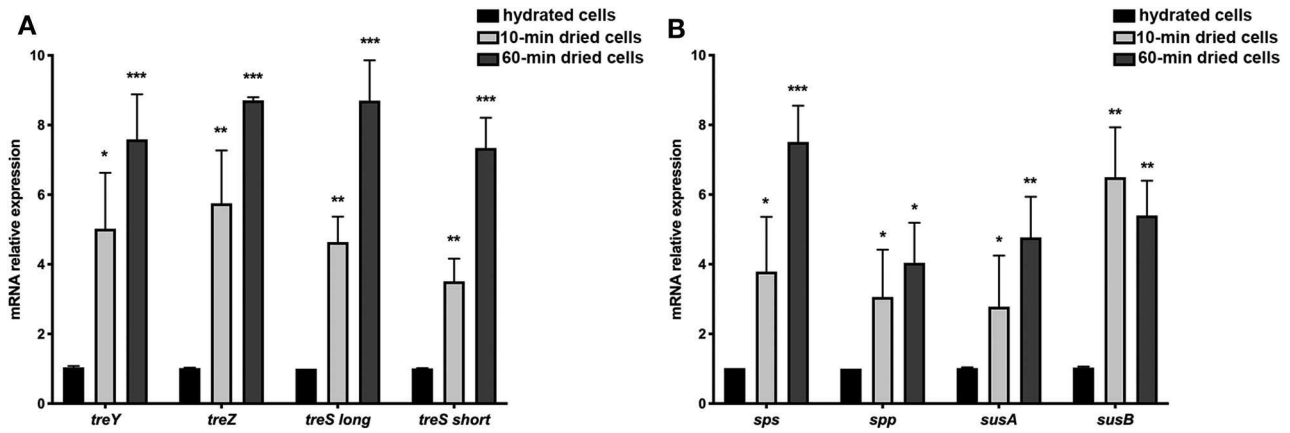

FIGURE 1 | Expression of trehalose and sucrose biosynthetic genes in 10- and 60-min dried *Chroococcidiopsis* cells. Gene expression of TreY/TreZ and TreS trehalose biosynthetic pathway genes **(A)**; gene expression of SPS/SPP and SUS sucrose biosynthetic pathways **(B)**. Values in hydrated cells were considered as control values and set to 1. Data represent mean ± SD ($n \geq 3$), $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

putative sucrose–phosphate phosphatase (SPP) and exhibited the highest similarity (BlastN output: query cover 99%, e-value 2e-127, and total score 469) with the gene of *Gloeocapsopsis* sp. AAB1 encoding a sucrose-6F-phosphate phosphohydrolase (GenBank: KJ183087.1).

Two *sus* genes, namely *susA* and *susB*, were identified (**Table 3**). The *susA* gene contained a 2,421-bp open reading frame coding a putative sucrose synthase (SUS) and shared the highest similarity (BlastN output: query cover 100%, e-value 0.0, and total score 1,854) with the *susA* gene of *Nostoc punctiforme* PCC 73102 (GenBank: AJ316589.1). The *susB* gene had a 2,412-bp open reading frame coding a putative sucrose synthetase (SUS) and shared the highest similarity (BlastN output: query cover 100%, e-value 0.0, and total score 2,209) with the orthologous gene of *Scytonema* sp. HK-05 (GenBank: AP018194.1: c1150884-1153295) and a high similarity (BlastN output: query cover 97%, e-value 0.0, and total score 1,043) with the *susB* gene of *Nostoc* sp. PCC 7120 (GenBank: AJ316584.1).

Expression of Trehalose and Sucrose Biosynthesis Genes Upon Desiccation

The expression of trehalose biosynthetic genes was evaluated by RT-qPCR in 10- and 60-min dried *Chroococcidiopsis* cells over silica gel and compared to their expression in liquid controls (**Figure 1A**). Regarding the TreY/TreZ pathway, after 10 min of desiccation, the expression of the *treY* and *treY* genes was induced 5- and 6-fold, respectively, and after 60 min, of desiccation, the two genes resulted induced 8- and 9-fold, respectively (**Figure 1A**). For the TreS pathway, the *treS long* gene showed high mRNA levels after 10 and 60 min of desiccation, whereas the expression of the *treS short* gene was significantly induced at both desiccation points although at a lower level compared to the *treS long* gene (**Figure 1A**).

Genes involved in sucrose biosynthesis were induced after 10 and 60 min of desiccation compared to liquid control cultures (**Figure 1B**). Regarding the SPS/SPP pathway, the expression of the *spp* gene was induced at both desiccation points, and the mRNA levels of the *sps* gene increased after 10 and 60 min of desiccation. For the SUS pathway, the *susB* gene was more highly induced after 10 min than after 60 min of desiccation, and the mRNA levels of the *susA* gene increased at both desiccation points although at a lower level compared to the *susB* gene (**Figure 1B**).

TABLE 4 | Trehalose and sucrose content in dried cells of *Chroococcidiopsis* sp. CCMEE 029 (mg/g dry weight).

Disaccharides	Liquid cultures	Dried cells (silica gel)	Dried cells (air-drying)
Trehalose	0.059 ± 0.102	1.934 ± 1.198	1.745 ± 0.163
Sucrose	1.127 ± 0.536	5.387 ± 1.233	4.850 ± 0.438

Data represent mean ± standard deviation ($n \geq 3$).

Trehalose and Sucrose Content in Dried Cells

In dried *Chroococcidiopsis* cells, the amount of trehalose and sucrose was quantified by HLPC analysis, and liquid cultures were used as a control (**Table 4**). Cells desiccated over silica gel for 3 months showed a trehalose and sucrose amount of about 1.934 and 5.387 mg/g dry weight, respectively. This value was 33- and 5-fold increased from that detected in hydrated cells, which for trehalose and sucrose was about 0.059 and 1.127 mg/g dry weight, respectively. Cells air-dried for 24 h showed a trehalose and sucrose content of about 1.745 and 4.850 mg/g dry weight, respectively (**Table 4**).

RNA Permanence Under Desiccation and Mars-Like UV Flux

The effects of short-term desiccation on RNA integrity was determined in 10- and 60-min dried cells *Chroococcidiopsis* by evaluating the cycle threshold (Ct) values in RT-qPCR

assay (**Figure 2**), considering that a reduced RNA copy number corresponded to a high Ct value. A progressive reduction of 16S rRNA copy numbers occurred in 10- and 60-min dried cells that showed average Ct values of 23 and 25, respectively, hydrated cells had an average Ct value of 22 (**Figure 2**).

The effects of long-term desiccation following exposure to Mars-like UV flux was determined in dried *Chroococcidiopsis* biofilms mixed with P-MRS and exposed to UV doses ranging from 5.5×10^3 kJ/m^2 to 5.5×10^5 kJ/m^2 (**Figure 2**). The absence of a reduction in the average Ct values with the increased UV doses suggests the permanence of a reduced 16S rRNA copy number (**Figure 2**).

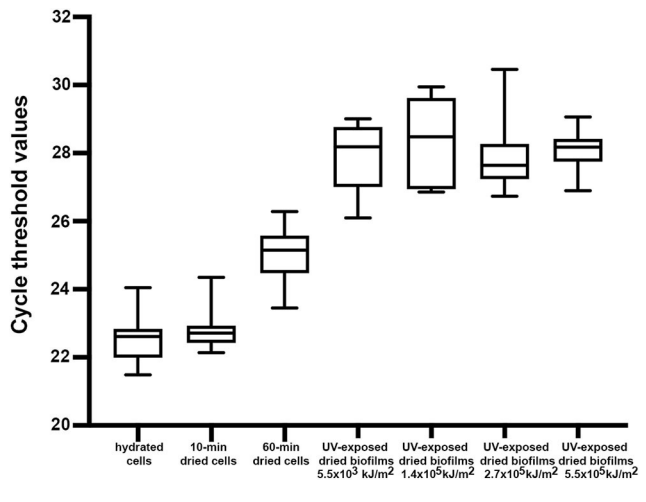

FIGURE 2 | Permanence of 16S rRNA in 10- and 60-min dried *Chroococcidiopsis* cells and in 7-year dried *Chroococcidiopsis* biofilms mixed with P-MRS, according to Ct values in the RT-qPCR assay. Samples: hydrated control cells; 10- and 60-min dried cells; 7-year dried biofilms mixed with P-MRS and exposed to increasing Mars-like UV radiation doses. Ct values are shown as medians (lines), 25th percentile to 75th percentile (boxes), and ranges (whiskers).

RNA Permanence Under Desiccation, Mars-Like UV Flux and Mars-Like Atmosphere

The Ct value method was used to evaluate the mRNA permanence in dried *Chroococcidiopsis* biofilms mixed with P-MRS and exposed to a Mars-like UV flux (5.7×10^5 kJ/m^2) combined with a Mars-like atmosphere and stored 7 years in the air-dried state. As shown in **Figure 3**, the four genes encoding sucrose biosynthetic enzyme were used as gene targets in determining Ct values in dried biofilms because they were previously reported to be induced upon desiccation (**Figure 2B**). Compared to hydrated cells, each gene showed reduced Ct value in 7-year dried biofilms exposed in the bottom-layer carrier, e.g., kept in the dark and exposed to a Mars-like atmosphere (**Figure 3**). When compared to dried biofilms exposed in the bottom-layer carrier, each gene showed slightly reduced Ct values in dried biofilms exposed in the top-layer carrier, i.e., exposed to 5.7×10^5 kJ/m^2 of a Mars-like UV flux combined with a Mars-like atmosphere (**Figure 3**). Similar results were obtained for the four genes encoding trehalose biosynthetic enzymes (not shown).

Cell Viability of Dried Biofilms Under Mars-Like UV Flux

In hydrated *Chroococcidiopsis* cells, the staining with the cell-impermeant SYTOX-Green stain revealed a few dead cells with damaged cell membranes showing SYTOX-Green stained nucleoids and red photosynthetic pigment autofluorescence (**Figure 4A**). In 7-year dried biofilms mixed with P-MRS and exposed to 5.5×10^3 kJ/m^2 of a Mars-like UV, about 20% of the alive cells were SYTOX-Green negative (**Figure 4B**). Only dead cells showing SYTOX-Green stained nucleoids and red photosynthetic pigment autofluorescence occurred in 7-year dried biofilms mixed with P-MRS and exposed to 1.4×10^5 kJ/m^2 and 2.7×10^5 kJ/m^2 (not shown) as well as to 5.5×10^5 kJ/m^2 (**Figure 4C**).

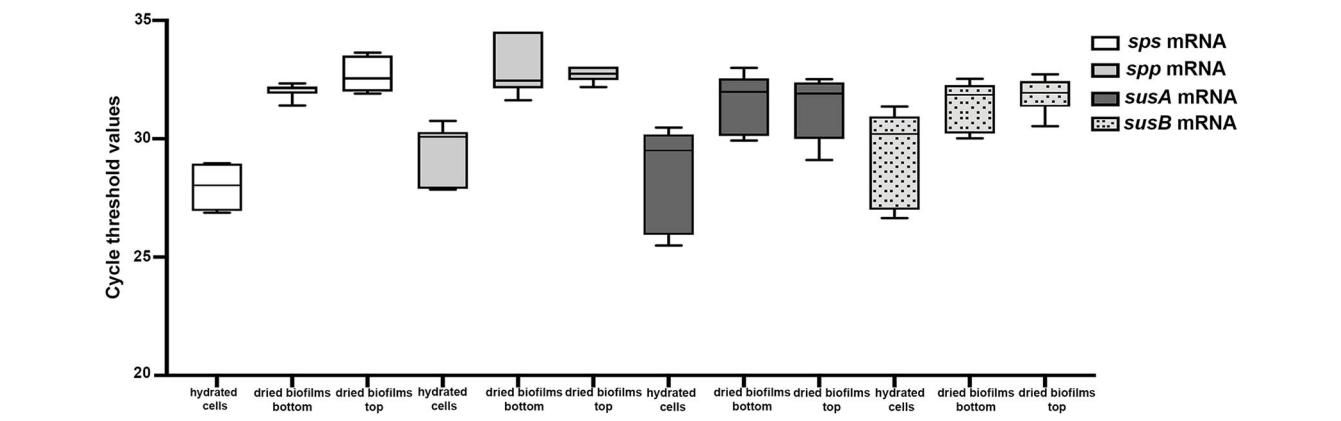

FIGURE 3 | Permanence of mRNA in 7-year dried *Chroococcidiopsis* biofilms mixed with P-MRS, according to Ct values in the RT-qPCR assay. Control: hydrated cells under laboratory routine growth conditions; bottom: dried biofilms exposed to Mars-like atmosphere; top: dried biofilms exposed to Mars-like UV flux (5.7×10^5 kJ/m^2) and Mars-like atmosphere.

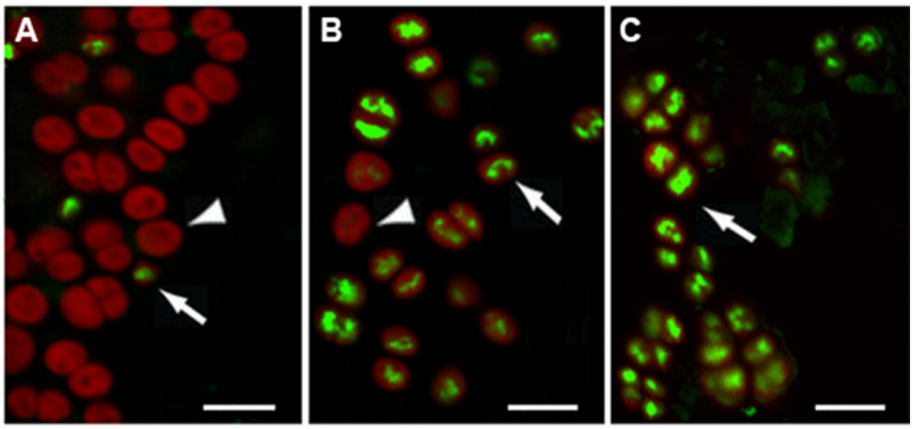

FIGURE 4 | Viability of 7-year dried *Chroococcidiopsis* biofilms mixed with P-MRS exposed to increasing Mars-like UV radiation doses, according to SYTOX-Green staining. Liquid control cultures showing live, SYTOX-Green-negative cells (arrowhead) and a few dead, SYTOX-green-positive cells (arrow) **(A)**; dried biofilms exposed to 5.5 × 10^3 kJ/m^2 showing live, SYTOX-negative cells (arrowhead); **(B)**; biofilms exposed to 5.5 × 10^5 kJ/m^2 showing dead, SYTOX-green positive cells with SYTOX-green stained nucleoids (arrow) and chlorophyll autofluorescence **(C)**. Scale bar = 10 μm.

DISCUSSION

In order to investigate the role of trehalose and sucrose in survivability and biomarker preservation under Mars laboratory simulations of the anhydrobiotic cyanobacterium *Chroococcidiopsis*, an *in silico* analysis of the genome of the CCMEE 029 strain was performed. The genome survey identified genes for trehalose synthesis according to the TreY/TreZ and TreS pathways as well as genes for sucrose synthesis according to the SPS/SPP and SUS pathways. The presence of the *treY* and *treZ* genes provided *Chroococcidiopsis* with the capability of catalyzing a two-step reaction to convert maltodextrins (maltooligosaccharides, glycogen, and starch) into trehalose. But the absence of a trehalase gene (*treH*) might contribute to trehalose accumulation. Indeed, the *treH* gene absence was reported for two desiccation-tolerant *Leptolyngbya* strains (Shimura et al., 2015; Murik et al., 2017) although *treH* mutants of *Anabaena* sp. PCC 7120 showed an increased trehalose amount and enhanced desiccation tolerance (Higo et al., 2006). The presence of two *treS* genes, encoding a long and short trehalose synthase, provided *Chroococcidiopsis* with the capability of transforming maltose into trehalose, according to the TreS pathway (Klähn and Hagemann, 2011). The genome survey of *Gloeocapsopsis* sp. UTEX B3054 and *Leptolyngbya ohadii* (Murik et al., 2017; Urrejola et al., 2019) revealed the presence of two *treS* gene, a feature suggested to be unique of desiccation-tolerant cyanobacteria (Murik et al., 2017). The long *treS* gene of *Chroococcidiopsis* sp. CCMEE 029 showed motifs encoding maltogenic amylase (IPR032091), maltokinase (IPR012811), and protein kinase (IPR011009) domains, responsible for trehalose production through glycogen degradation, that occurred in the long TreS homolog of *Leptolyngbya ohadii* (Murik et al., 2017).

The presence the *sps* and *sps* genes encoding a sucrose–phosphate synthase and sucrose–phosphate phosphatase provided *Chroococcidiopsis* with the capability of using the SPS/SPP sucrose biosynthetic pathway (Kolman et al., 2015). But the presence of two *sus* genes encoding a sucrose synthase also suggests the presence of the SUS pathway. Remarkably, Sus-encoding genes were identified during the genome survey of a selection of heterocyst-forming cyanobacteria, and they were present in the genome of a few unicellular cyanobacteria (Kolman et al., 2012). Indeed, representatives of the *Chroococcidiopsis* genus and heterocyst-differentiating cyanobacteria have been reported to be each other's closest living relatives (Fewer et al., 2002).

The relevance of trehalose and sucrose accumulation in the *Chroococcidiopsis* adaptation to dryness was supported by the expression of the identified genes during 10 and 60 min of desiccation over silica gel. Compared to the *treY* gene, the expression of the *treZ* gene was slightly higher after 60 min of dehydration, in agreement with the trehalose hydrolytic release (Avonce et al., 2006). The expression of the TreS-short encoding gene was 2-fold higher compared to TreS-long encoding gene as previously noticed in *Leptolyngbya ohadii* (Murik et al., 2017). Among the identified sucrose biosynthetic, the expression of *sps*, *sps*, and *susB* gens was induced after 10 and 60 min of desiccation over silica, whereas the *susA* gene was slightly induced only in 10 min dried cells. The low expression of this sucrose degradation–encoding gene might have contributed to increased sucrose content during desiccation.

Overall, the presence of multiple biosynthetic pathways might confer the advantage of accumulating sugars relevant for dryness adaptation under changeable environmental conditions leading to limited availability of a given substrate. As a result of this genomic feature, *Chroococcidiopsis* cells dried over silica gel for 3 months showed a trehalose and sucrose content about 33-fold (38 nmol/mg dry weight) and 5-fold (106 nmol/mg dry weight) increased compared to hydrated cells. Cells air-dried for 24 h had a trehalose and sucrose content 30- and 4-fold increased.

Indeed, a different level of desiccation tolerance might be reflected in terms of compatible solute content. For example,

Desmonostoc salinum CCM-UFV059 dried on silica gel had a trehalose and sucrose content of 40 and 15 nmol/mg dry weight, respectively (Viggiano de Alvarenga et al., 2020). *Anabaena* sp. PCC7120 did not recover desiccation on silica gel and showed no changes in sucrose content (Viggiano de Alvarenga et al., 2020), did not recover after 8 months of air-drying (Yoshimura et al., 2006), and showed a low trehalose amount (0.05–0.1% of dry weight) and a higher sucrose accumulation (1–2% of dry weight) (Higo et al., 2006).

In *Chroococcidiopsis*, the accumulation of trehalose and sucrose upon desiccation might have played a key role in cell survival and biomarker permanence in 7-year dried biofilms mixed with P-MRS and exposed to Mars laboratory simulations. Survivors were identified by SYTOX-Green staining after exposure to 5.5×10^3 kJ/m^2 of a Mars-like UV flux (**Figure 4**). It was previously reported that dried biofilms survived 1.5×10^3 kJ/m^2 of a Mars-like UV flux (Baqué et al., 2013; Mosca et al., 2019), and dried cells mixed with Mars regolith simulants died under 5.7×10^3 kJ/m^2 of a Mars-like UV flux (Baqué et al., 2016). Hence, in the present work, a tight contact between cells and minerals within the biofilm structure should have guaranteed the shielding against 5.5×10^3 kJ/m^2 of a Mars-like UV flux. Moreover, the occurrence of live cells in 7-year dried biofilms mixed with P-MRS exposed to 5.5×10^3 kJ/m^2 of a Mars-like UV (**Figure 4**), corresponding to 4 days on the Martian surface (Cockell et al., 2000), further supports the possibility that, during Mars's climatic history, a biofilm-like life form mixed with minerals could have survived a few hours' exposure to Mars UV flux while being transported from one protected niche to another (Westall et al., 2013).

On the other hand, although only dead cells occurred after 5.5×10^5 kJ/m^2, they showed the permanence of biomarkers: 16S RNA as revealed by RT-qPCR (**Figure 2**) and chlorophyll and genomic DNA as shown by CLSM analysis (**Figure 4**).

The drying process caused a decrease in the 16S rRNA copy number compared to hydrated cells although no further decrease occurred in 7-year dried biofilms mixed with P-MRS and exposed to increasing Mars-like UV (**Figure 2**). This might be due, in addition to the biofilm structure (Baqué et al., 2013), to the UV shielding provided by P-MRS and the trehalose presence acting as a free-radical scavenger (Benaroudj et al., 2001).

Moreover, the RNA stability was further reduced by 7 years of air-dried storage and, to a larger extent, by the exposure to Mars-like UV flux. In fact, 7-year dried biofilms mixed with P-MRS and exposed to a Mars-like UV flux (5.5×10^5 kJ/m^2) combined with a Mars-like atmosphere, showed an overall reduced copy number of a selection of mRNAs (**Figure 3**), corresponding to the induced expression of the sucrose and trehalose biosynthetic genes upon desiccation (**Figure 1**). For example, compared to dried samples kept in the dark, i.e., in the bottom-layer carrier of the exposure facility, a reduction of the copy number of the *sps* mRNA occurred in 7-year dried biofilms mixed with P-MRS and exposed to 5.5×10^5 kJ/m^2, i.e., in the top-layer carrier of the exposure facility (**Figure 3**).

These results are relevant because 5.5×10^5 kJ/m^2 (200–400 nm) corresponds to the UV dose expected during 1 year of exposure in low Earth orbit, a period of time generally planned for astrobiological experiments outside the International Space Station (de Vera and The Life Detection Group of BIOMEX/BIOSIGN, 2019; de Vera et al., 2019). Moreover, because this dose corresponds to 383 sols (half-year) on the Martian surface (Cockell et al., 2000), the detectability of biomarkers, such as RNA, genomic DNA, and chlorophyll, in dead biofilms suggests that biological signals might still be preserved over a long period of time if sufficiently stabilized under dryness and protected from UV radiation.

In conclusion, this work contributes to expand our knowledge on the adaptation strategies to extremely dry conditions and suggests that sucrose and trehalose accumulation might reduce macromolecular susceptibility to chemical and physical degradation taking place after cell death (Eigenbrode, 2008). This has implications for future life detection missions on Mars; moreover, the biomarker detectability in biofilms mixed with P-MRS exposed to a Mars-like UV flux followed by 7 years of air-dried storage is of interest in the context of the future BioSigN space mission, which will be performed outside the International Space Station in order to identify suitable biosignatures embedded in planetary analog minerals (de Vera and The Life Detection Group of BIOMEX/BIOSIGN, 2019).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT078991, MT078990, MT078993, MT078994, MT078996, MT078989, MT078992, MT078995.

AUTHOR CONTRIBUTIONS

DB and J-PV supervised the study. CF performed the experiments. MB prepared biofilm samples. SC performed the HPLC analysis. AN carried out the bioinformatic analyses. DB wrote the manuscript. All authors read and approved the final manuscript.

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