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Research article

Putative 3-nitrotyrosine detoxifying genes identified in the yeast *Debaryomyces hansenii*: *In silico* search of regulatory sequences responsive to salt and nitrogen stress

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ABSTRACT

Background: During salt stress, the yeast *Debaryomyces hansenii* synthesizes tyrosine as a strategy to avoid the oxidation of proteins. Tyrosine reacts with nitrogen radicals to form 3-nitrotyrosine. 3-nitrotyrosine prevents the effects of associated oxidative stress and thus contributes to the high halotolerace of the yeast. However, the mechanism of how *D. hansenii* counteracts the presence of this toxic compound is unclear. In this work, we evaluated *D. hansenii*'s capacity to assimilate 3-nitrotyrosine as a unique nitrogen source and measured its denitrase activity under salt stress. To identify putative genes related to the assimilation of 3-nitrotyrosine, we performed an *in silico* search in the promoter regions of *D. hansenii* genome.
Results: We identified 15 genes whose promoters had binding site sequences for transcriptional factors of sodium, nitrogen, and oxidative stress with oxidoreductase and monooxygenase GO annotations. Two of these genes, DEHA2E24178g and DEHA2C00286g, coding for putative denitrases and having GATA sequences, were evaluated by RT-PCR and showed high expression under salt and nitrogen stress.
Conclusions: D. hansenii can grow in the presence of 3-nitrotyrosine as the only nitrogen source and has a high specific denitrase activity to degrade 3-nitrotyrosine in 1 and 2 M NaCl stress conditions. The results suggest that given the lack of information on transcriptional factors in *D. hansenii*, the genes identified in our *in silico* analysis may help explain 3-nitrotyrosine assimilation mechanisms.

1. Introduction

Oxidative stress is one of the most harmful conditions for a cell. Reactive oxygen and nitrogen species are produced after different alterations of cellular homeostasis, such as changes in external or internal factors (pH, salt, temperature, oxygen levels and oxidants, metabolism, *etc.*), and react extremely rapidly with the components of the cell. For unicellular organisms exposed directly to environmental challenges, it is vital to respond immediately to protect themselves against primary stress and to avoid the initiation of secondary stress and consequently its damage. Oxidative stress occurs in halotolerant yeasts when they grow in the presence of sodium. This secondary stress is caused because of an increased demand for ATP to activate osmoregulatory mechanisms under salt stress [1], which accelerate the respiration and the consequent leakage of electrons and, in turn, lead to the overproduction of reactive oxygen and nitrogen species. One of the principal problems is the formation of the peroxynitrite species, which can inactivate proteins by mediating the nitration of tyrosine, thus affecting important cellular functions [2,3].

The damage by free radicals can be countered at three levels: (1) preventive: for example the mitochondrial uncoupling protein 2 decreases the proton electrochemical potential gradient, leading to increased oxidation of electron carrier pools, and decreases local oxygen concentration, thus decreasing the production of free radicals; (2) neutralization of free radicals: enzymes such as catalase, superoxide dismutase, and glutathione peroxidase, among others, participate to neutralize free radicals; (3) repair of damage caused by oxidation: for example, methionine sulfoxide oxidation can be reduced by the methionine sulfoxide reductases Msr-A and Msr-B. Despite these three levels of response, there are irreversible damages, such as the oxidation of aromatic amino acids, particularly tyrosine, which results in the

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formation of 3-nitrotyrosine (3-NT), a nondegradable compound [4]. In yeast, genes coding for oxygen and nitrogen radical-scavenging proteins, such as catalases, prevent damage to biomolecules [2,5,6]; however, no information is available on the repair of irreversible damages such as the production of 3-NT.

Growth of the moderately halotolerant yeasts *Saccharomyces cerevisiae* and *Debaryomyces nepalensis* under salt stress increases reactive oxygen species (ROS) production, protein carbonylation, and the specific activity of antioxidant enzymes [7,8]. In the halotolerant yeast *Debaryomyces hansenii,* the ROS production increases after salt stress [9,10]. This yeast has also been found to have a repair response and damage prevention to ROS and reactive nitrogen species, *e.g.*, increased expression of genes such as *DhAHP* and *DhARO4*; the former codes for alkyl hydroperoxide reductase [9], which is active against H_2O_2, organic peroxides and peroxynitrite [11], whereas the latter codes for a DAHP synthase, which is involved in the synthesis of the amino acid tyrosine [12]. The induction of *DhARO4* expression during salt stress increases the specific activity of *Dh*Aro4p. However, the levels of free tyrosine do not increase because it is rapidly oxidized to 3-NT [10]. We suggest that the rapid formation of 3-NT prevents the oxidation of tyrosine in proteins. However, once the 3-NT is formed, it is uncertain what *D. hansenii* does with the excess of 3-NT.

This work aims to define the 3-NT assimilation and detoxification mechanisms in *D. hansenii* exposed to hypersaline stress. We describe the organism's ability to assimilate free 3-NT and identify putative genes for its degradation. We also assess the correlation of transcriptional changes resulting from nitrogen, salt, and oxidative stress by analyzing the distribution and frequency of the corresponding binding sites in all putative gene promoter regions in the *D. hansenii* genome. To validate our results, we evaluated the expression of some genes identified by our search strategy that, given the lack of information regarding gene promoter annotation and regulation for *D. hansenii* genome, represents an *in silico* effort to elucidate novel response mechanisms to oxidative and nitrosative damage.

2. Materials and methods

2.1. Yeast strains and growth conditions

D. hansenii strain Y7426 (homologous to strain CBS 767) was obtained from the US Department of Agriculture, Peoria, IL. The capacity of *D. hansenii* to assimilate the oxidized compound 3-NT as the sole nitrogen source was evaluated on the basis of the results of previous assays that used 2 and 5 mM 3-NT as nitrogen source for the bacteria *Variovorax paradoxus JS171* and *Burkholderia* sp. strain JS165 respectively [13]; we determined that 10 mM 3-NT is the maximum concentration at which *D. hansenii* can grow with this poor source of nitrogen.

To obtain growth curves, the cells were grown overnight in YPD medium and were then re-inoculated in Erlenmeyer flasks containing fresh minimal medium without any sources of nitrogen such as amino acids and ammonium sulfate and supplemented only with 10 mM 3-NT. To evaluate the effect of the combination of the two main stressors in this work (NaCl and 3-NT), we prepared the minimal medium with 10 mM 3-NT and 1 M NaCl. From each of these cultures, 0.4 ml aliquots were taken and applied in triplicate to the wells of a 96-well plate. Growth was monitored spectrophotometrically (Bioscreen C model) using a 96-well plate reader. Absorbance was automatically recorded at 600 nm every 60 min during 110 h of incubation at 28°C with continuous shaking.

For denitrase activity assay and RT-PCR, *D. hansenii* cells were re-inoculated in Erlenmeyer flasks containing fresh YPD medium (control condition) or with 1 and 2 M NaCl (salt stress). The cells were collected by centrifugation when the OD reached 0.9 units at 600 nm.

2.2. Denitrase activity assay in cell extracts

The reduction of 3-NT by denitrase enzyme (removal of the NO_2 group from 3-NT) was evaluated according to *in vitro* assay of Zeyer and Kocher [14], in which the enzyme denitrase contained in a crude extract reacts to with commercial 3-NT, after the 3-NT disappears, it must be taken into account that also the absorbance of this compound decreases, and this diminution is recorded at 410 nm. After growth in YPD medium with or without 1 or 2 M NaCl, *D. hansenii* crude extracts were obtained [10]. The reaction medium [0.1 mM 3-NT, 0.4 mM NADPH, 4 mM $MgSO_4$] was added to a 3-ml quartz cuvette, and then the optical density was observed spectrophotometrically (DW2a-SLM-Aminco-Olis). The reaction was started by adding 120 μl of the crude extract, and absorbance changes at 410 nm were recorded for 2 min. To evaluate the specific activity of denitrase enzyme, p-nitrophenol and 2-nitrophenol were used as alternative nitrate substrates. Enzymatic activities were calculated using the molar extinction coefficients of 3-NT and 2-nitrophenol (4400 and 3470, respectively) and were then expressed as units (μmol of substrate consumed in 1 min) per mg of protein mass.

2.3. Searching for denitrase candidate genes

2.3.1. D. hansenii genome database

Genome sequences of *D. hansenii,* including a total of 6252 genes and their annotations in Gene Ontology (GO), were obtained from the Genolevures database (www.genolevures.org/deha.html; now at http://igenolevures.org). We considered the region 1000 bp upstream from the initiation ATG codon, *i.e.*, from positions -1 to -1000, as putative promoter regions for the construction of a putative promoter database.

2.3.2. Sequences for transcription factor binding sites

Using our promoter database, we mapped for the presence of seven binding sites (BS) (Table S1) as reported for *S. cerevisiae* [15,16]. The transcriptional factors (TFs) were related to the type of stress to which *D. hansenii* was exposed during its growth: NaCl stress (NaS), 3-NT as a poor nitrogen source (PNS), and the oxidative stress produced when the yeast grows in the presence of sodium (OSNa). We searched for two main TFs for each type of stress, with the basic consensus sequence for each BS with minimal modifications. These modifications were made to refine our search; for example, in the sequence WGATWR for both GLN3 and GAT1 BSs [17], we included the letter H at the end of GAT1 motif (*i.e.,* A, C, or T at the end) as suggested by Cornish-Bowden [18]. The BS sequences from this work, additional sequences for diverse stress types, and search tools used are available at http://www.deha.abacoac.org.

The *in silico* search was performed in three stages. We first identified 616 genes whose promoter region had BSs for TFs that respond to the three stresses: NaS (Msn2p/Msn4p and Sko1p), PNS (Gat1p and Gln3p), and OSNa (Skn7p and Yap1p). We also identified 1917 genes with sequences responding to OSNa and PNS stress. Next, we included in our search the definition of function for each gene from GO. Because the main function of denitrases is the catalysis of oxidoreductions, we looked for this annotation in both gene groups (616 and 1917 genes). We identified 104 genes with oxidoreductase activity. From this group of 104 genes, we searched with a more precise annotation for denitrase activity as monooxygenase enzymes that incorporate molecular oxygen, with NADP or FMN as cofactors and the inclusion of one atom of oxygen into the other donor. We found 15 genes with denitrase function.

2.4. RNA extraction and expression RT-PCR

The expression of some of the candidate denitrase genes obtained through the *in silico* search was evaluated by end-point RT-PCR.