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Molecular analysis of stress response of the marine yeast *Debaryomyceshansenii*

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Abstract:

Studies on gene expression are critical for understanding the biological consequences of stress. *Debaryomyceshansenii* is an industrially important yeast used in food processing and biotechnology. Its extremophilic characteristics serve in a variety of commercial applications. Its genetic makeup enables it to survive in hypersalinity, extreme temperatures, and toxic metal exposure. Interest in is natural resilience has inspired studies in microarrays, Next-Generation sequencing, and CRISPR tools. These studies have led to the isolation of regulatory sequences that can potentially be used for gene expression. This review explores the molecular analyses of individual stressors as well as the synergistic/ antagonistic effects of multiple stressors on the organism.

Keywords **—extremophile, ESR "Environmental Stress Response", osmotolerant, oxidative stress, CRISPR.**

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I. INTRODUCTION

The unicellular haploid ascomycete marine yeast, *D. hansenii*, is considered an extremophile since it can live in a wide range of: salinities (3% – 24% w/v, osmotolerant) [1], pHs (3 -10) [2-3], and temperatures (6°C-37°C) [4-5]. It can withstand heavy metal ions (Cobalt, Zinc, others) in its environment through responses, such as riboflavin production [6-11]. Many *Debaryomyces spp.* have been isolated from diverse provenances, including estuaries, bays, brine, cheeses, wine, plants, soil, and even from interdigital mycotic lesions in patients [1]. More recently, this organism has been isolated from arctic glaciers [12]. The organism has been taxonomically subdivided into two major subspecies: *D. hansenii var. fabryi* and *D. hansenii var. hansenii*[1]. It has been considered closely related to *Candida spp*. [13-15], supporting genetic interrelatedness among the ascomycete yeasts [11].

This yeast has been used in many diverse industrial settings, for example, to reduce industrial ecological impact. Donzella et al. [16] assessed the potential of the marine yeast *D. hansenii* (strain Mo40) isolated from industrial effluent as a platform strain for phytase, protease, and lipase production using industrial media containing high concentrations of sugars, urea, and NaCl. Another example was utilizing *D. hansenii* biomass as effluent to remove azo dyes Reactive Red 141 (RR141) and Reactive Blue 19 (RB19) from aqueous solutions [17].

For agricultural purposes, the antagonistic bioactivities of *D. hansenii* have been known and used as biocontrols against bacterial, fungi, and mold in various food items such as wine, cheese, sausage, meats, salads, and others. This ascomycete yeast can produce a cocktail of toxins and other secondary metabolites while innocuous to humans are only harmful to "pests" commonly found in perishables [18]. This method has been preferable to regularly used pesticides due to social demand on organically grown produce [19]. In addition to biocontrol, this yeast has been used as a probiotic in livestock, significantly improving their immune system[20-21]. It is capable of providing immune stimulation, increasing growth and survival rates in a wide variety of animals, like fish, crustaceans, mollusks, and recently in goats, to stimulate both immune and antioxidant responses. In some feed or food industries, obtaining *D. hansenii* biomass is a cheap and environmentally friendly way for potentially replacing unsustainable ingredients in food or nutrient processing, as this species is included in the QPS EFSA list [16].

D. hanseniis broad applications open up research to understand the gene expression responsible for its extremophilic characteristics. The well-known molecular techniques, the lack of pseudogenes or multi-copy genes, and manageable genome size make this yeast an ideal model organism for gene expression studies. Its facultative nature toward many environment signals, as previously stated, makes it challenging to evaluate stress conditions. Transcription studies in *D. hansenii* have identified some genes responsible for stress acclimation when exposed to sodium chloride (NaCl), cobalt (Co (II)), and pH changes [10,22-23]. Differences among stress responses have delineated a threshold of tolerance vs. preadaptation to environmental stimuli showing that the organism is better adapted to deal with some stressors than others [10,22]. Comparing the stress response of *D. hansenii* to those of other commonly known yeasts like *Saccharomyces cerevisiae* or *Candida albicans*[24-25] demonstrates that this organism is better acclimated to the perturbations of an industrial environment. Understanding stress response mechanisms can expand current applications of this yeast into areas of commercial interest[26]. This review intends to discuss changes in gene expression under conditions of stress.

II. REVISITINGMOLECULAR BEHAVIOUROF *D. HANSENII*UNDER STRESS

A. Overview of the concept 'General Stress Response'

When exposed to external stimuli, an organism's altered behaviour is commonly known as the stress response. Some common stressors elicit a universal response in all organisms. In most cases, several genes are upregulated or downregulated in response to stress, including (but not limited to) DNA replication and metabolism genes (e.g., POLα, POL30, CDC28) as well as heat shock proteins (e.g., HSP12, HSP26) [2,5]. For example, the Hsp70/ Hsp90 co-chaperone or the CDC37 chaperone complex is a feedback inhibition complex typically expressed during cell division and stress conditions (osmotic changes). Under stress conditions, this complex is activated by regulatory proteins (e. i. Msn2, Msn4) to alter transcriptional and posttranscriptional processes in the cell [5,27]. In addition to this, it's worth mentioning that Hsps' can upregulate after stress exposure to conditions other than thermal fluxes. Stress also impacts overall cellular metabolism. High salt conditions increase glycerol biosynthesis and other cytoplasmic processes to compensate for the osmotic imbalance. At the same time, nutrient starvation increases the transcription of genes related to carbon source utilization, amino acid metabolism, and lipid biosynthesis. This helps the cell acclimate by switching its energy source and making certain building blocks available when they may not be found in sufficient quantities in the environment [26]. These responses can be used as biological stress markers.

Interestingly, as conditions stabilize, the stressor can be assimilated, overcome, and in some cases even promote high yield or fast proliferation [22]. Due to environmental fluxes, multiple stressors, and prolonged exposure time, the stress response can adjust. To understand a stress response, one needs

to establish a baseline transcriptional behaviour for comparison.

B. *Genefunction under stress conditions*

Environmentally inducible genes are of great value to the biotechnological industry. Identifying the upregulation or downregulation of affected genes can allow a better understanding and a future manipulation of those genes for targeted purposes. *D. hansenii's* great diversity and incredible resilience have made it the object of gene expression studies[10,28]. Microarray studies of *D. hansenii*exposed to sodium chloride and the transcriptome analysis in the presence of cobalt enriched conditions have established parameters to understand better how this yeast reacts to drastic changes in its environment [22].

Constitutive stressors and induced tolerance can be differentiated by comparing environmental responses. Various studies have been done to elucidate stress-induced reactions of *S. cerevisiae*, *Schizosaccharomyces pombe*, and *D. hansenii* utilizing DNA microarrays for the whole genome transcription analyses [22,29-30]. These studies help determine possible responses of yeasts of similar clades. For example, it is known that the stress response to osmotic changes in *S. cerevisiae* is to commence the production of glycerol via the High Osmolarity Glycerol (HOG) pathway comprising of isoenzymes GPD1 and GPP1. It also commences trehalose synthesis employing a tri-enzymatic complex- TPS1, TPS2 TSL1/TPS3. These measures counterbalance dehydration and cytoplasmic leakage [3,6,25]. These and many other reactions have come to be known as 'Environmental Stress Response' (ESR) [24,29]. Specific promoter incorporated sequences ("CCCCT") [30], shared by many stress-induced genes, are responsible for the responses as mentioned above.

Precise and quantifiable conclusions can be drawn from RNA sequencing data by comparing pre and post-stress transcriptomes. Recent investigations into *D. hansenii's* genetic responses to short-term and prolonged stress have employed this tactic [10,31-32].

C. *Osmotic Stress*

In Gonzalez et al. [22], *D. hansenii* was exposed to high NaCl-induced osmotic stress (2M NaCl), and the expression of 1.7% of the genes was significantly changed. Functional annotation tied the initially upregulated genes to ribosomal RNA expression. Upregulation of these genes was maintained throughout the experiment, indicating that the cell had quickly completed its response to hyperosmolarity. This efficient acclimation sets *D. hansenii* apart from *S. cerevisiae*, which succumbs to osmotic shock when exposed to NaCl solutions greater than 2M [33]. *Candida spp*., on the other hand, seems more similar to*D. hansenii* in its response to NaCl [34]. Phylogenetic studies likewise have placed *D.hanseniispp* more closely to *Candida albicans* than *S. cerevicea* [20,34].

Upregulation of mitochondrial and nuclear genes in *D. hansenii* showed that respiration and metabolism were not inhibited. GPD1 (glycerol-3-phosphate dehydrogenase) and GPP1 (glycerol-3-phosphate phosphatase) are the genes responsible for glycerol production, which acts as an osmolyte [7,25,29,35] were identified at 0.5hr, but upregulation values were not statistically significant22. It is possible that the glycerol biosynthesis occurs at high speed after osmotic shock or occurs faster than the first measurements were done; hence the time point detected the decrease in activity. *D. hansenii* could retain glycerol better than *S. cerevisiae*[35]. This indicates that *D. hansenii* is preadapted to hypersalinity. Oher preadaptations include ENA1 (P-type ATPase, which mediates efflux and prevents accumulation of some salts in yeast) and protein synthesis. This response showed to be statistically significant even after 3 hours of exposure to hypersalinity [22].

Upregulation of ENA2, GPD2, and GPP1 in *S. cerevisiae*, and ENA1 and GPD1 in *C. albicans* continues throughout exposure to high osmolarity [30,34]. The main difference between species is the response time for the upregulation of stress-related genes. In contrast, *D. hansenii* is highly efficient in osmoregulation as glycerol production was not continued indefinitely. Its efficiency in

osmoregulation is perhaps due to its ability to better retain glycerol [25,35]. It has been documented that under saline stress, *D. hansenii* utilizes potassium (K+) from the medium instead of sodium chloride to elude hypertonic conditions. The yeast cells were not negatively affected by the ionic change; therefore, the selected NaCl concentration was within a viable- tolerant range signifying a preadaptation to the environment.

HWP1 is one of the genes responsible for the hyphal wall protein (mannoprotein) in yeast. It is most commonly associated with mating and adhesion in *C. albicans* [36]. It was found to be upregulated at all time points during stress. This upregulation could be indirect evidence of the preadaptation of *D. hansenii* to osmotic shock. Along with HWP1, upregulation of other genes like OLA1 (putative GTP binding protein), mitochondrial carrier protein, and even DNA synthesis genes indicates that the cell restored normal function of DNA replication mitochondrial function and cell division [10].

Contrary to expectations, osmotic stress-related genes like dihydroxyacetone kinase (DAK1), phosphoglucomutase (PGM2), and α,α trehalose phosphate synthase (TPS3) [37] were not significantly upregulated. Even heat shock proteins, notorious for their role in global stress response, were not significantly upregulated, supporting *D. hansenii's* hypersaline pre- adaptability. *D. hansenii's* preadaptation to hypersaline environments seems to be useful handling other stressors [38]. Though at extreme temperatures, this is not the case [5].

D. Oxidative Stress

Though heavy metal ions are necessary to complete biological processes like growth development and metabolism [25], high concentrations of heavy metals cause oxidative stress, leading to severe DNA damage, protein disruption, and lipid degradation, even at subtoxic levels of exposure [23,30]. Hydrogen peroxide (H2O2), heavy metals (iron, cobalt, copper, manganese, zinc), and toxic chemicals/drugs (cyclopentene, menadione, diamide) are the most widely used agents in studies to induce oxidative stress [23,38].

Guma- Cintron et al. [10] exposed *D. hansenii* to 5mM Cobalt (II) to study oxidative stress. 471 genes were significantly changed (four-fold and above) from 4,721 transcripts in their time-course transcriptome analysis. However, only 40% of those genes could be functionally annotated. Three distinct responses were identified: DNA synthesis, oxidative/ free radical response, and cell growth. This response is dissimilar to that of *S. cerevisiae* exposed to the same oxidative agent in Hosiner et al. [39], where out of 1,023 significantly identified genes, only 36 were upregulated, and 41 were downregulated. In a more exhaustive study by Hosiner et al. [39], nine additional oxidizing agents were included to obtain a typical oxidative stress response between *Saccharomyces* strains, yet no common denominator was found. In a study from Navarrete et al. [40], *Debaryomyces spp*. reacted more strongly to oxidative stress than *Saccharomyces*, supporting the transcriptional differentiation between species. Also, most recent studies show that osmotic stress has a prophylactic effect against oxidative damage [41].

E. Oxidative Response: Limitation of the Free Radicals

Limiting additional free radical generation while dealing with oxidative stress, dehydrogenase-like enzymes were severely downregulated, interrupting the Krebs Cycle and reducing superoxide production meant a decrease in the electron transport chain [10]. However, some of the essential enzymes like aconitase and succinate semialdehyde dehydrogenase (SSADH) are drastically affected by heavy metal toxicity. The GABA shunt, a well-known and highly preserved process was activated to restore balance and replace the loss of NADH [42]. One of the genes responsible for this pathway, UGA3, was upregulated (four-fold change) throughout the trial, confirming GABA shunt activation [10]. This pathway is composed of three (3) enzymes:

glutamate decarboxylase (GAD, gene- GAD1), GABA aminotransferase (GAT, gene-UGA1), and succinate semialdehyde dehydrogenase (SSADH, gene- UGA2) [42-43]. This alternate route to complete the Krebs Cycle is accomplished by converting glutamate into succinate, allowing the yeast to continue its metabolic course. Studies have shown this strategy to be vital for thermal tolerance in *S. cerevisiae* [5,43].

The non-enzymatic system of glutathione free radical scavenger [23,34,39,41]sulfiredoxin, an antioxidant protein for signalling catalytic reduction of oxidative modifications and lysine-histidine metabolism for 'protein carbonylation', was upregulated [1]. Enjalbert et al. [34] demonstrated this type of behaviour for *C. albicans* and in Jamieson [44] for *S. cerevisiae* under oxidative stress. *C. albicans* showed a decrease in superoxide dismutase and a fourfold upregulation in a glutathione (TTR1) system (TRX1- thioredoxin, TRR1- thioredoxin reductase) [34]. The intensity of the response varies according to the oxidative stressor [8,39]. Another well-known gene of the non-enzymatic pathway that was highly upregulated in *D. hansenii* was STB [5]. This transcription factor upon oxidative stress binds and regulates the expression of most genes of the pentose phosphate pathway and genes involved in the production of NADPH, a metabolite required for oxidative stress resistance [45].

F. DNA Synthesis, Cell division, and growth

The initial shock reaction of *D. hansenii* to cobalt (II) stimulated DNA synthesis and repair genes like RAD52 (which stimulates strand exchange by annealing and repairing DNA during vegetative growth), MSH2 (mismatch binding and repair), and SMC1 (chromosome segregator and DNA breakage repair), among others. This upregulation goes down after the first time point (0.5hr), and only a few genes are continuously upregulated throughout the extension of the experiment, like PLM2 and SYM1. These genes are part of the DNA damage response pathway enclosed in the stress response [46]. The chromatin-associated PML2, a putative transcription factor, is generally induced to respond to DNA damaging substances [41]. SYM1 is an integral mitochondrial inner membrane-bound protein capable of ethanol metabolism and is heat shock-induced [47].

Although the stress response to heavy metals severely affected the yeast population, proteins for vegetative growth were observed throughout the trial, and consequently, an incremental increase in cell density was observed. Paxillin-like protein 1 (PXL1), involved in actin cytoskeletal regulation and mediator of polarized cell growth [48], is constantly upregulated, which means that after the initial 'shock,' the cell could recuperate and establish a mechanism to battle the environmental disturbance regaining the capacity and comfort for reproduction.

III. STRESS RESPONSE COMPARISON

G. Osmotic vs. Oxidative stress response

The versatility of this organism can lead one to mistake resistance and tolerance for exaptation and assume physiological and biochemical responses toward stress are 'normal'. Expression studies have greatly helped differentiate taxing genetic activity from the environmental impact of stressors vs. non-stressors.

Gonzalez et al. [22] data exhibited deficient gene expression levels (109 genes) conversely Guma-Cintron et al. [10] cobalt (II) exposure, where the expression roughly tripled. Heavy metal showed 471 genes with a 4-fold change meaning that it possesses the capacity to survive heavy metals yet not by a predisposing mechanism like those used under NaCl. Only 1.72% of the genes were statistically relevant in response to NaCl, indicating a minor effect from high salinity. The osmotic stress data presented an inversely proportional up- and downregulation of genes (with an initial low in upregulation) which can be interpreted as a change in machinery to accommodate new conditions. This trend does not hold in the case of the oxidative stress response. Here a fourfold cut-off change had to be applied to manage the vast dataset. Initial upregulation was dominated by protein synthesis machinery,

mitochondrial functionality, cell wall structure, and carbohydrate and glycerol metabolism for osmotic stress; wherein oxidative stress DNA synthesis and repair, non-enzymatic oxidative stress response (suppression of superoxides), and metabolic pathways predominated the upregulation. Consistent upregulation was noted along between time points.

Notably, the number of unknown-annotated genes in both studies is remarkably high at various time points. It is consistently higher for oxidative stress, either up or downregulated, yet the downregulation monopolizes unannotated gene functions in osmotic stress. Even though a vague global understanding of the concept exists, details are lacking due to the inability to assign a function to the too many downregulated genes. Most of the unknown genes were downregulated during osmotic stress, 23% at 0.5hr, 46% at 3hr and 39% at 6hr, but only 8% at 3hr and 15% at 6hr were upregulated [22]. In this case, most annotated upregulated genes defined the organism as preadapted to the changes, but the amount of unknown downregulated genes (44 out of 109 significantly changed) still hides alternate routes and mechanisms that deal with the environmental variation.

In contrast, when the yeast was subjected to cobalt (II), to which it is not preadapted, the high percentage of unknown genes precluded any meaningful conclusions. Fifteen percent of unknown genes at 0.5hr and 1.5hr and 21% at 3hr were upregulated. Twenty-six percent, 19%, and 15% were downregulated. The number of unknown genes is consistently higher than all the known genes individually; this implies a strong response that's still not accounted for. More than half (60%) of the observed gene expression could not be explained, leaving a great set of uncertainty about how accurate the behavioural prediction is for the oxidative stress response. This opens up a new investigation opportunity to completely comprehend the complete stress response for *D. hansenii.*

Each stressor shows a specific panorama of dealing with an isolated event, but the synergistic effects of these stressors can show a different biological response. In Capusoni et al. [49] high NaCl (2M) environment was shown to depolarize the cell membrane reducing its permeability. This feature is advantageous to other environmental stressors like oxidative, thermal, or ion changes. So much so that it has been adapted as a pre-treatment for biocontrol induction [38]. Osmotic stressors have become a superpower for the yeast by managing its environment to further its adaptation to other damaging conditions. In Sui et al. [23], the preadapting treatment has been tried with oxidative agents like H2O2 for 30 mins. It showed the same results that Guma-Cintrónet al.[10] published regarding fast-acting non-enzymatic response and reactive oxygen species (ROS) production for defence. These studies were performed individually, but when the data is compared side by side, the general stress response developed in both scenarios shows a consistent pattern of specific gene activations and responses like anti-oxidative enzymes (glutathione radical scavengers), metabolic intermediates (ROS, free amino acids, alternative pathways), among others [5,10,41].

H. CRISPR in D.hansenii

The CRISPR- Cas9 system has been successfully utilized in model organisms like *Saccharomyces* [50-51], *Drosophilamelanogaster* [52], Zebrafish, and mice [53] to create 'knock-in – outs,' gene editing, mutants, streamline vectors (plasmid) for easy use, among others. The novel plasmid-based CRISPRCUG/Cas9 method was finally perfected for easy and efficient gene editing of the prototrophic strains of *D. hansenii* [54]. The plasmid creation for this yeast was successful due to highly specific gene functions. This new method has been successfully implemented to support multiplex gene engineering. This group [55] developed a system for studying the transcriptional regulation of the 26S proteasome, an essential ATP-dependent protease complex whose proper activity is vital for all cells and organisms. They developed a genetic approach to control the activity of this enzyme by deregulation of its essential subunits.

The mutant strains were sensitive to geno- and proteotoxic stresses as well as high salinity and osmolarity, suggesting a contribution of the proteasome to the extremophilic properties of *D. hansenii*. The developed CRISPR systems allow efficient *D. hansenii* genome engineering and should advance applications of this yeast.

IV. CONCLUSIONS AND FUTURE PERSPECTIVES

D. hansenii is an ascomycete yeast species known for its ability to grow rapidly on various substrates. This capability makes it a strong candidate for industrial, agricultural, and biotechnological applications, including bioproduction of chemicals and fuels, livestock immune-boosting, biocontrol and others. Additionally, *D. hansenii* has been shown to be tolerant to high ionic conditions, which may eliminate costly detoxification processes that use saline water. Impressively, this could potentially provide an economical solution for the renewable production of several important industrial commodities. Building on the successful example set by many other industrial activities, the biotechnology industry has progressed from an academic concept to bioindustry, continuing to evolve. To further boost biotechnology processes at an industrial level, synergistic collaborations and applications must continue to be trialed. Applications are often controlled in singular units, yet new research needs to push boundaries and try multifunctional assessment of nested effects on already industrially accepted *D. hansenii* strains.

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