Received: 7 September 2023 | Accepted: 15 December 2023

DOI: 10.1111/jpy.13436

RESEARCH ARTICLE

Ecophysiological and genomic approaches to cyanobacterial hardening for restoration

Beatriz Roncero-Ramos[1,2] | **Valentina Savaglia**[1,3] | **Benoit Durieu**[1] | **Isabelle Van de Vreken**[4] | **Aurore Richel**[4] | **Annick Wilmotte**[1]

[1]InBios-Molecular Diversity and Ecology of Cyanobacteria, University of Liège, Liege, Belgium

[2]Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Sevilla, Spain

[3]Laboratory of Protistology & Aquatic Ecology, Ghent University, Ghent, Belgium

[4]TERRA-Biomass and Green Technologies, University of Liège, Gembloux, Belgium

Correspondence
Beatriz Roncero-Ramos, InBios-Molecular Diversity and Ecology of Cyanobacteria, University of Liège, 4000 Liège, Belgium.
Email: broncero@us.es

Funding information
Université de Liège, Grant/Award Number: R.DIVE.0899-J-F-I; Junta de Andalucía, Grant/Award Number: PAIDI-DOCTOR 21_00571

Editor: D. Casamatta

Abstract

Cyanobacteria inhabit extreme environments, including drylands, providing multiple benefits to the ecosystem. Soil degradation in warm drylands is increasing due to land use intensification. Restoration methods adapted to the extreme stress in drylands are being developed, such as cyanobacteria inoculation to recover biocrusts. For this type of restoration method to be a success, it is crucial to optimize the survival of inoculated cyanobacteria in the field. One strategy is to harden them to be acclimated to stressful conditions after laboratory culturing. Here, we analyzed the genome and ecophysiological response to osmotic desiccation and UVR stresses of an Antarctic cyanobacterium, *Stenomitos frigidus* ULC029, which is closely related to other cyanobacteria from warm and cold dryland soils. Chlorophyll *a* concentrations showed that preculturing ULC029 under moderate osmotic stress improved its survival during an assay of desiccation plus rehydration under UVR. Additionally, its sequential exposure to these stress factors increased the production of exopolysaccharides, carotenoids, and scytonemin. Desiccation, but not osmotic stress, increased the concentrations of the osmoprotectants trehalose and sucrose. However, osmotic stress might induce the production of other osmoprotectants, for which the complete pathways were observed in the ULC029 genome. In total, 140 genes known to be involved in stress resistance were annotated. Here, we confirm that the sequential application of moderate osmotic stress and dehydration could improve cyanobacterial hardening for soil restoration by inducing several resistance mechanisms. We provide a high-quality genome of ULC029 and a description of the main resistance mechanisms (i.e., production of exopolysaccharides, osmoprotectants, chlorophyll, and carotenoids; DNA repair; and oxidative stress protection).

KEYWORDS
biocrusts, cyanobacteria, drylands, exopolysaccharide, genomics, resistance mechanisms, soil restoration, UV radiation

Abbreviations: CDS, coding sequence; COGs, clusters of orthologous groups; EPS, exopolysaccharide; GGPP, geranylgeranylpyrophosphate; KEGG, Kyoto encyclopedia of genes and genomes; LB-EPS, loosely bound EPS; MAAs, mycosporine-like amino acids; OCP, orange carotenoid-protein; PAR, photosynthetic active radiation; ROS, reactive oxygen species; SOD, superoxide dismutase; TB-EPS, tightly bound EPS; UN, United nations; UVR, ultraviolet radiation.

INTRODUCTION

Cyanobacteria are extremophiles that can survive in different ecosystems, including some of the most extreme on Earth, such as the McMurdo Dry Valleys in Antarctica (Zhang et al., 2015) or the Atacama Desert in Chile (Patzelt et al., 2014). In terrestrial ecosystems, they are often part of communities, known as biological soil crusts, or biocrusts, along with other organisms (lichens, mosses, fungi, heterotrophic bacteria, or microalgae), colonizing the first centimeter of soil surfaces (Weber et al., 2022). Biocrusts provide many ecosystem functions such as improving soil fertility (Barger et al., 2016), stability (Belnap et al., 2007; Chamizo et al., 2017), and biodiversity (Maestre et al., 2011), among others. The cyanobacteria located in these ecosystems have mechanisms to withstand environmental stresses, such as high and continuous light intensity and UV radiation (UVR), desiccation, freezing, or high salinity. The cyanobacteria produce an exopolysaccharide (EPS) matrix surrounding cells, which accumulates water, stabilizing the cell membranes during desiccation (Pereira et al., 2009). This EPS matrix also protects cell membranes from freezing-induced damage (Tamaru et al., 2005) and UVR (Chen et al., 2009), and it accumulates nutrients (Mager & Thomas, 2011), which is important for survival in oligotrophic environments. In order to prevent the production of reactive oxygen species (ROS) by UVA radiation, some cyanobacteria accumulate the pigment scytonemin in the EPS matrix (Soule et al., 2013). In addition, cyanobacteria produce a wide range of mycosporine-like amino acids (MAAs) that accumulate inside cells and prevent DNA damage by absorbing UVB radiation (Gao & Garcia-Pichel, 2011). Cyanobacteria in extreme environments are also exposed to high light intensity, which can lead to photooxidative damage by ROS formation (Muramatsu & Hihara, 2012). To revert or avoid ROS formation, they produce oxygen-scavenging proteins (Kvíderová et al., 2019) and the non-photochemical quencher orange carotenoid-protein (OCP; Muramatsu & Hihara, 2012). Another important stress factor in hot and cold deserts is salinity. The two major strategies to withstand the osmotic stress due to an increase of the external ion concentrations present in a highly saline environment or due to events of desiccation or freezing are the salt-out strategy and the production/uptake of osmoprotectants (Kvíderová et al., 2019). The type of osmoprotectants, also called compatible solutes, that cyanobacteria may produce or uptake is roughly correlated to their natural habitat and their salt tolerance range (Kirsch et al., 2019), mainly sucrose and trehalose in low salt tolerance strains, glucosylglycerol in medium salt tolerance ones, and glycine betaine and glutamate betaine for high salt tolerance strains (Mackay et al., 1984). However, there are a few exceptions to these patterns (Kirsch et al., 2019). If these protective strategies do not succeed, the strategies to revert cellular damage are activated, such as DNA repair mechanisms (Pathak et al., 2019) or chaperone production (Chatterjee et al., 2020).

Cyanobacterial ability to withstand extreme conditions makes these interesting organisms for different disciplines, such as astrobiology (Billi et al., 2022), or for soil restoration in drylands (Rossi et al., 2017). The publications focused on dryland soil restoration by cyanobacterial inoculation have increased during the last decades (Antoninka et al., 2020) because of the increase in degradation of dryland ecosystems due to climate change and anthropogenic impacts (Huang et al., 2016). In fact, the protection and restoration of terrestrial habitats have been included in the Sustainable Development Goals of the United Nations (UN) for 2030 (UN, 2015). Because cyanobacteria are the first colonizers of soils and thus participate in the first steps of biocrusts succession, soil restoration aims to induce the formation of biocrusts on degraded soils by different cyanobacteria (Weber et al., 2016). In laboratory conditions, the inoculated cyanobacteria can survive and colonize different types of degraded soils and induce biocrust formation (Adessi et al., 2021) thanks to their capacity to withstand the environmental stresses. However, when the restoration is tested in natural conditions in degraded dryland soils, the success inducing biocrust formation has been limited, until now, to inoculated sand dunes in cold deserts in China (Park et al., 2017). Román, Chamizo, et al. (2021) did not observe an increase in soil chlorophyll *a* (chl *a*) content, as a proxy of biocrust formation, compared to controls after inoculating cyanobacteria in field, even after hardening cultures by dehydration combined with a progressive increase of light intensity. In Bowker et al. (2020), the authors showed that a hardening protocol based on desiccation events, exposure to high light over time, and fluctuating temperatures on inocula composed of cyanobacteria, mosses, and lichens did not perform better than nonhardened ones. Conversely, Giraldo-Silva et al. (2020) observed an increase in chl *a* content in inoculated soils when cyanobacteria were pre-hardened by wet-dry cycles and a progressive increase in light intensity. However, they maintained a watering regime outdoors, ameliorating field conditions. This study investigates the resistance mechanisms of a cyanobacterial strain to stresses encountered in its natural environment, with the aim to develop a hardening method that could be applied in future field experiments with success.

For this goal, we followed a physiological and genomic approach. The selected strain, ULC029, isolated in one of the most extreme regions on Earth, continental Antarctica, is a thin homocytous filamentous cyanobacterium that was identified as *Leptolyngbya frigida* ANT.LH52B.3 at the time of isolation (Taton et al., 2006) but was later renamed as *Stenomitos frigidus*, which

belongs to a genus commonly observed in dryland biocrusts (Muñoz-Martín et al., 2019; Patzelt et al., 2014; Pushkareva et al., 2015; Williams et al., 2016). This strain shows a high 16S rRNA gene similarity (99.4%; Shalygin et al., 2020) with several *Leptolyngbya* strains isolated in warm semiarid biocrusts in SE Spain (Roncero-Ramos et al., 2019). Moreover, it has been shown that the relative abundance of these strains increases in incipient biocrusts with ecosystem degradation (Roncero-Ramos et al., 2020). Also, other thin filamentous strains identified as *Leptolyngbya* have already shown promising results in inducing biocrusts after soil inoculation (Mugnai et al., 2018). Yet, authors studying the impact of stress have shown that the exposure to individual or combined stress factors may produce a different response (Joshi et al., 2018; Rai et al., 2013). Some stress factors might activate mechanisms usually associated with another one. For example, osmotic stress (Dillon et al., 2002) or desiccation (Fleming & Castenholz, 2007) led to the production of the UV-screening pigment scytonemin. Therefore, we hypothesize that culturing cyanobacteria under multiple stress factors would improve the hardening method, leading to a better performance after inoculation in the field. To test our hypothesis, two ecophysiological assays were performed with cultures of ULC029 grown in the laboratory. First, we analyzed the influence of increasing salinity, and decreasing nutrient concentration on cyanobacterial growth, the efficiency of photosynthesis (F_v/F_m), and pigment production (chl *a*, carotenoids, and scytonemin). After characterizing the salinity tolerance of the strain, we analyzed the influence of a multiple-stress environment that included osmotic, desiccation, and UVR stresses. In this second assay, we also quantified the osmoprotectants (sucrose and trehalose) and the exopolysaccharides. Finally, to identify all the potential resistance mechanisms of the strain ULC029, we sequenced and assembled the genome after a hybrid, which reduced the error rate and gaps and, thus, increased genome assembly quality (Koren et al., 2012). We identified the presence or absence of a selection of 140 genes known to be involved in cyanobacterial resistance mechanisms to environmental stress.

MATERIALS AND METHODS

Cyanobacterial strain and culture conditions

The unicyanobacterial strain *Stenomitos frigidus* ULC029 (synonym *Leptolyngbya frigida*; Figure S1 in the Supporting Information), from the BCCM/ULC Cyanobacterial Collection (Liège, Belgium) was isolated from a benthic microbial mat in the lake 52 (76°23′ S, 69°24′ E) in the Larsemann Hills in the Prydz Bay region of East Antarctica in 1998 (Taton et al., 2006). It was identified as *L. frigida* by morphological analysis and sequencing of the 16S rRNA gene and the ITS rRNA region between the 16S and 23S rRNA genes (ITS rRNA region; Taton et al., 2006), but it was later reclassified to *S. frigidus* (Shalygin et al., 2020). A maximum likelihood tree (Figure S2 in the Supporting Information) built with all 16S rRNA gene sequences showing more than 96.3% similarity to ULC029 showed its close relatedness (circa 90%) with other strains from extreme biotopes (Antarctica and semiarid and dry environments). The climatic conditions in the Larsemann Hills where ULC029 was isolated are characterized by a maximum snow annual precipitation of 250 mm, strong katabatic winds, and air temperatures that range from maximum values of 4 or even 10°C in summer and mean monthly temperatures between –15 and –18°C in winter (Sabbe et al., 2004). When the lakes partially melt in summer, the water temperature can increase up to 8°C. The lake 52 where the mat sample was collected is oligotrophic and oligosaline (conductivity: 138 $\mu S \cdot cm^{-1}$). The pH was 6.7 and the alkalinity was 0.1 $meq \cdot L^{-1}$ (Sabbe et al., 2004).

ULC029 was cultured at 12°C under a continuous irradiance of 6 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$ and shaking at 80 rpm (IKA KS 4000). The culture medium was BG11 (Rippka et al., 1979).

Salinity thresholds of *Stenomitos frigidus* ULC029

To determine the salt tolerance of the strain, it was cultured in BG11 with increasing concentrations of NaCl: 0, 0.5, 0.2, 0.7, 1.8, and 2.5 M. A homogeneous starting inoculum was prepared by centrifuging the cyanobacterial biomass at 6360 *g* for 7 min. The supernatant was discarded, and the pellet was resuspended in BG11 to reach a proportion of 20:80 of ULC029 pellet (solid) in BG11 (liquid). Then, six different inocula were prepared by mixing 1 mL of the prepared suspension of 20% of ULC029 in BG11 and 1 mL of solutions with increasing concentrations of NaCl in sterilized milliQ H_2O. The final concentration of all compounds in BG11 after mixing the suspension with the increasing concentrations of NaCl was half for all of them. Twelve multiwell plates were used to carry out the experiment. Each well was filled with 2 mL of inoculum, and four replicates were set up for each treatment and time point (0 h, 2 h, 21 d, and 32 d; Figure S3 in the Supporting Information). All multi-well plates were incubated at 12°C under an irradiance of 6 $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$. At each time point, four replicates of each treatment were used to measure the efficiency of the photosynthesis (F_v/F_m) and the concentration of three pigments: chl *a*, carotenoids, and scytonemin. At the end of the experiment,

the cultures were subjected to one of the two additional steps: either a UVR treatment or a recovery phase. The first treatment was carried out by placing the multi-well plates containing four replicates of each treatment at day 32 inside a dark box provided with UVA, UVB, and PAR lamps. The total irradiance received by the cultures was 10 and 2 $W \cdot m^{-2}$ of UVA and UVB respectively, and 11 μmol photons $\cdot m^{-2} \cdot s^{-1}$ of PAR. The plates were left in the box for 3 days, and after that, the same measurements as before were performed. For the second treatment, the biomass of four replicates of each treatment was transferred at day 32 to new multi-well plates filled with 2 mL of BG11 and was allowed to recover for 33 days. Then, the same two measurements were performed. Note that the BG11 medium used in the recovery phase was not diluted in half.

Would a mild salinity stress improve the resistance of *Stenomitos frigidus* ULC029 to desiccation and UVR?

When ULC029 was cultivated in BG11/2 + 0.05 or 0.2 M NaCl, its growth and recovery appeared to improve, as did its resistance to UVR, following the data from Experiment 1. To better understand the potential effect of NaCl on the capacity of ULC029 to withstand stress, we carried out a second experiment with two media: BG11 (as a control) and BG11 + 0.2 M NaCl (the highest salt concentration that allowed ULC029 to grow in Experiment 1; Figure S3). During a pre-adaptation phase, ULC029 was cultured in flasks with either of the culture media (periodically refreshed) for more than 4 months. As in Experiment 1, the biomass was centrifuged and resuspended in fresh medium to obtain two homogeneous inocula with the same quantity of biomass. Two milliliters of each inocula were filtered on GF/F filters of Ø 25 mm (Whatman) for each replicate. Four replicates per treatment and measurement were set up. Three consecutive treatments were performed: the control, which consisted of analyzing samples just after filtering; the desiccation treatment; and the rehydration + UVR treatment. The desiccation treatment consisted of transferring the filters into a chamber filled with 100 g of silica gel at the bottom to keep an HR of 10%, which allowed us to desiccate the filters in less than 1 day. Temperature and % RH inside the desiccation box were monitored with an iButton Hygrochron logger (Maxim Integrated, USA) every 5 min (Figure S4 in the Supporting Information). The filters were kept desiccated for 1 week. The desiccation chamber was opened, and four replicates were kept for each measurement. The rest of the filters were transferred to multi-well plates filled with 2 mL of BG11 medium to rehydrate them. Plates were placed into the UV box with the same irradiances as in Experiment 1 and left inside for 4 days. After that, the last replicates were taken and all measurements were performed on them. For this experiment, additional variables were measured: the exopolysaccharides and the osmolytes (trehalose and sucrose) content.

Culture monitoring and physiological measurements

In both experiments at each time point (including the initial inoculum), the culture growth was monitored by quantifying the chl *a* content as a proxy of photosynthetic biomass. It was extracted by mixing the biomass of each replicate with acetone 90% in a proportion of 1:5, vortexing, and leaving it for 24 h in darkness at 4°C (following Castle et al., 2011; Giraldo-Silva et al., 2020). After that, the samples were centrifuged (6360 *g*, 7 min at 4°C), and the absorbance at 680 and 750 nm was measured with a UV–visible spectrophotometer (SPECORD 50, Analytik Jena). The quantity of two other pigments was also measured in the same extract at 490 (carotenoids) and 384 nm (scytonemin). The trichromatic equation was used to correct for the interferences of the other pigments (Garcia-Pichel & Castenholz, 1991). The maximum photochemical quantum yield of photosystem II (F_v/F_m), considered as an indicator of photosynthesis efficiency and cellular stress, was measured with a PAM fluorometer (AquaPen AP-C, Photon Systems Instruments, CZ) after culture adaptation in the dark for 15 min.

Three additional variables were measured in Experiment 2, using four replicates per treatment and time point, and the EPS was extracted to find out if production was affected by the addition of NaCl to the medium, desiccation, and/or the rehydration + UVR treatment. The extractions were divided into two categories, the loosely bound EPS (LB-EPS) and the tightly bound EPS (TB-EPS), following Rossi et al. (2018). Extracting the LB-EPS was done by adding 3 mL of miliQ H_2O, vortexing, and incubating the filters for 15 min at 20°C. After that, samples were centrifuged (4000 *g*, 20 min, 9°C), the supernatant was transferred to a new tube, and the same process was repeated twice, ending up with a final extract of 9 mL, classified as LB-EPS fraction. After that, 3 mL of 0.1 M Na_2EDTA were added to the filters, vortexed, and incubated overnight at 20°C. After centrifuging as described above, this step was repeated twice, decreasing the incubation time to 20 min. At the end, we had an extract of 9 mL, classified as the TB-EPS fraction. All the extractions were quantified by the phenol-sulfuric acid method (Dubois et al., 1956), measuring the absorbance (488 nm) with a spectrophotometer (SPECORD 50, Analytik Jena). The total quantity of EPS was obtained by summing up both fractions. The other two variables measured in Experiment 2

were the quantification of two osmolytes: trehalose and sucrose. Filters were freeze-dried, eluted, and measured with a Dionex System HPAED-PAD (High-performance Anion Exchange Chromatography with Pulsed Amperometric Detection) equipped with a CarboPAC PA100 column of 4×250 mm. The eluents used were: (A) HPLC-grade water + 100 mM NaOH and (B) HPLC-grade water + 100 mM NaOH + 600 mM of sodium acetate. The system was operated at 30°C with a selected flow rate of 1 mL · min^{-1}.

DNA extraction and (meta) genome sequencing

A fresh culture of ULC029 in BG11 was used to extract genomic DNA using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the recommendations of the manufacturer. Three tubes with 1 mL of the culture were centrifuged (4211 *g* for 5 min); the supernatant was discarded and washed again in BG11 several times to get rid of as many heterotrophic bacteria as possible, as it was not an axenic culture. In the end, we extracted the DNA from these three pellets. The integrity of the gDNA was checked by electrophoresis and the DNA quality and concentration were quantified with NanoVue (Biochrom). Finally, the three extractions were mixed to obtain enough DNA (>5 μg) and sent to the sequencing platform (GIGA Genomics, University of Liege).

Both short and long reads of the gDNA were sequenced. Library preparation was performed using Nextera XT libraries (for Illumina sequencing) and Native barcoding genomic DNA kit (EXP-NBD114/LSK109) for Nanopore sequencing. Short reads (2×300 bp, 100× coverage) were sequenced by the Illumina Miseq v3 sequencing platform. Long reads sequences (coverage 100×) were obtained using a MinION Flow Cell R9.4.1 (Oxford Nanopore Technologies, UK).

Bioinformatic analyses

As the ULC029 culture was not axenic, its genomic dataset was treated as a metagenome and binned to obtain cyanobacterial-specific contigs. The Illumina reads were filtered and trimmed using iu-filter-quality-minoche from Anvi'o (Eren et al., 2013), and their quality was checked with fastQC (Andrews, 2010). After checking the Nanopore sequences quality with NanoPlot (v1.29.0; De Coster et al., 2018), NanoFilt (v2.6; De Coster et al., 2018) was used to filter out reads with a quality score lower than 10 and a length shorter than 500 bp. Long reads were assembled using Flye (metagenome mode; v2.9; Kolmogorov et al., 2020). Afterward, filtered Illumina sequences were aligned to the assembled genome with bwa (v0.7.12; Li & Durbin, 2009). The obtained sam files were converted to bam format and indexed using Samtools (v1.3.1; Danecek et al., 2021). Then, the assembled genome was polished with pilon (v1.23; Walker et al., 2014), using the indexed bam files as input. Binning was performed with CONCOCT (v1.1; Alneberg et al., 2014). Finally, we used GTDBtk (Parks et al., 2018) to determine the taxonomic placement of the bins and CheckM (v.1.1.6; Parks et al., 2014) to assess their completeness and contamination level. The assembly statistics were obtained with QUAST (default settings; v2.3; Gurevich et al., 2013). The genome was plotted using Proksee (Stothard & Wishart, 2005) and manually edited with Inkscape v1.2. The genome assembly has been deposited in GenBank (Bioproject number: PRJNA1006388).

Genome annotation

A contig database was created using Anvi'o (Eren et al., 2015, 2021). Then, a first annotation was performed using a web version of Dfast (Tanizawa et al., 2016). Afterward, genes in the database were annotated with functions from the NCBI's Clusters of Orthologous Groups (COGs) using DIAMOND (fast mode; Buchfink et al., 2021) and with KEGG (Kyoto Encyclopedia of Genes and Genomes) identifiers using GhostKOALA (Kanehisa et al., 2016). Gene annotations and sequences are available in the Supporting Information: Tables S1 and S2 in the Supporting Information, respectively.

Identifying genes related to stress resistance

Genes known to be involved in resistance mechanisms against stress (Al-Hosani et al., 2015; Chatterjee et al., 2020; Chrismas et al., 2016, 2018; Ferreira & Garcia-Pichel, 2016; Klicki et al., 2018; Kopf et al., 2015; Kvíderová et al., 2019; Mosca et al., 2021; Napoli et al., 2021; Pathak et al., 2019; Pereira et al., 2009, 2019; Rajeev et al., 2013; Shang et al., 2019; Shimura et al., 2015; Sinetova & Los, 2016; Soule et al., 2013; Ye et al., 2021) were searched in the generated contig database and classified in the following groups: (i) Production of the EPS matrix, (ii) Photoprotection, (iii) Oxidative stress protection, (iv) DNA repair, and (v) Osmotic stress protection. When genes were not located in the database, they were searched manually using tblastn against the ULC029 assembled genome with an e-value threshold of 1e-5 and a score higher than 50 bits (following Pearson, 2013). The queries used can be located in the supplementary material (Table S3 in the Supporting Information). Alignments were validated by checking if the Pfam functional protein domains determined with SMART (normal mode; Letunic & Bork, 2020) were the same as those from the query.

Statistical analyses

In Experiment 1, results were analyzed to determine the effect of the increasing NaCl concentration on the pigments' concentrations at different time points individually. Values of each time point were tested for normality and homoscedasticity with the Shapiro–Wilk (shapiro.test function, stats package) and Levene (function leveneTest, car package) tests, respectively. A two-way ANOVA (anova function, car package) followed by the Tukey post hoc test (TukeyHSD function, stats package) was applied when data followed a normal distribution and was homoscedastic; otherwise, the Kruskal–Wallis test (package conover.test) was applied. Moreover, the pigments contents after incubating the cultures under UVR from days 32 to 35 were analyzed by applying a one-way ANOVA (anova function, car package) and the Tukey post hoc test (TukeyHSD function, stats package) for chl *a* and carotenoid values independently.

Data obtained in Experiment 2 were also tested for normality and homocedasticity, as previously described. To determine the influence of the desiccation and rehydration+UVR treatments in cyanobacteria cultured in media with different NaCl concentrations, the following variables were analyzed: chl *a*, carotenoids, scytonemin, trehalose, sucrose, and EPS content. In all cases, we performed a two-way ANOVA (anova function, car package) followed by the Tukey post hoc test (package lsmeans).

Figures were plotted using the ggplot2 package and manually edited using Inkscape v1.2. All analyses were performed with R 4.0.5 (R Core Team, 2017).

RESULTS

Determining the salt tolerance of *Stenomitos frigidus* ULC029

Stenomitos frigidus ULC029 could grow and survive for 32 days in BG11/2 medium with a NaCl concentration up to 0.7 M (Figure 1a). After 32 days, the highest chl *a* concentration was observed in the medium with 0.2 M NaCl, followed by 0.05 and 0 M. For NaCl concentrations of 1.8 and 2.5 M, the chl *a* concentration decreased with time and was null after 1 month. Moreover, there was no recovery, as no chl *a* was detected after transferring and incubating the cultures in BG11 without NaCl for another 33 days. Interestingly, the highest chl *a* values after recovery were in cultures growing in 0.05 and 0.2 M NaCl, followed by 0 M NaCl (Figure 1a). Such high values of chl *a* concentration may be caused by the higher nutrient availability provided by the non-diluted BG11 used for recovery in comparison with the BG11/2 medium used in the previous phase. However, although *S. frigidus* kept producing chl *a* in BG11 + 0.7 M NaCl during the recovery, the amount of chl *a* was significantly lower than with lower salt concentrations (Figure 1a). Therefore, 0.7 M NaCl is the maximum concentration that *S. frigidus* can withstand among the tested concentrations. In Figure 1b, the carotenoid concentration pattern is very similar to the one followed by chl *a*. In conclusion, the highest concentrations of chl *a* were obtained in 0.2, 0.05, and 0 M of NaCl (Figure 1b) after cultivation for 32 days, but also when a subsequent recovery was performed in 0 M NaCl for 33 days. Scytonemin was not detected in any treatments of Experiment 1.

F_v/F_m values (~0; Figure S5 in the Supporting Information) showed that *Stenomitos frigidus* was photosynthetically inactive in 0.7, 1.8, and 2.5 M NaCl during the growth phase. Only the strain cultured in 0.7 M NaCl could recover after 33 days in BG11 medium, and it became photosynthetically active again (F_v/F_m 0.3; Figure 1). Cultures in BG11/2 with 0.2 M NaCl were also photosynthetically inactive at day 32 (~0; Figure S5), but they could fully recover and reach the same values as at time 0 (~0.2; Figure S5). Finally, cultures with a low salinity stress (0.05 M NaCl) and the controls (0 M NaCl) were always photosynthetically active, and both followed a similar pattern with the highest value at day 21 (0.3 and 0.4, respectively; Figure S5). Moreover, Figure S5 shows that culturing *S. frigidus* in diluted BG11 did not cause stress to the cyanobacterium, as the F_v/F_m values are similar to those shown 33 days after being transferred to non-diluted BG11.

When cultures were exposed to UVR after 32 days of cultivation in media with different NaCl concentrations, both chl *a* and carotenoid concentrations were significantly lower compared to the non-exposed replicates (Figure 1c,d). However, cultures previously grown in media with 0 and 0.05 NaCl did not show stress and were photosynthetically active even after UVR exposure (Figure S5). Cultures in 0.2 M NaCl had higher F_v/F_m values, and thus were more photosynthetically efficient, after 3 days of UVR compared to the control, which was measured before UVR exposure (Figure S5).

Ecophysiological response of *Stenomitos frigidus* ULC029 to multiple stress factors (osmotic stress pretreatment, desiccation, and UVR)

Contrary to the results obtained in Experiment 1, none of the variables measured showed significant differences between the cultures cultivated in medium with 0 or 0.2 M NaCl in the absence of other stresses (Control treatment; Figure 2). However, significant differences were observed in most of variables after desiccating the cultures for 1 week and after the 4 days of rehydration and UVR exposure.

Chlorophyll *a* concentration did not decrease compared to the control after maintaining the cultures

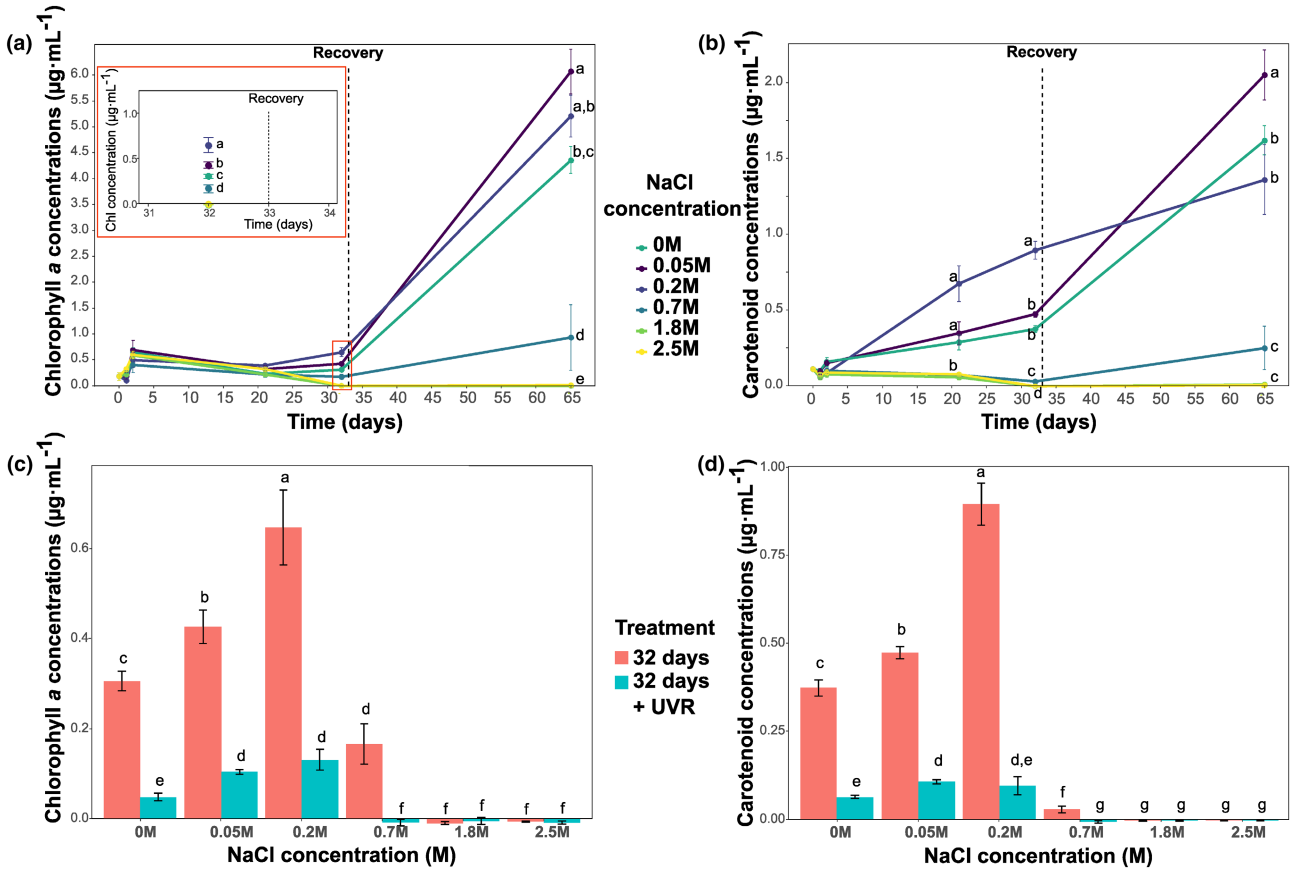

FIGURE 1 Pigment contents produced by *Stenomitos frigidus* ULC029 cultured at different concentrations of NaCl during 32 days of incubation and after subsequent recovery in BG11 medium for 33 days in Experiment 1: (a) chlorophyll *a* and (b) carotenoid concentrations during the 32 days of incubation; (c) chlorophyll *a* and (d) carotenoid concentrations after 32 days compared with the same cultures exposed to 3 additional days of UVR. All data are means ($n = 4$). The error bars represent $\pm SE$ and letters indicate significant differences ($p < 0.05$) among treatments. Test performed, *df*, and critical values are, respectively: (a) 32 days (TukeyHSD, 12, 4.20); (a) recovery and (b) 20 days and recovery (TukeyHSD, 18, 4.49) and 33 days (Conover-Iman, 18, 20.64); (c and d) (TukeyHSD, 12, 4.20).

desiccated at 10% HR for 1 week after a pre-treatment in BG11 with 0.2 M NaCl (Figure 3). This pre-treatment did not prevent the chl *a* from decreasing after the rehydration + UVR treatment; however, the concentration was significantly higher (0.4 $\mu g \cdot mL^{-1}$; Figure 2) than in the absence of NaCl pre-treatment (0.03 $\mu g \cdot mL^{-1}$; Figure 2). The different pre-treatments did not seem to influence the carotenoid concentrations after desiccation, but they were again significantly higher in the rehydrated-UVR exposed cultures pretreated with 0.2 M NaCl (Figure 2). Interestingly, in this experiment, we detected the production of scytonemin for the first time (Figure 2) after rehydrating and exposing the cultures to UVR (Figure 2). A significantly higher concentration was observed in cultures preincubated with 0.2 M NaCl (0.31 $\mu g \cdot mL^{-1}$; Figure 2) compared to 0 M NaCl.

The production of EPS increased after desiccation in the absence of a NaCl pre-treatment. However, it increased further after rehydration and UVR exposure regardless of the pre-treatment (Figure 2). The total content of EPS was always significantly lower when *Stenomitos frigidus* was pre-grown in medium with 0.2 M NaCl, except for the control (Figure 2). The loosely bound fraction of EPS was significantly different between salinity pre-treatments (Figure S6 in the Supporting Information). This fraction was always significantly higher in cultures pre-treated with 0 M NaCl, even in the control treatment (Figure S6). The only significant increase of EPS after desiccation compared to the control was overserved in the LB-EPS fraction of the 0 M NaCl pre-treatment (Figure S6). After the rehydration + UVR treatment, both EPS fractions significantly increased compared with the control and desiccation treatments (Figure S6).

The production of the osmoprotectants trehalose and sucrose was significantly higher after the desiccation and rehydration + UVR treatments in the absence of a NaCl pre-treatment, except for the control treatments in which there were no differences (Figure 2). Their concentrations significantly increased after desiccation compared to the control for both pre-incubation types, followed by a significant decrease after rehydration + UVR, whatever the pre-treatment type (Figure 2). The highest concentrations were observed

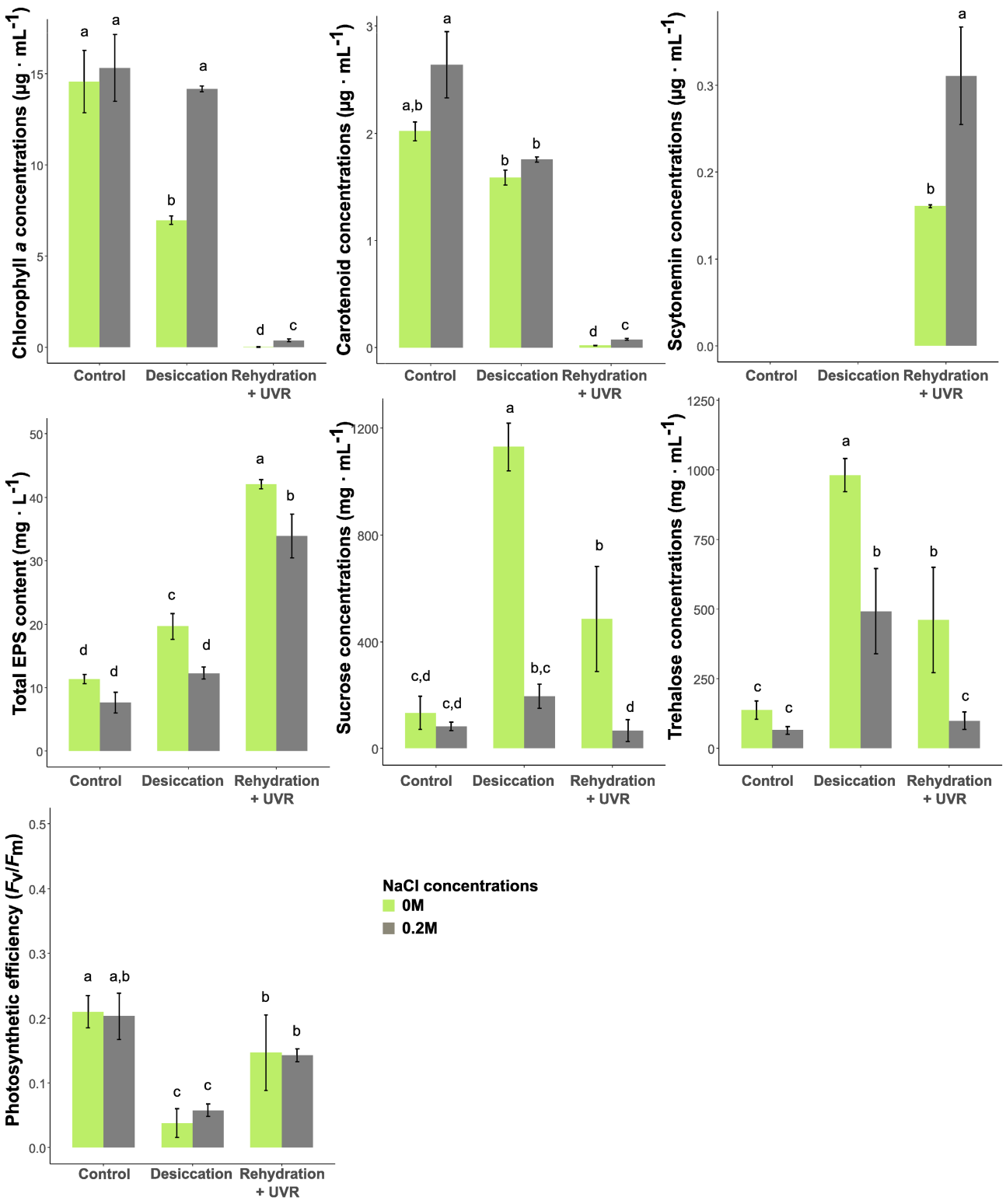

FIGURE 2 Influence of 1 week of desiccation and subsequent 4 days of rehydration combined with UVR exposure on *Stenomitos frigidus* ULC029 cultured in BG11 with 0 or 0.2 M NaCl compared with the control (initial values before desiccation) on different variables: chlorophyll *a*, carotenoids, scytonemin, and total exopolysaccharides contents; sucrose and trehalose concentrations; and the photosynthetic efficiency (F_v/F_m). All data are means ($n = 4$). The error bars represent $\pm SE$ and letters indicate significant differences ($p < 0.05$) among treatments. The test performed, *df* and critical values are in all cases: TukeyHSD, 18, and 4.49, respectively.

after desiccation of the strain pre-grown without NaCl, namely for sucrose (1130.3 mg · mL^{-1}), followed by trehalose (981 mg · mL^{-1}; Figure 2).

The efficiency of the photosynthesis (F_v/F_m) did not show differences when *Stenomitos frigidus* had been pre-cultured with or without 0.2 M NaCl in any treatment

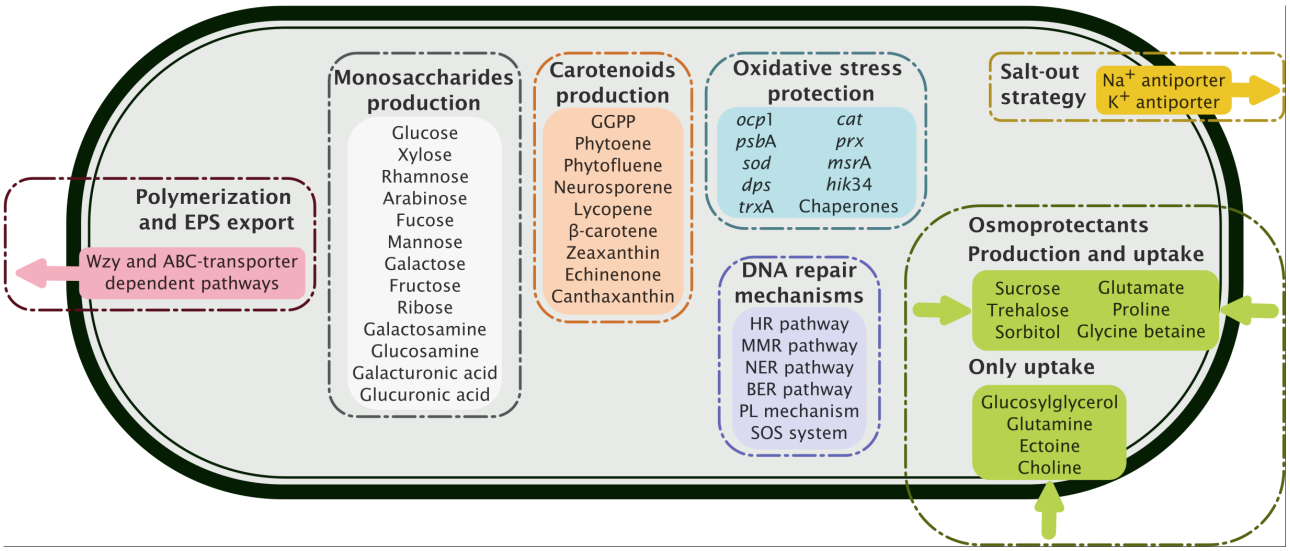

FIGURE 3 Complete pathways related to stress resistance mechanisms observed in ULC029.

TABLE 1 Genome characteristics obtained from results produced by Quast, DFAST, anvi'o, and CheckM software.

	***Stenomitos frigidus* ULC029**
Contigs	3
Largest contig (bp)	6,320,863
Genome size (bp)	6,403,461
GC (%)	50.68
No. of CDSs	5587
No. of rRNA operons	3
No. of tRNA	47
No. of CRISPRS	15
Coding ratio (%)	80.6
COGs assignation	4042
KEGGs assignation	2026
CheckM completeness (%)	99.29
CheckM contamination (%)	0.35

Abbreviation: CRISPRS, Clustered Regularly Interspaced Short Palindromic Repeats.

(Figure 2). However, the strain was photosynthetically inactive after desiccation (~0; Figure 2), as expected, and started recovering its initial values of F_v/F_m after rehydration and UVR treatment (Figure 2).

Mechanisms to cope with stress potentially observed in the genome of ULC029

Genome assembly

After sequencing and combining short and long reads of *Stenomitos frigidus* ULC029, we obtained eight bins (Table S4 in the Supporting Information). One of them was identified by CheckM as a cyanobacterium, with a completeness of 99.29% and a contamination of 0.35% (Table 1). This genome was assigned to *S. frigidus* ULC029 and had a total length of 6,403,461 bp and a GC% of 50.68 (Table 1). It was composed of 1 long contig (6,320,863 bp) and 2 short contigs (Figure S7 in the Supporting Information), with a coding ratio of 80.6% (Table 1). Gene annotation by DFAST resulted in 5587 CDSs, six rRNAs, and 47 tRNAs (Table 1). GTDBtk classification placed the genome in the *Stenomitos* cluster (see Figure S7) with *S. frigidus* ULC018 (GCF_003003795.1), which showed the closest ANI value (79.22%) and was isolated from a neighboring lake microbial mat in the same Antarctic region (*Stenomitos* sp. ANT.LH53B.2; Taton et al., 2006).

Mechanisms to cope with osmotic, desiccation, and UVR stress

Genome annotation was enhanced after using the COG and KEGG databases, which resulted in 4042 and 2026 annotated genes, respectively (Table S1). Among them, we identified 140 genes coding for proteins known to be involved in resistance mechanisms to desiccation, UVR, and salinity stress (Table S5 in the Supporting Information): production of the EPS matrix, photoprotection, oxidative stress protection, DNA repair, and osmotic stress protection. One hundred thirteen of these were annotated with the COGs and/or KEGGs databases, and 27 were identified using tblastn against the protein sequence of interest downloaded from NCBI (Table S3).

We detected genes coding for proteins related to the production of the following monosaccharides that could compose the EPS matrix: glucose (*glg*AC),

xylose (*uxs*), rhamnose (*rfb*ABCD), arabinose (*uxe*), fucose (*fcl*), mannose (*man*A), galactose (*gal*E), fructose (*pgi*), ribose (*rpi*A), glucosamine (*glm*U), glucuronic acid (*ugd*), galactosamine (*wbp*P), and galacturonic acid (*cap*1J; Tables S3 and S5). Although three pathways were described in cyanobacteria to assemble and export polysaccharides (Pereira et al., 2019), only two of them were complete in this genome: the Wzy (*wza*, *wzb*, *wzc*, *wzx*, and *wzy*) and the ABC-transporter (*kps*TMED) dependent pathways (Tables S3 and S5). Two of the main proteins of the synthase-dependent pathway could not be annotated—AlgE and AlgK—while Alg8 and Alg44 were observed (Table S5).

The photoprotection strategies of cyanobacteria focused on the production of pigments (carotenoids and scytonemin) and of mycosporine-like amino acids. The genes encoding enzymes involved in the production of most carotenoids were annotated: *crt*BEHOPQRW and *cru*A (Table S5). Therefore, the list of carotenoids that *Stenomitos frigidus* ULC029 could potentially produce included at least: geranylgeranylpyrophosphate (GGPP), phytoene, phytofluene, neurosporene, lycopene, β-carotene, zeaxanthin, echinenone, and canthaxanthin (Table S5). Concerning the synthesis pathway of scytonemin, we could not find all the proteins and genes involved. The annotated genes composing the scytonemin operon (Klicki et al., 2018) were *trp*ABCDEFG and *tyr*A, which are involved in the biosynthesis of tryptophan and p-hydroxyphenylpyruvic acid, respectively, and *aro*BG, which is involved in the shikimic acid pathway (Table S5). The genes coding for proteins involved in the last steps of the scytonemin biosynthesis, *scy*ABDE, were assigned by tblastn (Tables S3 and S5), except for the gene *scy*C. Additionally, the genes belonging to the *ebo* cluster, involved in the translocation of scytonemin to the periplasm, were not annotated (Table S3). Finally, all the genes (*mys*ABE) coding for enzymes involved in the synthesis of MAAs were assigned by tblastn, except for *mys*C, which encodes the protein producing mycosporine-glycine, the branching point for the rest of MAAs.

Several oxidative stress protection mechanisms were identified in the ULC029 genome. First, the gene *ocp*1, coding for the orange-carotenoid-binding protein, involved in nonphotochemical quenching and thermal dissipation of high light energy (Chrismas et al., 2018), was assigned by tblastn (Tables S3 and S5). Another annotated gene was *psb*A (Table S5), which codes for a chlorophyll-binding protein (D1) of PSII. This gene is induced by light stress and would confer oxidative stress protection to the photosynthetic system (Kopf et al., 2015). One of the mechanisms against reactive oxygen species, the superoxide dismutase (*sod*), was also annotated (Table S5), specifically, the combination of Fe and Mn superoxide dismutase (SOD), which is the type found in filamentous non-heterocystous cyanobacteria (Napoli et al., 2021). Other genes related to oxidative damage protection that were detected were: a methionine sulfoxide reductase (*msr*A), the Mn containing catalase (*cat*), the DNA-binding protein (*dps*), and peroxiredoxin (*prx*). Several genes coding for molecular chaperones involved in protein folding were annotated too: *dna*KJ, *gro*El/ES, *htp*G, and *clp*B, as well as two of their regulatory factors, *hik*34 and *trx*A (Table S5).

We identified four DNA repair mechanisms in the genome, as shown in Table S5. First, the homologous recombination pathway, composed of the genes *hol*B (DNA polymerase III), *rec*QG (ATP-dependent DNA helicase), *rec*J (single-stranded-DNA-specific exonuclease), *ssb* (single-strand DNA-binding protein), *rec*ORF (recombinational DNA repair proteins), *rec*A (recombinase), *uvr*ABC (hollyday junction enzymes), and *pri*A (primosomal protein). The genes *hol*B, *rec*J, and *ssb* are also involved in the mismatch repair (MMR) pathway, together with the genes *lig* (DNA ligase), *uvr*D (DNA helicase), *mut*SL (ATPases), *exo*VII (exonuclease VII), and *dam* (site-specific DNA-adenine methylase). The nucleotide excision repair (NER) pathway was also detected: *uvr*ABCD and *lig*, *dpol* (DNA polymerase I), and *mfd* (transcription-repair coupling factor). The base excision repair (BER) pathway was also complete in the genome: *fpg* (nucleotide glycosylase), *nei* (endonuclease VIII), *nth* (endonuclease III), *xth* (exonuclease III), *mpg* and *alk*A (DNA-3-methyladenine glycosylase and II), *mut*Y (A/G-specific adenine glycosylase), and *udg* (uracil-DNA glycosylase), as well as *lig* and *dpol*. Another DNA repair mechanism, *p*L, coding for a DNA photolyase, which corrects DNA damage caused by solar light, and *lex*A, a transcriptional repressor involved in the regulation of the SOS repair system, was also observed.

Salt-out strategy and the osmoprotectant production and uptake (Table S5) involves the genes coding for the Na^+ and K^+ antiporters (*nh*A and *ktr*AB, respectively) and the *ktr*E gene (that contributes to the import of K^+ into the cells), and these were detected in the genome. However, only one member of the *kdp* gene family (*kdp*D), related to the import of H^+ into cells, was detected. The *kdp*D gene is involved in the activation of the rest of the *kdp* genes. A second resistance strategy in highly saline environments is the synthesis and/or uptake into the cells of osmoprotectants. *Stenomitos frigidus* ULC029 has the main genes involved in the production of the following osmoprotectants: sucrose (*spp* and *sps*), trehalose (*tre*YZX and *glg*ABC), sorbitol (*srl*D), glutamate (*glt*S), proline (*pro*ABC), glycine betaine by the choline pathway (*bet*AB), and the glycine pathway (*GNMT* and *DMT*). However, this strain would not be capable of

producing glycosylglycerol, as both genes coding for the enzymes involved in its production pathway are missing (*ggp*S and *ggp*P TS4), nor ectoine, because the gene coding for the L-ectoine synthase (*ect*C) was not detected (Table S3). The mechanisms for osmoprotectant uptake that were detected are: ABC-type sucrose/trehalose/glucosylglycerol transport system (*ggt*A), ABC transporter for glutamine and glutamate (*gln*HPQ and *glu*A), neutral aa transporter N-I type (*nat*ABCDE) and one of the genes required for the N-II type transporter of acidic and polar aa (*nat*F), glycine betaine and proline transporter (*opu*ABC), and ABC transporter for ectoine, glycine betaine, proline and choline uptake (*pro*XWV).

DISCUSSION

The strain ULC029 belongs to a cluster of 16S rRNA gene sequences with two lineages, one containing Antarctic strains (Rego et al., 2019; Taton et al., 2006) and one with strains from Spanish arid deserts and a dry Siberian steppe (Temraleeva, 2018; Roncero-Ramos et al., 2019; Figure S2). The similarity between the sequences of ULC029 and CAU10 was 99% (1367 positions), which may indicate that they belong to the same species and which underlines the relevance of this experimental data for soil restoration strategies. Although taxonomic uncertainties and the recent establishment of the genus *Stenomitos* complicate the comparison with literature data, it seems probable that similar resistance mechanisms are present in the thin filamentous genera from the Leptolyngbyaceae (Strunecký et al., 2022).

Several resistance mechanisms to abiotic stress factors have been already reported for strains belonging to the genus *Leptolyngbya*, in which ULC029 was previously included, such as the production of photoprotective pigments, carotenoids, and scytonemin (Kokabi et al., 2019; Lin & Wu, 2014); the production of mycosporine-like amino acids and the identification of the related genes (Rossi et al., 2012; Shimura et al., 2015); the production of trehalose (Shimura et al., 2015), proline (Lin & Wu, 2014), sucrose, glycogen, and glucosylglycerol (Keshari et al., 2019) as compatible solutes during events of osmotic stress or desiccation; and the increase of the activity of the ROS-scavenger enzymes peroxidase and superoxide dismutase after drought stress (Lin & Wu, 2014). In this study, we analyzed *Stenomitos frigidus* ULC029 for this strain's ecophysiological response to osmotic, desiccation, and UVR stress. Moreover, we identified a high-quality genome for this strain, being the second one identified as *S. frigidus* that is available in public databases, and screened it for genes related to cellular stress responses.

The response of *Stenomitos frigidus* to osmotic stress and UVR exposure

We observed that *Stenomitos frigidus* ULC029 could not tolerate NaCl concentrations higher than 0.7 M, and its optimal NaCl concentration was between 0.05 and 0.2 M (Figure 1). Thus, it belongs to the cyanobacterial group with low tolerance to salinity (Mackay et al., 1984). We also have shown that after 1 month of cultivation in BG11/2 medium with 0.2 and 0.7 M NaCl, *S. frigidus* ULC029 showed signs of stress (Figure S5). However, it was resilient, showing a full recovery of the F_v/F_m values after the osmotic stress and low nutrient concentration ceased (recovery phase, Figure S5). The similarity among F_v/F_m values of cultures in 0 M NaCl medium also showed that decreasing the nutrient concentration in the media (BG11/2) did not cause higher stress compared to BG11 (recovery phase, Figure S5). However, the great increase in chl *a* concentration 33 days after recovery in 0 M NaCl BG11, compared to the previous time points within the incubation in BG11/2, could show that apart from its capacity to recover, under a higher availability of nutrients, its growth can be increased (Figure 1a). Moreover, although a decrease in chl *a* concentration was generally observed at all salinities and after 3 days of exposure to UVR, cultures growing in media with 0.2 M NaCl showed the highest chl *a* concentration after UVR (Figure 1c). However, they also showed higher stress, that is, lower photosynthetic efficiency, than cultures growing in 0 and 0.05 M NaCl (Figure S5). Carotenoid concentrations showed a similar pattern to chl *a* concentration (Figure 1) and are related to the production of primary carotenoids, those bound to the photosystems and participating in the photosynthesis process (Mulders et al., 2015; Rehakova et al., 2019). Primary carotenoids that *S. frigidus* ULC029 could produce were zeaxanthin and echinenone (Table S5; Figure 3). However, it has been shown (Mulders et al., 2015; Rehakova et al., 2019) that primary carotenoids are usually degraded under stress and replaced by secondary carotenoids that are involved in photoprotection, such as canthaxanthin or β-carotene, for which pathways are complete in ULC029 genome as well (Table S5; Figure 3). Therefore, the carotenoid composition before and after UV radiation could be different with increasing concentrations of secondary carotenoids after UVR. Finally, as the addition of 0.2 M NaCl was stressing the strain but it could still survive and grow, we selected this concentration to further explore the influence of osmotic stress on the cyanobacterial resistance to desiccation and UVR in a second experiment. Moreover, we did not select the diluted medium (BG11/2) because it was not stressing the cyanobacteria by means of photosynthetic efficiency, but the biomass production could have been affected by the lower nutrient availability.

Performance of *Stenomitos frigidus* under multiple stress factors

In Experiment 2, after 4 months of pre-adapting ULC029 to the osmotic stress induced by 0.2 M NaCl, there were no significant differences in the variables measured compared to non-pre-treated cultures (Control treatment, Figure 2). Concerning chl *a* and carotenoid concentration, we found significant differences between cultures in 0 and 0.2 M NaCl BG11/2 in Experiment 1 (Figure 1a). The main difference between both experiments was that in Experiment 1 cyanobacteria were cultured for 1 month in BG11/2 and in Experiment 2 for more than 4 months in BG11. We hypothesize that the longer pre-adaptation period in Experiment 2 caused of the lack of differences in pigment concentrations in the Control treatment in Experiment 2 (Figure 2). Moreover, this hardening pre-treatment by osmotic stress did activate resistance mechanisms that allowed ULC029 to withstand better the subsequent desiccation and UVR treatments compared to the non-acclimated ones, as was shown by a significantly higher concentration of chl *a* and similar F_v/F_m values (Figure 2).

The EPS production increased significantly after desiccation, rehydration, and UVR (Figure 2), which aligns with previous work (Li et al., 2022; Shang et al., 2019; Wu et al., 2021). Given the genome annotation results (Tables S3 and S5), the two pathways that are complete and could be triggered to assemble and export EPS are the Wzy and the ABC-transporter-dependent pathways (Figure 3). Furthermore, according to the genome annotation, the EPS matrix could be composed of glucose, xylose, rhamnose, arabinose, fucose, mannose, galactose, fructose, ribose, glucosamine, glucuronic acid, galactosamine, and galacturonic acid. The possibility of producing an EPS matrix with such a complex composition of monosaccharides could also favor the colonization of the inoculated soils by a more diverse microbial community (Chamizo et al., 2019). Also, most of these sugars have been observed in artificially induced biocrusts (Román, Chamizo, et al., 2021; Román, Roncero-Ramos, et al., 2021), and some of them have been related to a protective role under desiccation, such as the galacturonic acids (Tamaru et al., 2005). Despite the increase in EPS production after desiccation and UVR stress, the hardening treatment by osmotic stress coincided with a significantly lower EPS concentration compared to non-acclimated cultures (Figure 2). This is in line with the controversy already reported by others (Cruz et al., 2020) about the increasing and decreasing evolution of EPS production found in different cyanobacteria cultivated with NaCl.

As for the production of osmoprotectants, the strain we used was capable of significantly increasing the concentration of both sucrose and trehalose after 1 week under high dehydration stress (Figure 2), as had been previously shown by others (Raanan et al., 2016). Sucrose concentration values were slightly higher than the trehalose ones in most cases (Figure 2), suggesting that sucrose might have a more important role in the response to stress than trehalose in this cyanobacterium. Both osmoprotectants would be acting against cellular osmotic unbalance due to dehydration (Potts, 1994) by preventing protein denaturation (Borges et al., 2002) and stabilizing membrane structure (Hincha & Hagemann, 2004). After 3 days of rehydration and UVR exposure (Figure 2), the concentration of both osmoprotectants significantly decreased. Fagliarone et al. (2020) have shown that *Chroococcidiopsis* sp. desiccated cells under UVR could maintain a basal number corresponding to the sucrose and trehalose production pathways. This suggests that if we had exposed *Stenomitos frigidus* to UVR in a desiccated instead of a rehydrated state, the concentrations of sucrose and trehalose might have been higher. The decrease in osmoprotectants was larger in pre-treated cultures (0.2 M NaCl), with concentration values similar to those before desiccation (Control; Figure 2). Contrary to previous work (Keshari et al., 2019), the accumulation of both osmoprotectants in all pre-treated cultures was lower than in those not pre-treated (0 M NaCl; Figure 2). This has been previously shown in *Anabaena fertilissima* (Swapnil & Rai, 2018), in which the production of sucrose and trehalose increased with NaCl concentration until a maximum value was reached (276% and 280%, respectively, of control), and the production started to decrease. These authors also tested how the addition of other molecules caused osmotic stress, such as the addition of Na_2SO_4, which showed a lower toxicity than NaCl and induced a higher production of sucrose and trehalose. Therefore, using different molecules to induce osmotic stress in the pre-adaptation medium could help increase the production of these osmoprotectants in *S. frigidus* as well. *Stenomitos frigidus* ULC029 had all the genes required for the production and/or uptake of trehalose and sucrose, also of sorbitol, glucosylglycerol, glutamate, proline, ectoine, and glycine betaine (Table S5; Figure 3). The lower production of sucrose and trehalose under osmotic stress (Figure 2) might be counteracted in *S. frigidus* ULC029 by the synthesis of any of these other osmoprotectants. However, the ability to accumulate glucosylglycerol and glycine betaine is normally considered an indicator of a haloterant or halophilic cyanobacterium (Pade & Hagemann, 2014), which is not the case for ULC029, as it was not able to tolerate NaCl concentrations higher than 0.7 M (Figure 1). This deviation from the classical pattern has already been shown in marine cyanobacterial species lacking the genes for glucosylglycerol, such as *Crocosphaera watsonii* and *Trichodesmium erythraeum* (Pade & Hagemann, 2014). Our results

support the importance of enlarging the cyanobacterial genome databases and suggest the need for revising the cyanobacterial classification by osmotic resistance based on the type of osmoprotectant produced, first by performing metabolomics analyses to determine if, after exposure to different stress factors, such as salinity, these genes are expressed.

Finally, both pigments measured (carotenoids and scytonemin) showed significantly higher values after desiccation and UVR exposure in cultures pre-treated with NaCl (Figure 2). Our hypothesis is that in Experiment 1, the carotenoids probably mostly consisted of secondary carotenoids synthetized after desiccation and UVR stress treatments. Despite the significantly higher concentration of carotenoids in cultures pre-treated with NaCl in Experiment 2, the carotenoid concentrations decreased in both cases after desiccation and UVR treatments (Figure 2). These results contrast previous work, which has shown increases in carotenoid concentrations after UV radiation (Kokabi et al., 2019) and desiccation (Lin & Wu, 2014) in strains belonging to the *Leptolyngbya* genus. Carotenoids serve as photoprotectants and quenchers (Llewellyn et al., 2020). The decrease in primary carotenoids might be related to the decrease in chl *a*, as they are involved in the photosynthesis process and are usually degraded under stress (Mulders et al., 2015; Rehakova et al., 2019). Lin and Wu (2014) discussed how only drought-tolerant cyanobacteria showed an increase in carotenoids after desiccation. As *Stenomitos frigidus* ULC029 is a freshwater cyanobacteria, its production of secondary carotenoids might not be activated under desiccation stress, which could explain the unexpected decrease of total carotenoids after desiccation and UVR stress.

Interestingly, the production of scytonemin was detected by the extraction method used after the desiccation and UVR treatments in both cultures in Experiment 2 (Figure 3). In contrast, there was no production of scytonemin in Experiment 1 after exposure to UVR. However, after the desiccation and UVR treatments in Experiment 2, scytonemin increased in both cultures and was significantly higher in the strain pre-acclimated with 0.2 M NaCl (Figure 3). Thus, it seems that a previous desiccation step could be necessary to trigger the production of scytonemin in this strain, and its production could be also enhanced by adding preliminary osmotic stress. Desiccation and osmotic stress in fact trigger similar physiological responses in cyanobacteria (Billi & Potts, 2002), especially during the early stages of air drying. It has been previously shown (Dillon et al., 2002; Fleming & Castenholz, 2007) that scytonemin production is affected by periodic exposures to desiccation, which agrees with our results and could be explained by scytonemin's probable role in the stabilization of the EPS matrix (Gao, 2017). Conversely, to our knowledge, this is the first time that an increase in scytonemin production linked to a preliminary osmotic stress has been observed. Previously, this was not detected; for example, Bennett and Soule (2022) showed no activation of the genes related to scytonemin production after osmotic stress in *Nostoc punctiforme* ATCC 29133, similar to our results in Experiment 1. This finding supports the hypothesis that scytonemin production can be induced by a more generalized environmental stress in cyanobacteria (Dillon et al., 2002; Rehakova et al., 2019). However, after analyzing the genome annotation, we could not find all genes related to scytonemin production, especially *scy*C and the *ebo* cluster (Table S3). The former gene is involved in a key step of scytonemin biosynthesis (Ferreira & Garcia-Pichel, 2016) and the latter in the translocation of scytonemin to the periplasm (Klicki et al., 2018). This might be based on gaps in the genome assembly or the removal of these genes during the genome assembly (Zhou et al., 2014).

Future prospects for cyanobacterial hardening

Previous research (Giraldo-Silva et al., 2019, 2020; Román, Roncero-Ramos, et al., 2021) showed that a hardening pre-treatment could improve soil cyanobacteria growth in field conditions, using chl *a* concentration as a proxy. Our work shows that hardening pre-treatments could be improved by using a multiple-stress hardening treatment for *Stenomitos frigidus* ULC029 (Figure 3). The cultivation of ULC029 with NaCl osmotic stress, but growth (Figure 1), had a higher resistance to desiccation and UVR. This was shown by similar or higher values of chl *a* concentration in pre-treated cultures compared with cultures without osmotic stress (Figure 3). The resistance mechanisms involved were not identified by our ecophysiological measurements, as neither EPS nor sucrose nor trehalose concentrations were higher in cultures under osmotic stress compared with controls (control; Figure 3). However, the genome annotation shows the capacity of this strain to produce many other osmoprotectants, enzymes related to oxidative stress—such as the superoxide dismutase (SOD), the orange carotenoid protein (OCP), and the chlorophyll-binding protein (D1), several DNA repair pathways (Tables S3 and S5; Figure 3)—which were not analyzed during our experiments, and could have reduced osmotic stress. Yet, the exposure of *S. frigidus* ULC029 to UVR after desiccation (Figure 3) or without this step (Figure 1), showed that the production of scytonemin was triggered, especially under osmotic stress (Figure 3). Moreover, EPS production was significantly enhanced after desiccation and UVR exposure in both cases (Figure 3). In view of these results, we propose a hardening treatment based on the application of sequential stress factors to the cultures, which would always include at least one stress factor

related to osmotic unbalance (osmotic or desiccation stress), and if UVR is applied, that application should be more effective afterward. However, more studies analyzing the response of cyanobacteria to multiple stress factors are required to fully understand the mechanisms that contribute to their survival in extreme environments, and thus, how they should be applied as nature-based biotechnological tools for soil restoration or as biofertilizers in agriculture. In addition, the number of cyanobacterial genomes in public databases with high completeness and low contamination values is low (Cornet et al., 2018): only seven to date, if we only consider polar strains (Pessi et al., 2023). None of these genomes belongs to the genus *Stenomitos*, which is closely related to *Leptolyngbya* sp. BC1307 (Chrismas et al., 2018). The release of more high-quality genomes would improve our knowledge of the resistance mechanisms that could be activated under stress, as well as enable the application of transcriptomic analyses to monitor cyanobacterial responses to stress.

CONCLUSIONS

More efficient and effective hardening methods of cyanobacterial inocula are required to improve biocrust restoration. In this work, we tested a feasible and easy-to-do hardening method for *Stenomitos frigidus* ULC029 based on a pre-treatment of adding salt to the culture medium to induce osmotic stress. This method allowed the strain to maintain its chl *a* concentration under a severe desiccation event, followed by rehydration with UVR exposure. The latter induced an expected decrease of chl *a* concentration values, which were, however, slightly higher in cultures under osmotic stress. Here, we show that the sequential application of multiple stress factors to cyanobacterial cultures induced the production of three of the main mechanisms needed for survival in dryland soils: scytonemin, carotenoids, and EPS. Additionally, we provide a new high-quality genome for this Antarctic strain, which could be used in future studies to deepen our knowledge of its resistance mechanisms by, for example, performing transcriptomics.

AUTHOR CONTRIBUTIONS

Beatriz Roncero-Ramos: Conceptualization (lead); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); resources (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Valentina Savaglia:** Conceptualization (equal); writing – review and editing (equal). **Benoit Durieu:** Formal analysis (equal); writing – review and editing (equal). **Isabelle Van de Vreken:** Formal analysis (equal). **Aurore Richel:** Formal analysis (equal); funding acquisition (supporting). **Annick Wilmotte:** Conceptualization (equal); funding acquisition (equal); investigation (equal); resources (equal); writing – review and editing (equal).

ACKNOWLEDGMENTS

BRR was supported by the IPD-STEMA Programme and the Special Funds for Research (R.DIVE.0899-J-F-I, University of Liège), and by the Junta de Andalucía (PAIDI-DOCTOR 21_00571), VS and BD by the PhD FRIA fellowship from the FRS-FNRS, and AW is Senior Research Associate of the FRS-FNRS. We would like to thank Anne Catherine Ahn and Kim Beets for their help with the cultivation of the strain of the BCCM/ULC cyanobacterial culture collection, Emmanuel Mignolet for the building of the UV box, Elie Verleyen for borrowing the UV sensor, and Luc Cornet for discussion of the genomic data.

ORCID

Beatriz Roncero-Ramos https://orcid.org/0000-0002-7253-8374

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Roncero-Ramos, B., Savaglia, V., Durieu, B., Van de Vreken, I., Richel, A., & Wilmotte, A. (2024). Ecophysiological and genomic approaches to cyanobacterial hardening for restoration. *Journal of Phycology*, *00*, 1–18. https://doi.org/10.1111/jpy.13436