

RESEARCH ARTICLE

Perchlorate-specific proteomic stress responses of *Debaryomyces hansenii* could enable microbial survival in Martian brines

Jacob Heinz¹  | Joerg Doellinger²  | Deborah Maus³  | Andy Schneider²  | Peter Lasch²  | Hans-Peter Grossart^{4,5}  | Dirk Schulze-Makuch^{1,4,6,7} 

¹Center for Astronomy and Astrophysics, RG Astrobiology, Technische Universität Berlin, Berlin, Germany

²Robert Koch-Institute, Centre for Biological Threats and Special Pathogens, Proteomics and Spectroscopy (ZBS6), Berlin, Germany

³Robert Koch-Institute, Metabolism of Microbial Pathogens (NG2), Berlin, Germany

⁴Department of Plankton and Microbial Ecology, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Stechlin, Germany

⁵Institute for Biochemistry and Biology, Potsdam University, Potsdam, Germany

⁶GFZ German Research Center for Geosciences, Section Geomicrobiology, Potsdam, Germany

⁷School of the Environment, Washington State University, Pullman, Washington, USA

Correspondence

Jacob Heinz, Center for Astronomy and Astrophysics, RG Astrobiology, Sekr. ER 3-2, Technische Universität Berlin, Hardenbergstr. 36A, 10623 Berlin, Germany.
Email: heinz@tu-berlin.de

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Abstract

If life exists on Mars, it would face several challenges including the presence of perchlorates, which destabilize biomacromolecules by inducing chaotropic stress. However, little is known about perchlorate toxicity for microorganisms on the cellular level. Here, we present the first proteomic investigation on the perchlorate-specific stress responses of the halotolerant yeast *Debaryomyces hansenii* and compare these to generally known salt stress adaptations. We found that the responses to NaCl and NaClO₄-induced stresses share many common metabolic features, for example, signalling pathways, elevated energy metabolism, or osmolyte biosynthesis. Nevertheless, several new perchlorate-specific stress responses could be identified, such as protein glycosylation and cell wall remodulations, presumably in order to stabilize protein structures and the cell envelope. These stress responses would also be relevant for putative life on Mars, which—given the environmental conditions—likely developed chaotropic defence strategies such as stabilized conformations of biomacromolecules or the formation of cell clusters.

INTRODUCTION

Life as we know it requires energy and access to CHNOPS (carbon, hydrogen, nitrogen, oxygen, phosphorus, sulfur), trace elements, and liquid water. On Mars, energy would be provided to putative life chemically or via sunlight, carbon is accessible through the thin but CO₂-rich atmosphere, and other essential elements are abundant in the regolith (Clark et al., 2021).

Availability of liquid water, however, is strongly restricted due to the low atmospheric pressure of approximately 6 mbar and mostly subzero temperatures on Mars (Martínez & Renno, 2013). One of the few possibilities to generate liquid water in the Martian near surface is the formation of temporarily stable brines via deliquescence, a process in which a hygroscopic salt absorbs water from the atmosphere and dissolves within that water (Hallsworth, 2020). It has been

shown that deliquescent water is sufficient to drive the metabolism of halotolerant methanogenic archaea (Maus et al., 2020). Intriguingly, several hygroscopic salts have been detected on Mars (Davila et al., 2010). Among those are very deliquescent and freezing point depressing perchlorates (ClO_4^-), which are widely distributed on the Martian surface (Clark & Kounaves, 2016) but appear in natural environments on Earth only occasionally in hyperarid deserts (Catling et al., 2010; Kounaves et al., 2010).

Brines formed via deliquescence provide diverse challenges for microbial life. High salt concentrations lead to osmotic stress and reduced water activity, which is a measure for the amount of unbound water molecules in a solution available for biological processes (Hallsworth et al., 2021). Furthermore, salts can induce ion-specific stresses like interferences with the cell's metabolism or changes in cell permeability through variations in ionic hydration shells (Waajen et al., 2020). Some anions like perchlorate additionally evoke chaotropic stress, that is, they destabilize biomacromolecules like proteins (Ball & Hallsworth, 2015), presumably through nonlocalized attractive dispersion forces (Hyde et al., 2017). In *Pseudomonas putida*, it has been shown that chaotropic solute-induced water stress mainly leads to upregulation of proteins involved in stabilization of biological macromolecules and membrane structure (Hallsworth et al., 2003). Furthermore, it has been demonstrated that chaotropic effects can be neutralized to some extent by the presence or bioproduction of kosmotropes or compatible solutes (Bhaganna et al., 2010; Cray et al., 2015). However, detailed research on proteomic responses to chaotropic stress induced by perchlorate is still lacking.

Here, we present a proteomic study investigating the perchlorate-specific stress response of *Debaryomyces hansenii* to evaluate the physiological adaptations required for microorganisms to thrive in the Martian near surface. The halotolerant yeast *D. hansenii* has been chosen as a model organism as it has been described earlier to tolerate the highest perchlorate concentrations reported to date (Heinz et al., 2020, 2021). This yeast provides a large metabolic toolset to counteract salt stress, such as the high-osmolarity glycerol (HOG) pathway, which enables stress signalling and concomitant biosynthesis of glycerol (Prista et al., 2016), which acts as compatible solute and antagonizes osmotic as well as chaotropic stress (Bhaganna et al., 2016). Its close relation to the intensively studied bakery yeast *Saccharomyces cerevisiae* greatly facilitates the annotation of proteins and thus prediction of their functions.

It has been found previously that the eukaryotic species thus far investigated showed a higher perchlorate tolerance than prokaryotes (Heinz et al., 2020). Even though an evolutionary development of eukaryotes on Mars might be considered unlikely due to the

relatively short habitable window of Mars, it can be assumed that Martian microorganism—if they exist—would have adapted over longer time scales to the increasing aridity, salinity, and perchlorate concentrations (Davila & Schulze-Makuch, 2016) and developed defence strategies at least as efficiently and complex as observed in *D. hansenii*, which is not even exposed to high perchlorate concentrations in its natural environment.

For the investigation of the proteome of *D. hansenii*, we choose a recently developed proteomics protocol called Sample Preparation by Easy Extraction and Digestion (SPEED) which enables sample-type independent deep proteome profiling with high quantitative accuracy and precision (Doellinger, Blumenscheit, et al., 2020; Doellinger, Schneider, et al., 2020).

This is the first study investigating perchlorate-specific stress responses with an untargeted proteomic approach to provide novel and fundamental understanding of the required cellular adaptation mechanisms for life in perchlorate-rich, chaotropic habitats on Earth, Mars, and beyond.

EXPERIMENTAL PROCEDURES

Microbial cultures

The halotolerant yeast *D. hansenii* (DSM 3428) was obtained from the Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures. A stock culture was grown aerobically without shaking at 25°C (optimum growth temperature) in liquid DMSZ growth medium #90 (3% (w/v) malt extract, 0.3% (w/v) soya peptone) and was frequently re-inoculated. Additionally, four different salt-containing liquid growth media (DSMZ #90) were prepared having a molal (mol/kg) salt concentration of either 1.5 mol/kg NaClO_4 , 2.4 mol/kg NaCl , 2.4 mol/kg NaClO_4 , or 3.9 mol/kg NaCl . The latter two concentrations (2.4 mol/kg NaClO_4 and 3.9 mol/kg NaCl) represent the almost highest concentrations of the respective salt enabling growth of *D. hansenii*. The maximum growth-enabling concentrations reported to date are 2.5 mol/kg NaClO_4 and 4.0 mol/kg NaCl (Heinz et al., 2021). We choose slightly lower concentrations to guarantee reproducible growth of the cultures and to generate sufficient biomass for protein extraction. The other two salt concentrations (1.5 mol/kg NaClO_4 and 2.4 mol/kg NaCl) represent moderate salt concentrations of the respective salt (i.e. approx. 62 mol% of the respective maximum salt concentrations enabling growth). The availability of two treatments with the same molal salt concentrations (2.4 mol/kg NaCl and 2.4 mol/kg NaClO_4) allowed for an additional comparison of cellular stress responses to the two different salt species at the same osmolality. The growth media were prepared by mixing the media

components, the respective salt and water, followed by pH adjustment (pH \sim 5.6) and sterile filtration. All treatments (no salt, 1.5 mol/kg NaClO₄, 2.4 mol/kg NaCl, 2.4 mol/kg NaClO₄, and 3.9 mol/kg NaCl) were inoculated as biological triplicates, that is, for each treatment three different samples were inoculated. The salt-free treatment and the samples containing 1.5 mol/kg NaClO₄ and 2.4 mol/kg NaCl were inoculated with the salt-free stock culture. Hence, the two saline treatments with moderate salt concentrations (1.5 mol/kg NaClO₄ and 2.4 mol/kg NaCl) experienced a salt shock after inoculation. Since the respective salt shock would be too intense in 2.4 mol/kg NaClO₄ and 3.9 mol/kg NaCl treatments to enable growth, these samples were inoculated with long-term adapted cultures already grown at the respective salt concentration (Figure 1A).

Sample preparation for proteomics

Protein extraction was conducted using the recently developed filter-aided Sample Preparation by Easy Extraction and Digestion (fa-SPEED) protocol (Doellinger, Schneider, et al., 2020). Cells were centrifuged for 3 min at $5.000 \times g$ after reaching late exponential growth phase, which is approximately 1 day for salt-free treatments, 3 days for 1.5 mol/kg NaClO₄ and 2.4 mol/kg NaCl, 6 days for 2.4 mol/kg NaClO₄, and 7 days for 3.9 mol/kg NaCl (Figure 1B). Cell pelleting in 3.9 mol/kg NaCl samples was incomplete (turbid supernatant) but sufficient for further protein extraction. The reason for incomplete pelleting is presumably an electrostatic repulsion of cells because dilution of additional test samples with water did not result in larger pellets but gently stirring with a grounded metal rod before centrifugation did. The cell pellets were washed three times with phosphate buffer saline (PBS) followed by cell lysing with 50 μ l trifluoroacetic acid (TFA) for 3 min at 70°C. Afterwards, samples were neutralized with 500 μ l 2 M tris(hydroxymethyl)aminomethane (TRIS) solution. After adding 55 μ l reduction/alkylation buffer (100 mM tris(2-carboxyethyl)phosphine/400 mM 2-Chloracetamid), the samples were incubated at 95°C for 5 min.

Protein concentrations were determined by turbidity measurements at 360 nm using GENESYS™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific). The 50 μ g of proteins was diluted to 40 μ l using a 10:1 (v/v) mixture of 2 M TrisBase and TFA, mixed with 160 μ l acetone and incubated for 2 min at RT. For samples containing less than 50 μ g proteins per 40 μ l sample, the volumes of sample and acetone were increased at constant sample/acetone ratio until 50 μ g protein/sample were reached. Afterwards, proteins were captured on Ultrafree®-MC (0.5 ml) centrifugal devices, 0.2 μ m, PTFE (Merck) at $5000 \times g$ for 2 min. The samples were washed successively with 200 μ l 80% (v/v)

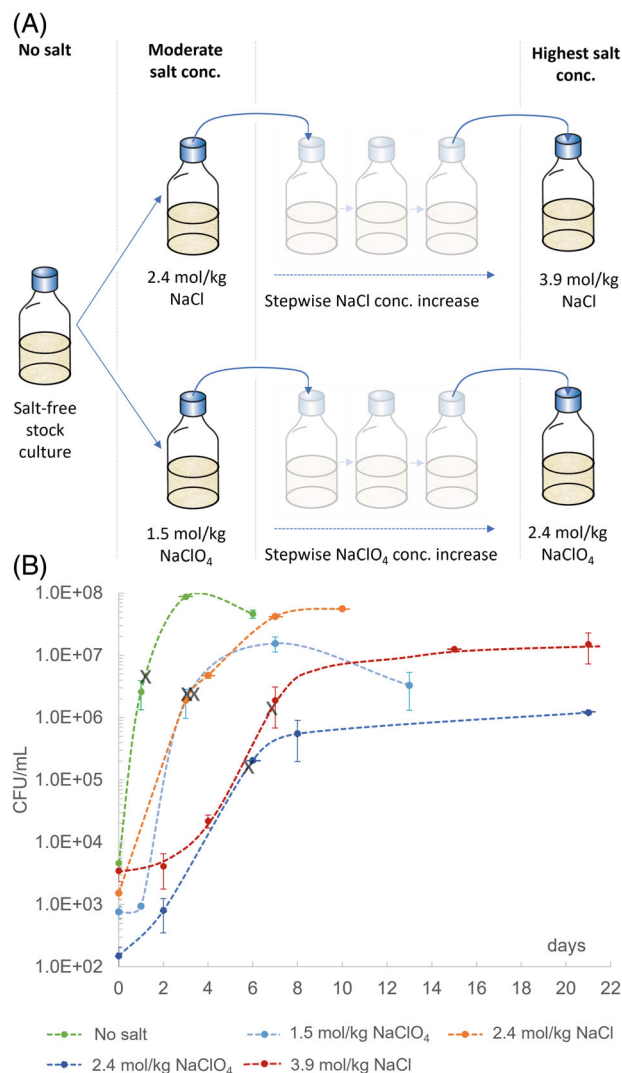


FIGURE 1 Workflow of the inoculation procedure and corresponding growth curves. (A) A salt-free stock culture of *D. hansenii* was frequently re-inoculated into fresh growth medium. An aliquot of this culture was used to inoculate growth media with moderate salt concentrations (2.4 mol/kg NaCl and 1.5 mol/kg NaClO₄). To obtain cell growth at even higher salt concentrations, a stepwise concentration increase was needed for each inoculation step. The maximum salt concentrations used in this study were 3.9 mol/kg NaCl and 2.4 mol/kg NaClO₄. (B) Growth curves of all samples used in this study ($n = 3$). Cells were harvested for protein extraction in the late exponential growth phase of the respective treatments (label with 'X').

acetone, 200 μ l 100% acetone and 200 μ l n-pentane at $5000 \times g$ for 2 min each.

Subsequently, 40 μ l of digestion buffer (50 mM ammonium bicarbonate) containing trypsin (1:25 [enzyme-to-protein ratio] Trypsin Gold, Mass Spectrometry Grade [Promega]) was added to the filter containing the proteins followed by incubation at 37°C for 20 h. The sample solution containing the digested proteins was centrifuged at $5.000 \times g$ for 2 min and the filter was washed subsequently with 40 μ l digestion buffer

containing 0.1% (v/v) TFA. The 10% (v/v) TFA solution was added until the pH of the samples reached approximately 2. Peptides were desalted using the Pierce™ Peptide Desalting Spin Columns (Thermo Scientific) according to the manufacture's protocol no. 2162704. The desalted samples were dried in a vacuum concentrator. The dried peptides were dissolved in 0.1% (v/v) formic acid and quantified by measuring the absorbance at 280 nm using an Implen NP80 spectrophotometer (Implen, Munich, Germany).

Liquid chromatography and mass spectrometry

Peptides were analysed on an EASY-nanoLC 1200 (Thermo Fisher Scientific, Bremen, Germany) coupled online to a Q Exactive™ HF mass spectrometer (Thermo Fisher Scientific). One microgram of peptides was separated on a PepSep column (15 cm length, 75 µm i.d., 1.9 µm C18 beads, PepSep, Denmark) using a stepped 30 min gradient of 80% (v/v) acetonitrile (Solvent B) in 0.1% (v/v) formic acid (Solvent A) at 300 nl/min flow rate: 5%–11% (v/v) B in 2:49 min, 11%–29% (v/v) B in 18:04 min, 29%–33% (v/v) B in 3:03 min, 33%–39% (v/v) B in 2:04 min, 39%–95% (v/v) B in 0:10 min, 95% (v/v) B for 2:50 min, 95%–0% (v/v) B in 0:10 min and 0% (v/v) B for 0:50 min. Column temperature was kept at 50°C using a butterfly heater (Phoenix S&T, Chester, PA, USA). The Q Exactive™ HF was operated in a data-independent (DIA) manner in the m/z range of 345–1650. Full scan spectra were recorded with a resolution of 120,000 using an automatic gain control (AGC) target value of 3×10^6 with a maximum injection time of 100 ms. The full scans were followed by 62 DIA scans of dynamic window widths using an overlap of 0.5 Th (Doellinger, Blumenschein, et al., 2020). DIA spectra were recorded at a resolution of 30,000 using an AGC target value of 3×10^6 with a maximum injection time of 55 ms and a first fixed mass of 200 Th. Normalized collision energy (NCE) was set to 27% and default charge state was set to 3. Peptides were ionized using electrospray with a stainless-steel emitter, I.D. 30 µm (PepSep, Denmark) at a spray voltage of 2.1 kV and a heated capillary temperature of 275°C.

Data analysis and statistical information

Protein sequences of *Debaryomyces hansenii* (UP000000599, downloaded 16/10/20), were obtained from UniProt (UniProt Consortium, 2019). A spectral library was predicted for all possible peptides with strict trypsin specificity (KR not P) in the m/z range of 350–1150 with charge states of 2–4 and allowing up to one missed cleavage site using ProSight (Gessulat

et al., 2019). Input files for library prediction were generated using EncyclopeDIA (Version 0.9.5) (Searle et al., 2018). The data were analysed using the predicted library with fixed mass tolerances of 10 ppm for MS¹ and 20 ppm for MS² spectra using the 'robust LC (high accuracy)' quantification strategy. The false discovery rate was set to 0.01 for precursor identifications and proteins were grouped according to their respective genes. The resulting pg_matrix.tsv file was used for further analysis in Perseus (Version 1.6.5.0) (Tyanova et al., 2016).

The same programme was used to z-normalize protein abundances followed by ANOVA (FDR = 0.01) and post hoc testing (FDR = 0.05). Subsequently, the abundances of biological triplicates were median averaged, and the relative log₂-fold changes of the salt-containing (saline) treatments compared to the salt-free control were calculated. The results were filtered for significant pairs of the salt-free samples and at least one of the saline treatments and were then plotted into a hierarchical clustered heatmap. Additionally, volcano plots have been generated with the same software after *t*-test of the z-normalized protein abundances. Protein groups of interest were annotated and analysed with the STRING database (<https://string-db.org/>) (Szklarczyk et al., 2021) regarding enriched metabolic pathways and the formation of functional protein clusters.

RESULTS

In order to distinguish the perchlorate-specific stress response of *D. hansenii* to the stress caused by NaCl, proteomes of cell cultures containing either NaClO₄, NaCl or no additional salts in growth medium DSMZ #90 were analysed. Two different salt concentration regimes were investigated (Figure 1A). At moderate salt concentrations (1.5 mol/kg NaClO₄ and 2.4 mol/kg NaCl), growth was obtained by inoculation with a salt-free culture to provoke a salt shock response. However, the highest salt concentrations used in this study (2.4 mol/kg NaClO₄ and 3.9 mol/kg NaCl) only enabled growth when cells were long-term adapted to stepwise increasing salt concentrations (Figure 1A). All samples were prepared as biological triplicates and cells were harvested in the late exponential growth phase in order to obtain sufficient biomass for protein extraction (Figure 1B). It should be noted that while 2.4 mol/kg NaClO₄ is already close to the growth-limiting NaClO₄ concentration (similar growth rate to 3.9 mol/kg NaCl), 2.4 mol/kg NaCl represents a readily feasible NaCl concentration with a growth rate similar to 1.5 mol/kg NaClO₄.

In total, 2713 proteins were detected representing a bulk coding sequence coverage of approximately 43%. Through analysis of variance (ANOVA, FDR ≤ 0.01) of

the z-normalized protein abundances, the expression of 1099 proteins was found to be significantly different between the five different treatment types (one salt-free control and four salt-exposed treatments). The salt concentration (moderate vs. high) had a stronger impact on the intensity of protein expression than the type of anion as can be seen from the comparison of protein abundances of all replicates, which show similar protein expressions for the same salt concentration regimes (Figure 2A). This is confirmed by the principal component analysis (PCA), which revealed a clear clustering of the replicates of each treatment in dependence on salt concentration and type of anions (Figure 2B). While

the physiological response to different salt concentrations clustered along principal component 1 and explains 55% of the observed differences, the salt species had a lower impact on the variability (15%), as treatments exposed to chloride or perchlorate spread along the principal component 2.

Post hoc testing ($FDR \leq 0.05$) revealed 1068 proteins to be significantly regulated in at least one of the salt-exposed samples compared to the salt-free treatment. The log₂-fold changes of these proteins in the saline treatments compared to the salt-free control were plotted in a heatmap with upregulated proteins coloured red and downregulated protein shown in

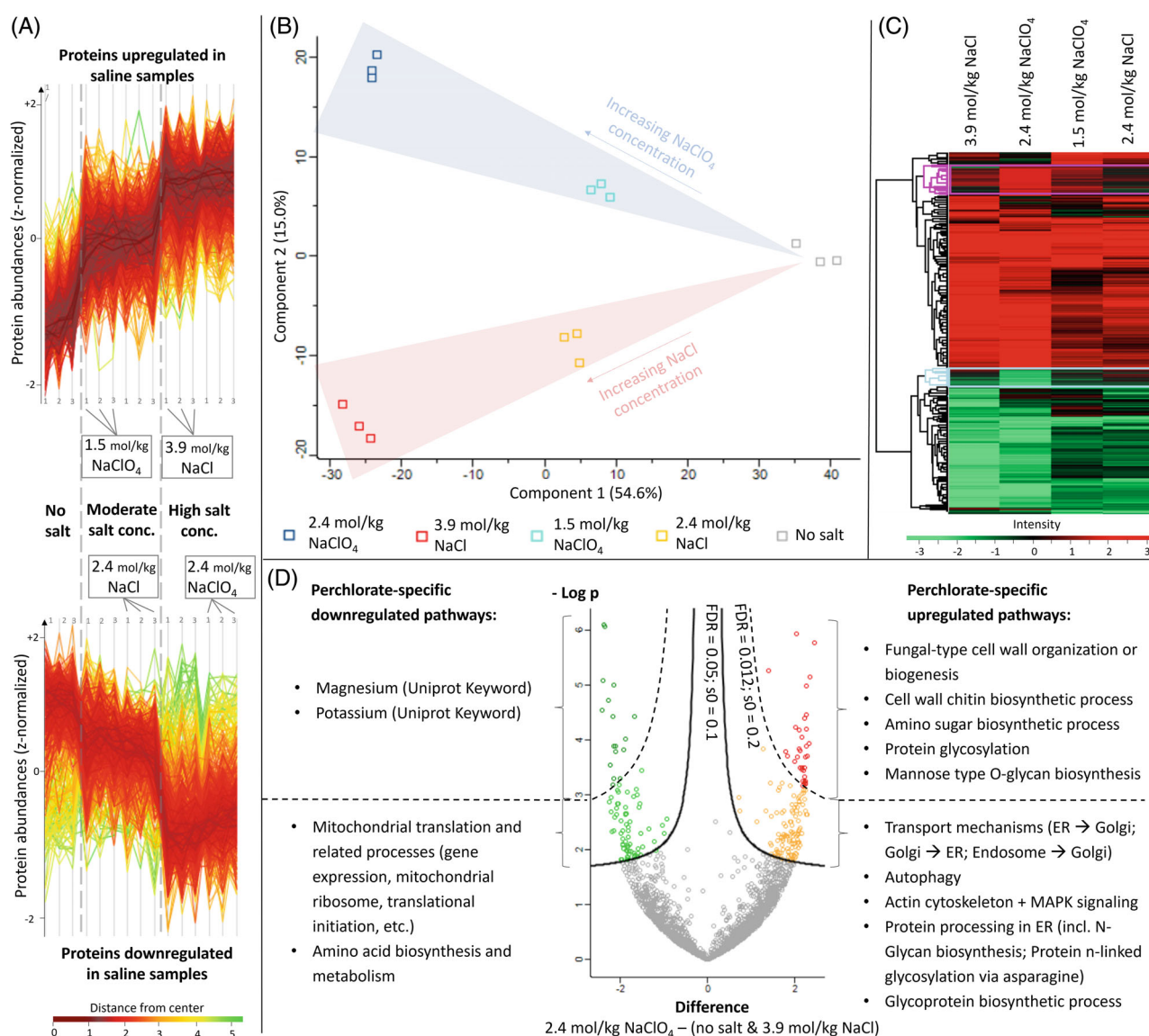


FIGURE 2 Results of the proteomic analyses. (A) Abundances of upregulated (upper plot) and downregulated (lower plot) proteins expressed in all investigated samples (three replicates for each treatment as indicated in the space between the two plots). (B) Principal component analysis (PCA) demonstrating clear clustering of all biological triplicates in dependence of salt concentration and type of anion. (C) Heat map including all proteins passing ANOVA ($FDR \leq 0.01$) and post hoc test ($FDR \leq 0.05$) generated by the Perseus software after hierarchical clustering. Upregulated proteins (compared to the salt-free treatment) are coloured red and downregulated proteins are shown in green. Two exemplarily perchlorate-specific clusters are highlighted in pink for upregulated and in cyan for downregulated proteins. (D) Volcano plot visualizing perchlorate-specific regulated proteins with a high ($FDR \leq 0.012$) and a lower significance ($0.012 \leq FDR \leq 0.05$). Significantly regulated metabolic pathways were analysed with the STRING database.

green (Figure 2C). The heatmap revealed two major clusters, one containing the proteins predominantly upregulated in all saline treatments compared to the salt-free control, and a second cluster with downregulated proteins. This indicates that the overall stress response is relatively similar in all treatments. However, both main clusters also contain proteins that are substantially more regulated in the 2.4 mol/kg NaClO₄ sample than in all other treatments. The two largest subclusters containing proteins of this category are highlighted in pink (for upregulated proteins) and cyan (for downregulated proteins) in Figure 2C. Proteins in these subclusters represent the perchlorate-specific stress response, which apparently manifests only after long-term adaptation to high perchlorate concentrations, as protein expression patterns in the 1.5 mol/kg NaClO₄ treatment are coinciding more with the NaCl than with the 2.4 mol/kg NaClO₄ treatment.

This enables the possibility to investigate the significance of perchlorate-specific protein expression patterns by a volcano plot that presents the differences of the z-normalized protein abundances in the 2.4 mol/kg NaClO₄ treatment and the control samples (salt-free and 3.9 mol/kg NaCl) versus the logarithmic *p* value after a *t*-test (Figure 2D). The resulting perchlorate-specific regulated proteins include the ones from the heatmap subclusters (marked pink and cyan in Figure 2C) and additional proteins from smaller subclusters. Proteins were fed into the STRING database (Szklarczyk et al., 2021) which predicts physical and functional protein–protein interactions and identifies significantly enriched (FDR ≤ 0.05) metabolic pathways that assort into protein clusters (Figure 3A, and Table S1). The physiological interpretation of the perchlorate-specific enriched pathways is summarized in Figure 3B and discussed in detail below.

In order to better evaluate the specificity of perchlorate-induced stresses, they should be compared to non-perchlorate-specific stress responses, which are equally expressed in NaCl- and NaClO₄-stressed cultures (i.e. protein expression depends predominantly on the concentration of the respective salt, but not on the type of salt). For this purpose, proteins that show significant expression (FDR ≤ 0.05) in all salt-exposed treatments compared to the salt-free control have been analysed with the STRING database. The results of this approach are discussed below and summarized in Figure 4 and Table S1.

DISCUSSION

Salt stress response shared by NaCl and NaClO₄

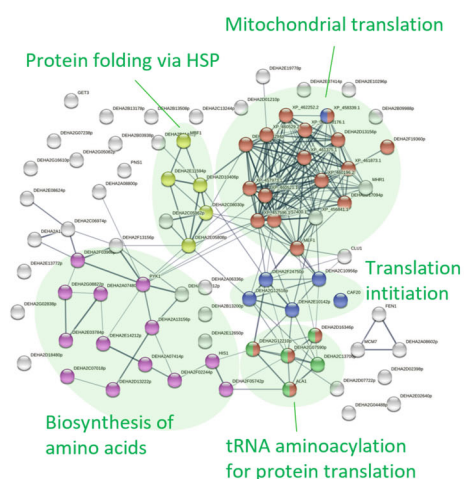
The stress responses shared equally by all saline treatments (i.e. non-perchlorate-specific responses) encompassed several metabolic stress response pathways,

previously well described for *D. hansenii* and its close relative, the intensively investigated yeast *S. cerevisiae* (Hohmann, 2002; Prista et al., 2016). Only the most prominent pathways detected in this study are described below. Any form of environmental stress in yeasts is usually communicated from the cell envelope to the nucleus via signalling pathways such as mitogen-activated protein kinase (MAPK) pathways (Sharma et al., 2005) accompanied by a rearrangement of cytoskeletal arrays (Samaj et al., 2004). Several proteins involved in these signalling pathways were found to be upregulated in all saline treatments (see Figure 4 and Table S1). For example, the upregulated serine/threonine-protein kinase CLA4 (DEHA2B12430p) modulates the expression of biosynthesis of glycerol (Joshua & Höfken, 2019), an important osmoprotectant in *D. hansenii* (Prista et al., 2016), which can decrease the intracellular water activity more efficiently than other compatible solutes (de Lima Alves et al., 2015).

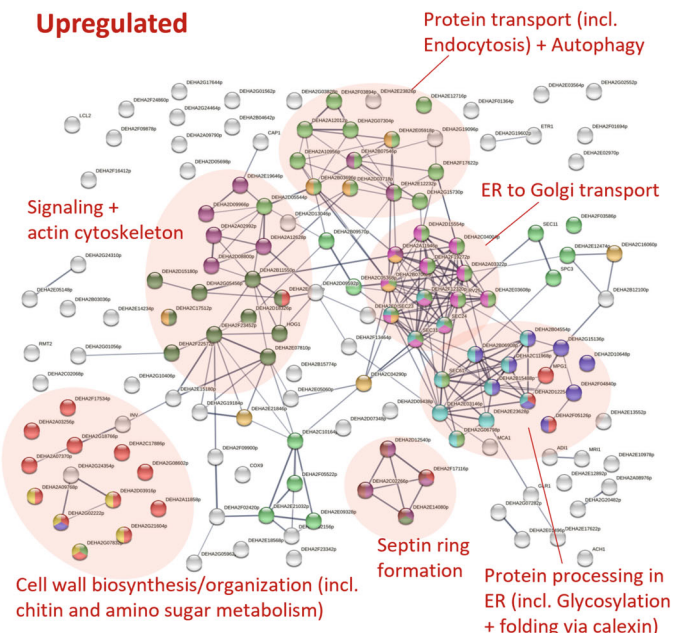
As a consequence of the received signal, the cell's energy metabolism is upregulated to induce certain stress responses. In our experiments, several energy-releasing pathways were upregulated equally in NaCl- and NaClO₄-stressed samples, such as the TCA cycle. Furthermore, processes in the peroxisomes showed upregulation including the energy-releasing beta-oxidation of fatty acids indicated by upregulation of the multifunctional beta-oxidation protein DEHA2A08646p, the acyl-coenzyme A oxidase POX1 (DEHA2D17248p), and the peroxisomal long-chain fatty acid import protein DEHA2B08646p. In yeasts, also the glyoxylate cycle, a variation of the TCA cycle (Duntze et al., 1969), takes place in the peroxisomes as well as in the cytoplasm, and correspondingly the two key enzymes, that is, malate synthase (DEHA2E13530p) and isocitrate lyase (ICL1 DEHA2D12936p), were upregulated.

The energy released from these processes is presumably needed to guarantee survival under enhanced osmotic cell stress. For example, the amount of biosynthesized glycerol is increased as can be observed by the upregulation of the glycerol lipid metabolism, which includes proteins that participate in the formation of glycerol, for example, several significantly enriched glycerol-3-phosphate dehydrogenase complex proteins. In addition to the accumulation of glycerol, osmotic stress in *D. hansenii* can also be antagonized through ion transmembrane transporters (Breuer & Harms, 2006). Even though not being incorporated in a significant enrichment, we found several ion transporter proteins to be upregulated, such as the ATPase-coupled cation transmembrane transporters DEHA2G09108p and DEHA2C02552p (Table S1). These two cation transporters showed the highest significance upon all proteins upregulated in the saline treatments (see volcano plot in Figure 4). The provision of sufficient ATP required for the functioning of these transporters constitutes to the enhanced cellular energy demand.

(A) Downregulated



Upregulated



(B)

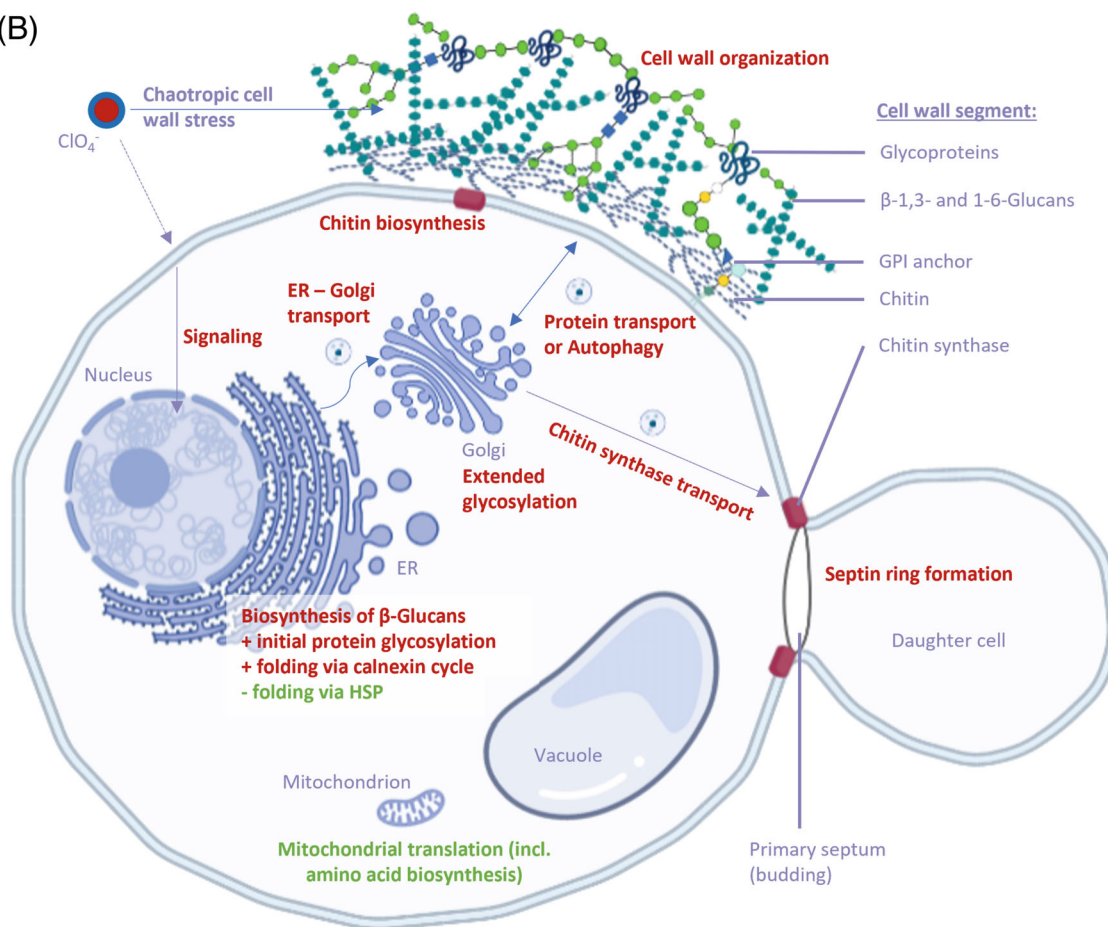


FIGURE 3 Perchlorate-specific stress responses. (A) STRING database calculated interactions of all downregulated (left) and upregulated (right) proteins involved in the perchlorate-specific stress response according to the volcano plot (Figure 2D, all proteins with FDR ≤ 0.05). Coloured proteins indicate significantly enriched metabolic pathways (FDR ≤ 0.05) and are annotated in Table S1. The most prominent pathways are encircled. (B) A mother cell and a budding daughter cell of *Debaryomyces hansenii* displaying the most relevant metabolic pathways with perchlorate-specific upregulations (red) and downregulations (green) as explained in the main text. Created with BioRender.com

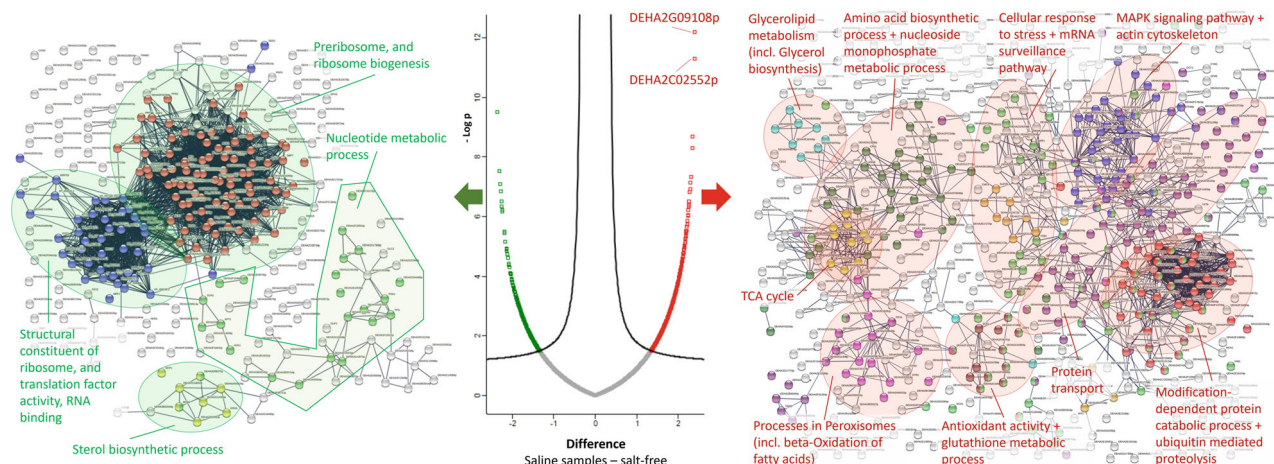


FIGURE 4 Stress response shared by all salt-exposed samples. Volcano plot (at the centre) of proteins significantly up- (red) or downregulated (green) in all salt-exposed samples compared to the salt-free control, and STRING database analyses of up- (right) and downregulated (left) pathways. A selection of significantly ($FDR \leq 0.05$) regulated pathways is colour-coded and the most prominent protein clusters are encircled and labelled. All other (less prominent) enrichments as well as the annotation of the colour codes are provided in Table S1. In the volcano plot, the two ATPase-coupled cation transmembrane transporters DEHA2G09108p and DEHA2C02552p are labelled, which have the highest significance upon all upregulated proteins.

Osmotic stress usually induces oxidative stress to a some extent, for example, by production of reactive oxygen species (ROS) in the mitochondria (Petrovic, 2006). Hence, it is expected that proteins involved in oxidative stress responses are regulated under salt stress conditions as well. Indeed, we found antioxidant activity and glutathione metabolic processes to be upregulated equally in NaCl- and NaClO₄-stressed samples, including the enzymes catalase (DEHA2F10582g) and peroxidase (DEHA2A02310p) enabling cell protection against oxidative stress.

The significantly enriched pathway forming the most pronounced and condensed upregulated protein cluster contains proteins involved in modification-dependent protein catabolic processes and ubiquitin-mediated proteolysis (Figure 4). Osmotic and induced oxidative stresses can cause protein misfolding (Schnitzer et al., 2022). Proteins, which cannot be refolded by chaperones, are degraded by the proteasome of the cell (Jackson & Hewitt, 2016). The so generated amino acids can then be reused for cellular amino acid metabolism, which forms a protein subcluster interwoven with the TCA cycle (Figure 4), indicating that the amino acids liberated by proteolysis feed the energy metabolism. Additionally, the recycling of amino acids via proteolysis conserves energy as compared to amino acid de novo biosynthesis.

Most of the stress responses described above require protein transport, for example, for post-translational modifications in the ER or the Golgi apparatus, for the transfer of proteins to their place of activity, or for the excretion of cell wall proteins. Consistently, many of the proteins upregulated similarly

in response to NaCl- and NaClO₄-induced stress are involved in protein transport mechanisms.

More than half of the proteins non-perchlorate-specifically downregulated (i.e. similarly downregulated in NaCl- and NaClO₄-stressed samples) are structural ribosomal constituents and have translation factor activity or are involved in the cytosolic (pre)ribosome biogenesis and related pathways such as the nucleotide metabolism (Figure 4). Ribosome biogenesis is a complex and very energy-demanding process (Albert et al., 2019). Consequently, for saving energy, ribosome biogenesis is downregulated under various stress conditions (Shore et al., 2021). A transient reduction in ribosome biogenesis and translation together with the accumulation of glycerol has also been detected in *Candida albicans* upon salt stress (Jacobsen et al., 2018). While ribosome biogenesis was generally downregulated in our experiments, we consistently observed upregulation of the ribosome-recycling factor RRF1 (DEHA2F14630g) which allows the ribosome to unbind from mRNA after the release of the generated polypeptide and to be reused for new translation processes instead of the energy-consuming de novo biosynthesis of ribosomes (Kiel et al., 2007).

Upregulation of ribosome synthesis occurs only in response to favourable growth conditions and enables the cell to grow faster (Mayer & Grummt, 2006), while downregulation of translation via depletion of the ribosomal population is known to prolong the lifespan of cells (Steffen et al., 2008). Consistently, we found that the downregulation of ribosome biosynthesis and concomitant translational processes coincided with slower cell growth under salt stress conditions (Figure 1B).

The only other significantly enriched downregulated pathway forming a protein cluster that is physiologically not directly connected to the ribosome assembly and translational processes is the biosynthesis of ergosterol (Figure 4), a component of fungal cell membranes (Jordá & Puig, 2020). In *S. cerevisiae*, the downregulation of the ergosterol biosynthesis has already been described earlier as response to hyperosmotic stress (Montañés et al., 2011). It has been hypothesized that it results from increased uptake of Na^+ and/or a decreased Na^+ extrusion in a plasma membrane environment with elevated levels of ergosterol (Montañés et al., 2011). Furthermore, sterol biosynthesis is a highly energy-consuming process (Hu et al., 2017), and its downregulation might constitute, similar to the downregulation of the ribosome biogenesis, an energy-saving approach.

Perchlorate-specific stress response

In a previous study, we demonstrated that *D. hansenii* has the highest microbial perchlorate tolerance reported to date (Heinz et al., 2020). However, subsequent experiments revealed that the tolerance towards NaClO_4 (2.5 mol/kg) was still more than one third lower than towards NaCl (4.0 mol/kg), even though the water activity was substantially higher in the NaClO_4 -containing growth medium (0.926) than in the NaCl -rich medium (0.854) (Heinz et al., 2021). Differences in salt tolerances were interpreted by the authors to account from the chaotropic stress exerted by the perchlorate anion. This interpretation is strongly supported by our proteomic investigations as explained below.

As a chaotropic ion, perchlorate is destabilizing biomacromolecules such as proteins (Salvi et al., 2005) or glycan (i.e. polysaccharide) macromolecules (Williams & Hallsworth, 2009). The fungal cell wall of *D. hansenii*'s close relative, *S. cerevisiae*, consists of approximately 85% glycans (incl. 1%–2% chitin) and 15% cell wall proteins (Lesage & Bussey, 2006). Hence, it can be expected that the presence of chaotropic perchlorate induces cell wall stress in addition to the stresses caused by NaCl . Our results suggest that yeast cells counteract this chaotropic stress to a certain extent by increasing the bioproduction rate of cell wall components.

For example, chitin metabolic processes (incl. the synthesis of its amino sugar precursors) were found to be significantly upregulated under perchlorate stress conditions (Figure 3) and show a higher significance for the perchlorate-specific stress response than other metabolic pathways (Figure 2D). Although being a minor component of fungal cells wall, chitin provides important structural stability (Brown et al., 2020). Chitin is produced by chitin synthases, such as the upregulated DEHA2D03916p, to a lesser degree directly in the

lateral cell wall and to a higher extent in the primary septum during cell budding (Lesage & Bussey, 2006), presumably to protect the emerging nascent cell (Brown et al., 2020). This highlights the importance of enriched chitin synthesis already during budding under perchlorate stress which otherwise might chaotropically destabilize the nascent cell envelope. This also explains the upregulation of septin proteins (Figure 3), which provide structural support during cell division at the septum (Douglas et al., 2005).

Another metabolic pathway upregulated under perchlorate-specific stress conditions is the glycosylation of proteins. Studies have shown that N-glycosylation is stabilizing proteins (Shental-Bechor & Levy, 2008) also with respect to chaotropic denaturation (Kern et al., 1992). The glycosylation-induced increase in protein stability affects both intracellular proteins as well as cell wall proteins. However, in contrast to intracellular proteins which are usually N-glycosylated in the ER with only 9–13 glycan residues, cell wall proteins experience an extensive additional glycosylation (including O-glycosylation) in the Golgi apparatus resulting in a highly branched structure containing as many as 200 glycan residues (Lesage & Bussey, 2006). We found that one of the most pronounced and densest perchlorate-specific upregulated protein clusters contained proteins involved in the ER to Golgi vesicle mediated transport (Figure 3A). This suggests that a large part of the upregulated glycosylation processes is applied to cell wall proteins in the Golgi. Furthermore, three of the upregulated proteins involved in protein glycosylation are O-mannosyltransferases involved in O-glycosylation, which is essential for cell wall rigidity (Gentsch & Tanner, 1997) and also upregulated upon heat stress (Hamiel et al., 2009).

The glycosylated cell wall proteins are transported from the Golgi apparatus via vesicle-mediated transport to the cell wall. A protein that needs to be highlighted in this context is the upregulated Chs5-Arf1p-binding protein DEHA2G07832p whose homologue in *S. cerevisiae* mediates export of chitin synthase 3 from the Golgi apparatus and the transport to the plasma membrane in the bud neck region (Trautwein et al., 2006) confirming the importance of cell wall chitin metabolic processes under perchlorate stress. Misfolded proteins or proteins chaotropically denatured despite stabilizing glycosylation might be autophagically degraded explaining the upregulation of proteins involved in autophagy (Figure 3).

Among the perchlorate-specific upregulated proteins are several proteins that are involved in cell wall biogenesis and remodulation. Apart from the chitin synthases, these are, for example, the two glycosidases DEHA2G21604p (glycoside hydrolase family 16, CRH1 homologue in *S. cerevisiae*) and DEHA2G18766p (Glucan 1,3-beta-glucosidase BGL2)

responsible for glucan cross-linking and chain elongation in the cell wall (Cabib et al., 2007; Taff et al., 2012). Stronger cross-linking of cell wall components and concomitant disability to separate cells after cell division might explain the formation of cell chains of *Hydrogeothermus marinus* (Beblo-Vranesovic et al., 2017) and of large cell aggregates of *Planococcus halocryophilus* (Heinz et al., 2019) when exposed to perchlorate stress.

In comparison to the upregulated cell wall biosynthesis and organization as well as the protein glycosylation, all downregulated perchlorate-specific processes have a lower significance (Figure 2D, with the exception of the Uniprot keywords ‘magnesium’ and ‘potassium’, which are very general assignments that do not provide profound information on underlying stress responses). The most pronounced downregulated protein subcluster contains proteins involved in mitochondrial translation and is physiologically linked to the simultaneously downregulated amino acid biosynthesis, the tRNA aminoacylation for protein translation, and the translation initiation (Figure 3A). Apart from energy-saving aspects similar to the above described downregulation of cytosolic translation observed under shared NaCl- and NaClO₄ stress conditions, it has been observed that changes in mitochondrial translation accuracy modulate cytoplasmic protein quality control (Suhm et al., 2018). For example, it has been found that decreasing mitochondrial translation output coincides with cytoplasmic protein folding (Andréasson et al., 2019), which seems plausible under chaotropic stress conditions that promote destabilization of protein tertiary and quaternary structures.

Therefore, it might be surprising at first, that the machinery for protein folding via heat shock proteins (often acting as chaperons) is downregulated under perchlorate stress conditions (Figure 3). However, protein folding is regulated by two major folding pathways. The general pathway is mostly mediated by 70-kDa heat shock proteins (Hsp70), while the second pathway, called the calnexin cycle, is dedicated for N-glycosylated proteins and requires, among others, the action of the proteins calnexin (or its homologue calreticulin) and disulfide isomerase (Kozlov & Gehring, 2020). Since we observed a high degree of protein glycosylation in the perchlorate-specific stress response, it seems likely that not the heat shock protein mediated folding pathway is upregulated under perchlorate stress, but rather the calnexin cycle. Indeed, we detected perchlorate-specific upregulations of the disulfide isomerase DEHA2E23628p, and the calnexin homologue DEHA2E03146p as part of the of the protein processing in the ER cluster (Figure 3A, Table S1).

The reduced mitochondrial translation might also be explained by the prevention of proteotoxic stress within the mitochondria as mitochondrial encoded proteins cannot be stabilized by glycosylation which takes place exclusively in the ER and Golgi apparatus

(intramitochondrial glycosylation is under debate; Guo et al., 2021; however, this process has not yet been described for yeast cells), and therefore might be denatured more easily under perchlorate stress. Following this argumentation, the mitochondrial translation might be downregulated to the minimal required performance to avoid accumulation of denatured proteins within the mitochondria.

The role of perchlorate-induced oxidative stress

Due to the high oxidation state (+7) of the chlorine atom in the centre of the perchlorate anion, it is expected that perchlorate exhibits a stronger oxidation stress response than observed in the NaCl-containing samples. Indeed, there is evidence that several genes in microorganisms from sediments of hypersaline ponds increase both the resistance to perchlorate and to oxidative stress induced by hydrogen peroxide (Díaz-Rullo et al., 2021). Furthermore, increased levels of lipid peroxidation after growth of different species of cyanobacteria in perchlorate-containing growth media were interpreted as results of oxidative stress (Rzymiski et al., 2022). However, the authors did not investigate whether the oxidative stress is perchlorate-specific or would be observed to a similar extent by other salts (e.g. NaCl) as well.

From all proteins potentially involved in oxidative stress response (e.g. superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, glutaredoxin, glyoxalase, or thioredoxin), in our experiments only the glutathione reductase GLR1 (DEHA2E13442p) was observed to be perchlorate-specifically upregulated with a low significance (FDR > 0.012; Table S1). GLR1 is involved in a multiplicity of cellular functions including besides the protection of cells from oxidative damage also amino acid transport as well as DNA and protein synthesis (Collinson & Dawes, 1995). This indicates that in our experiments the oxidative stress response is not substantially upregulated under perchlorate stress compared to NaCl-induced stress, even though earlier studies indicated that chaotropic-induced stress induces oxidative damage more strongly than does osmotic stress (Cray et al., 2015; Hallsworth et al., 2003).

The above-mentioned reduction of the mitochondrial translation activity might also be interpreted as an attempt of the cell to minimize ROS production during aerobic respiration. Indeed, previous studies indicated that perchlorate induces oxidative stress to mitochondria by enhanced ROS production (Zhao et al., 2011; Zhao et al., 2014). Yet, the missing comparisons with NaCl or other solutes made it impossible for the authors of these studies to prove that the increased ROS levels resulted from perchlorate-specific reactions and would not be provoked by other salts as well. If the reduced

mitochondrial translation activity observed in our experiments would be a result of an enhanced oxidative stress, a concomitant downregulation of respiratory chain proteins would be expected to occur. However, we did not observe a conclusive downregulation of these kinds of proteins. For example, while the cytochrome c oxidase (COX) assembly mitochondrial protein DEHA2C13244p was downregulated, the COX subunit 9 was upregulated under perchlorate-specific stress conditions.

In summary, the proteomic data suggest that antioxidant activity is important for survival under salt stress conditions (to a similar extent in NaCl- and NaClO₄-stressed cells), but the oxidative stress induced specifically by perchlorate seems to play only a minor role compared to the chaotropic stress. This is in accordance with previous experiments demonstrating that the more oxidatively reactive (but less chaotropic) chlorate anion (ClO₃[−]) can be better tolerated by *D. hansenii* than perchlorate, which indicates the oxidative character alone cannot account significantly to the additional stress exhibited by NaClO₄ compared to NaCl (Heinz et al., 2021). A possible explanation for this phenomenon is that perchlorate is astonishingly stable in solution under ambient temperatures (Urbansky, 1998) due to the reduction rate-limiting oxygen atom transfer (Ren & Liu, 2021). These additional stressors (chaotropicity, and potentially to a minor degree also oxidative stress) presumably require different or more distinct stress signalling, which likely explains the upregulation of proteins involved in signalling and the actin cytoskeleton organization pathways (Figure 3) in addition to the signal transduction proteins expressed similarly in NaCl- and NaClO₄-stressed cell cultures (Figure 4).

The most relevant of the above-described perchlorate-induced chaotropic stress responses are graphically summarized in Figure 3B. In particular, cell wall genesis is a very energy-consuming process (Hu et al., 2021), but also intensive protein glycosylation required for protein stability under perchlorate stress costs additional energy compared to non-chaotropic conditions. While in the non-chaotropic NaCl-stressed samples, most of the energy provided by the stress-adapted cell metabolism can be used to counteract osmotic and induced oxidative stresses, in perchlorate-containing samples a substantial part of the cellular energy demand is required for counteracting chaotropic stress resulting in a lower NaClO₄ tolerance of *D. hansenii* compared to NaCl.

Consequences for microbial habitability of perchlorate-rich environments on Mars

This study provides new insights for putative life on Mars if it exists in perchlorate-rich regions, which have

been identified during the exploration of Mars (Clark & Kounaves, 2016; Hecht et al., 2009; Lauro et al., 2021). Protein-stabilizing glycosylation and cell wall reorganization are major stress responses emerging only after long-term adaptations to high perchlorate concentrations, while being not significantly expressed after perchlorate-shock at moderate salt concentrations. Hence, it is likely that biomacromolecules and cell envelopes of putative Martian microorganisms exposed to perchlorate-rich brines would evolve stable conformations and, hence, prefer covalent bounds and cross-linking over looser electrostatic interactions, hydrogen bonding or hydrophobic effects. Additionally, cell components susceptible to chaotropic stress might be stabilized by the attachment of polymers similar to stabilization effects via protein glycosylation as observed in our experiments.

Furthermore, previous microscopic observations (Beblo-Vranesovic et al., 2017; Heinz et al., 2019) indicated that larger cell aggregates are more likely to occur (possibly due to cell wall rearrangements and cross-linking) under perchlorate stress than single cells. Consequently, cell clusters or biofilms might be considered as potential macroscopic visible biosignatures on Mars; however, metabolomic changes under perchlorate stress should be investigated in upcoming experiments as well in order to identify potential perchlorate-specific biomarkers on the molecular level.

The presented results are also important for in situ resource utilization (ISRU) technologies to support a human outpost on Mars (Billi et al., 2021). Oxygen and food production by phototrophic microorganisms and the recycling of waste material in perchlorate-rich Martian soil might be conducted by ‘chaotolerant’ (Zajc et al., 2014) organisms, because they would likely possess a metabolic toolset for stabilization of biomacromolecules similar to *D. hansenii*. Alternatively, genes responsible for increased biomacromolecular stability might be used in synthetic biology to create perchlorate resistance strains that can thrive in perchlorate-rich Martian soil without the necessity for perchlorate remediation (Díaz-Rullo et al., 2021).

The presence of perchlorates might be even beneficial for enzymatic activities at the low temperatures prevailing on Mars due to a reduced enthalpy of activation owing to chaotropic effects of perchlorate salts (Gault et al., 2021). Furthermore, the chaotropicity of perchlorate might also extend the temperature window for activity of microorganisms to subzero temperatures, as chaotropes enable flexibility of cellular macromolecules, which is crucial for growth under frigid conditions (Chin et al., 2010).

Our data indicate that perchlorate-induced oxidative stress is not substantially higher than for other salts like NaCl. However, this might be only true for the Martian subsurface, because close to the surface, cosmic radiation penetrates the soil, usually within 0.7–0.9 m soil

depth depending on the type of regolith (Röstel et al., 2020). UV radiation, on the other hand, is effectively shielded by a few microns of dust (Mancinelli & Klovstad, 2000). The cosmic radiation can decompose perchlorates present in the Martian regolith to far more reactive oxychlorine species such as hypochlorite which exhibit a strong oxidative stress to cells (Quinn et al., 2013). While these components might not be able to infiltrate dormant lifeforms in the absence of liquid water (Hallsworth, 2021), they become an active oxidizing stressor for cells as soon as liquid water is provided, for example, by deliquescence. Therefore, we conclude that microbial cells close to the Martian surface, which are exposed to perchlorate salts and cosmic radiation require cellular adaptation against cosmic radiation, low water activities, chaotricity and oxidative stress, while at soil depths deep enough to shield cosmic radiation perchlorate-exposed organisms have to struggle neither with cosmic radiation nor with oxidative stress induced by perchlorate decomposition products (because perchlorate itself is not exposing a significant oxidative stress to cells according to our results).

CONCLUSIONS

The results of this study revealed perchlorate-specific microbial stress responses never described in this context before. Even though NaCl- and NaClO₄-induced stress responses in *D. hansenii* share several metabolic features, we identified enhanced protein glycosylation, folding via calnexin cycle and cell wall biosynthesis or remodulation as a counteractive measure to perchlorate-induced chaotropic stress, which generally destabilizes biomacromolecules. At the same time, mitochondrial translation processes are downregulated under perchlorate-specific stress. When applying these physiological adaptations, cells can increase their perchlorate tolerance substantially compared to perchlorate shock exposure. These findings make it likely that putative microorganisms on Mars could draw on similar adaptation mechanisms enabling survival in subsurface perchlorate-rich brines.

AUTHOR CONTRIBUTIONS

Jacob Heinz performed growth experiments with *D. hansenii*. Jacob Heinz and Andy Schneider conducted protein extraction. Jacob Heinz, Joerg Doellinger, Deborah Maus, and Peter Lasch accomplished proteomic analysis. All authors, including Jacob Heinz, Joerg Doellinger, Deborah Maus, Andy Schneider, Peter Lasch, Hans-Peter Grossar, and Dirk Schulze-Makuch, contributed to the interpretation of results. Jacob Heinz wrote the manuscript with input from all authors.

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CONFLICT OF INTEREST


The authors declare no competing financial interests.


DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD033237.

ORCID

Jacob Heinz  <https://orcid.org/0000-0002-8237-4713>

Joerg Doellinger  <https://orcid.org/0000-0001-8309-082X>

Deborah Maus  <https://orcid.org/0000-0002-9688-2663>

Peter Lasch  <https://orcid.org/0000-0001-6193-3144>

Hans-Peter Grossar  <https://orcid.org/0000-0002-9141-0325>

Dirk Schulze-Makuch  <https://orcid.org/0000-0002-1923-9746>

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