Electronic Journal of Biotechnology 29 (2017) 1–6

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

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

Daniela E. Castro [a,*], Miguel Murguía-Romero [b], Patricia E. Thomé [c], Antonio Peña [a], Marissa Calderón-Torres [b,*]

[a] Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, Circuito Exterior s/n, Ciudad de México, Mexico
[b] Unidad de Biomedicina, Facultad de Estudios Superiores-Iztacala, Universidad Nacional Autónoma de México, Ave de los Barrios #1, Col. Los Reyes Iztacala, Tlalnepantla, Estado de México, Mexico
[c] Unidad Académica Puerto Morelos, Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Apartado Postal 13, Puerto Morelos, Quintana Roo, Mexico

ARTICLE INFO

Article history:
Received 27 February 2017
Accepted 6 June 2017
Available online 15 June 2017

Keywords:
Extremophiles
Free tyrosine
Halotolerance
Irreversible damages
Neutralization of free radicals
Nitrogen source
Osmoregulatory mechanisms
Oxidative stress
Salt-tolerant yeast
Transcriptional factors (TF)
Tyrosine synthesis

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 formation of 3-nitrotyrosine (3-NT), a nondegradable compound [4]. In yeast, genes coding for oxygen and nitrogen radical-scavenging

* Corresponding authors.
E-mail addresses: dcastro@uw.edu.mx (D.E. Castro), mcalderon@campus.iztacala.unam.mx (M. Calderón-Torres).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.06.003

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. *D. hansenii* was grown in YPD medium with or without 1 or 2 M NaCl or 10 mM 3-NT. Genes that do not code for denitrase enzyme, as indicated by GO, and that have one or two BSs for all TFs used in the search were defined as negative controls.

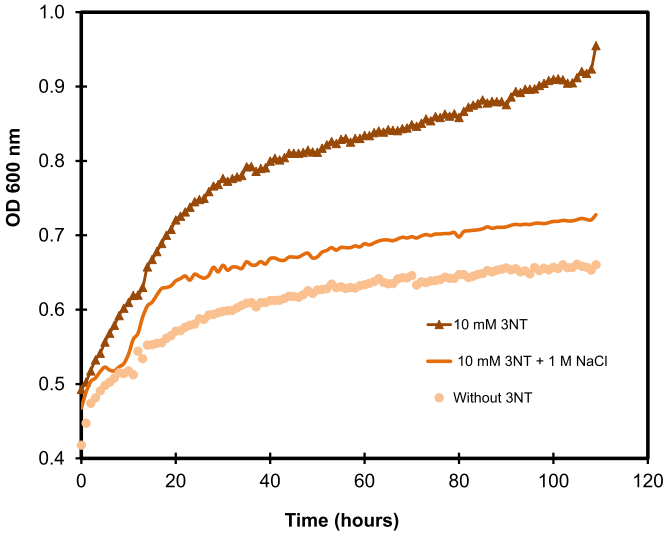

Fig. 1. *Debaryomyces hansenii* grown in the presence of 3-NT. Yeast cells were grown in fresh medium with or without 10 mM 3-NT and in the presence of 1 M NaCl and 10 mM 3-NT. Growth curves were followed in an automatic spectrophotometer at 600 nm during 110 h. The data are representative of three independent experiments.

Total RNA was extracted following the modified hot phenol protocol of Schmitt et al. [19]. RNA quality was verified by electrophoresis on 1.5% formaldehyde agarose gels. The sequences of forward and reverse primers used to evaluate the expression of genes were designed using the software program Primer 3 v.0.4.0. cDNA was synthesized using SuperScript III (Invitrogen) following the manufacturer's instructions. PCR conditions were as follows: initial denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 52–56°C (depending on the gene sequence) (Table S2), and extension at 72°C for 40 s; and finally 5 min on the last cycle. The RT-PCR products were verified by electrophoresis on DNA agarose gels. The intensity of the bands was quantified by densitometry using the Image Lab 5.2.1 software program (BioRad) and was normalized to *D. hansenii* actin gene expression. To determine a change in expression, the intensity value of the control band (YPD medium) was taken as reference, and the intensity value of each treatment (YPD + sodium or YPD + 3-NT) was divided by the reference value. The ratios of scanned bands are represented as means ± SD and were analyzed using Tukey test comparisons. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. 3-NT assimilation and recycling

We first tested and found that *D. hansenii* could grow with 3-NT as the sole nitrogen source. The growth rate was double of that in a medium without 3-NT. We also tested the effect of the presence of sodium and 3-NT at the same time, observing a severe growth restriction (Fig. 1), with OD values ranging from 0.5 to 0.6 at 600 nm. Considering the severity of using both stressors together, for our later evaluations of denitrase activity assay, we used YPD as control medium, adding NaCl after (as in Section 2.1).

In the crude extracts of *D. hansenii* grown in YPD medium with and without 1 and 2 M NaCl, we observed a high denitrase activity under conditions of salt stress (Fig. 2). In the presence of 2-nitrophenol and p-nitrophenol, the denitrase activity decreased five-fold compared to that in the presence of 3-NT but increased slightly in the presence of p-nitrophenol with 1 M NaCl (Fig. 2).

3.2. Validation of denitrase genes

To confirm that our *in silico* search is an optimal approach to predict the genetic expression of *D. hansenii* under NaS and PNS stress growth conditions, we evaluated the expression of some of these genes by RT-PCR. First we evaluated the expression of negative control genes, DEHA2G10054g and DEHA2F25564g, which did not change in the presence of 10 mM 3-NT; only the former had an expression above 1 (Fig. 3a). Conversely, the genes DEHA2E24178g and DEHA2C00286g, both candidates coding for denitrase enzymes, showed an increased expression, the former with the highest expression in all three conditions and the latter in 2 M NaCl and PNS stress (Fig. 3b). However, the genes that only coded for putative enzymes with oxidoreductase function showed a different expression; the gene DEHA2G08162g had an increased expression in the presence of 1 M NaCl and 10 mM 3-NT, but not under 2 M NaCl stress, while the

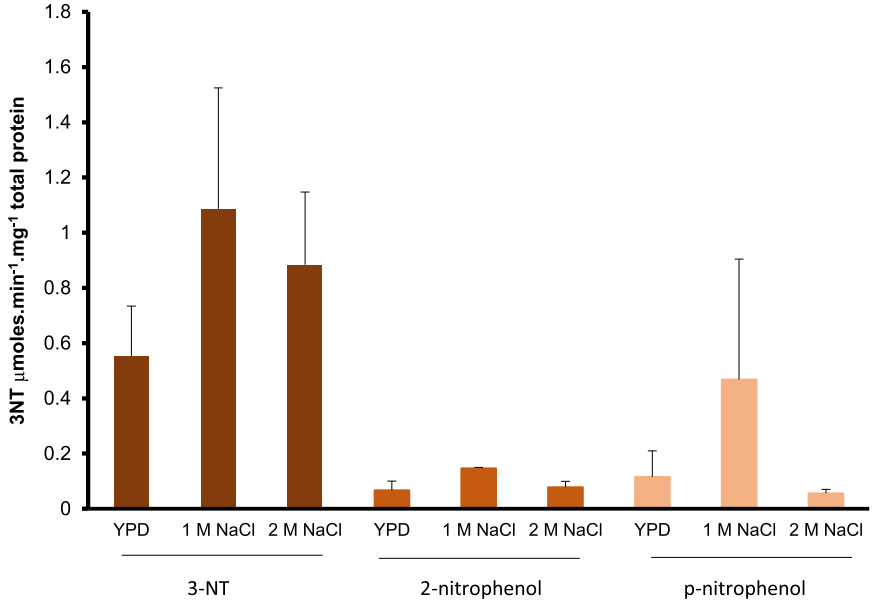

Fig. 2. Denitrase activity in cell extracts of *Debaryomyces hansenii* under salt stress. Cells were grown in YPD medium with 1 or 2 M NaCl. Total protein extracts were evaluated for denitrase activity with 3-NT and with 2-nitrophenol and p-nitrophenol as alternative nitrated substrates. Denitrase activity is reported as units (µmoles of substrate consumed min^{-1}) per mg of total protein. Results are the mean values of three independent experiments + SD.

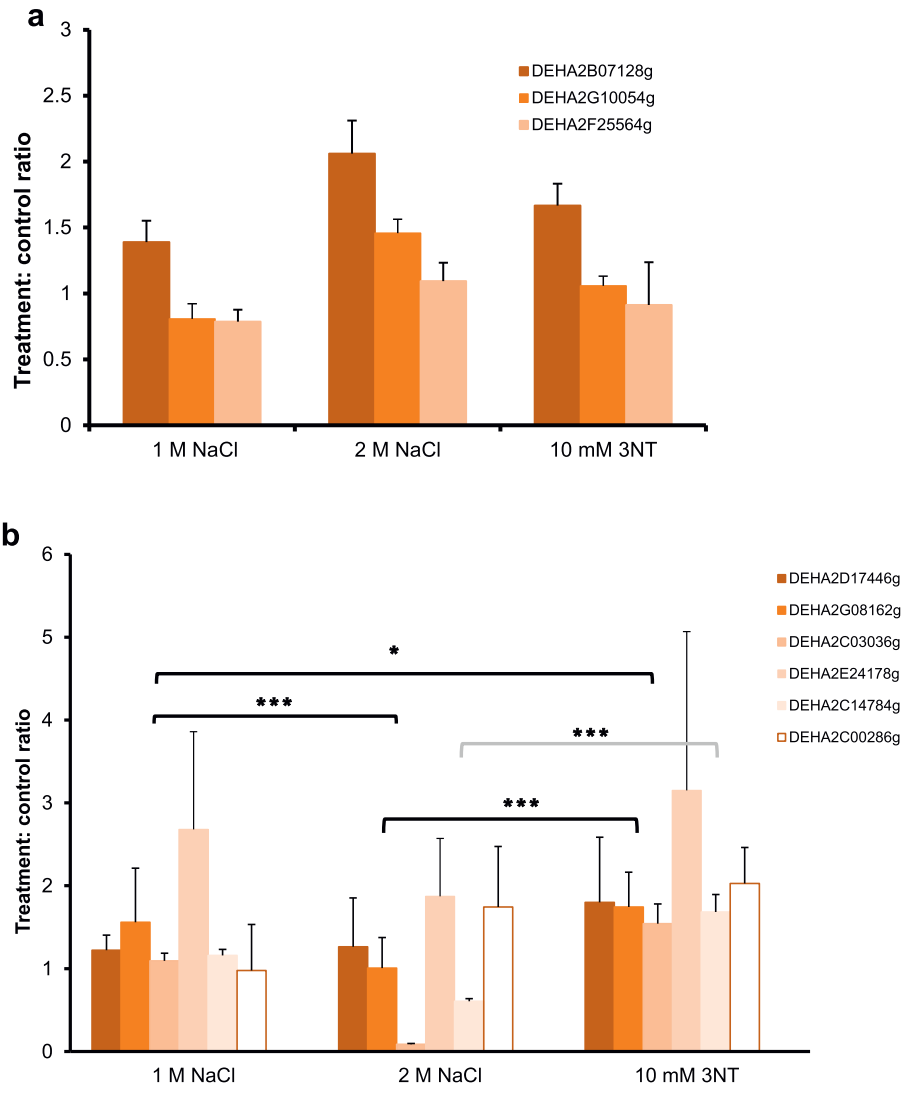

Fig. 3. RT-PCR expression of candidate genes identified *in silico*. Total RNA from *D. hansenii* grown in YPD medium with 1 and 2 M NaCl or 10 mM 3-NT was used for RT-PCR analysis. The expressions of negative control (a) and genes identified as oxidoreductase and denitrase (b) were calculated from the densitometry analysis of the bands. Changes in expression levels are the ratio of the intensity value of the control band (YPD medium) to the intensity value of each treatment's band (YPD + sodium or YPD + 3-NT). Ratio values are the means of three biological replicates + SD. Significantly different ratios are indicated by * ($P < 0.05$) and *** ($P < 0.001$).

expression of DEHA2C14784g only increased under NPS stress. Both DEHA2D17446g and DEHA2C03036g showed an expression above 1 only with 3-NT treatment Fig. 3b). The latter results corroborate that our *in silico* search of BS frequency for specific TF, coupled to function definition, can be useful as a tool to predict gene expression.

We assessed a possible correlation among the expressions of these genes and the number and position of BSs in all promoter regions (Table 1). From this table, a correlation between the number of GAT1 and the increase in gene expression in all three conditions was apparent, particularly in the presence of 10 mM 3-NT. In addition,

Table 1
Frequency of binding sites in the promoter regions of selected genes and their expression under NaCl and 3-NT stress.

Gene	GO annotation for function	Transcription Factor Frequency[a]					Expression[b]		
		Yap1	Msn2/Msn4	Sko1	Gat1	Gln3	1 M NaCl	2 M NaCl	10 nM 3-NT
DEHA2G08162g	Oxidoreductase activity	1	1		1	1	+	eq	+
DEHA2D17446g	Oxidoreductase activity	1	1		3		eq	eq	+
DEHA2C03036g	Oxidoreductase activity	1			1	1	eq	–	+
DEHA2E24178g	Monooxygenase activity	2			3	1	+	+	+
DEHA2C14784g	Oxidoreductase activity	3					eq	–	+
DEAH2C00286g	Monooxygenase activity	1			3		+	+	+
Negative control									
DEHA2G10054g	Fatty acid elongase	1					eq	+	eq
DEHA2F25564g	Subunit of the stator stalk of mitochondrial F1F0 ATP synthase	1					eq	eq	eq
DEHA2B07128g	POL5 protein			1	2		+	+	+

[a] indicates the number of BS copies in the promoter.
[b] indicates the expression ratio of genes (without changes [eq], positive [+], or negative [-]) over control conditions, calculated for cells treated with 1 or 2 M NaCl or 10 mM 3-NT, respectively (see Fig. 3).

we noted a synergistic association between the number of NPS and OSNa stress BSs and the increase in gene expression. Furthermore, we evaluated DEHA2B07128g expression, which codes for a POL5 protein, that has only two BSs for GAT1, and we still observed an increase with 10 mM 3-NT treatment; however, no change of expression occurred in the negative control's DEHA2F25564g and DEHA2G10054g, which have no BS and have only one for OSNa, respectively (Fig. 3a and Table 1).

4. Discussion

In this work, we demonstrate that *D. hansenii* can grow in the presence of 10 mM 3-NT as the sole nitrogen source, showing elevated denitrase activity under salt stress. This enzymatic activity is consistent with the growth properties of *D. hansenii,* which has been described to commonly occur in media rich in proteins, such as cheese [20,21]. In the cheese-making process, for example, there are different steps and biochemical transformations such as salting and acidification, which induce protein changes. Hence, it is very likely that at some stage of the process, 3-NT emerges as a subproduct of protein oxidation, and therefore, the denitrase activity of *D. hansenii* is indispensable to eliminate it. We postulate that this enzymatic activity is essential for this yeast's survival when grown in diverse stress conditions in which 3-NT is produced and may have biotechnological use for the reduction of this compound in oxidized organic substrates. In this scenario, and returning to our initial goal of finding 3-NT detoxification genes, it is important to consider that gene expression is complex because it implies the transcription of genes whose promoters have responsive elements not only to salt or oxidative stress but also to metabolite degradation or assimilation.

Despite the complexity of gene regulation, we searched for the genes that impart *D. hansenii* its capacity to transform 3-NT into a metabolic compound of easy assimilation. For the search, we considered the following two important facts: the existence of considerable investigation concerning *S. cerevisiae* gene expression and regulation [22,23,24] and the absence of studies on TFs of *D. hansenii*. So we designed a novel search strategy, looking *in silico* for genes associated with BS sequences of common TFs described in *S. cerevisiae* as responsive to stress in all promoter regions of *D. hansenii* genome. Our proposal is innovative and can be useful as a tool to predict gene expression because it suggests that the localization and enumeration of the BSs for TFs in the promoter region of a gene determines the expression of a gene. For example, we counted the number of GATA sequences, which are the BS of the GAT1 TF, which is activated when there is a change to a poorly assimilated nitrogen source such as oxidized 3-NT. We then selected five genes with GATA sequences in their promoter and experimentally confirmed the increase in the expression levels of the five genes. This implies that their expression increases when *D. hansenii* is grown in the presence of 3-NT. Moreover, we observed that the increase in the expression levels correlates with a larger number of GATA sequences in the promoter region. In this regard, De Hertogh et al. [25] were pioneers in the annotation and classification of the proteins of four unconventional yeasts, among them *D. hansenii*, through a comparative BLAST analysis, and they compared each of the 24,165 hemiascomycete proteins against a database of *S. cerevisiae* proteins. In addition, Nikolauo et al. [26] performed a reciprocal BLAST search of the orthologous genes of *S. cerevisiae* involved in signaling pathways and TFs in 13 species of fungi and yeasts, including *D. hansenii*. Tsankov et al. [27] used the motif sequences of diverse DNA-binding proteins of *S. cerevisiae* to study chromosome organization of 12 Hemiascomycota yeast species, including *D. hansenii*. These works support our *in silico* search employing BS sequences for NaCl, nitrogen, or oxidative stress in *S. cerevisiae*.

When *D. hansenii* is grown in 1 M NaCl and 10 mM 3-NT, there is an evident increase in the expression of the genes that code for putative oxidoreductases and denitrases. We postulate that this expression depends on the TFs Yap1p and Gat1p as they are involved in the regulation of oxidative and nitrogen stress, respectively. This observation supports our original assumption that salt stress produces an associated secondary oxidative stress condition [9,10]. We did not observe the same correlation in gene expression between salt concentration and NaCl stress TFs because when MSN2/MSN4 BS was present in the promoter region in two of the six genes examined, only one gene's expression levels increased. We speculate that either there are other TFs that activate gene expression in the presence of NaCl or the expression of this gene is indispensable under different stress conditions. We favor the second possibility because the expression level of this gene was higher than that of the other genes tested.

The increase in the gene expression of putative oxidoreductases and denitrases leads us to consider that *D. hansenii* has a system of catabolic repression of nitrogen (NCR) similar to that of *S. cerevisiae,* offering *D. hansenii* an advantage of survival in adverse stress conditions, such as high salt and PNS. We argue in favor of the possibility that in *D. hansenii*, a NRC response is functional as this system involves the participation of Gln3p and Gat1p as genes transcription activators for the assimilation of PNS [28,29]. In the presence of a good nitrogen source, Gln3p and Gat1p are restricted to the cytoplasm by interaction with Ure2p; however, when a change to PNS occurs, the two TFs are translocated to the nucleus to activate the NCR genes [30]. There are few studies that have demonstrated the presence of genes related to NCR responses during salt stress, such as in *Hansenula polymorpha* [31] or *D. hansenii,* where it has recently been confirmed that *DhGFZ3* gene (encoding a putative negative TF of GATA) functions as a negative repressor of nitrogen regulation [32]. However, our proposal of a functional NCR system in *D. hansenii* is very likely because we found that *D. hansenii* can grow in a PNS such as 3-NT.

The fact that the growth of *D. hansenii* in the presence of sodium and 3-NT increases the mRNA levels of genes such as DEHA2E24178g and DEHA2C00286g provides an opportunity for the biotechnological development of gene therapy, specifically in degenerative diseases such as type-2 diabetes [33] or Alzheimer's [34], in which there is an accumulation of nitrated proteins, such as 3-NT, which causes significant changes in protein function. There is a particular interest in finding organisms that naturally degrade 3-NT because, to date, there is no clear evidence of detoxification systems that can help eliminate 3-NT in mammals, and *D. hansenii* may offer such a possibility. Furthermore, it has been shown that the bacteria *Burkholderia* sp. JS165 and *Variovorax paradoxus* JS171 can grow in 2–5 mM 3-NT and have specific 3-NT deaminase and HNPA denitrase activities [13]; however, our results show that *D. hansenii* can grow at higher 3-NT concentrations than *Burkholderia* sp. and *V. paradoxus.*

Although the GO function annotations of the genes DEHA2E24178g and DEHA2C00286g indicated that both code for putative denitrases and belong to the same family of monooxygenases as it was annotated in Genolevures (GL3R0157), only DEHA2E224178g has 24% of similarity to a *S. cerevisiae* (YH-R176w) flavin-containing monooxygenase (EC 1.14.13.8); therefore, further research is necessary to confirm that the proteins encoded by these two genes have denitrase specific activity.

5. Conclusions

We report the ability of *D. hansenii* to grow in a medium with the oxidized compound 3-NT as the sole nitrogen source. We also show that protein extracts have specific denitrase activity for 3-NT degradation, increasing remarkably in 1 and 2 M NaCl stress conditions. These results support that *D. hansenii* develops NCR gene response when grown in a PNS such as 3-NT.

We demonstrate that the use of known BS sequences of common *S. cerevisiae* TFs analyzed *in silico* can be an adequate tool for the identification of genes potentially involved in the metabolism of 3-NT in *D. hansenii*.

We also suggest that an *in silico* search reduces labor time and costs in the laboratory because in most works where massive analysis of gene expression are first applied, for example, the sequencing of total RNA where actively transcribed genes are found, it is not until later that the researcher can ask which and how many TFs activated the transcription of the genes found. We propose that this question should be asked first, followed by the evaluation of a few genes' expression, as it is presented in our results. Here, we provide evidence that a less time-consuming approach is possible.

Conflict of interest

The authors declare no conflict of interest.

Financial support

This work was partially financed by grants PAPCA 2013 No 38 and IN226716 from DGAPA-UNAM (to M.C.T.) and IN202114 (to A.P.) from DGAPA-UNAM.

Acknowledgments

We specially thank Dr. Nandini Sarma for her assistance on the English style and valuable comments about the work. We also thank Dr. Martha Calahorra for technical assistance and BA Elvira Rosales Abundiz for her assistance on the English style.

Supplementary data

http://dx.doi.org/10.1016/j.ejbt.2017.06.003

References

[1] Petrovič U. Role of oxidative stress in the extremely salt-tolerant yeast *Hortaea werneckii*. FEMS Yeast Res 2006;6:816–22. http://dx.doi.org/10.1111/j.1567-1364.2006.00063.x.

[2] Sahoo R, Bhattacharjee A, Majumdar U, Ray SS, Dutta T, Ghosh S. A novel role of catalase in detoxification of peroxynitrite in *S. cerevisiae*. Biochem Biophys Res Commun 2009;385:507–11. http://dx.doi.org/10.1016/j.bbrc.2009.05.062.

[3] Song BJ, Abdelmegeed MA, Henderson LE, Yoo SH, Wan J, Purohit V. Increased nitroxidative stress promotes mitochondrial dysfunction in alcoholic and nonalcoholic fatty liver disease. Oxid Med Cell Longev 2013;781050. http://dx.doi.org/10.1155/2013/781050.

[4] Jung T, Hohn A, Grune T. The proteasome and the degradation of oxidized proteins: Part II-protein oxidation and proteasomal degradation. Redox Biol 2014;2:99–104. http://dx.doi.org/10.1016/j.redox.2013.12.008.

[5] Kim HJ, Jung HY, Lim CJ. The pap1 (+) gene of fission yeast is transcriptionally regulated by nitrosative and nutritional stress. FEMS Microbiol Lett 2008;280: 176–81. http://dx.doi.org/10.1111/j.1574-6968.2007.01056.x.

[6] Pedrajas JR, Carreras A, Valderrama R, Barroso JB. Mitochondrial 1-Cys-peroxiredoxin/thioredoxin system protects manganese-containing superoxide dismutase (Mn-SOD) against inactivation by peroxynitrite in *Saccharomyces cerevisiae*. Nitric Oxide 2010; 23:206–13. http://dx.doi.org/10.1016/j.niox.2010.06.004.

[7] Kumar S, Kalyanasundaram GT, Gummadi SN. Differential response of the catalase, superoxide dismutase and glycerol-3-phosphate dehydrogenase to different environmental stresses in *Debaryomyces nepalensis* NCYC 3413. Curr Microbiol 2011;62:382–7. http://dx.doi.org/10.1007/s00284-010-9717-z.

[8] Paumi CM, Pickin KA, Jarrar R, Herren CK, Cowley ST. Ycf1p attenuates basal level oxidative stress response in *Saccharomyces cerevisiae*. FEBS Lett 2006;586:847–53. http://dx.doi.org/10.1016/j.febslet.2012.02.010.

[9] Chao HF, Yen YF, Ku MS. Characterization of a salt-induced *DhAHP*, a gene coding for alkyl hydroperoxide reductase, from the extremely halophilic yeast *Debaryomyces hansenii*. BMC Microbiol 2009;182. http://dx.doi.org/10.1186/1471-2180-9-182.

[10] Calderón-Torres M, Castro DE, Montero P, Peña A. *DhARO4* induction and tyrosine nitration in response to reactive radicals generated by salt stress in *Debaryomyces hansenii*. Yeast 2011;28:733–46. http://dx.doi.org/10.1002/yea.1903.

[11] Chen L, Xie Q, Nathan C. Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. Mol Cell 1998; 1:795–805. http://dx.doi.org/10.1016/S1097-2765(00)80079-9.

[12] Calderón-Torres M, Peña A, Thomé PE. *DhARO4*, an amino acid biosynthetic gene, is stimulated by high salinity in *Debaryomyces hansenii*. Yeast 2006;23:725–34. http://dx.doi.org/10.1002/yea.1384.

[13] Nishino SF, Spain JC. Biodegradation of 3-nitrotyrosine by Burkholderia sp. strain JS165 and Variovorax paradoxus JS171. Appl Environ Microbiol 2006;72(2): 1040–4. http://dx.doi.org/10.1128/aem.72.2.1040-1044.2006.

[14] Zeyer J, Kocher HP. Purification and characterization of a bacterial nitrophenol oxygenase which converts ortho-nitrophenol to catechol and nitrite. J Bacteriol 1988;170:1789–94. http://dx.doi.org/10.1128/jb.170.4.1789-1794.1988.

[15] Badis G, Chan ET, Bakel HV, Pena-Castillo L, Tillo D, Tsui K, et al. A new library of yeast transcription factor motifs reveals a widespread function for Rsc3 in targeting nucleosome exclusion at promoters. Mol Cell 2008;32(6):878–87. http://dx.doi.org/10.1016/j.molcel.2008.11.020.

[16] Fordyce PM, Gerber D, Tran D, Zheng J, Li H, De-Risi JL, et al. *De novo* identification and biophysical characterization of transcription-factor binding sites with microfluidic affinity analysis. Nat Biotechnol 2010;28:970–5. http://dx.doi.org/10.1038/nbt.1675.

[17] Minehart PL, Magasanik B. Sequence and expression of *GLN3*, a positive nitrogen regulatory gene of *Saccharomyces cerevisiae* encoding a protein with a putative zinc finger DNA-binding domain. Mol Cell Biol 1991;11:6216–28. http://dx.doi.org/10.1128/MCB.11.12.6216.

[18] Cornish-Bowden A. Nomenclature for incompletely specified bases in nucleic acid sequences. Recommendations 1984. Nomenclature Committee of the International Union of Biochemistry (NC-IUB). Proc Natl Acad Sci U S A 1986;83: 4–8. http://dx.doi.org/10.1073/pnas.83.1.4.

[19] Schmitt ME, Brown TA, Trumpower BL. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res 1990;18:3091–2. http://dx.doi.org/10.1093/nar/18.10.3091.

[20] Mounier J, Goerges S, Gelsomino R, Vancanneyt M, Vandemeulebroecke K, Hoste B, et al. Sources of the adventitious microflora of a smear-ripened cheese. Journal of Applied Microbiology 2006;101:668–81. http://dx.doi.org/10.1111/j.1365-2672.2006.02922.x.

[21] Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, et al. Genome evolution in yeasts. Nature 2004;430:35–44. http://dx.doi.org/10.1038/nature02579.

[22] Hughes TR, de Boer CG. Mapping yeast transcriptional networks. Genetics 2013;195: 9–36. http://dx.doi.org/10.1534/genetics.113.153262.

[23] Costanzo M, Baryshnikova A, Bellay J, Kim Y, Spear ED, Sevier CS, et al. The Genetic Landscape of a Cell. Science 2010;327:425–31. http://dx.doi.org/10.1126/science.1180823.

[24] Taymaz-Nikerel H, Cankorur-Cetinkaya A, Kirdar B. Genome-wide transcriptional response of *Saccharomyces cerevisiae* to stress-induced perturbations. Front Bioeng Biotechnol 2016;4:1–19. http://dx.doi.org/10.3389/fbioe.2016.00017.

[25] De Hertogh B, Hancy F, Goffeau A, Baret PV. Emergence of species-specific transporter during evolution of the hemiascomyte phylum. Genetics 2006;172:771–81. http://dx.doi.org/10.1534/genetics.105.046813.

[26] Nikolaou E, Agrafioti I, Stump M, Quinn J, Stansfield I, JP Brown A. Phylogenetic diversity of stress signaling pathways in fungi. BMC Evol Biol 2009;9:44. http://dx.doi.org/10.1186/1471-2148-9-44.

[27] Tsankov AM, Thompson DA, Socha A, Regev A, Rando OJ. The role of nucleosome positioning in the evolution of gene regulation. PLoS Biol 2010;8:e1000414. http://dx.doi.org/10.1371/journal.pbio.1000414.

[28] Coffman JA, Rai R, Cunningham T, Svetlov V, Cooper TG. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol Cell Biol 1996;16:847–58. http://dx.doi.org/10.1128/MCB.16.3.847.

[29] Georis I, Feller A, Vierendeels F, Dubois E. The yeast GATA factor Gat1 occupies a central position in nitrogen catabolite repression-sensitive gene activation. Mol Cell Biol 2009;29:3803–15. http://dx.doi.org/10.1128/MCB.00399-09.

[30] Beck T, Hall MN. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 1999;402:689–92. http://dx.doi.org/10.1038/45287.

[31] Rodríguez C, Tejera P, Medina B, Guillén R, Domínguez A, Ramos J, et al. Ure2 is involved in nitrogen catabolite repression and salt tolerance via Ca^{2+} homeostasis and calcineurin activation in the yeast *Hansenula polymorpha*. J Biol Chem 2010; 285:37551–60. http://dx.doi.org/10.1074/jbc.M110.146902.

[32] García-Salcedo R, Casamayor A, Ruiz A, González A, Prista C, Loureiro-Dias MC, et al. Heterologous expression implicates a GATA factor in regulation of nitrogen metabolic genes and ion homeostasis in the halotolerant yeast *Debaryomyces hansenii*. Eukaryot Cell 2006;5:1388–98. http://dx.doi.org/10.1128/EC.00154-06.

[33] Anderson EJ, Kypson AP, Rodriguez E, Anderson CA, Lehr EJ, Neufer PD. Substrate-specific derangements in mitochondrial metabolism and redox balance in the atrium of the type 2 diabetic human heart. J Am Coll Cardiol 2009;54:1891–8. http://dx.doi.org/10.1016/j.jacc.2009.07.031.

[34] Sultana R, Poon HF, Cai J, Pierce WM, Merchant M, Klein JB, et al. Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. Neurobiol Dis 2006;22:76–87. http://dx.doi.org/10.1016/j.nbd.2005.10.004.