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Proteomics of long-term acclimation of the desert cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 in perchlorate-rich medium and its implications for *in situ* resource utilization on Mars

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ABSTRACT

Space exploration demands sustainable technologies to minimize reliance on Earth-based resources. The extreme-tolerant cyanobacterium *Chroococcidiopsis* sp. CCMEE 029 exhibits remarkable resistance to perchlorate salts ubiquitously found on Martian soil, holding promise for in situ resource utilization. Exploring the proteomic responses to this chaotropic agent is fundamental for understanding the mechanisms of salt-stress response in cyanobacteria and to develop biotechnologies based on local resources to support human outposts. Hence, using liquid chromatography–tandem mass spectrometry, we analyzed the protein expression after 21 days of cultivation in the presence of increasing perchlorate concentrations with triplicates per experimental condition ($|\log_2 FC| \geq 0.5$; $FDR \leq 0.05$). This study shows that this cyanobacterium displays a broad suite of enzymatic and non-enzymatic mechanisms for mitigating reactive oxygen species, as well as formaldehyde assimilation capacity from the RuMP pathway. The upregulation of polyhydroxybutyrate and siderophore biosynthesis further supports its suitability for putative bioplastic production, nutrient mobilization, and plant protection in extraterrestrial environments. Overall, *Chroococcidiopsis* sp. CCMEE 029 modulates its metabolism to maintain energy balance, support growth, and synthesize biotechnologically relevant compounds. This versatility underscores its suitability as a candidate for Martian bioprocessing, where robustness, low resource requirements and multifunctionality are paramount. By providing molecular insights into cyanobacterial stress responses, this research advances our understanding of microbial adaptation to environmental constraints occurring on Mars and lays the groundwork for optimizing microbial biotechnologies to support astronauts in long-term missions on the red planet.

1. Introduction

The next stage of human space exploration is approaching with NASA's Artemis program, which aims to return humans to the Moon as a stepping stone toward establishing outposts on Mars (NASA, 2024). However, sustaining long-duration manned missions in deep space by continuously supplying life-support consumables from Earth is unrealistic. A critical component of developing self-sufficient extraterrestrial outposts is in situ resource utilization (ISRU), an approach encompassing a broad range of technologies that enable the use of local materials at mission destinations (Volger et al., 2020). Biology-driven use of local resources harnessing microorganisms, known as Bio-ISRU, has gained momentum: microorganisms can act as self-reproducing modifiable factories to produce biomass, oxygen, food and pharmaceutical compounds through ISRU (Averesch et al., 2021; 2023). The concept of living off-the-land will be even more important on Mars rather than the Moon, since its increased distance results in greater transport costs, travel time and failure risks. However, microorganisms growing in Martian regolith may be harmed by the presence of perchlorates, which were detected at varying concentrations as 0.5 % (2.4 mM) at the

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NASA's Phoenix landing site or the 1 % (20 mM) at Gale Crater (Clark and Kounaves, 2016; Sutter et al., 2017). On Earth, the perchlorate origin derives from photochemical reactions in the atmosphere, where it accumulates in dry environments subjected to scarce precipitation, so that the largest perchlorate concentrations ranging from 0.03 wt % to 0.6 % have been reported in the Atacama Desert (Catling et al., 2010; Michalski et al., 2004). Despite the high oxidation state (+7) of the chlorine atom, perchlorates are powerful oxidants only when heated, while they are rather stable at room temperature and lower temperatures (Urbansky, 1998). As with any high salt concentration, perchlorates can cause osmotic stress, and due to their highly chaotropic nature they destabilize biochemically important macromolecules and trigger oxidative stress (Urbansky, 1998).

However, most microorganisms likely cannot make use of raw Martian soil, which is poor in organics, fixed nitrogen and readily available minerals (Verseux et al., 2016). Hence, a Bio-ISRU strategy to "power" bio-production intends to use cyanobacteria as primary producers to feed bacteria used in biotechnological productions (Rothschild, 2016). In this context, rock-weathering cyanobacteria are promising candidates due to their capability of using minerals present in the Lunar and Martian regolith simulants as growth substrates (Olsson-Francis et al., 2010). In order to contribute to support long-term human presence on Mars, it was proposed to use biomass of cyanobacteria cultivated with Martian regolith as feedstock for bacteria or as plant biofertilizer (Verseux et al., 2016). In such an endeavor, the cyanobacterium *Anabaena* sp. PCC 7938 has been identified among other *Anabaena* strains as the best candidate for growth on Mars regolith simulant and for yielding biomass to feed *Escherichia coli* (Ramalho et al., 2022a).

Attempts to cultivate *Anabaena* sp. PCC 7938 with increasing amounts of Martian regolith simulant identified as optimal a 3 mM Na-perchlorate concentration, corresponding to 50 g/L Martian regolith containing 0.6 wt % perchlorate (Ramalho et al., 2022b).

A recent screening of 17 cyanobacterial strains identified five strains as the most perchlorate-resistant, among which are three *Chroococcidiopsis* strains (Rzymski et al., 2022). Compared to *Anabaena* sp. PCC 7938, the desert strain *Chroococcidiopsis* sp. CCMEE 029 can tolerate up to 100 mM Na-perchlorate (Billi et al., 2021) and as a proof-of-concept for supporting bioproduction systems, the biomass yielded after 3-week cultivation with 2.4 mM perchlorate (provided as a mixture of Mg- and Ca-perchlorate) was used to grow *E. coli* (Fernandez et al., 2023). Notably, Ca-perchlorate is a strong chaotropic agent, while Na-perchlorate is a weak chaotropic agent (Cray et al., 2013). A defence strategy against perchlorate stress based on cell aggregation has been reported for *Chlamydomonas reinhardtii* through multi-omics analysis (Zhang et al., 2023). A recent transcriptome analysis also revealed the toxicity and defense mechanisms of the exposure to perchlorate in *Synechocystis* (Zhang et al., 2025).

A first insight into *Chroococcidiopsis* sp. CCMEE 029′s short- and long-term acclimation to perchlorates was obtained during a 3-week cultivation with 2.4 mM perchlorate (Fagliarone et al., 2024). Besides a progressive increase in ROS production throughout the whole period, genes for the biosynthesis of the compatible solutes trehalose and sucrose were overexpressed during the first 2 weeks, whereas genes involved in the antioxidant defence and oxidative DNA damage repair remained upregulated throughout the 3-week period (Fagliarone et al., 2023). However, there is still a gap in knowledge on the mechanisms used by cyanobacteria to reprogram their metabolism for long-term acclimation to perchlorate salts, which represents a limit for developing ISRU-based technologies. Besides being feedstock for biotechnological relevant bacteria, cyanobacteria grown on perchlorate-rich Martian soil could be an undeciphered reservoir of secondary metabolites of relevance for agriculture on Mars since they could be beneficial for plant growth, nutrient availability, or even for enhancing the overall productivity of crops (Macário et al., 2022). Indeed, salt-stressed cyanobacteria have been shown to activate secondary metabolic pathways, producing valuable molecules such as antibiotics, volatile organic compounds, biostimulants and biofertilizers in response to abiotic stress (Macário et al., 2022; Santini et al., 2021).

In the present work, the long-term acclimation to perchlorate of *Chroococcidiopsis* sp. CCMEE 029 was investigated for the first time by using a proteomic approach combining unbiased SPEED-based sample preparation and liquid chromatography tandem mass spectrometry (LC-MS/MS) through data-independent acquisition (DIA) (Doellinger et al., 2020, 2023). Hence, *Chroococcidiopsis* sp. CCMEE 029 was grown for 21 days in the presence of increasing perchlorate concentrations, namely 2.4 mM, 12 mM and 24 mM, in the 60 and 40 % mixture of Mg- and Ca-perchlorate as reported by the NASA's Phoenix Mars Lander (Hassler et al., 2014). These concentrations were selected because in the range of the perchlorates detected on the Martian surface and ranging from 2.4 mM to 20 mM (Clark and Kounaves, 2016; Sutter et al., 2017) and supported by previously reported growth of this cyanobacterium with Martian regolith simulant containing 2.4 mM (Fernandez et al., 2023). A focus was given on the perchlorate effects on photosynthesis and carbon metabolism, enzymatic and non-enzymatic antioxidant defense as well as on biosynthetic gene clusters by integrating a bioinformatic genome mining analysis.

2. Material and methods

2.1. Test organism and sample preparation

Chroococcidiopsis sp. CCMEE 029 was isolated by Roseli Ocampo-Friedmann from cryptoendolithic growth in sandstone in the Negev Desert (Israel) and maintained at the University of Rome Tor Vergata, as part of the Culture Collection of Microorganisms from Extreme Environments (CCMEE) established by E. Imre Friedmann. Cells were cultivated for 21 days in BG-11 medium containing increasing perchlorate concentrations (2.4, 12 and 24 mM) provided as a 60 % and 40 % mixture of Mg- and Ca-perchlorate and in standard BG-11 medium as control. To prepare a BG-11 medium containing 2.4 mM perchlorate the following 10X stock solution was prepared: 1.60 g/L of $Mg(ClO_4)_2 \cdot 6H_2O$ and 2.20 g/L of $Ca(ClO4)_2 \cdot 4H_2O$. Consequently, 10X stock solutions were prepared for 24 mM and 12 mM perchlorate. BG-11 medium composition (containing 0.075 g/L $MgSO_4{\cdot}7H_2O$ and 0.036 g/L $CaCl_2{\cdot}2H_2O$) was not modified before perchlorate salts were added, no antibiotics were present in the medium. Cyanobacterial cultures (three replicates) were incubated for 21 days, at 25 °C under a constant photon flux density of 10 μmol/m^2/s, provided by a white LED light (4000 K, OSRAM, MI, Italy). A constant photon flux was used for higher biomass yield (Fleischhacker-Daffert et al., 2024), with no shaking. These conditions were maintained identical across the treatments. Cyanobacterial growth was monitored by determining the optical density at 730 nm (OD_{730}) (Clark et al., 2018).

Samples were prepared using filter-aided Sample Preparation by Easy Extraction and Digestion (fa-SPEED) (Doellinger, et al., 2020). Cells were lysed with 50 μL trifluoroacetic acid (TFA) for 3 min at 70 °C. Afterwards, samples were neutralized with 500 μL 2 M tris(hydroxymethyl)aminomethane (TRIS). After adding 55 μL reduction/alkylation buffer (100 mM tris(2-carboxyethyl) phosphine/400 mM 2-Chloracetamide), the samples were incubated at 95 °C for 5 min. Protein concentrations were determined by turbidity measurements at 360 nm using an Implen NP-80 (Implen, Munich, Germany). 50 μg of proteins were diluted to 40 μL using a 10:1 (v/v) mixture of 2 M TrisBase and TFA, mixed with 160 μL acetone and captured on Ultrafree®-MC (0.5 mL) centrifugal devices, 0.2 μm, PTFE (Merck) at 5000 × *g* for 2 min. The samples were washed successively with 200 μl 80 % (v/v) acetone, 200 μL 100 % acetone and 200 μL n-pentane at 5000 × *g* for 2 min each. Afterwards, 40 μL of digestion buffer (50 mM TEAB) containing trypsin (1:10 [enzyme-to-protein ratio] Trypsin Gold, Mass Spectrometry Grade [Promega]) was added to the filter followed by incubation at 37 °C for 1 h. The peptides were eluted by centrifugation at 5.000 × *g* for 2 min and the filter was washed subsequently with 40 µL 0.1 % (v/v) TFA.

2.2. Liquid chromatography and mass spectrometry

Peptides were analyzed on an Evosep One (Evosep, Odense, Denmark) liquid chromatography system coupled online via the CaptiveSpray source to a timsTOF HT mass spectrometer (Bruker Daltonics, Bremen, Germany). 1 µg of peptides were manually loaded onto Evotips Pure (Evosep) and separated using the 60 samples per day (SPD) method on the respective performance column (8 cm x 150 µm, 1.9 µm, Evosep). Column temperature was kept at 40 °C using a column toaster (Bruker Daltonics) and peptides were ionized using electrospray with a CaptiveSpray emitter (20 µm i.d., Bruker Daltonics) at a capillary voltage of 1750 V. Spectra were acquired in diaPASEF mode in the m/z range of 100–1700 and in the ion mobility (IM) range of 0.65 – 1.35 Vs/cm^2. Each scan cycle comprised of 12 diaPASEF scans, which consisted of two IM windows with variable isolation window widths adjusted to the precursor densities using py_diAID (Skowronek et al., 2022) covering the m/z range of 350 – 1150. The collision energy was decreased as a function of the IM from 59 eV at $1/K_0 = 1.6$ Vs/cm to 20 eV at $1/K_0 = 0.6$ Vs/cm. The accumulation and ramp times were specified as 100 ms.

2.3. Data analysis

Protein sequences of *Chroococcidiopsis* sp. CCMEE 029 were obtained from the NCBI database (taxid:155,894, downloaded 01/05/24). Spectral libraries were predicted using the deep-learning algorithm implemented in DIA-NN (version 1.9.2) (Demichev et al., 2020) with strict trypsin specificity (KR not P) allowing up to one missed cleavage site in the m/z range of 350 – 1150 with charge states of 2 – 4 for all peptides consisting of 7–30 amino acids with enabled N-terminal methionine excision and cysteine carbamidomethylation. The LC-IMS-MS/MS data were analyzed in DIA-NN (version 1.9.2) using default settings including a false discovery rate (FDR) of 1 % for precursor identifications with enabled “match between run” (MBR) option. The resulting pg_matrix.tsv (Lib.Q.Value = 1 %) file was used for further analysis in Perseus (version 2.1.3) (Tyanova et al., 2016). Relative protein quantification was done based on log(2)-transformed values, and missing values were imputed from normal distribution, without prior data normalization, as recommended by best practices for DEA methods in proteomics for DIA data employing the DIA-NN software (Peng et al., 2024). The resulting matrix was therefore queried to Perseus to perform principal component analysis. Subsequently, to perform the differential expression analysis, the limma package (Kammers et al., 2015) was employed creating six contrasts, namely 2.4 mM vs CTRL, 12 mM vs CTRL, 24 mM vs CTRL, 12 mM vs 2.4 mM and 24 mM vs 12 mM. We considered to be differentially expressed all the proteins with a $|\log_2 FC| \geq 0.5$; and significant with FDR ≤ 0.05 (calculated with Benjamini–Hochberg correction method).

Results were then filtered for significant values and functional enrichment of differentially expressed proteins (DEPs) was evaluated through the STRING database version 12.0.0 (Szklarczyk et al., 2021) and Kyoto Encyclopedia of Genes and Genomes (KEGG) and pathview package (Luo et al., 2013).

2.4. Genome mining for biosynthetic gene clusters

In order to identify possible biosynthetic gene clusters in *Chroococcidiopsis* sp. CCMEE 029 genome (Accession: GCF_023558375.1), we employed three different specific annotation softwares, namely antiSMASH v8.0.1 bacterial version with “relaxed” detection strictness parameter and with every extra feature activated, while SanntiS and GECCO were run with default parameters (Carrol et al., 2021; Blin et al., 2025; Sanchez et al., 2023).

Moreover, we functionally annotated protein-coding genes with the eggNOG-mapper v2.1.12 tool and EggNog5 database, Tmbed, SignalP6 and Phobius softwares (Bernhofer and Rost, 2022; Cantalapiedra et al., 2021; Huerta-Cepas et al., 2019; Käll et al., 2004; Teufel et al., 2022).

Figures were generated with Plotly, Canva, antiSMASH, Shinycircos-V2.0, GeneViewer and Perseus softwares (van der Velden, 2025; Wang et al., 2023).

3. Results

3.1. Reduced cyanobacterial growth in perchlorate-rich medium

The growth of *Chroococcidiopsis* sp. CCMEE 029 was evaluated after 21-day cultivation with BG-11 medium containing increasing perchlorate concentrations, namely 2.4 mM, 12 mM and 24 mM, provided as a 60 and 40 % mixture of Mg- and Ca-perchlorate. The readings of the optical density (OD 730 $_{\text{nm}}$) showed that the presence of 2.4 mM perchlorate ions reduced the growth to about 85.4 % compared to that in control BG-11 medium. A further growth reduction occurred in the presence of 12 mM and 24 mM resulting in OD 730 $_{\text{nm}}$ values corresponding to about 56.9 % and 25.2 % relative to the control, respectively (Fig. 1A).

3.2. Principal component analysis and global proteomic analysis

To gain insights into the global acclimation process of *Chroococcidiopsis* sp. CCMEE 029 we used a differential expression analysis (DEA), KEGG pathway and gene set enrichment analysis (GSEA) along with an investigation of the presence and expression of biosynthetic gene clusters (BGCs). We identified 3272 protein groups, representing approximately 60 % of the total coding sequences of this cyanobacterium. A distinct clustering between the salt-grown cyanobacterial cells and the control was shown by the Principal Component Analysis (PCA), that supported high similarity between the replicates (Fig. 1B). The first principal component (PC1) accounted for 52.2 % of the variance, the second principal component (PC2) accounted for 12.6 % variance and the third principal component (PC3) accounted for 6.2 % of the variance. These components effectively separated the control replicates from the experimental conditions into 3 main groups. The control and 2.4 mM perchlorate replicates were clearly distinguished, whereas the 12 mM and 24 mM perchlorate conditions appeared more homogeneous, reflecting similar expression profiles. This indicates high consistency among replicates and suggests a different perchlorate-stress response at 2.4 mM compared to 12 mM and 24 mM treatments, as illustrated in Fig. 1B.

3.3. Higher perchlorate concentrations result in increased downregulation of proteins

The differential expression analysis (DEA) revealed that, compared to control, 125 proteins were upregulated in cyanobacterial cells grown in each salt-containing BG-11 medium. This indicates a core response to perchlorate-induced stress (Fig. 2). Furthermore, 183 proteins were upregulated only in cyanobacterial cells grown in 12 mM and in 24 mM perchlorate-containing media, which suggests a common response to higher perchlorate-concentrations. Specifically, 24 mM perchlorate positively induced the highest number of differentially upregulated proteins (629), followed by 12 mM (355) and 2.4 mM (251) (Fig. 2).

On the other hand, the expression profiles of 107 proteins revealed downregulation when the cyanobacterium was grown in each one of the perchlorate-containing media. In terms of treatments, the same trend of upregulated proteins is followed by the downregulated ones. In fact, higher salt concentrations result in more downregulated proteins, while the 2.4 mM treatment showed the least number of negatively expressed proteins relative to the controls.

In particular, in the DEA results against 2.4 mM, we noticed a slight upregulation in both 24 mM and 12 mM treatments respectively of 211 and 87 proteins, corresponding to a decrease of 76.5 % and 85 %

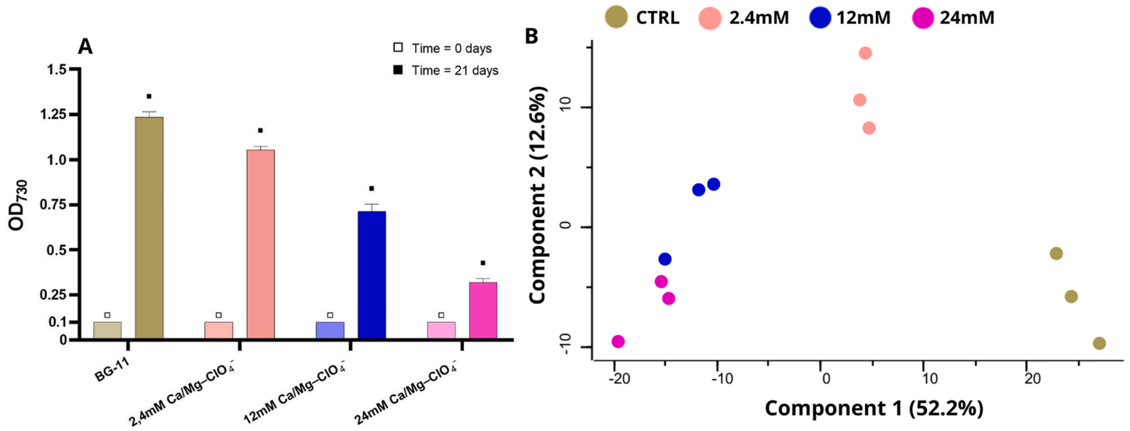

Fig. 1. Effects of cultivation for 21 days in increasing perchlorate concentrations on *Chroococcidiopsis* sp. CCMEE 029. Cell densities of cyanobacterial cultures grown in BG-11 medium with 2.4 mM, 12 mM and 24 mM perchlorate ions (A). PCA of the proteome obtained after cultivation with different perchlorate concentrations (B).

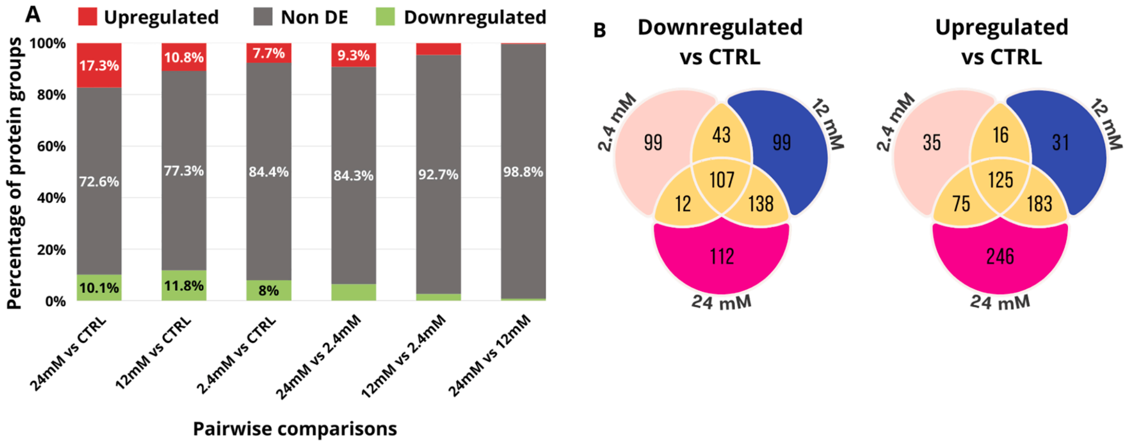

Fig. 2. Fractions of upregulated, downregulated and non-differentially expressed (Non DE) proteins in each pairwise combination of *Chroococcidiopsis* sp. CCMEE 029 cultivated for 21 days with increasing perchlorate concentrations (and control) (A). Venn diagrams for up- and down-regulated proteins against the controls (B).

compared to the control.

Whereas 211 and 87 proteins were downregulated in the 24 mM and 12 mM treatments, corresponding to a reduction of 43 % and 78 % relative to control. Finally, in the 24 mM versus 12 mM comparison we observed a slight deregulation, resulting in only 12 upregulated and 26 downregulated proteins, in accordance with replicates treatments' distribution in PCA (Fig. 1B).

3.4. *Different concentrations result in different long-term acclimation to perchlorate*

The GSEA analysis, conducted in pairwise comparisons, revealed common trends in major biological processes (BPs) such as (i) cation and (ii) metal ion transport, which resulted upregulated in each perchlorate-containing medium compared to control. Similarly, we observed commonly downregulated BPs including: (iii) photosynthesis and related biosynthetic pathways, like pigment, chlorophyll and porphyrin BPs; (iv) glutamine family amino acid metabolic process, involved in glutamine, glutamate, arginine and proline biosynthesis; (v) nucleotide biosynthesis and metabolism, and (vi) nitrate transmembrane transport, directly linked to bicarbonate transmembrane transport (Fig. 3).

The 12 mM and 24 mM perchlorate concentrations showed downregulated enriched BPs in respect to 2.4 mM, such as nitrate and cation transmembrane transport and nucleotide biosynthetic process, while the 12 mM treatment resulted in the upregulation of antibiotic metabolic/biosynthetic process (Supplementary Fig. 1).

The 24 mM vs 12 mM enrichment showed a wide repertoire of downregulated BPs related to cell wall, polysaccharide and lipid biosynthesis. On the other hand, translation, amide biosynthetic process and photosynthesis were upregulated (Supplementary Fig. 2).

The most involved cellular compounds/string compartments enrichments in cyanobacterial cells grown with perchlorates were mainly represented by the outer membrane-bounded periplasmic space, cell envelope (upregulated) and photosystem (downregulated) (Supplementary Table 1).

Moreover, the KEGG pathway enrichment analysis showed a common downregulation in photosynthesis and biosynthesis of amino acids pathways in each perchlorate-containing medium compared to control, while 2.4 mM caused mainly an upregulation in nonribosomal peptide structures and biosynthesis of siderophores through nonribosomal peptide synthase, starch and sucrose metabolism. Interestingly, phenylalanine, tyrosine and tryptophan biosynthesis pathways in 2.4 mM and 12 mM were downregulated, like 2-oxocarboxylic acid metabolism in 2.4 mM (Supplementary Fig. 3).

3.5. *Altered photosynthesis and carbon metabolism rerouting in long-term acclimation to perchlorate*

After 21 days of cultivation in BG-11 medium with increasing perchlorate content, the photosynthetic activity-related proteins were greatly affected under the higher salt concentration. We observed downregulation of photosystems, antenna proteins, cytochromes and

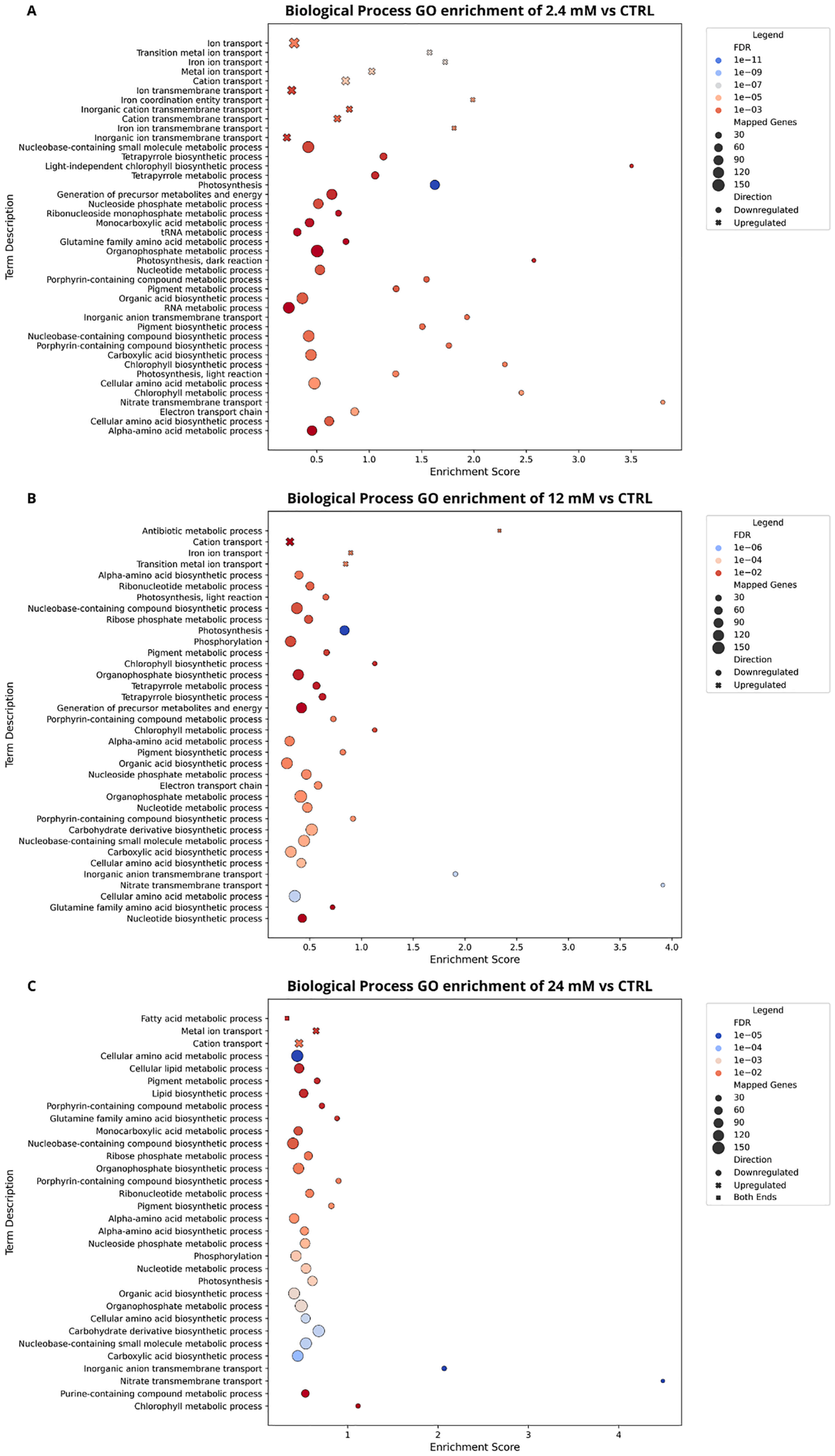

Fig. 3. GSEA results of Biological Process category (BP) of *Chroococcidiopsis* sp. CCMEE 029 cultivated under perchlorates at 2.4 mM vs CTRL (A); 12 mM vs CTRL (B); 24 mM vs CTRL (C).

ATPase, with only the exception of PsbC, PsbU and AtpH, which were mainly upregulated in the 24 mM treatment. Consequently, carbon fixation by Calvin-Benson-Bassham cycle (CBB) key genes were similarly downregulated. Among the different carbon-metabolism related pathways, the pentose phosphate pathway (PPP) resulted generally upregulated in each perchlorate concentration. In particular, we observed high upregulation of ribulose monophosphate pathway proteins (KEGG module: M00345), with a $\log_2$ fold-change (log2FC) of 1.38, 1.6 and 1 for HxlA (Hps) and 3.33, 5.26 and 1.96 for HxlB (Phi) (shown in Supplementary Fig. 4) and of its transcription factor HxlR 1.38, 1 and 0.64 log2FC (24 mM, 12 mM and 2.4 mM vs CTRL, respectively).

On the other hand, the starch and sucrose metabolism was particularly upregulated in higher salt concentrations, indicating a higher metabolic flux towards fructose and glucose, the latter possibly providing a substrate for the trehalose biosynthesis. Finally, 2-oxocarboxylic acid metabolism was directed towards energy production rather than arginine biosynthesis (Supplementary Fig. 5).

3.6. Key DEPs in cyanobacteria grown in perchlorate-rich medium

Based on GSEA, KEGG pathways, orthology information from EggNog5 database manual inspection and literature review, DEPs/pathways/clusters related to salt-stress and carbon metabolism were explored. In particular, a 12-gene cluster was found to be highly upregulated (Fig. 4). This cluster is found to be mainly associated with a methylglyoxal-induced oxidative stress response, and includes the *gloA* gene and its orthologue. In addition, a putative GLX3-like ortholog, containing the functional domains of glyoxalase 3 (IPR029062, IPR032633, IPR050325; GO:0019,172 glyoxalase III activity) was identified, which allows the direct formation of D-lactate from methylglyoxal. Moreover, a tautomerase-encoding gene (IPR014347, IPR032710, IPR037401, IPR004370) and a dioxygenases-containing-domain gene (IPR004360) were identified, together with the *mdlC* gene encoding for benzoylformate decarboxylase (phenylglyoxylate dehydrogenase) which catalyzes the formation of benzaldehyde and CO_2 from phenylglyoxylate. Finally, this cluster included the *betA* gene, which is involved in the formation of the osmoprotectant betaine, as well as the *yhbO* gene, which encodes a deglycase that repairs proteins glycated by glyoxal and methylglyoxal (Abdallah et al., 2016).

3.7. Differentially expressed enzymatic and non-enzymatic antioxidant proteins during long-term acclimation to perchlorate

A considerable variety and number of upregulated antioxidant proteins was identified in *Chroococcidiopsis* cultivated for 21 days in the presence of perchlorates. Among them, manganese superoxide dismutase, manganese catalase, peroxiredoxin, glutaredoxin, aldo-keto reductases, flavodoxin IsiB, glutathione transferases, PsbC3 (IsiA-like) and alcohol dehydrogenase (representing short-chain dehydrogenases/reductases) were identified. A detailed list of NCBI accessions with their log2FC, KEGG IDs, EC numbers is present in the Supplementary Table 1.

Moreover, additional general-stress-related genes, like *dps, mrgA*, and *dps-like* bacterioferritin resulted in a high upregulation, except for one *dps*, whose expression in the 2.4 mM perchlorate was 4-fold less expressed relative to the control. Similarly, HtrA, HhoB, Ctp and BepA proteases were upregulated. Regarding the non-enzymatic antioxidant defense, out of five orange carotenoid proteins present in *Chroococcidiopsis* sp. CCMEE 029 (OCP), three were consistently upregulated across the treatments, while one was downregulated at 2.4 mM and 12 mM perchlorate and one was not detected.

Moreover, five proteins related to plant chlorophyll a/b binding proteins associated with high-light inducible proteins (containing the characteristic domain, IPR022796) were identified. Out of these, four were upregulated in all the perchlorate treatments, with higher log2FC in 12 mM and 24 mM, while one was not observed. Results showed also the presence and upregulation (in 12 mM and 24 mM perchlorate) of proteins related to the production of polyhydroxybutyrate (PHB). Regarding polyamine biosynthesis-related proteins, results showed SpeA and SpeB to be upregulated, redirecting arginine towards putrescine production. Similarly, three protein orthologs of BetA and BetI were upregulated, promoting betaine formation.

Notably, proteins involved in DNA-repair, such as in SOS, NER, UVER, BER and HR and MMR pathways, were not found to be differentially regulated. While transporters related to anion efflux pumps, like ClcA were slightly upregulated with a log2FC of 0.72, 0.56, 0.49 (24 mM, 12 mM and 2.4 mM vs CTRL, respectively), while the most upregulated transporter represented by the YbhSFG operon.

An in depth investigation of regulators; cell wall and envelope-related; transporters; toxin-antitoxin systems; transmembrane and

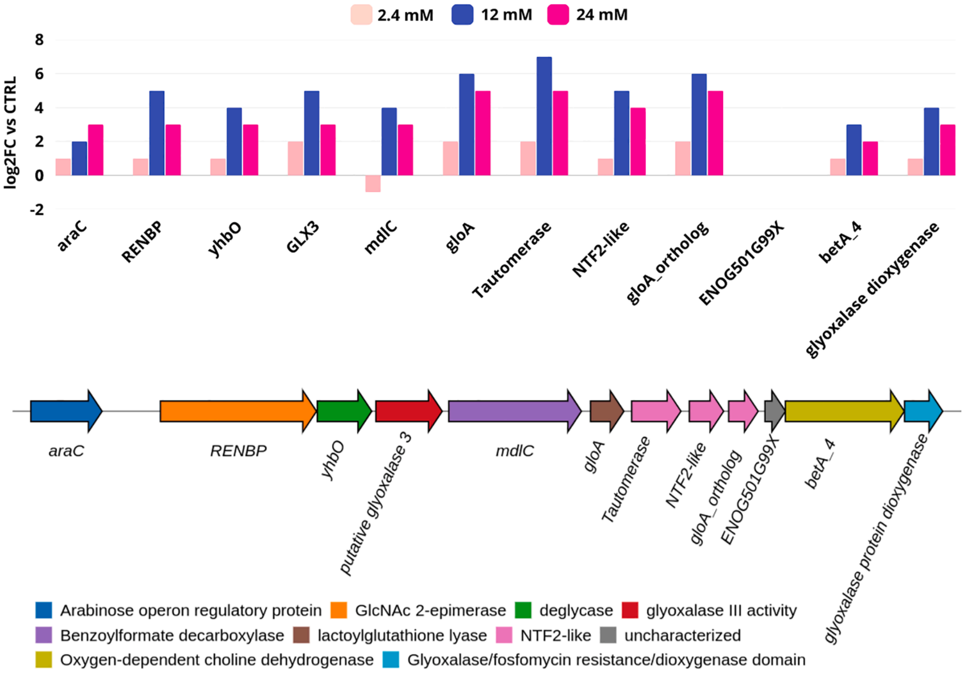

Fig. 4. Perchlorate-stress related upregulated gene cluster in *Chroococcidiopsis* sp. CCMEE 029 cultivated with increasing perchlorate concentrations vs control.

secreted proteins is available in the Supplementary file 1.

3.8. *Secondary metabolites in cyanobacteria grown in perchlorate-rich medium*

The genome mining of *Chroococcidiopsis* sp. CCMEE 029 revealed a wide distribution of biosynthetic gene clusters (BGCs), both in number and type. Specifically, a total of 22 clusters were identified, consisting of eight different types: four saccharide, six terpene (including precursors), two NRP-Polyketide, two NRP, one RiPP and one alkaloid, plus six unclassified clusters.

3.9. *Upregulation of a NRPS-metallophore/polyketide hybrid BGC during long-term acclimation*

After predicting BGCs, an interesting upregulation was identified regarding a nonribosomal peptide-metallophore type 1 polyketide (NRPS-metallophore/T1PKS) secondary metabolite biosynthetic gene cluster shared by all the experimental treatments (Fig. 5).

In this cluster, by inspecting the protein-protein interactions computed by StringDB, multiple copies of siderophore nonribosomal peptides such as LgrD, GrsB and PchB were identified along with potentially interacting proteins. In fact, the MbtH, AcpT, LgrE, LgrD and GrsB proteins formed cross interactions among them (Supplementary Fig. 6A); at the same time, a cluster of phenylalanine, tyrosine and tryptophan biosynthesis related genes was identified (Supplementary Fig. 6B) as generally downregulated, except for PchB.

4. Discussion

This study provides the first proteome-wide analysis of the response and defense mechanisms employed by *Chroococcidiopsis* sp. CCMEE 029 when cultivated for 21 days in the presence of perchlorate, a highly chaotropic salt occurring in the Martian soil that can impair Bio-ISRU in support of human outposts (Cockell, 2022).

Our results indicate that this cyanobacterium does not rely on isolated biochemical responses, but instead undergoes a large-scale metabolic reorganization enabling its persistence under extremely challenging conditions (Fig. 6). This global response reveals a level of metabolic flexibility and resilience that suggests many use-cases to exploit it in foreseen space biotechnologies and synthetic biology (Onofri et al., 2025).

The effect of 21-day cultivation in the presence of perchlorate caused the suppression of the cell growth at the higher salt concentration as a consequence of the impairment of photosystems, which reduced the efficiency of carbon fixation (Zhang et al., 2025), hence triggering the rerouting of carbon metabolism and activating alternative pathways such as the RuMP formaldehyde-fixing route. This shift suggests that this cyanobacterium could possess a previously unrecognized methylotrophic potential, enabling it to convert toxic intermediates like formaldehyde into useful metabolic products (Lee et al., 2021; Zhang et al., 2017). The presence of such pathways is especially valuable from a life support standpoint, since closed-loop recycling of carbon, including toxic or “waste” molecules, is essential for any sustainable extraterrestrial bioprocessing system (Kumar et al., 2019). If confirmed, this cyanobacterium could be a particularly suitable candidate for plant-growth promotion in the context of space agriculture.

In parallel with such metabolic flexibility, *Chroococcidiopsis* sp. CCMEE 029 deploys a broad suite of stress-acclimation mechanisms to maintain redox balance, protect cellular structures, and withstand osmotic fluctuations. These responses include the activation of enzymatic and non-enzymatic antioxidant systems, the modulation of osmoprotective transporters, and the reorganization of intracellular storage compounds. Notably, perchlorate exposure upregulates the machinery for PHB synthesis, a bioplastic precursor, indicating that environmental stress can enhance the production of compounds that are valuable for in situ resource utilization (Batista et al., 2018). This observation aligns well with current efforts to exploit cyanobacteria as low-input bioplastic producers for future extraterrestrial habitats, even though further research will be required to confirm such production (Carpine et al., 2020; Koch and Forchhammer, 2021; Rueda et al., 2022).

Another key finding is the activation of a siderophore-associated NRPS/T1PKS-metallophore biosynthetic cluster. Given that iron is one of the major limiting nutrients, the ability to mobilize iron through siderophore production could support both cyanobacterial growth and plant-associated nutrient acquisition in space agriculture (De Pascale et al., 2021; Hakim et al., 2021; Michel and Pistorius, 2004). Moreover, the potential production of such siderophores could often confer antimicrobial properties, suggesting that in the context of crop farming on Mars *Chroococcidiopsis* sp. CCMEE 029 may provide protection against microbial pathogens while simultaneously improving plant nutrient availability. In this way, the cyanobacterial natural stress responses directly overlap with beneficial traits for future attempts to confer perchlorate resistance to microorganisms relevant to space biotechnologies and life-support systems, sustaining human outposts on Mars (Cockell, 2022).

Also, most cyanobacteria possess a variable number of OCP involved in ROS scavenging by non-photochemical quenching, acting as photoprotectors of photosystem II (Kirilovsky and Kerfeld, 2012, 2016; Sedoud et al., 2014). This increased carotenoid production has also been observed in a wide range of cyanobacteria subjected to perchlorate salts, pointing out their importance in limiting and preventing damage to the photosynthetic apparatus (Rzymski et al., 2022). Moreover, since salt-stress negatively affects protein functionality by disrupting their folding, *Chroococcidiopsis* upregulates proteases such as HtrA and HhoB, Ctp and BepA, which maintain the integrity of the outer cell membrane and degrade abnormally exported proteins (Narita et al., 2013; Noone D et al., 2001). Similarly, the upregulation of transporters continues to maintain cellular osmotic homeostasis, with the most pronounced activation likely observed during early responses rather than after prolonged exposure.

Finally, the presence of an upregulated gene cluster related to methylglyoxal detoxification, together with genes possibly involved in aromatic compounds degradation, suggests that *Chroococcidiopsis* possesses a genetic toolset conferring a high level of metabolic plasticity, enabling both survival and detoxification. In fact, this is also reflected by the absence of differential regulation in DNA repairing mechanisms, either because the damage never occurred or because DNA had already been repaired. Furthermore, the presence in the BG-11 medium of the chelating agent sodium EDTA might have altered perchlorate toxicity in the experimental conditions, although the fact that this cyanobacterium can grow in water containing Martian regolith simulants or water-released minerals (supplemented with urine as a nitrogen source) and with 2.4 mM perchlorate ions (provided as a 60 % and 40 % mixture of Mg- and Ca-perchlorate) further supports its relevance for ISRU biotechnologies (Fernandez et al., 2023).

Overall*, Chroococcidiopsis* sp. CCMEE 029 reorganizes its metabolism in ways that preserve energetic balance, maintain growth potential, and produce compounds of biotechnological importance. This versatility underscores its suitability as a candidate for Martian bioprocessing, where robustness, low resource requirements and multifunctionality are paramount. A limitation of this study is that it did not discriminate perchlorate-specific effects from a more general ionic/osmotic stress due to salt loading; addressing this will require targeted experimentations with different salts like $CaCl_2$ or $MgCl_2$, as previously reported (Heinz et al., 2022).

5. Conclusion

This work demonstrates that *Chroococcidiopsis* sp. CCMEE 029 can be cultivated for a prolonged period in the presence of perchlorate thanks to a complex, coordinated acclimation process involving metabolic

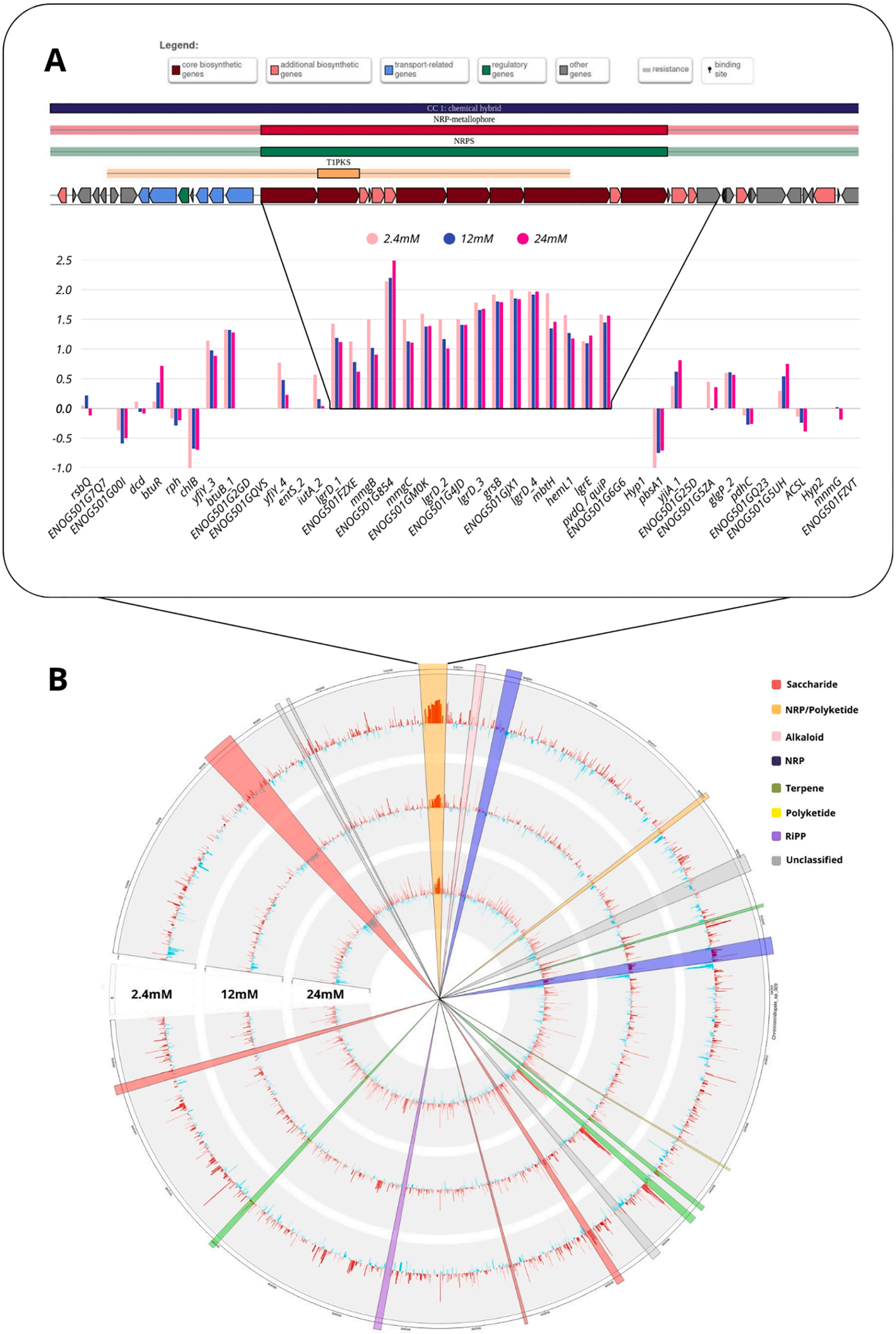

Fig. 5. Focus on the upregulated NRPS-metallophore/T1PKS hybrid BGC across every perchlorate treatment activated to regain iron lost from thylakoid breakdown (A). Circos plot showing genome-wide protein expression (in log2FC) of *Chroococcidiopsis* sp. 029 cultivated with different perchlorate concentrations vs control and landscape of different kinds of BGCs encoded in its genome (highlighted in different colors) (B).

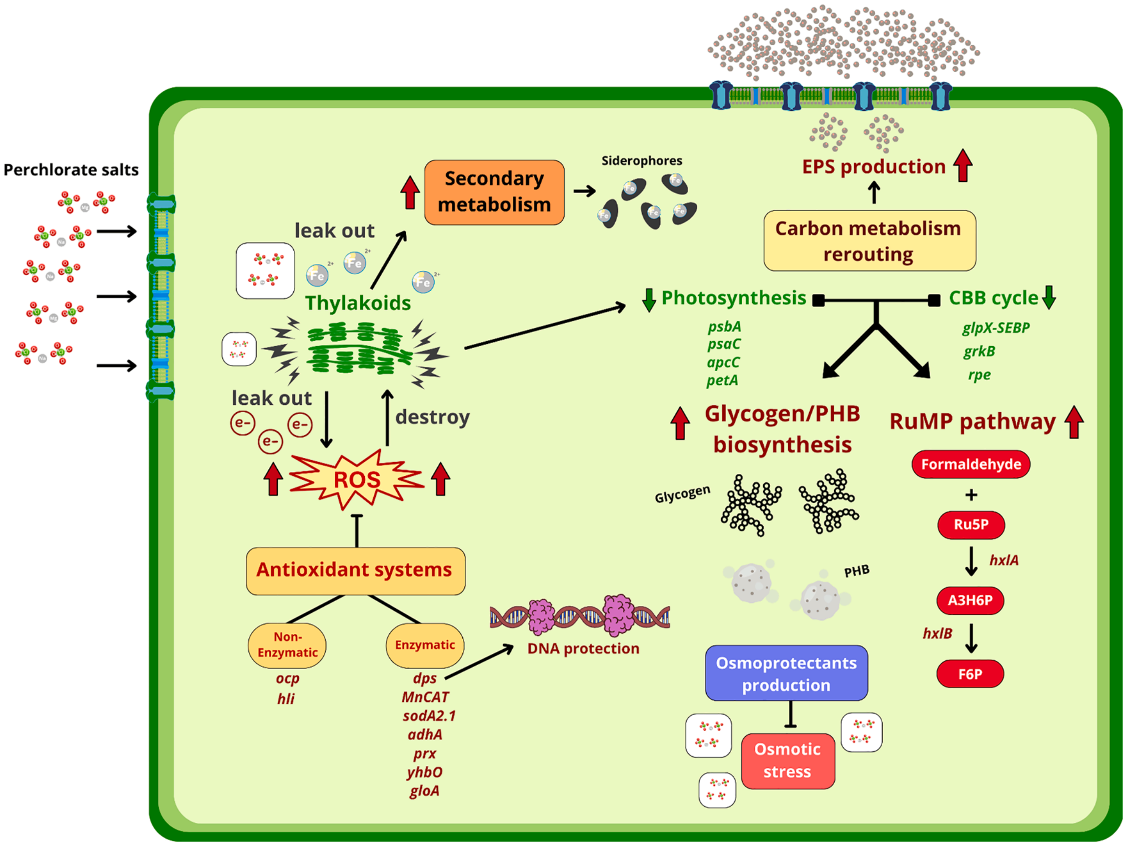

Fig. 6. Graphical representation of the main response and defense mechanisms of *Chroococcidiopsis* sp. CCMEE 029 cultivated for 21 days in perchlorate-rich medium. Perchlorates destabilize the thylakoid membranes, leaking out electrons and iron ions, thus causing ROS production furtherly affecting photosystems. Consequently, photosynthesis and CBB cycle are downregulated, inducing a carbon metabolism shift towards the ribulose monophosphate pathway (RuMP) and glycogen/PHB/exopolysaccharides (EPS) putative production for osmoprotection and detoxification. Antioxidant systems are activated to cope with ROS stress, involving both enzymatic and non-enzymatic mechanisms, while the lost iron from the photosystems decay is recollected by siderophores putatively produced through secondary metabolism.

rewiring, oxidative stress management, osmotic regulation and the activation of pathways linked to valuable biotechnological outputs. This provides a first prerequisite for employing this cyanobacterium in Bio-ISRU. Nevertheless, additional investigations are required for transforming this study's findings into applications in ISRU like additional experimentations to define the attenuations of the Martian surface conditions needed to support cyanobacterial growth.

The discovery of a functional formaldehyde-fixing capacity highlights a possible methylotrophic nature, which will need further testing to be confirmed, while the induction of PHB and siderophore biosynthesis positions this organism as a promising candidate for applications in bioplastic generation, nutrient mobilization and plant protection in extraterrestrial settings. Overall, the cyanobacterial resilience and metabolic flexibility support its potential use in future bioreactor systems for space agriculture and ISRU, where maintaining productivity is essential.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (http://proteomecentral.proteomexchange.org) (Perez-Riverol et al., 2025) partner repository with the dataset identifier PXD074715.

Scripts used to analyze the Mass-spectrometry data, annotations and supplementary material are available at the dedicated github repository GabrieleRigano99/Chroococcidiopsis_029_Perchlorates_proteomics.

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CRediT authorship contribution statement

Gabriele Rigano: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Joerg Doellinger:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Peter Lasch:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Giorgia Di Stefano:** Methodology, Formal analysis,

Investigation. **Beatriz Gallego Fernandez:** Methodology, Formal analysis, Investigation. **Loredana Santo:** Supervision, Project administration, Funding acquisition. **Daniela Billi:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they do not have competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2026.100580.

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