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Genetic and genomic characterisation of thermotolerance in *Kluyveromyces marxianus*

Thesis presented by

Noemi Montini, MSc.

for the degree of

Doctor of Philosophy

in

Microbiology

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Declaration

I, Noemi Montini, declare that I have not obtained a degree from University College Cork, National University of Ireland, Cork or elsewhere on the basis of this Ph.D. thesis and that the results presented in this thesis were derived from experiments undertaken by myself. Most of the research presented in this thesis was carried out at University College Cork but some data in the research chapters III and IV presented as chapters in this thesis were generated in collaborations with other researchers from Chalmers University of Technology. Specifically, the RNA sequencing for all conditions and differential expression (DE) analysis for the high temperature condition in Chapter III and the orthology inference in Chapter IV in was provided the collaborators at Chalmers University of Technology. The contribution of collaborators to those chapters is indicated at each chapter.

Acknowledgments

I gratefully acknowledge the financial support of the CHASSY program, funded by the European Union. I would also like to thank to all the partners within the project who contributed with ideas and suggestions to my research throughout the years.

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Abstract

This thesis was carried out as part of the Horizon 2020 project CHASSY (http://chassy.eu/), funded by the European Union. The CHASSY project was a multi-partner research collaboration focused on unlocking the potential of yeast as a microbial cell factory. The research scope of CHASSY included investigating the basis of stress adaptation response in various yeast species, including *K. marxianus*, in order to engineer robustness in industrial strains. In fact, microbial cell factories face suboptimal growth conditions during the bioprocesses that cause stress on the cell and hinder productivity. Stress responses and adaptation mechanisms are a complex phenomenon and involve numerous genes and processes in yeast. Therefore, a better understanding of these is needed in order to design more robust yeast strains and broaden their application in industrial biotechnology. In this context, *K. marxianus* possesses valuable physiological traits: the yeast is thermotolerant, has a rapid growth rate and is able to utilise a wide range of substrates. Particularly promising for biotechnological applications, is its innate thermotolerance, with certain strains being able to grow up to 45 °C. Since thermotolerance is desirable from an industrial standpoint due to reduced cooling cost and contamination risk, *K. marxianus* thermotolerance mechanisms were investigated in this study. However, since a wide physiological variability is known between *K. marxianus* strains, and the physiological studies available focus on different strains and conditions, a systematic physiological comparison was needed in order to efficiently compare the data. Therefore, batch cultivations in bioreactors were carried out with three strains: the haploids CBS 6556 and NBRC 1777 and the diploid CBS 397 in Chapter III. Growth characteristics of each strain were compared under two growth temperatures (30 °C and 40 °C) in order to investigate the physiological response of different *K. marxianus* strains to elevated temperature. NBRC 1777 was the fastest growing strain at 30 °C among the three compared, while both CBS 6556 and CBS 397 showed an almost doubled growth rate at 40 °C compared to 30 °C, indicating that higher growth temperatures are preferred by some *K. marxianus* strains. NBRC 1777 instead showed decreased biomass yield on substrate at 40°C and higher oxygen uptake rate, in addition to slower growth, suggesting that these parameters are linked to growth under high temperature.

Subsequently, *K. marxianus*' thermotolerance response was further investigated under long-term exposure to stress, since adaptation mechanisms are better observed under these premises. Specifically, the haploid strain CBS 6556 was subjected to chemostat cultivation under three industrially relevant stresses, namely: high temperature, low pH and high osmolarity, which allowed the simulation of long term stress. The analysis of the transcriptional changes in response to the three stresses revealed that, while some biological processes are involved in the response to all tested stresses, there are no genes upregulated under all three stress condition, which suggests a stress-specific response. The physiological data collected under high temperature stress revealed that the sugar uptake rate is higher in CBS 6556 under high temperature stress, likely to fulfil a higher energy demand to cope with the condition. In addition, and contrary to what might be expected, analysis of the transcriptional responses showed that the genes involved in the respiratory activity, also involved in energy generation, were downregulated. This strategy could be used by *K. marxianus* to control reactive oxygen species (ROS) accumulation, a phenomenon naturally occurring under high temperature stress, and contributing therefore to its innate thermotolerance.

A collaborative study within CHASSY analysing *K. marxianus* and other yeast species' transcriptomic data, found that evolutionarily young genes are proportionally more responsive, in yeast, to growth under stressful conditions than core genes shared by many species. In the study, it was therefore postulated that such genes could be important for long-term adaptation to stress. This hypothesis was tested in *K. marxianus* in regards to thermotolerance in Chapter IV by identifying and studying species-specific genes that showed upregulation during high temperature growth. Twelve such genes were identified and eleven were successfully inactivated using CRISPR-mediated mutagenesis. One gene, *KLMX_70384*, was shown to be required for competitive growth at high temperature. *KLMX_70384* was predicted to encode an 83AA peptide and RNA-Seq and Ribo-Seq were used to confirm transcription and translation of the gene. Although the precise function of KLMX_70384 remains unknown, the generic structure and short sequence suggest RNA-binding activity. Since *KLMX_70384* is among the genes that arose *de novo* in *K. marxianus* post the speciation event that separated *K. marxianus* and *K. lactis*, this confirmed the hypothesis that evolutionary young genes contribute to the unique traits of *K. marxianus'*, such as thermotolerance.

In yeast, high temperatures trigger ROS accumulation, therefore the thermal stress response is tightly connected to the oxidative stress response. This is suggested by evidences of a crosstalk between signalling mechanisms for the two stresses. In Chapter V, a physiological and genomic comparison was carried out between two *K. marxianus* strains: ATCC 26548 and UCC01, the latter was derived from ATCC 26548 but displays a thermosensitive phenotype. The comparison of the two genome sequences showed a high similarity between the two strains, with only 1012 variants between the two genomes, suggesting that UCC01 spontaneously accumulated mutations which resulted in thermosensitivity. A physiological comparison via bioreactor cultivation under high temperature and different aeration levels (0.5 and 2 L/min), revealed that UCC01's thermosensitive phenotype is linked to oxygen availability. An accumulation of ROS was measured at 45 °C in UCC01 under 2L/min aeration and, accordingly, single nucleotide polymorphisms (SNPs) analysis found five SNPs-containing genes of which four (*CYC8*, *SWI1*, *BNR1* and *YAK1*) with functions related to oxidative and general stress response, and one unannotated gene KLMX_30726. Although individual deletion of these genes in ATCC 26548 did not reproduce a thermosensitive phenotype, deletion of *CYC8* and *YAK1* resulted in an oxidative stress-sensitive phenotype, confirming the existence of a crosstalk between oxidative and thermal stress responses in *K. marxianus*, possibly linked to its innate thermotolerance.

Finally, trehalose metabolism was investigated in *K. marxianus* in Chapter VI, since trehalose is considered a thermoprotectant allowing yeast survival to heat stress. Recent studies also demonstrated a more complex role of trehalose, such as regulating central carbon metabolism and cell homeostasis. Knock-out of different enzymatic subunits of the trehalose synthase complex is known to generate diverse phenotypes in various yeast species. In addition, several genes of trehalose metabolism were found upregulated under long term temperature stress by differential expression (DE) analysis in *K. marxianus*. Here, we investigated the individual roles of *TPS1* and *TPS2*, enzymatic subunits of the trehalose synthase complex, on *K. marxianus* thermotolerance by construction and analysis of knock-out mutants. The deletion of *TPS1* was not found to be lethal in *K. marxianus*, unlike in *S. cerevisiae*, but yielded a thermosensitive phenotype. Moreover, deletion of *TPS2* yielded a thermosensitive phenotype exclusively on galactose. The thermosensitive phenotype was found to be

linked to the accumulation of trehalose-6- phosphate (T6P) under high temperature, which deregulates the galactose metabolism genes. In accordance, *GAL1* and *GAL7* genes were found to be upregulated, and *PGM2* strongly upregulated in the Δ*tps2* mutant under high temperature on galactose. The study shows evidence of T6P playing a role in regulating central carbon metabolism in *K. marxianus*; it also reveals a previously unknown layer of metabolite repression acting on galactose genes in this yeast, linked to thermotolerance. The results presented in this thesis contribute to better the current understanding of the many mechanisms involved *K. marxianus'* thermotolerance, providing a knowledge resource to further develop this yeast for cell factory applications.

List of publications derived from this thesis

Published

Montini, N., Doughty, T. W., Domenzain, I., Fenton, D. A., Baranov, P. V, Harrington, R., Nielsen, J., Siewers, V., & Morrissey, J. P. (2022). Identification of a novel gene required for competitive growth at high temperature in the thermotolerant yeast *Kluyveromyces marxianus*. *Microbiology*, 1–13. https://doi.org/10.1099/mic.0.001148

In preparation for submission

Montini, N., Donzella, L., Huff, F., Akinola, J. Sousa, M. J., Louis, E.F. and Morrissey, J.P. A primer on *Kluyveromyces marxianus*

Montini, N., Belloch-Molina, C., Morrissey, J. Physiological comparison of *K. marxianus* strains grown under industry-relevant conditions in bioreactors

Montini, N. and Morrissey, J. Insights on the interaction between trehalose biosynthesis and galactose metabolism in *K. marxianus.*

Collaboration

Doughty, T. W., Domenzain, I., Millan-Oropeza, A., **Montini, N.**, de Groot, P. A., Pereira, R., Nielsen, J., Henry, C., Daran, J. M. G., Siewers, V., & Morrissey, J. P. (2020). Stress-induced expression is enriched for evolutionarily young genes in diverse budding yeasts. *Nature Communications*, *11*(1), 1–9. https://doi.org/10.1038/s41467-020-16073-3

List of abbreviations

A600nm, OD600nm	Absorbance at 600 nm
CDS	Coding sequences
CER	CO2 emission rate
DCW	Dry cell weight
DE	Differential expression
DO	Dissolved oxygen
DT	Duplication time
ESR	Environmental stress response
GO	Gene onthology
gRNA	Guide RNA
GSA	Gene set analysis
HML	Hidden MAT left
HMR	Hidden MAT right
HPLC	High performance liquid chromatography
HSP	Heat shock protein
HSR	Heat shock response
MAT	Mating type locus
MLST	Multi-locus sequencing analysis
mtDNA	Mitochondrial DNA
ORF	Open reading frame
OUR	O_2 uptake rate
Pi	Pyrophosphate
PI	Propidium iodide
qO_2	Oxygen consumption rate
RFO	Raffinose family of olysaccharides
ROS	Reactive oxygen species
RPM	Repetition per million

RQ	Respiratory quotient or respiratory coefficient
S	Grams of substrate
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
T6P	Trehalose 6 phosphate
TPS	Trehalose synthase complex
μO_2	Oxygen consumption rate
μs	Glucose uptake rate
VVM	Gas volume flow per unit of liquid volume per minute (ll-1min-1)
WGD	Whole genome duplication
X	Grams of biomass DCW
$Y_{X/S}$	Biomass yield on substrated (g/g)

Chapter I

Overview on stress tolerance in yeast

1. Environmental changes cause a stress response in yeast.

Yeast cells adapt to respond to the challenge of an ever-changing environment in their natural niches, such as changes of seasonality and nutrients availability (Gasch, 2000). Yeast face even more challenging conditions when applied in traditional food fermentations (Aceituno et al., 2012; Gibson et al., 2008; Marullo et al., 2009; Matallana & Aranda, 2017; Sharma et al., 2018), or in modern biotechnology as microbial cell factories (Chen et al. 2016; Liu et al. 2020; Zeng et al. 2018). Industrial fermentations are particularly challenging on production strains, since they often present suboptimal conditions for growth, different from the natural environment to which the yeast has naturally adapted. This disrupts the cells' homeostasis and generates stress which negatively affects growth and, consequently, industrial productivity. For this reason, understanding and engineering of the yeast stress tolerance has been the focus of many studies, with the ultimate goal of obtaining robust industrial strains (Guirimand et al. 2020; Lehnen, Ebert, and Blank 2019; Saini et al. 2018, Fu et al. 2019; Zhang et al. 2019). To react to the stress caused by the changes in their environment, yeast have evolved sensing strategies and signal transduction cascades, conserved among many species (Estruch; 2000). Although the main stress response mechanisms are found conserved among the species (Gasch et al., 2000), different species demonstrate diverse mechanisms to withstand the same stress. This could be due to convergent adaptation and tolerance mechanisms evolved over the years (Brion et al., 2016; Doughty et al., 2020). The complexity and diversity of such mechanisms have been the reasons why yeast stress tolerance is a long-standing unresolved puzzle to date. Information on yeast stress tolerance mechanisms, especially of non-saccharomyces species such as *K. marxianus* are scarce, due to the limited availability of large scale omic studies, the only recent advances in genome sequencing techniques allowing to easily sequence large genomes, and the traditional focus on the yeast *S. cerevisiae.*. This section provides an overview of the main industrial stresses encountered by yeast strains during growth in non-optimal environments, together with a description of their negative effects on cell homeostasis. In addition, the conserved mechanisms of stress sensing and signal transduction are described, including the known transcription regulators which actuate yeast stress

response. Most of the available literature is based on studies with *S. cerevisiae*, and are the main focus of the stress response mechanisms described in this chapter. However where possible comparisons with *K. marxianus* and the closely related *K. lactis* are discussed.

1.1 Effects of environmental stress on cell homeostasis

Environmental stresses can cause various disruptions to yeast cell homeostasis but mainly act through damaging various cell macromolecules; the main effects of environmental stress on the yeast cell are summarized in Figure 1.

Protein denaturation and formation of aggregates is commonly observed in yeast subjected to stress (Saini et al., 2018; Weids et al., 2016). Misfolded protein aggregates induce in turn endoplasmic-reticulum (ER) stress, a source of reactive oxygen species (ROS) accumulation which can cause cell death (Knupp et al., 2018; Yu et al., 2016). Yeast respond to the potentially lethal ER stress by inducing autophagy and non-selective autophagy (also called macroautophagy) of the ER itself in subcellular structures called autophagosomes (Gross & Graef, 2020; Mizuno et al., 2020), as a strategy to counterbalance ER expansion and reduce the misfolded protein aggregates (Høyer-Hansen & Jäättelä, 2007). Macroautophagy of ER, vacuole and mitochondria is a common response observed under different stresses (Cebollero & Reggiori, 2009; Ishii et al., 2018; Kiffin et al., 2006; Lin et al., 2019; Palmer et al., 2007; Song & Kumar, 2012; Yang et al., 2020; Yang & Klionsky, 2010). The mechanism of macroautophagy is well characterized in *K. marxianus*, for which (Yamamoto et al., 2015) identified all the autophagy related genes (ATG) homologues to *S. cerevisiae*. The study found that the corresponding ATG genes' primary protein sequences were shorter that those of *S. cerevisiae*, and conferred them superior thermostability. The same study reported that autophagic activity is enhanced under high temperature in *K. marxianus*. Macroautophagy was also found to play a role in cell survival during ethanol production on inulin (Gao et al., 2015) and during high temperature fermentation (Fu et al. 2019) in *K. marxianus*. In particular the genes *ATG8*, *ATG3* and *ATG7*, involved in autophagosome assembly, were found upregulated. Crystal structures of KmAtg18 and the complex Atg1-Atg13 are also available (Fujioka et al., 2014; Watanabe et al., 2012).

Oxidative stress can causeDNA-damage and insurgence of mutations (Hori et al., 2009; Kawanishi et al., 2001; Shor et al., 2013; Yan et al., 2014). Accordingly, in *K. marxianus* high temperature conditions and the accumulation of fermentation

inhibitors induced upregulation of genes involved in DNA damage repair as well as cell cycle progression (Fu et al., 2019). Moreover, environmental factors such as temperature and osmotic stress, or elevated ethanol concentrations, can damage the cell wall, essential for the integrity of the cell, and induce a cell wall stress response (Bermejo et al. 2008; García et al. 2017; Sanz et al. 2017). Accordingly, it has been reported that cell wall biogenesis process genes were found to be upregulated in *K. marxianus* after adaptive evolution to increasing concentrations of ethanol (Mo et al., 2019), to repair the solvent induced damage.

Finally, the accumulation of toxic free radical oxygen species (ROS) induces the peroxidation of several essential cell components, namely proteins, lipids, and nucleic acids (Herrero et al. 2008). *K. marxianus* is not immune to ROS accumulation under stressful conditions, ultimately resulting in disruption of homeostasis (Fu et al. 2019; Li et al. 2019).

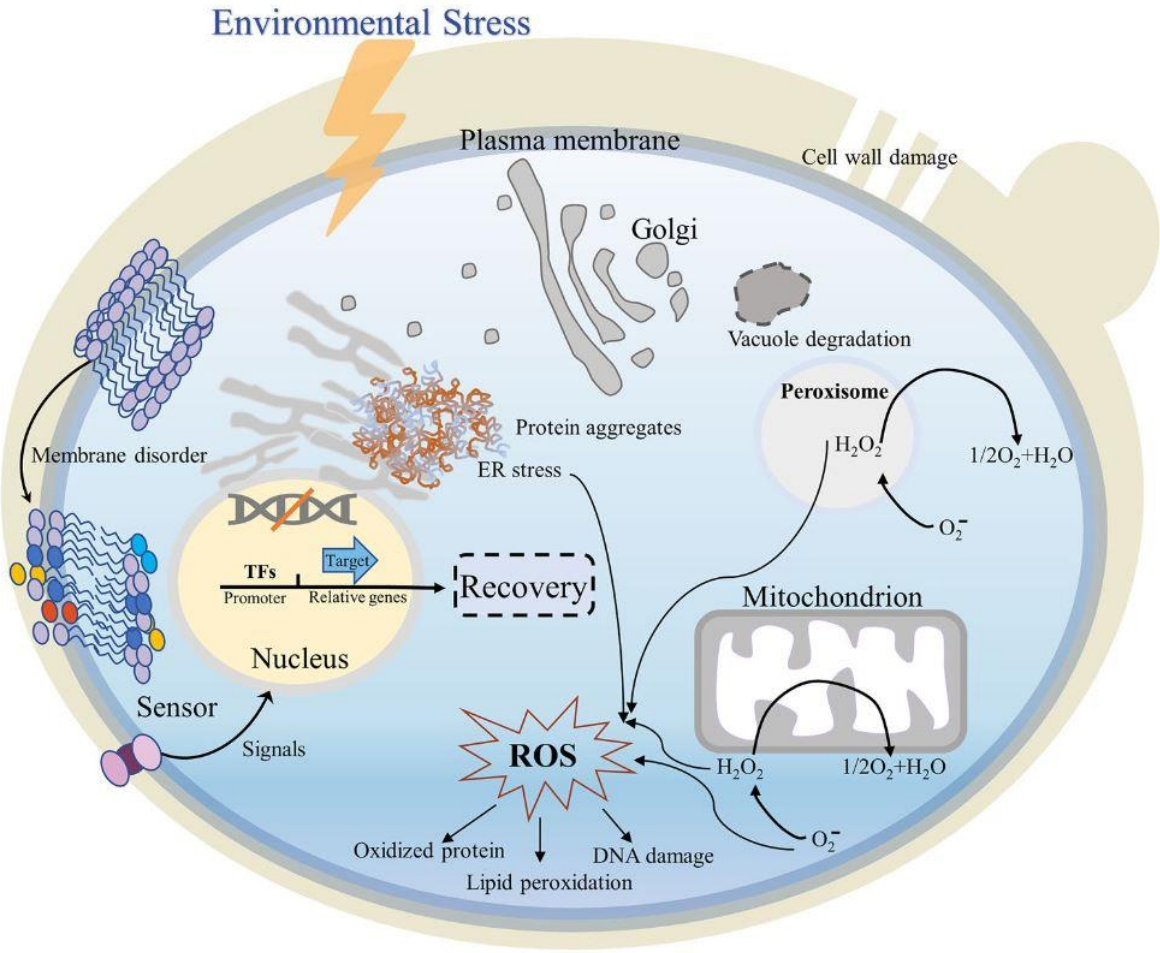

Figure 1. A schematic of the stress-induced damage and stress response in yeasts. Environmental stress causes cell wall damage, plasma membrane disorder, aggregation of denatured proteins, increase of ROS level and endoplasmic reticulum (ER) stress. In response to these damages, the ESR process is initiated, membrane stress sensors first pass the stress signals to stimulate key signalling pathways and contributing biosynthesis pathways. With the cooperation of these functional pathways, yeasts try maintain its homeostasis. Figure taken from Lin et al. (2021).

1.2 Industry-associated stresses

Industrial fermentations are particularly challenging on the yeast strains because conditions are sub-optimal for growth. The non-ideal conditions required by biotechnological processes translates into a variety of stresses that microbial cell factories have to overcome. Traditional application in food fermentations cause a series of stresses in yeast, among which oxidative stress, hyperosmotic shock and starvation, which have been extensively reviewed (Gibson et al., 2008; Matallana & Aranda, 2017). Similarly, both *K. marxianus* and *K. lactis*, traditionally employed in the dairy industry because of their β-galactosidase activity and the ability to metabolise

lactose (Rodicio & Heinisch, 2013; Tofalo et al., 2014), face challenges when used in biotechnological applications such as the valorisation of cheese whey for production of β-galactosidase enzyme and bioethanol (Alves et al., 2019; Saini, Beniwal, & Vij, 2017; Sharma et al., 2018).Whey fermentation causes oxidative and osmotic stress on the strains due to the elevated concentration of sugar and proteins of this substrate (Becerra et al., 2006; Saini et al., 2017).

One of the recent applications of yeast as microbial cell factories is for bioethanol production. However, ethanol concentration reached during fermentation can strongly inhibit the growth of yeast cells and their ethanol production, resulting in ethanol stress that mostly affects the cell membrane (Lei et al. 2007; Ne Cot et al. 2007; Zhao and Bai, 2009). *K. marxianus* is particularly sensitive to ethanol stress, to which several physiological and genomic factors have been attributed, such as lack of free fatty acids and ergosterol content (da Silveira et al., 2020), or the onset of oxidative stress, redox imbalance and ROS species accumulation (Fu et al., 2019). Ethanol production from lignocellulosic biomass instead of grain-based feedstocks is more sustainable andcheaper ; however, yeast cells face potential toxicity of many by-products released from the necessary lignocellulose pre-treatment process, such as acetic acid or furfurals (Zhao & Bai, 2009). In *K. marxianus*, exposure to such growth inhibitors induces genes involved in maintaining redox balance, NAD(P)+/NAD(P)H homeostasis or NAD+ synthesis (Wang et al., 2018).

Perhaps the most common stress encountered during industrial fermentations is high temperature stress. In fact, cell growth is naturally exothermic, leading to temperature increase in the fermentations vessels, which need cooling down to temperature that allows for physiological growth (Tibayrenc et al., 2011). The new generation processes of one-pot bioethanol production from lignocellulosic biomasses require high temperatures for the process of simultaneous saccharification and fermentation (SSF), causing heat stress on the cells (Della-Bianca & Gombert, 2013; Eardley & Timson, 2020; Zabed et al., 2016). Moreover, higher fermentation temperatures are preferred in order to reduce both cooling costs and contamination risks, for which *K. marxianus* is particularly suited since it is naturally thermotolerant (Cunha, Castro, and Roberto, 2020.; Malairuang et al., 2020; Yanase et al., 2010). As the stresses faced during biotechnological fermentations ultimately impact the process yield and efficiency, an important requirement for microbial cell factories is robustness to the stress conditions (Zhou, 2017).

1.3 Sensing and signalling of cellular stress

Environmental changes generate stress, which has an immediate effect on the cells' homeostasis and is sensed by sensor proteins triggering the stress response. The first phase of the stress response is through a signalling cascade which, in turn, triggers the expression of the stress response genes. The sensing mechanisms can vary depending on the kind of stress: external stresses affecting the integrity of the cell membrane (i.e. osmotic, heat, ethanol) are sensed by a group of surface sensors with a transmembrane domain: Wsc1, Wsc2, Wsc3, Mid2 and Mtl2 (Dupres et al., 2009; Rodicio et al., 2008). The osmotic stress response is triggered by two cell membrane proteins: Sln1 (Bermejo et al., 2008; Posas et al., 1996) and Sho1, also identified in *K. lactis* (Siderius et al., 2000): the same sensing mechanism can be assumed for *K. marxianus*, since the identification and characterization of the Hog1 homolog which, if deleted hindered the osmotolerance of the strain (Qian et al., 2011). An immediate effect of thermal and oxidative stresses is the accumulation of unfolded proteins, which cause the initiation of the Unfolded Protein Response (UPR) pathway. One of the prime UPR signalling sensors is Inositol-requiring enzyme 1 (IRE1), an ER membrane embedded protein with dual enzyme activities: kinase and endoribonuclease, which controls the synthesis of the basic leucine-zipper (bZIP)-containing transcription factor Hac1 (Bashir et al., 2021; Guerra-Moreno et al., 2019; Schröder et al., 2003). In *K. lactis*, unlike *S. cerevisiae*, the Ire1/Hac1 system seems independent from the presence of inositol in the medium, indicating their function might differ (Hernández-Elvira et al., 2018). The oxidative stress response in *S. cerevisiae* yeast is mediated by the transcription factor Yap1 (see section 1.4.3), which responds to H_2O_2 only in the presence of the sensor and signal transducer Gpx3 (Rodrigues-Pousada et al., 2019).

The stress sensing activates signalling cascades culminating in transcriptional regulators inducing genes of the stress response (Figure 2). The response to environmental stress involves several signalling pathway, which often overlaps in their function and are overall conserved among yeasts. The main stress signalling pathways involve the mitogen activated protein kinase (MAPK) cascades and the cyclic adenine monophosphate/ protein kinase A (cAMP/PKA) pathway (Figure 2). Two of the six MAPK pathways have a role in stress response induction: the HOG pathway for glycerol synthesis in response to osmotic stress, and the CWI pathway responsive to cell wall damage. These pathways dynamically interact with each other:

the sensors of the CWI pathway sense environmental stresses while simultaneously repressing the cAMP/PKA pathway, the cAMP/PKA pathway can negatively regulate the transcription factors Msn2/4, known to trigger yeast's stress response, and finally Rho1 (upstream regulator of the CWI pathway) can control YAP1/SKN7 transcription factors. (Lin et al., 2021).

1.3.1 MAPK-pathway

The common mitogen-activated protein kinase (MAPK) pathway contains a three-component signal transduction cascade in which an activated MAPK kinase kinase (MAPKKK or MEKK) activates a MAPK kinase or a MAPK/ERK kinase (MAPKK or MEK), which then activates a MAPK. The activated MAPK has a phosphorylation of a conserved motif in its activation loop, which causes a substantial conformational rearrangement. MAPKs phosphorylate a diverse set of well-characterized substrates, including transcription factors, translational regulators, MAPK-activated protein kinases (MAPKAP kinases), phosphatases, and other classes of proteins, regulating metabolism, cellular morphology, cell cycle progression, and gene expression in response to a variety of extracellular stresses and molecular signals (Chen and Thorner, 2007).

Among the responses mediated by the MAPK cascades, are the Hog1-mediated responses. Hog1 controls gene expression of several hundred genes for glycerol accumulation, signal fidelity and cell cycle arrest under hyperosmotic conditions; and its deletion confers osmosensitivity (Babazadeh et al., 2014). While in *S. cerevisiae* a *hog1Δ* mutant seems to show sensitivity exclusively to osmotic stress, in *Candida albicans*, Hog1 has additional roles, including resistance to oxidative stress (Morales-Menchén et al., 2018). The same roles have been shown for *K. marxianus hog1Δ* mutant (Qian et al., 2011).

Another stress response mediated by the MAPK cascades, is the CWI pathway,which is highly conserved in the yeast kingdom. The CWI signalling pathway is activated by cell wall damage sensing proteins,and is governed by the mitogen-activated protein kinase (MAPK) Slt2 (also called MPK1), and works through the G protein Rho1, which activates a set of effectors. Collectively, these effectors regulate several processes, including: β-glucan synthesis, cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site. Other phenotypes associated with mutations in CWI gene pathway include sensitivity to mating

pheromone and cell wall antagonists such as calcofluor white, and actin polarization defects (Levin, 2005; Sanz et al., 2017; Udom et al., 2019). This pathway is generally conserved in *K. lactis*, with minor differences given the lack of genome duplication. In factonly three intramembrane sensor proteins are known in *K. lactis* instead of five, and only one isozyme for the downstream effectors Rom2 and Mkk1 (Backhaus et al., 2011; Rodicio et al., 2008).

Another MAPK-type mechanism with a role in stress tolerance is represented by Fus3/Kss1. Although these two MAPK-type proteins have roles in cell wall remodelling and are sensitive to pheromone, participating in mating process (Saito, 2010), a crosstalk between the two and the HOG-mediated pathways for osmostress resistance is known (Babazadeh et al., 2014).

1.3.2 cAMP/PKA

The cAMP/PKA pathway processes most of the yeast glucose-induced signalling (Conrad et al., 2014). Protein kinase A (PKA) is a heterotetramer that is catalytically inactive in the absence of glucose. In the presence of glucose, adenylate cyclase (AC) is activated and produces cAMP from ATP. In turn, cAMP binds and activates PKA. Active PKA influences a wide variety of targets in yeast cells. Globally, it positively regulates the cellular functions associated with fermentative growth and biomass accumulation and negatively regulates respirative growth, use of alternative carbon sources, stationary phase or stress response (Conrad et al., 2014). An intermediate component in the PKA-mediated regulation of gene expression is the Rim15 protein kinase, which is a regulator of four major transcription factors, including Msn2/Msn4, with a role in activating gene expression associated with stress response. In addition, the PKA pathway demonstrates a crosstalk with the CWI and the HOG-mediated pathways mentioned above (García et al., 2017; Gutin et al., 2015).

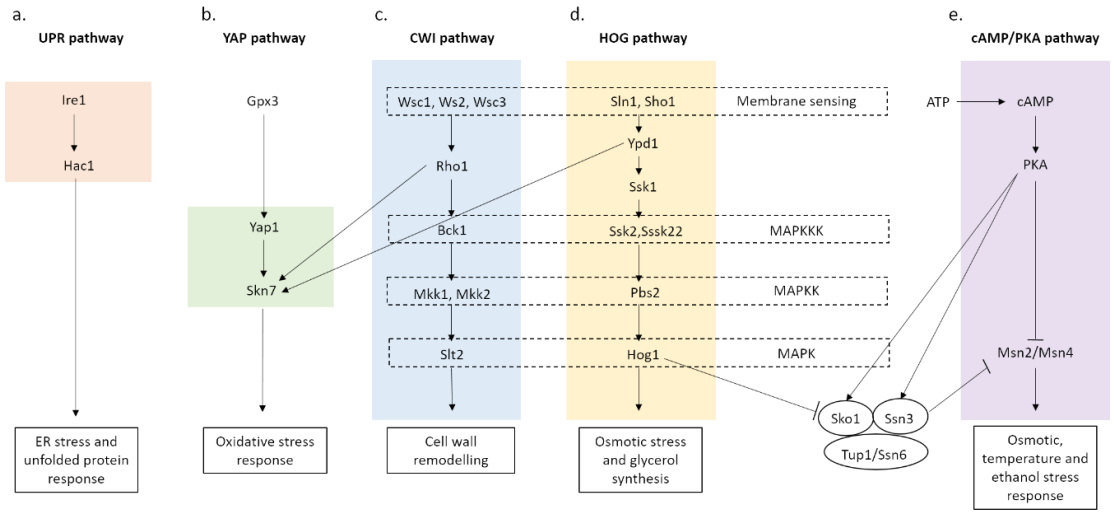

Figure 2. The major yeast stress signalling pathways. Schematic representation of the major player of the stress signalling pathway (see description in text). From left to right: the unfolded proteins response pathway (UPR) to cope with ER stress (**a**), the Yap1 pathway for oxidative stress response (**b**), the two MAPK patways: cell wall integrity pathway (CWI) (**c**), and the Hog1 pathway for osmotic stress (**d**) and finally the cAMP/PKA pathway which modulates activity of general stress transcription factors Msn2/Msn4 (**e**). Crosstalk relationship between pathways, including positive (the arrows) and negative (the T-lines) regulations are reported. Indicated in the boxes are the main effects of the pathway on cellular processes. Adapted from Gutin et al. (2019) and Lin et al. (2021).

1.4 The actuators of the stress response: the transcriptional factors.

Transcriptional regulation is an important process by which cells adapt to stress, achieved by the binding of transcription factors (TF) to target sequences in the DNA. In particular, several stress-responsive genes are controlled by stress-response elements (STREs), conserved DNA sequences recognised by the transcriptional factors Msn2 and Msn4. These factors, by binding a STRE element, mediate a protein kinase-dependent gene expression (Grant et al., 2000; Kandror et al., 2004). While Yap1/Skn7 are known to be involved in regulating the genes for oxidative stress response, a well-studied high temperature stress associated transcription factors in *S. cerevisiae* is Hsf1. Figure 2 provides a graphical overview of the main, conserved transcriptional factors and their associated stress response..

Given Hsf1's strong association with high temperature stress response, it will be described in the dedicated section Another way gene transcription might be regulated under stress is described in greater detail in Chapter V andis by chromatin remodelling, which determines the access of transcription factors on DNA binding sites (Koda et al., 2021); this is mediated by the SWI/SNF remodelling complex (Section 1.4.4).

1.4.1 Msn2/Msn4

In *S. cerevisiae,* the general stress response transcription factors Msn2 and Msn4 play an important role in a variety of environmental stresses, such as osmotic stress, thermal stress and high ethanol concentration. These factors bind to the STRE elements and mediate PKA-dependent gene expression (Grant et al., 2000; Kandror et al., 2004).. Msn2 and Msn4 are activated by a broad signalling cascade involving both the Hog1 pathway and the cAMP pathway (Gutin et al., 2015) (see figure 2). The deletion of both *MSN2* and *MSN4, which conjunctively regulate hundreds of genes,* results in sensitivity to oxidative, thermal and osmotic stresses (Hasan et al., 2002; Sadeh et al., 2011). *MSN2* and *MSN4* also contributes significantly to upregulation of heat shock proteins (HSPs)(Laia Castells-Roca et al., 2011). Moreover, *MSN2/MSN4,* together with *SNF1,* have a role in autophagy during ER-stress (Mizuno & Irie, 2021; Vlahakis et al., 2017). In *K. lactis* and *K. marxianus*, *MSN2/MSN4* have a single orthologue: *MSN2* and, unlike ScMsn2, KlMsn2 has acquired a role in mating type switch (Solieri et al., 2021).

1.4.2 Snf1

The AMP-dependent protein kinase Snf1 regulates the transcription of more than 400 genes. Active Snf1 causes the translocation to the cytoplasm of the inhibitor Mig1, responsible for the repression of over 90 genes mostly involved in glucose repression (Nicastro et al., 2015). Snf1 is linked to stress response, but only indirectly (Sanz, 2003). This is due to its interaction with Hsf1 for the activation of several heat responsive genes following glucose starvation (Hahn et al., 2004), and because it determines the cellular localization of the general stress transcription factors Msn2/Msn4 under conditions of carbon source starvation (Mayordomo et al., 2002). Nonetheless, *snf1Δ* mutant strains show an osmosensitive and thermosensitive phenotype, recovered by Msn2/4 overexpression, indicating its role in yeast stress response (Estruch, 2000; Hedbacker & Carlson, 2008). Moreover, Snf1 by negatively regulating Mig1 and Mig2, consequently promotes Atg39 expression and ER-phagy during ER-stress (Mizuno et al., 2020). In both *S. cerevisiae* and *K. lactis*, the SNF1/MIG1 pathway has been linked to cell wall synthesis and integrity, since deletion mutants display sensitivity to cell wall stressors, which can be suppressed by deletion of the glycolytic enzyme Pfk1, suggesting a compensatory effect given by the

accumulation of the intermediate glucose-6P, principal component of the cell wall (Backhaus et al., 2013; Rippert et al., 2017).

1.4.3 Yap1/Skn7

Yap1 and Skn7 are transcription factors which co-operatively control a number of oxidative stress-responsive genes, including *SOD1* and *SOD2*, encoding for superoxide dismutases, *TRX2* and *TRR1* encoding for thioredoxin and thioredoxin reductase, *TSA1* and *AHP1* encoding for peroxiredoxins and *GPX2*, encoding for a glutathione peroxidase. Several genes are regulated by Yap1 in a Skn7-independent manner, especially those involved in the glutathione system, such as *GSH1* and *GSH2*, and *GLR1*, encoding for a glutathione reductase. Under physiological conditions, Yap1 localizes to the cytoplasm and, upon exposure to oxidative stress, accumulates in the nucleus (Rodrigues-Pousada et al., 2019). The nuclear localization is made possible by the interaction with Gpx3 (D'Autréaux & Toledano, 2007). The same mechanism is known in *K. lactis*, with the addition of Hap1, which interacts with Yap1 attenuating the oxidative stress response under hypoxia conditions (González-Siso et al., 2009). On the other hand, Skn7 is a nucleic protein which seems to participate only in the response to peroxide. It has been shown that Skn7, in cooperation with Hsf1, is involved in the upregulation of *HSP* genes during oxidative stress. Furthermore, its role in regulating the cell wall synthesis, cell cycle, and osmotic stress response has also been reported (Choowong Auesukaree, 2017; Mulford & Fassler, 2011). This mechanism appears conserved in *K. lactis* (González-Siso et al., 2009).

1.4.4 Swi1

Swi1 is part of the SWI/SNF chromatin remodelling complex in yeast. Consequently, it regulates the access to translation of many transcription factors (TF), specifically stress-related (Dutta et al., 2021). A recent study found that Swi1 can generate a prion in yeast, which generates a range of distinct phenotypic effects on cells under stressful conditions (Du et al., 2020; Hines et al., 2011). In *K. lactis*, the SWI/SNF enzymatic subunit Snf2 has been linked to glucose metabolism regulation and transport (Cotton et al., 2012).

2. Heat stress sensing and response in yeast.

As outlined in the previous sections, one of the main challenges encountered by microbial cell factories is the high temperatures faced during biotechnological processes. Yeasts are able to respond to increasing temperatures by altering their cell functions. For instance, via starting a rapid physiological adaptation called heat shock response (HSR) (Castells-Roca et al., 2011; Krakowiak et al., 2018; Morano et al., 2012; Mühlhofer et al., 2019), consisting of mainly conserved changes in gene expression (Gasch et al., 2017). This change in expression brings to the repression of the protein biosynthetic activity (Cherkasov et al., 2015; Mühlhofer et al., 2019) and the induction of a battery of genes encoding for heat shock proteins (HSPs). Many HSPs function as molecular chaperones to protect thermally damaged proteins from aggregation, unfold aggregated proteins, and refold damaged proteins or target them for efficient degradation (Kregel, 2002; Verghese et al., 2012). Physiological changes such as the synthesis of protective compounds (Gancedo & Flores, 2004; Sugiyama et al., 2000a) and cell wall restructuring (Guyot et al., 2015; Imazu & Sakurai, 2005) also contribute to the cellular response to high temperature stress. This section outlines the main steps of the HSR and the main physiological changes yeasts undergo during the heat stress response.

2.1 Sensing temperature stress: Msn2 Msn4 and Hsf1 transcriptional factors

As mentioned in the previous section, the Msn2 and Msn4 transcription factors drive transcription of a large set of genes (200–300) in response to a variety of environmental stresses, including heat shock proteins (HSP) genes (Laia Castells-Roca et al., 2011); however, the Msn2/Msn4 regulon is distinct from that controlled by Hsf1, which has a role under physiological conditions as well as under thermal stress.

Heat shock transcription factor Hsf1 is a protein evolutionarily conserved from yeast to humans, known to bind the heat shock element (HSE) of target genes (Sakurai & Takemori, 2007), in addition to regulating transcription and protein homeostasis under normal physiological conditions (Solís et al., 2016). Hsf1 has a winged helix-turn-helix conformation that recognizes HSE upstream of genes encoding chaperones and other cytoprotective HSPs. Under non-stressful conditions, Hsf1 exists primarily as a non-DNA-binding monomer in either regulating nucleus or cytoplasm and controls the expression of 18 genes, controlling basal chaperone activity under physiological

conditions. In response to thermal or other proteotoxic stress, the protein trimerizes and acquires the capacity for high-affinity DNA binding (Solís et al., 2016). It was shown that Hsp70 basally binds Hsf1, negatively regulating it. Hsp70 transiently releases Hsf1 in response to thermal stress and then rebinds it following exposure to sustained stress, thereby constituting a two-component feedback loop (Zheng et al., 2016). Although overexpression of *HSP90* recovered the thermosensitive phenotype of a partially inactivated Hsf1 in *S. cerevisiae* (Truman et al., 2007), there is no evidence in budding yeast of a physical association, and regulation mechanism, between the two proteins in non-stressed cells. Nonetheless, the Hsp70/Hsp90 chaperone complex represses the transcriptional activation of Hsf1 under non-stress conditions. During heat shock, the accumulation of unfolded or damaged proteins may titrate the chaperone machinery from Hsf1, allowing the derepression of the transcription factor (Verghese et al., 2012). The transcription factors KmHsf1 and KmMsn2 of *K. marxianus* promote both cell growth and ethanol fermentation when expressed in *S. cerevisiae* at high temperatures. Transcriptomic analysis revealed different regulatory mechanisms of KmHsf1 and KmMsn2 in *S. cerevisiae*, by regulating a different pool of genes compared to the *S. cerevisiae* native homolog transcription factors. Based on these different genes upregulation profiles, the proposed mechanism is that KmHsf1 increased ethanol production at high temperatures in *S. cerevisiae* by limiting excessive ATP consumption via regulating transporter activity and promoting the uptake of glucose; while KmMsn2 may promote ethanol fermentation at high temperatures by regulating genes associated with glucose metabolism and glycolysis/gluconeogenesis. In addition, KmMsn2, may also regulate genes associated with lipid metabolism to improve membrane fluidity (Li et al., 2017).

2.2 Chromatin reorganization and mRNA-processing for heat stress response

An effective response to environmental changes requires rapid rearrangement of gene expression patterns, in order to adapt to the new conditions. Chromatin remodelling is critical for this process and allows for induction of genes involved in stress responses. Heat shock genes in particular undergo extensive nucleosome rearrangements upon induction. Chromatin remodelling is carried out by a large class of ATP-dependent chromatin remodelling complexes, divided into families by homology of their protein subunits: SWI/SNF family (SWI/SNF and RSC), ISWI family (ISWI1 and ISWI2),

CHD family (Chd1), INO80 family (INO80 and SWR1) (Erkina et al., 2010). The SWI/SNF complex in particular plays a dual role of activation and repression of genes during heat shock, in particular thanks to Snf2, which is recruited to Hsf1 target genes (Erkina et al., 2010; Shivaswamy & Iyer, 2008). Other complexes such as RSC, INO80 and ISW1 participate in chromatin remodelling of the cAMP-dependent protein kinase *TPK1*p under thermal stress conditions, eliciting a large transcriptional response: RSC and INO80 contribute to repression via nucleosome positioning on the *TPK1* promoter under normal conditions, while SWI/SNF participates in the eviction of nucleosomes after heat stress (Reca et al., 2020)

Changes in transcription rate and mRNA stability also shape the yeast response to stress, with mRNA profiles of the environmental stress response genes being affected by these processes (Castells-Roca et al. 2011). An mRNA flow through polysomes, p-bodies, and/or other mRNP particles such as stress granules is described during various stress responses including heat stress, although the exact dynamics are still unclear (Cowart et al., 2010). The cAMP-dependent protein kinase subunits Tpk2 and Tpk3, co-localize with stress granules induced by severe heat stress affecting translational response to heat stress (Barraza et al., 2017), such that might be a way mRNA localization affect the heat stress response.

2.3 The HSPs and protein folding

High temperatures and other physiological stresses, can make protein conformation unstable, and cause denaturation and aggregation, with proteotoxic effect on the cell. This results in synthesis of yeast's many cytoprotective genes commonly called heat shock proteins (HSPs), part of which encode for molecular chaperone, protein; with the capacity to shield, fold, or unfold targets (Verghese et al., 2012). The main described yeast chaperones are Hsp70 and Hsp90. The Hsp70 chaperone functions through an ATP/binding-dependent cycle to bind and shield short hydrophobic regions of polypeptides from the aqueous environment, while the remainder of the protein folds. Successive cycles of binding and release result in substrate folding to the native state. Hsp70 increases its substrate refolding efficiency via interaction with co-chaperones (Abrams et al., 2014). While Hps70 recognizes unfolded protein adjuvating their first stage of folding, Hsp90 functions primarily in the "final" maturation of proteins and the assembly of complex macromolecular structures. Hsp90 can interact with a group of substrates, termed "client" proteins, which include

many kinases and transcription factors and co-chaperones (Verghese et al., 2012). Hsp70 and Hsp90 coordinate their chaperoning action over time, in a mechanism known as the Hsp70-Hsp90 cascade (Morán Luengo et al., 2019). Other chaperones are described in yeast, such as Hsp104 which uniquely recognizes misfolded proteins within aggregates, small HSPs and chaperonines, extensively reviewed in (Verghese et al., 2012). Notably, chaperone systems are also present in yeast in the ER secretory pathway and in the mitochondrial matrix, to assist in the import and folding of protein substrates. These are regulated by specific stress responsive pathways: the unfolded protein response for ER, called ERAD (Nakatsukasa, 2021) and the retrograde response for mitochondria, which leads to a reconfiguration in the expression of a subset of nuclear genes for changes in the mitochondrial state (Jazwinski, 2013; Ždralević et al., 2012); but both employ similar chaperones and co-chaperones to what that described for the cytosolic systems (Verghese et al., 2012). The similarity between *K. lactis* and *S. cerevisiae* UPR and chaperones system is discussed in (Hernández-Elvira et al., 2018).The ancient origin of HSR in yeast, suggests that the same systems can be found in *K. marxianus,* although not characterized so far in the species. Recently, the prefoldin Pfd4 in *K. marxianus* has been described to improve ethanol fermentation in presence of cellulolytic-derived inhibitors (Zhang et al., 2021). Prefoldins are co-chaperones specifically helping cytoskeleton proteins folding, and could increase stress resistance to various stresses including high temperature. In addition, molecular chaperones such as Gtt1 and Atx1 were found upregulated in *K. marxianus* under high temperature stress (Li et al., 2019b).

Although the majority of the studies focus exclusively on chaperones during heat stress response, recent studies revealed that the response to proteotoxic stress is composed of two sides: one mediated by chaperones, which helps protein folding and counteracts aggregation; and one composed of anapleurotic reactions of protein turnover, counterbalancing the aggregation. In fact, the majority of upregulated genes during the heat stress response appear to have the function of guaranteeing constant protein levels for important metabolic processes under stress conditions and, thus, counterbalancing increased degradation as well as aggregation. According to the same study, higher temperatures would activate a third kind of heat stress response: phase transition and aggregation of proteins, followed by a regulatory shutdown of translation and growth (Mühlhofer et al., 2019).

2.4 Accumulation of trehalose and glutathione

As a response to high temperature, yeast accumulate protective compounds such as trehalose and glutathione. Both these compounds have a protective role in multiple stresses other than heat stress, such as osmotic and oxidative stress. The accumulation of trehalose observed in yeast under heat stress (Argüelles, 1997; Gibney et al., 2015; Mahmud et al., 2010) was previously thought to act solely as a thermoprotectant from enzyme denaturation (De virgilio et al., 1994). More roles were recently attributed to the trehalose biosynthetic pathway and to its precursor, trehalose-6P (Petitjean et al., 2015). These roles include regulating central carbon metabolism (Fraenkel & Nielsen, 2016), heat stress (Conlin & Nelson, 2007; Gancedo & Flores, 2004), and in general contributing to cell homeostasis (Kim et al., 2020), which will all be discussed in detail in Chapter VI of this thesis. Accordingly, trehalose biosynthesis was reported to be regulated by both heat shock proteins (Elliott, Haltiwangert, and Futcher, 1996; Hottiger et al., 1992) and cAMP kinase (Trevisol et al., 2014) under stress, and by glucose availability even under standard conditions (Apweiler et al., 2012), confirming the role of this metabolite in central cellular processes.

Glutathione indirectly protects the cells during heat stress by offering a reducing power against ROS accumulation, a sudden effect of temperature stress that will be discussed in the following section (Section 3). Glutathione is synthesized in two enzymatic reactions by the enzymes γ-glutamylcysteine (γ-GC) synthetase (Gsh1) and glutathione synthetase (Gsh2) and reversibly binds the proteins' sulfhydryl groups preventing oxidative damage (Suzuki et al., 2011). Expression of *GSH1* and *GSH2* is induced in yeast by heat shock stress in a Yap1-dependent manner, with subsequent increases in intracellular glutathione content (Laman Trip & Youk, 2020; Sugiyama et al., 2000b). Although not under heat stress, glutathione accumulation has been reported in *K. marxianus* in response to ethanol and osmotic stress (Saini, Beniwal, & Vij, 2017), suggesting the same role in protection against the oxidative damage.

2.5 Cell-wall damage

As well as affecting internal cellular processes, heat stress also impacts the cell wall integrity. The yeast cell is delimited by a plasma membrane, and an outer layer represented by a cell wall composed of glucose and mannose polysaccharides and N-acetylglucosamine, with an intervening periplasmic space containing numerous secreted enzymes and membrane-associated surface proteins (Klis et al., 2002).

Alteration of the ergosterol composition of the cell membrane achieved upon high temperature adaptation increases the strain's thermotolerance (Caspeta et al., 2014). Heat stress, which causes cell wall damage, is known to be sensed through the outer cell membrane sensors and activate the cell wall integrity pathway (CWI) described in the previous section (see paragraph 1.3.1). Unsurprisingly, mutants for members of the CWI pathway display a thermosensitive phenotype (Levin, 2005). There is often a crosstalk among yeast stress response pathways, and the CWI pathway is no exception: the transmembrane protein sensor Wsc1 mediates crosstalk with the cAMP/PKA for heat stress response through the downstream activation of Slt2/Mpk1 in the CWI pathway (Fuchs & Mylonakis, 2009). Moreover, a recent study showed that the RNA exosome complex is a regulator of cell wall stability upon heat stress, by influencing glycosylation of the cell wall proteins (Novačić et al., 2021). As discussed in section 1.3.3, the SNF1/MIG1 pathway has a role too in *K. lactis'* cell wall integrity and cell wall stress tolerance.

3. Oxidative and temperature stress are interconnected in yeast

Temperature stress induces oxidative stress in yeast, probably because the metabolic changes induced by heat stress response cause a redox imbalance at the level of the mitochondrial membrane, which results in accumulation of ROS species (Fedoseeva et al., 2017; Zhang et al., 2015). Several studies have shown that augmenting the ROS detoxification ability by overexpressing antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) improves thermotolerance (Nai-Xin Lin et al., 2021; Xu et al., 2018). Conversely, cells lacking antioxidant enzymes are hypersensitive to heat shock (Davidson et al., 1996); indicating that the responses to oxidative and temperature stress are strongly interconnected, and the oxidative stress aspect cannot be ignored when investigating thermotolerance. Several studies have shown that *K. marxianus* itself activates a strong oxidative stress response when dealing with growth under high temperature (Fu et al., 2019; Lertwattanasakul et al., 2011; Li et al., 2019a). Transcription of the oxidative stress response genes in yeast is mainly under the control of Yap1 and Skn7 transcription factors (described in the section 1.4.3), both also involved in the osmotic stress response mediated by Sln1 membrane sensor. However, the oxidative stress response signalling cascade seems to be independent of Sln1 (Ikner & Shiozaki, 2005; Mulford & Fassler, 2011). Moreover, although in *S. cerevisiae* there is controversy over whether Hog1 kinase is the main transducer of the

signal of oxidative stress or not (Ikner & Shiozaki, 2005), in *K. marxianus* a *hog1Δ* mutant is sensitive to oxidative stress, indicating its role in the response; but the gene seems not to play a role in thermotolerance (Qian et al., 2011). Below the main effects and response strategies to ROS accumulation in yeast are mentioned. The correlation between oxidative stress and heat stress response in *K. marxianus* is investigated in more details in Chapter V of this thesis.

3.1 ROS accumulation and detoxification strategies

Reactive oxygen species (ROS) are oxidants derived from molecular oxygen during the cell metabolism, principally respiration (Chung 2017; Herrero et al., 2008). Excessive accumulation of ROS, called oxidative stress, damages the main biological macromolecules such as nucleic acids, proteins and lipids (Farrugia & Balzan, 2012) and can influence protein biosynthesis (Topf et al., 2018) and mitochondrial biogenesis (Bouchez & Devin, 2019).

Under physiological conditions, and under heat stress, the leakage of electrons from the mitochondrial respiratory chain is the major intracellular source of ROS (Davidson & Schiestl, 2001). The yeast antioxidant defence includes a number of protective enzymes that are present in different subcellular compartments and can be upregulated in response to ROS exposure. Superoxide dismutase (SOD) is a major antioxidant enzyme that specializes in metabolizing superoxide radicals into molecular oxygen and H_2O_2, which is further converted to water by catalase (CAT), peroxiredoxins, and glutathione peroxidases (Morano et al., 2012). In addition to its antioxidant role, Sod1 has recently been described to play roles as a nuclear transcription factor, RNA binding protein, a synthetic lethal interactor and a signal modulator in glucose metabolism (Chung, 2017), indicating the centrality of the antioxidant response to the cellular homeostasis. In addition to the antioxidant enzymes, yeast have non-enzymatic defences against oxidative stress, typically consisting of small molecules that can act as free radical scavengers, such as ascorbic acid and glutathione (See section 2.4) (Morano et al., 2012).

4. Sudden stress response versus stress adaptation.

Chapters III, IV and V of this work investigate various aspects of *K. marxianus* responses to long term stress, as a way to understand the mechanisms of this natural trait for exploitation in biotechnology. Long term temperature stress was achieved

using chemostat fermentations as opposite to heat shock methods, allowing investigation of adaptive mechanisms to stress. In fact, acute heat shock response started by *S. cerevisiae* strains immediately after a sudden change of temperature, were seen to differ from that following adaptation to high temperature. In particular fewer HSPs, less protein degradation and downregulation of TCA, PPP and aa metabolism was observed during temperature adaptation compared to heat shock. The same study identified differences in transcriptional factors' upregulation under the two responses, indicating thermotolerance is an inherently different response from the heat shock response (Shui et al., 2015). Moreover, genes upregulated during HSR does not correlate with the genes needed for adaptation to high temperature (Gibney et al., 2013)

One recent theory is that different yeast species have evolved different adaptation strategies, dictated by their need to survive in diverse natural niches, through the evolution of species-specific genes (Brion et al., 2016; Doughty et al., 2020; Tirosh et al., 2011). Moreover, different yeast species display different metabolic responses under high temperature stress (Lehnen, Ebert, and Blank, 2018), indicating the variability of the responses. Genome rearrangements producing loss of heterozygosis in some *K. marxianus* strains probably originated by domestication during application in the dairy industry, and could be another form of evolutionary adaptation to stress during industrial fermentations (Ortiz-Merino et al., 2018). These elements taken together, diverge from the concept of a common environmental stress response (ESR) theorized for ascomycetes yeast (Gasch, 2007), maybe suggesting that the general pathways activated by the response to various stresses might be conserved, but differ in specific genes and regulation patterns, and generate a peculiar response in each species. This variability of adaptation systems among non-saccharomyces yeasts constitutes a resource for exploitation of natural traits in the development of industrial strains (Rebello et al., 2018), provided that the underlying mechanisms are first understood and described in detail.

5. Approaches to study stress response in yeast and space for improvement

Studying yeast stress responses are challenging because it involves transcriptional changes of hundreds of genes (Gasch et al., 2000), many of which are genes are often compounded in central metabolism and there is cross-talk between the regulatory pathways. Thus, researchers have employed systems biology and omic techniques to

characterize the transcriptome and/or proteome-wide stress-induced changes (Auesukaree et al., 2009; Chen et al., 2016; Li et al., 2019; Wang et al., 2018), in an attempt to obtain an holistic picture of the stress resistance phenomenon. Metabolomic analysis are also a valid resource to investigate stress response (Lehnen, Ebert, and Blank, 2019; Walther et al., 2010).

However, different yeasts seem to have developed specific stress adaptation mechanisms involving species-specific genes, with no homology in other species (Brion et al., 2016; Doughty et al., 2020). Since traditional annotation techniques are homology-based (Sinha et al., 2020), this represents a limitation when studying stress response in non-saccharomyces yeast(Brion et al., 2016). Furthermore, small proteins (proteins below 90 aa) are most often not annotated (Hao et al., 2018; Yagoub et al., 2015), which represents another challenge for the study of stress adaptation in yeast, where a significant part of stress responsive genes (between 41 and 69%) are evolutionary young genes, on average shorter than previously annotated coding sequences (Blevins et al., 2021; Doughty et al., 2020). This limitation is often overcome through utilisation of deep sequencing approaches, which combine proteomics or Ribo-sequencing with transcriptomics (Blevins et al., 2021; Yagoub et al., 2015) and allow for discovery and annotation of previously unknown genes (Fenton et al., 2022).

Other novel approaches to study and improve a strain's stress tolerance involve single cell sequencing methods, which allow one to account for heterogeneity of physiological response to stress among a cell population (Gasch et al., 2017; Zhang et al., 2017), and genome-wide reciprocal hemizygosity mapping (RH-seq).The latter involving a mutagenesis screen in an interspecies hybrid background allowing to identify which variation between the species contributes to the thermotolerance trait (Abrams et al., 2021).

When it comes to *K. marxianus*, the yeast represents a promising microbial cell factory (Patra et al., 2021) due to innate traits, which will be discussed in detail in Chapter II, among which elevated thermotolerance is particularly relevant. However, the mechanism behind this trait remain mostly unknown, due both to the limited genomic and omic data available for *K. marxianus* (Diniz et al., 2017; Jia et al., 2021; Lertwattanasakul et al., 2011; Li et al., 2019a), and physiological data available presents a wide strain and experimentalconditions variability (Table 1), which represents an obstacle in establishing reference data for comparison of strains and

conditions. Chapters III, IV and V of this thesis will try to elucidate aspects of *K. marxianus* thermotolerance, taking an omic approach to investigate some of its high temperature adaptation mechanisms.

Table 1 Variability of strains and condition used for physiological investigations of *K. marxianus* yeast. The table reports the variety of *K. marxianus* strains, and the diverse cultivation conditions utilized in physiological investigation of the species, which makes it difficult to compare the parameters for stress tolerance studies. Where available, the main growth parameters are indicated.

Study	Strain	Conditions	Main findings	MMAX (H-1)	DT	$Y_{x/s}$ (GG-1)
(FLEMING ET AL., 1993)	IMB3	Flask Sucrose 10% 45°C	Sucrose hydrolysing activity Ethanol accumulation	-	-	-
(HOEKSTRA ET AL., 1994)	CBS 6556	Flask Chemostat glucose	Ribosomal proteins translation rate varies with growth rate in chemostat	1.1 h-1	-	-
(BELLAVER ET AL. 2004)	CBS 6556	-Batch -chemostat with glucose pulse Aerobic 30C	No ethanol production in any condition (no crabtree effect)	0.44 h-1	1.57	0.49
(FONSECA ET AL., 2007)	ATCC 26548	-Batch -Chemostat Glucose 1% Aerobic 30C	No crabtree High growth rate Low metabolites sectretion	0.56h-1	1.24	0.52
(WILKINS ET AL., 2008)	IMB2 IMB4 IMB5	Anaerobic Xylose 40C, 45C	Comaprison of yield of xyilitol and ethanol between the three strains	-	-	-

(ROCHA, ABRAHÃO-NETO, AND GOMBERT 2011)	CBS 6556 CBS 397 CBS 712	Flask Glucose,Sucrose,Lactose 30C, 37C	Cbs 6556 shows max growth rate of all three at 37C on sucrose	0.59 0.55 0.31	0.66 0.52 0.47	-	0.44 0.40 0.46	0.44 0.34 0.55
(SIGNORI ET AL., 2014)	CBS 712	Chemostat Mixed glucose/xylose 30C, 41C Various oxygen limiting	Oxygen level influences xylose fermentation	-		-	0.17, 0.11	
(WANG ET AL., 2018)	YHJ010	Flasks YPD+inhibitors 42C	Transcriptomic analysis of response to various inhibitors	-		-	-	
(FU ET AL. 2019)	DMKU3-4210	Flask YPD anaerobic 45C	Transcriptome and metabolimc analysis following a fermentatiojn arrest at elevated temperature	-		-	-	
(LEHNEN, EBERT, AND BLANK 2019)	ATCC 748 CBS 2080 CBS 712	Flask Airtight 40C-47C	Ideal growth temperature 40C, differences in metabolic flux between K mar and other species at high temp	0.94		-	0.55	

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Chapter II

A primer on *Kluyveromyces marxianus*

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JA wrote section 5

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JM coordinated the work and edited the manuscript

1. Introduction

Kluyveromyces marxianus is a budding yeast belonging to the sub-phylum *Saccharomycotina*, sometimes referred to as the "budding yeast subphylum", which diverged from other ascomycetous fungi about 450 million years ago (Shen et al., 2020). Within the sub-phylum, there is a single order, the *Saccharomycetales*, which contains thirteen families including the *Saccharomycetaceae*. This family comprises 11 genera (Kurtzman & Sugiyama, 2015; Shen et al., 2018), including the *Kluyveromyces* genus. The *Kluyveromyces* and *Saccharomyces* genera are in the same family but separated prior to the whole genome duplication, and *Kluyveromyces*, *Lachancea* and *Eremothecium* cluster together forming what is sometimes referred to as the "KLE clade" (Kurtzman, 2003; Marcet-Houben & Gabaldón, 2015). Within the *Kluyveromyces* genus, there are six species (Figure 1A). *K. marxianus* is traditionally associated with fermented dairy products (Figure 1B), which permits its GRAS (US FDA– generally regarded as safe: Bulletin of the IDF N° 495/ 2018) and QPS (European Food Safety Authority – Qualified Presumption of Safety) qualification, but strains have been isolated from diverse environments including insects, soil, and plants (Am-In et al., 2008; Varela et al., 2019). In fact, the natural environment and reservoir for the species is likely to be plant material, with very-well studied lactose-utilising strains being a subset of this larger population. This is reflected by the species' ability to assimilate several plant polymers, while lactose assimilation is restricted to some strains. Although this single-celled eukaryotic microorganism is generally found in a yeast-like shape, its cell morphology can vary in relation to cultivation conditions, in particular to growth rate, and it is able to assume an elongated, filamentous and pseudohyphal form (Figure 1C)(Burke, 2011). *K. marxianus* is a homothallic yeast, capable of switching mating type and self-mating in response to adverse environmental conditions (Yarimizu et al. 2013). Mating is immediately followed by sporulation without undergoing mitosis and thus the yeast is generally isolated from the environment as a haploid (Hanson & Wolfe, 2017). Its genome comprises 8 chromosomes and is approximately 10Mb long (Lertwattanasakul et al., 2015). The yeast's natural endurance to common industrial stresses and the recent advances in the development of molecular biology tools to perform strain engineering, make *K. marxianus* a promising microbial cell factory

candidate for sustainable production of biochemicals. The following review treats future perspectives for this yeast's application in the bioeconomy sector.

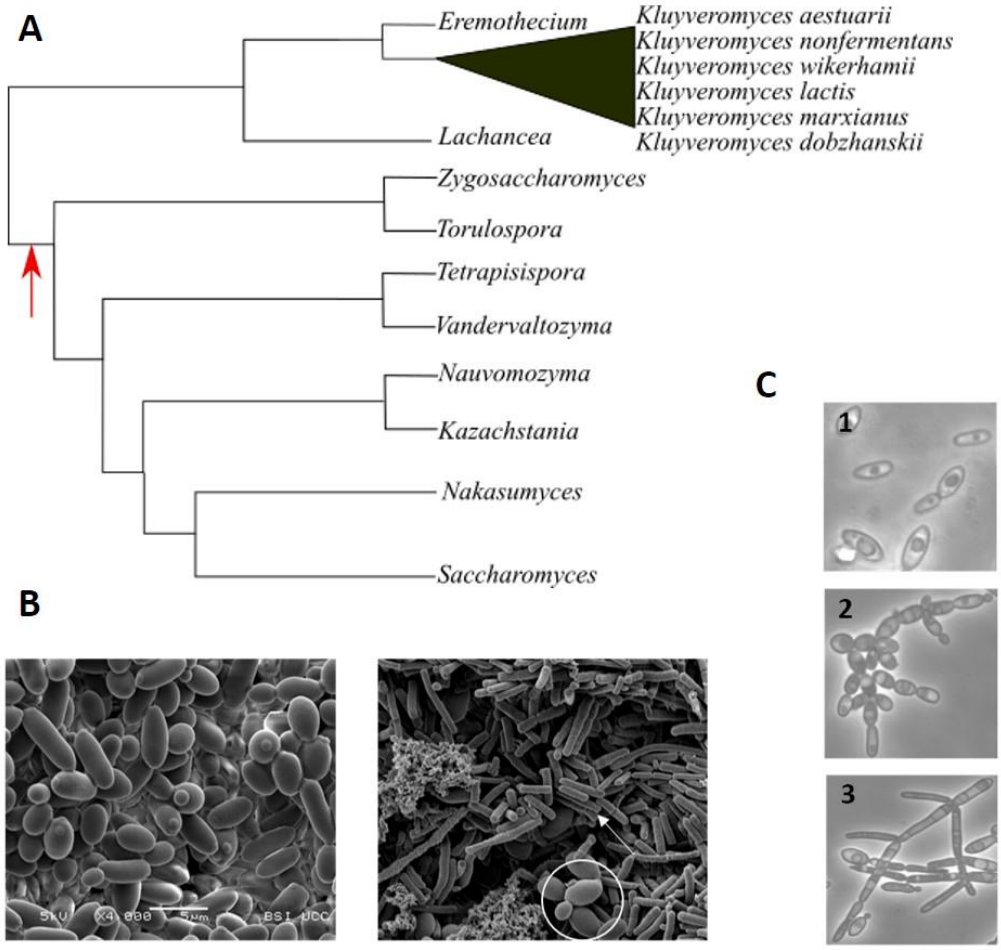

Figure 1 Morphology and phylogeny of *Kluyveromyces marxianus.* A Phylogenetic tree of the *Saccharomycetacee* family. Schematic representation of the main genera, based on the phylogenetic tree inferred by (Shen et al., 2016). The tree was inferred from the concatenation-based analysis of 1233 single-copy orthologues. The Whole Genome Duplication event is indicated by a red arrow. The species belonging to the *Kluyveromyces* genus are specified. **B** Electron microscopy images of *K. marxianus* cells (left) and *K. marxianus* cells – circled in white - in a kefir matrix (right). The bacteria of the kefir consortium are indicated with a white arrow (Gethins, Crotty and Morrisey, unpublished). **C** *K. marxianus* can assume different morphologies depending on strain and culture conditions. Represented are microscope images of elongated (1), pseudo-hyphae (2) and filamentous (3) cells (Burke, 2011)

2. Carbon assimilation

K. marxianus can be found in diverse ecosystems. This results from the ability of the yeast to access a wide range of sugars and polymers present in plants. These include structural polymers such as cellulose, hemicellulose and pectin, and reserve

carbohydrates called fructans and RFOs (Raffinose Family Oligosaccharides). The enzymatic digestion of such polymers generates the wide variety of monosaccharides that the yeast is able to metabolize. In fact, in addition to glucose, favoured by the cell because of the easy access into glycolysis, *K. marxianus* is able to metabolize the hexose sugars fructose and galactose, as well as the pentose sugars xylose and arabinose, major components of cellulosic material. In addition, *K. marxianus* possess the enzymatic activity necessary to access monomers of several disaccharide sugars, such as sucrose, cellobiose and lactose. However, unlike other baker yeasts, *K. marxianus* is not able to metabolize maltose, likely due to the lack of transporter activity. The metabolism and transport of the mentioned sugars are described in the following sections, and the process of carbon assimilation in *K. marxianus* is summarized in Figure 2.

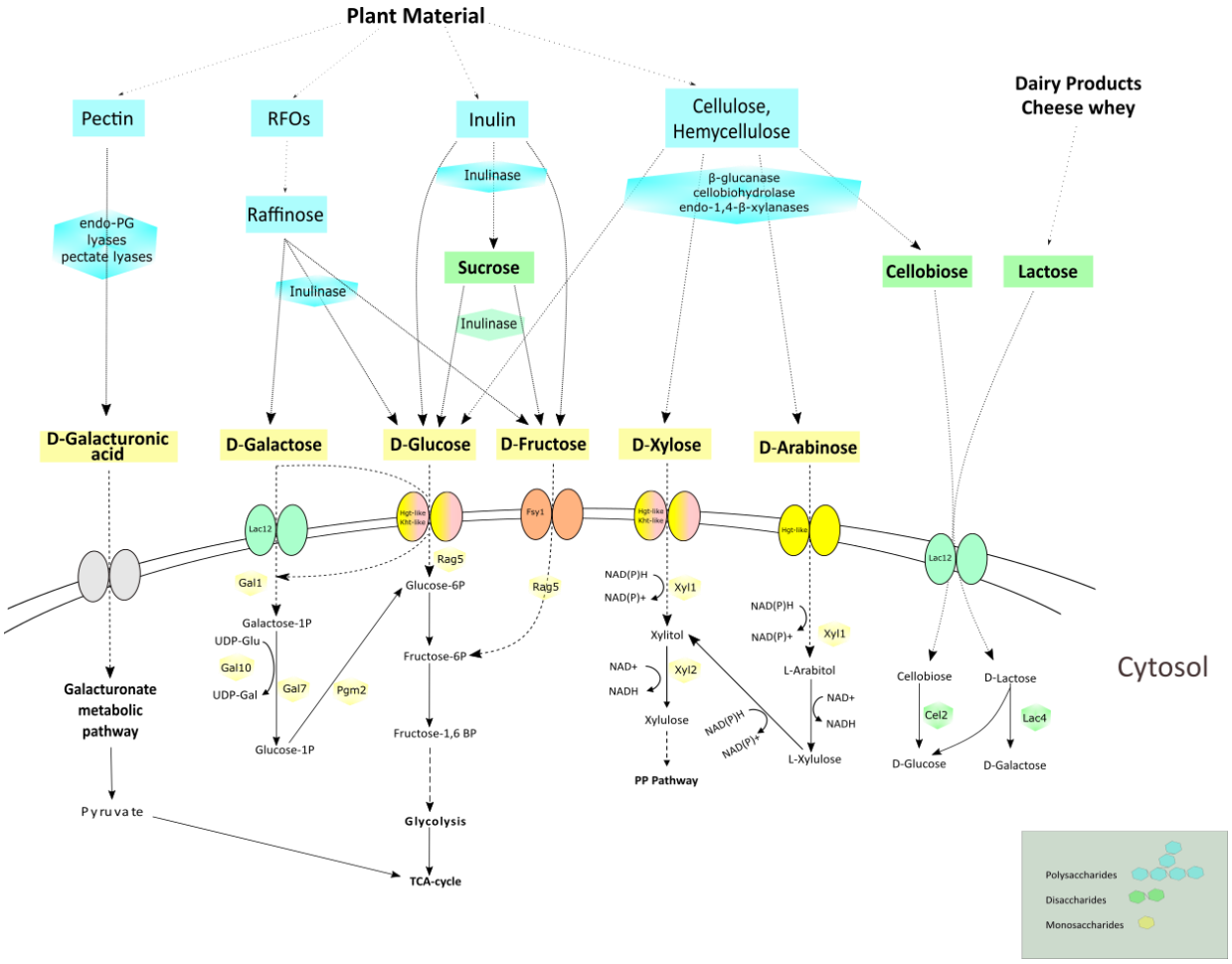

Figure 2 Carbon uptake and assimilation in *K. marxianus*. *K. marxianus* is able to access polymers deriving from plant materials and dairy products. Polysaccharides, disaccharides and monosaccharides are respectively shown in blue, green and yellow squares. **Breakdown of polymers and disaccharides.**

Enzymes involved are shown in hexagonal shape. Cellulose and hemicellulose are degraded by cellulases and hemicellulases (β-glucanase, cellobiohydrolase, endo-1,4- β-xylanases) into the disaccharide cellobiose and monomers of glucose, fructose, xylose and arabinose – these monomers are present also as free sugars in this feedstock (not shown). Inulin is broken down by inulinases into sucrose, glucose and fructose. Inulinase is also responsible for the cleavage of raffinose into glucose and melibiose. Pectin is cleaved by pectinases (endo-PG, lyases and pectate lyases) into galacturonic acid. **Transport system**. Transporters responsible for the sugar uptake are reported on the membrane lipid bilayer. Lac12 permease (in light green) is responsible for the uptake of lactose, cellobiose and galactose. Both Hgt-like and Kht-like transporters (in yellow and pink) carry glucose, xylose, galactose, while only Hgt-like transporters (in yellow) enable the entrance of arabinose in the cell and Fsy1 (in orange) uptakes fructose. Galacturonic acid enters through an unknown transporter (in grey). **Metabolism.** Lactose is split in glucose and galactose by a β-galactosidase encoded by *LAC4*. Cellobiose is degraded in two molecules of glucose by a β-glucosidase encoded by *CEL2*. Galactose is converted in glucose-6-P in the Leloir pathway and enters glycolysis. Fructose is phosphorylated by Hxk2 (Rag5) and, as glucose, is then metabolized via glycolysis. Xylose and Arabinose assimilation occurs through the aldose reductase pathway continuing then to the pentose phosphate pathway. Galacturonic acid is metabolized via the galacturonate metabolic pathway producing pyruvate.

2.1 Plant Polysaccharides

2.1.1. Pectin, cellulose and hemicellulose

Pectin is a polysaccharide-based polymer that is commonly found in fruit and vegetables. It consists of a backbone of galacturonic acid and rhamnose with side chains of arabinose, fucose, galactose and xylose (Martins et al., 2020). The pathway for D-galacturonic acid catabolism is not described in *K. marxianus*, but the fungal metabolic pathway was characterized (Martens-Uzunova & Schaap, 2008; Rippert et al., 2021) and ultimately yields pyruvate, which can enter the TCA cycle, and L-glyceraldehyde, which is converted into glycerol using NADPH as the electron donor. There are several classes of enzymes known to break the α-1,4-glycosidic linkage between pectin monomers: lyases, pectate lyases and polygalacturonases (Geralda Da Silva et al., 2005). *K. marxianus* can degrade pectin through the production of specific hydrolytic enzymes, named pectinases (Arrizon et al., 2011; Vicente-Magueyal et al., 2020). The enzyme endopolygalacturonase (endo-PG) is also abundantly secreted from *K. marxianus* and plays a role in the process of fruit ripening and spoilage and in cocoa processing (Jia & Wheals, 2000; Schwan et al., 1997; Serrat et al., 2002). Although a D-galacturonic acid transporter is not described in *K. marxianus*, (Benz et al., 2014) reported the characterization of a *Neurospora crassa* transporter whose heterologous expression in *S. cerevisiae* allowed for galacturonic acid transport. It is plausible that one of the sugar transporters described in section 2.4 could transport galacturonic acid, but this remains to be determined.

Cellulose and the heteropolysaccharide hemicellulose play an integral role as structural components of the plant cell wall. The two are organized in microfibrils tied into a network by cross-linking glycans that form hydrogen bonds with them. An additional network of pectin polysaccharides confers robustness to the cell wall structure (Houfani et al., 2020). Cellulose is exclusively composed of β-glucose monomers in a straight-chain linked by □-1-4 bonds. On the other hand, hemicellulose, which represents 20-30% of the dry weight of wood, is a cross-linked polymer composed of various monomers: six-carbon sugars like glucose, galactose, mannose; and five-carbon sugars, xylose and arabinose. Breakdown of cellulose and hemicellulose (components of lignocellulosic biomass) is, in a natural environment, primarily carried out by enzymatic activities secreted by microorganisms like cellulolytic bacteria (e.g. *Caldicellulosiruptor thermophiles, Caldicellulosiruptor bescii*) or filamentous fungi (e.g. *Trichoderma* spp., *Aspergillus* spp.). *K. marxianus* was reported to produce an extracellular B-glucanase (endo-β-1,3(4)-glucanase) with limited lignocellulolytic activity (Lopes et al., 2014). It has also been extensively engineered to improve the degradation of lignocellulosic polymers via over-expression of heterologous genes with cellulolytic activity (Hong et al., 2007; Zhou et al., 2018). Although the capacity for *K. marxianus* to degrade the polymers is limited, it is naturally able to consume the released disaccharide cellobiose and also hexose and pentose monomers (which comprise respectively 50-60% and up to 40% of available sugars in lignocellulosic biomass). Thus, it has the potential to thrive in a mixed microbial community degrading plant biomass.

2.1.2 Fructans and Raffinose Family Oligosaccharides (RFOs)

K. marxianus is also able to access water-soluble storage carbohydrates present in plants named fructans and Raffinose Family Oligosaccharides (RFOs). Inulin, is a common plant fructan and comprises a β(2,1) fructose polymer expanding from a sucrose (glucose αβ1-2) fructose) core. It is metabolized by enzymes called inulinases, which break down inulin into a mixture of oligofructoses, sucrose, fructose and glucose (Gustavo Graciano Fonseca et al., 2008; Niness, 1999; Rouwenhorst et al., 1990; Vicente-Magueyal et al., 2020). In *K. marxianus*, the enzyme inulinase (sometimes referred to as invertase) is encoded by *INU1* and is responsible for the

cleavage of inulin. Inu1 is an exo-inulinase that acts on the non-reducing end, liberating glucose and then fructose monomers from inulin. It is partially secreted and partially remains associated to the cell wall making this specific localization most likely ecologically beneficial for efficient scavenging of hydrolysed products, like sucrose into glucose and fructose (Rouwenhorst et al., 1990, Lertwattanasakul et al., 2011). Raffinose Family Oligosaccharides (RFOs) are non-reducing α(1,6) galactosyl (Gal) extensions of Sucrose, commonly found in vegetables. The trisaccharide raffinose (Galα1,6 Glcα1,2 βFru) is the smallest RFO and further elongation with Gal residues leads to the four unit stachyose (Galα1,6Galα1,6Glcα1,2βFru), which can also be further extended (Sengupta et al., 2015; Van den Ende, 2013). *K. marxianus* is also able to grow using raffinose as a sole carbon source (Hua et al., 2019; Lertwattanasakul et al., 2011). In *S. cerevisiae*, raffinose is hydrolysed by invertase (cleaves the glucose αβ1-2) fructose bond generating melibiose and fructose) and an α-galactosidase called Mel1 (cleaves the α(1-6) bond in Melibiose generating galactose and glucose). *MEL1* has been well-studied in *S. cerevisiae* (Álvarez-Cao et al., 2019) and has been identified in some other yeast species (Ventakesh et al., 2021), but is not encoded in the *K. marxianus* genome. In *K. marxianus,* inulinase cleaves raffinose to melibiose and glucose but since *K. marxianus* appears to lack either an extracellular or intracellular β-galactosidase, melibiose cannot be used by this yeast.

2.3 Assimilation of monosaccharides and disaccharides

The metabolic networks employed for the metabolism of hexoses are conserved in yeast and mainly consist of glycolysis (for glucose and fructose) and the Leloir pathway (for galactose). The final product of both the aforementioned pathways is pyruvate. The fate of pyruvate, which could be metabolised by fermentation to ethanol or in the TCA cycle for aerobic respiration, is strain-dependent (Rodrigues, Ludovico, & Leão, 2006, Fonseca et al., 2008, Rocha et al., 2011, Lane et al., 2011, Fonseca et al. 2013). Glucose tightly regulates the assimilation of other sugars and *K. marxianus* shows an intrinsic glucose repression system, in which the orthologous genes of *S. cerevisiae MIG1* and *HXK2* (in *K. marxianus MIG1* and *RAG5*) play a fundamental role in the regulation of the expression of genes implicated in the metabolism of alternative carbon sources (Ahuatzi et al., 2007; Lane et al., 2011; Nurcholis et al.,

2018). Rag5 is also the only *K. marxianus* identified hexokinase (Hua et al., 2019) and it is responsible for the first glycolytic step of conversion of fructose to fructose 6-P. Beside glucose and fructose, galactose is another hexose sugar metabolized by *K. marxianus*. It enters glycolysis after being converted to glucose-6-P by the enzymes of the Leloir pathway. In *K. lactis* and likely in *K. marxianus* too, Gal1 is a bifunctional protein and shows a regulatory function similar to *S. cerevisiae*'s Gal3: it is needed for *GAL* gene induction (Meyer et al., 1991).

K. marxianus can efficiently assimilate pentose sugars, named xylose and arabinose, under aerobic conditions (Rodrussamee et al., 2011). *K. marxianus* seems to have a different mechanism of glucose repression when it comes to xylose utilization compared to *S. cerevisiae*, not depending on Mig1, but on another unknown glucose repression factor (Hua et al., 2019). The assimilation of the pentoses xylose and arabinose in *K. marxianus* shares a partial overlap and occurs through the aldose reductase pathway. Xylose is first reduced in xylitol by Xyl1, then oxidised to xylulose by Xyl2 and then phosphorylated to xylulose 5-P by Xyl5. The xylulose 5-P enters in the pentose phosphate pathway (PPP). Arabinose is firstly converted to L-arabinitol by Xyl1. It joins then the xylose metabolic pathway via conversion into xylitol via a NAD+-dependent oxidative reactions and a NAD(P)H-dependent reductive reaction (Rodrussamee et al. 2011; Zhang et al. 2017). Under aerobic conditions, NADH is recycled to NAD+ in the mitochondrial respiratory chain, but in case of oxygen limitation, the incapability to oxidise NADH, leads to a cofactor imbalance and accumulation of xylitol. Consequently, *K. marxianus* wild type strains are not able to anaerobically grow on xylose (Rodrussamee et al., 2011).

Among the disaccharides commonly used by yeast are: sucrose, maltose, cellobiose and lactose. As previously mentioned, *K. marxianus* is not able to metabolize maltose, while sucrose is digested extracellularly by a secreted enzyme with inulinase activity; the derived monosaccharides glucose and fructose enter the cell through separate transporters and follow distinct metabolic pathways (Figure 2). Cellobiose and lactose follow distinct metabolic routes, but *K. marxianus* does not present a functioning

cellobiose transporter. Therefore, its function was overtaken by Lac12, as outlined in the following section.

2.4 Sugar transporters

Yeasts' sugar metabolism is highly dependent on sugar uptake process mediated by various transporters belonging to the Major Facilitator Superfamily (MFS). In yeast like *S. cerevisiae*, glucose transport is carried out by the hexose transporter (HXT) family (Rintala et al., 2008). This family includes around 20 high and low affinity glucose transporters, though not all are glucose transporters. Apart from these, a few other sugar transporters are known in *S. cerevisiae*, and these include a galactose and a maltose transporter (Lagunas, 1993). Other yeast such as *Yarrowia lipolytica* present a second family of hexose transporters in addition to HXT, described as the HGT family (Lazar et al., 2017).

In *K. marxianus*, glucose transport is carried out by both *KHT*s transporters, belonging to the HXT family, and by HGTs transporters with various affinites (Varela et al., 2019a). KHTs and HGTs in *K. marxianus* are present in expanded clusters of tandem copies with the copy number varying across different strains (between 5 and 6 copies), pointing out that evolution at these loci is recent and ongoing. The differences in copy numbers between strains is due to recombination events and elimination of genes with redundant function in some species. The copy expansion of HGT and KHT represents a peculiarity of *K. marxianus*, given that the closest *K. lactis* harbours only two copies (*KHT1* and *KHT2)* of low affinity transporters, in some strains recombined in a single copy, *RAG1* (Weirich et al., 1997), and a single transporter (*HGT1*) for high affinity glucose uptake (Billard et al., 1996). In addition, the *Kluyveromyces* clade encodes for the transporter Fsy1, allowing for the assimilation of fructose (Galeote et al., 2010).
The expansion of KHTs and HGTs, and the fact that this yeast can be mainly isolated from plants, suggests an adaptive mechanisms consisting of accessing the lignocellulosic biomass through secretion of enzymes such as inulases and pectinases, with release of a variety of monomers and disaccharides, and a concomitant expression of a pool of transporters to carry the released sugars inside the cell to support its growth. Accordingly, KHTs and HGTs transporters in *K. marxianus* are reported to have a broad specificity for both hexose and pentose sugars deriving from plant

sources (Donzella et al., 2021; Varela et al., 2019a). In fact, one copy of the HGT family, *HGT1* (also known as *KmAXT1*) is described as a high affinity glucose, xylose and arabinose and low affinity galactose transporter (Knoshaug et al., 2015; Varela et al., 2019b , Donzella et al., 2021). Another member of the HGT family (KMAR_10531) has similar capability. Finally, two other members of the HGT family are capable of xylose and arabinose import with a low affinity, and five of the KHTs are able to perform low affinity xylose transport (Donzella et al., 2021).

A unique feature of *K. marxianus* is the ability of metabolise the disaccharide lactose (Gethins et al, 2016; Morrissey et al., 2015). This peculiarity of the *Kluyveromyces* genus is likely the result of selective pressures imposed by human domestication (Varela et al., 2019b). Lactose utilization is enabled by a lactose permease, *LAC12*, and the *LAC4* gene, encoding for a β-galactosidase for the hydrolysis of lactose into glucose and galactose. *LAC12* underwent a copy number expansion in *K. marxianus*. Of these, only the ancestral copy encodes a functional lactose transporter, while the other copies are able to transport alternative carbon sources such as galactose (Varela et al., 2017) and cellobiose (Varela et al., 2019b). Cellobiose is transported by most *Kluyveromyces* species by the specific cellobiose transporter Cel1 but this has been lost in *K. marxianus* over the course of evolution (Varela et al. 2019b).

3. Genetics and genomics of *K. marxianus*:

3.1 Genome structure

K. marxianus is a homothallic yeast. As such, it is capable of switching mating type and self-mate, in response to adverse environmental conditions (Yarimizu et al. 2013).

As is typical for budding yeast that have not undergone the WGD, *K. marxianus*' genome is ~10.9 Mb in size and comprises 8 chromosomes (Lertwattanasakul et al., 2015). There is quite a high degree of nucleotide sequence divergence, up to 3%, as well as variation in ploidy and aneuploidy between strains (Ortiz-Merino et al., 2018).

To date, 17 *K. marxianus* genomes have been published, with only 3 strains (DMKU-1042, FIM1 and NBRC 1777) presenting a fully sequenced, annotated and assembled genome on a chromosomal level (Inokuma et al., 2015; Lertwattanasakul et al. 2015; Mo et al., 2019). The estimated the number of protein-coding genes in *K. marxianus* is in the range of 4952-5081, lower than the ~6000 in *S. cerevisiae*. Fenton et al.,

(2022) employed a combination of transcriptomics techniques and ribosome profiling for genome annotation, resulting in an updated and more accurate genome annotation of DMKU3-1042 strain. This includes 171 previously unannotated genes. These sequences and the improved annotation are now available for *K. marxianus* on the GWIPS-viz genome browser (www.riboseq.org).

3.2 Mating type and life cycle

K. marxianus is a homothallic species whereby cells can self-mate and form diploids which then undergo meiosis to produce haploid spores. The spores germinate to produce vegetative cells of mating type *MAT***a** or *MAT*α. *K. marxianus* possess three mating-type (*MAT*) loci: one expressed *MAT* locus and two silent (cryptic) loci: *HML* (Hidden MAT Left) and *HMR* (Hidden MAT Right). The silenced loci provide the templates to switch the active locus between the *MAT**a*** and the *MAT*α forms, thereby also switching the cells between the **a** and α type. In *Kluyveromyces HMR***a** is located on a different chromosome to *MAT* and *HML*α. The reversible and programmed DNA-rearrangement process which allows mating type switch occurs thanks to dsDNA break to the *MAT* locus, induced by endonucleases. Unlike *S. cerevisiae*, where a single endonuclease initiates the switch between *MAT***a** and *MAT*α, in *Kluyveromyces* two domesticated transposases, α3 and Kat1, are required. The *Kluyveromyces* hobo/Activator/Tam3 (hAT) transposase 1 (Kat1) drives the switch from *MAT***a** to *MAT*α, and the α3 transposase of the mutator-like (MULE) family the switch from *MAT***α** to *MAT***a** **(Solieri et al., 2021)**. *K. marxianus'* life cycle is represented in Figure 3A.

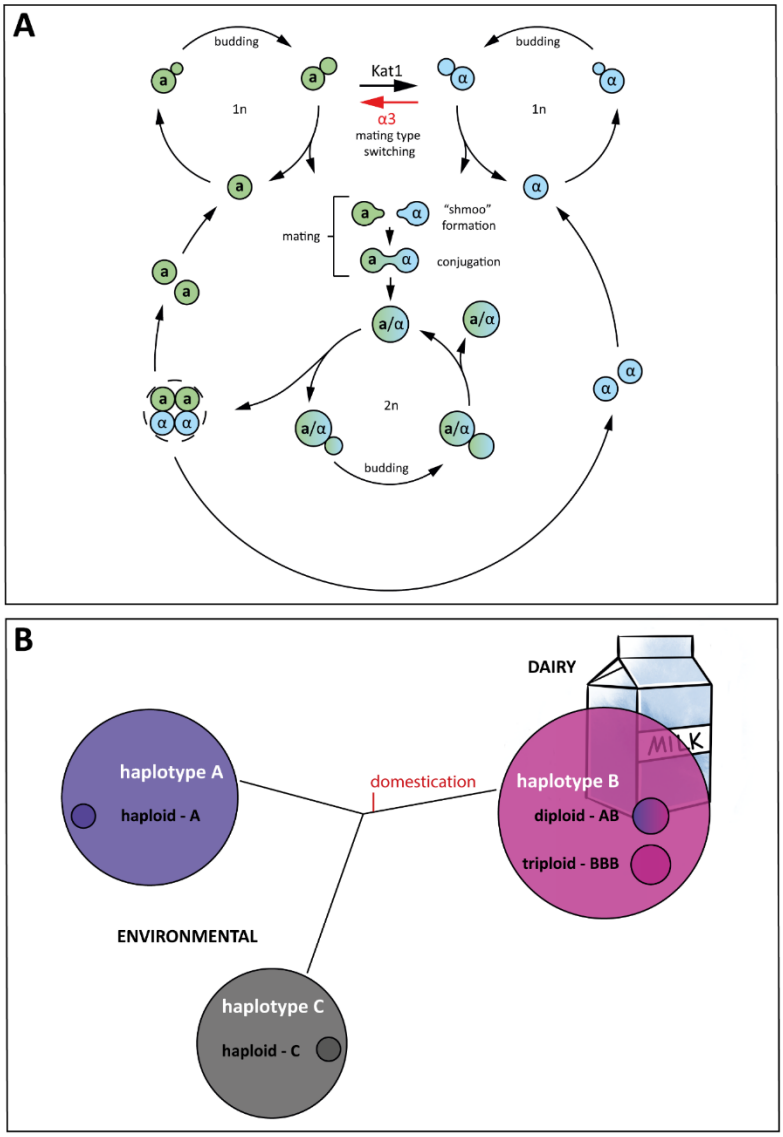

Figure 3: Life cycle and population structure of *K. marxianus*.

A: Transitions in the life cycle of *K. marxianus*. *K. marxianus* cells exist as one of two mating types - **a** or α. Mating type switching between the two can occur, whereas Kat1 catalyses the switch from *MAT***a** to *MAT*α and α3 the switch from *MAT*α to *MAT***a.** This schematic shows the transitions in ploidy that occur in the life cycle of *K. marxianus*: Haploid **a** or α cells can mate to form a diploid (**a**/ α), which can undergo meiosis, producing haploid spores that germinate under favourable conditions to become vegetative, haploid cells of either mating type again (closing the life cycle). All three cell types of *K. marxianus* (a, α , and a/ α) are capable of undergoing mitotic cell division (budding). **B:** Simplified phylogenetic tree of *K. marxianus* haplotypes. The tree shows the division of *K. marxianus* strains based on the haplotype, which reflects the isolation environment of the strain. Purple background indicates environmental strains, haploid and belonging to the A-haplotype; grey backgrounds indicates environmental strains, haploid and belonging to the C-haplotype; pink background indicates diploid (of AB haplotype) or triploid (of BBB haplotype), all dairy strains. Interestingly, no haploid B-haplotype strains are known so far. The tree is based on Ortiz-Merino *et al.*, 2018.

3.3 The dairy haplotype

In order to optimize the best *K. marxianus* industrial strain, it is interesting to know whether the yeast's ploidy is dependent on domestication and, if so, if a link exist between the ploidy of the strains and their industrial suitability. So far, a single genetic trait has been associated with a specific sub-populations in *K. marxianus* – the ability of efficient uptake and assimilation of lactose (Ortiz-Merino et al., 2018; Varela et al., 2017; Varela et al., 2019b; Varela et al., 2019a). Dairy strains of *K. marxianus* include a dairy-specific haplotype – haplotype B, which could be an indicator of domestication. Whereas haplotype A and C are found in "natural" non-dairy isolates (Ortiz-Merino et al., 2018). Dairy strains also frequently show aneuploidy and contain long genomic tracts exhibiting loss of heterozygosity (LOH) and they only exist in diploid (AB genotype) or triploid (BBB genotype) form (Figure 3). It is thought that the dairy AB diploids have formed by mating of a haploid A and B, but no haploid B haplotype strains could be found to date (Ortiz-Merino et al., 2018). The mechanism of domestication appears to have selected a particular haplotype (the B haplotype) that either mated with strains of the A haplotype to form allodiploids, or with itself, forming autotriploids. Furthermore, the dairy strains, with the AB-haplotype can be considered intra-species hybrids, as they are similar enough to mate but sporulation seems to be hindered. A representation of *K. marxianus* haplotypes and their relation with the isolation environment is presented in Figure 3B.

4. Physiology and stress response

K. marxianus' metabolism can be described as respiratory-fermentative, meaning energy is produced by the TCA cycle following oxidative phosphorylation, as well as by the fermentation of sugars into ethanol (Hagman et al., 2014; Merico et al., 2007). *K. marxianus* is classified as Crabtree-negative and does not generally produce ethanol during aerobic growth (Fonseca et al., 2008; Lane et al., 2011). In contrast, increased aeration stimulates the TCA cycle, ensuring an efficient electron flux without the production of ethanol (Sakihama et al., 2019). *K. marxianus* cannot grow under strictly anaerobic conditions, and the ethanol formation is linked to oxygen limitation (Fonseca et al., 2008). The absence of a mechanism of sterol uptake was pinpointed as responsible for *K. marxianus* oxygen requirement. Introduction of heterologous squalene-tetrahymanol cyclase, an enzyme allowing a sterol surrogate synthesis, restored *K. marxianus* growth at high temperature under anaerobic condition (Dekker

et al., 2021). *K. marxianus* is notable for a high growth rate, doubling almost twice as fast as *S. cerevisiae* under optimum conditions (Fonseca et al., 2008). A genomic and transcriptomic study suggested that the upregulation of genes involved in respiratory chain, ATP and glucose transport during the fastest phase of growth, has a role in its fast growth rate (Yu et al., 2021). Recent genome-scale metabolic models (GEMs), which can be used to predict the yeast's carbon source utilization and growth rates under different growth conditions (Domenzain et al., 2021; Marcišauskas et al., 2019), are a valuable resource to study metabolism of *K. marxianus*. These models represent a complete source of *K. marxianus* metabolic reactions under specific growth conditions, and are useful resources for rational strain engineering.

Among the traits making *K. marxianus* a promising candidate for biotechnology applications are its robustness to a wide variety of stresses, such as elevated temperatures, salt and cell wall stress (Lane et al. 2009). Since robustness is an important requirement for a production strain, understanding the basis of a strain's response to stress is fundamental to improve it. Listed below are the main studies exploring the yeast's physiological response to a series of stresses traditionally encountered during industrial fermentations.

4.1 Ethanol tolerance

K. marxianus can only tolerate low concentrations of ethanol, up to 6%. The transcriptional response of *K. marxianus* to moderate amounts of ethanol was found to cause a reduction of flux through the main metabolic pathways, downregulation of ribosome biogenesis and upregulation of heat shock protein genes. Moreover, unsaturated fatty acid in the cell membrane and ergosterol biosynthesis were decreased. The fact that free fatty acid and ergosterol biosynthesis under high ethanol stress remained unchanged compared to unstressed cells, indicates that cell membrane composition could affect *K. marxianus'* ethanol tolerance (Hermano Santos Diniz et al., 2017). Oxidative stress and ROS accumulation were also identified as the main causes of a growth arrest in a *K. marxianus* during ethanol fermentation at high temperature (Fu et al., 2019), indicating this too could be a contributing factor to *K. marxianus*' low ethanol tolerance.

4.2 Thermotolerance

One of the most interesting traits of *K. marxianus* is its thermotolerance, with strains being able to grow under temperature as high as 50°C (Banat et al. 1992; Lertwattanasakul et al. 2011). The ability to grow at higher temperature is essential for industrial fermentations such as bioethanol production, where a 5°C only increase of the fermentation temperature results in lower production costs (Abdel-Banat et al. 2009). Several omic-studies have focused on understanding the basis of *K. marxianus* robustness to high temperature, but the roots of this complex phenomenon remain unclear. Heat stress usually results in the accumulation of intracellular reactive oxygen species (ROS) and redox imbalance. Accordingly, transcriptomic and metabolomics analysis of *K. marxianus* growing at high temperature showed ROS accumulation and a lower $NADH/NAD^+$ ratio. A drastic remodelling of the central carbon metabolism has also been observed, probably for ROS scavenging purposes. In particular, downregulation of glycolytic and TCA enzymes and consequential pyruvate accumulation have been measured (Fu et al. 2019; Lertwattanasakul et al. 2011; Wang et al. 2018). The glycolytic enzyme Glucose-6-phosphate 1-dehydrogenase (ZWF), which is the first step of the pentose phosphate pathway, is upregulated at high temperatures, possibly to cope with ROS accumulation by increasing NADPH availability (Fu et al., 2019). In the same study, various genes belonging to the major facilitator superfamily were found to be upregulated, including iron and some of the glucose transporters. *K. marxianus* aldehyde dehydrogenase was also upregulated at high temperature, as acetic acid production is a common way yeasts use to regulate intracellular reducing equivalent such as NADPH for ROS scavenging (Fu et al., 2019). Further responses to high temperature stress observed in *K. marxianus* are upregulation of genes involved in cellular DNA repair mechanisms, protein degradation and mitochondrial respiratory chain (Fu et al., 2019; Lertwattanasakul et al., 2011).

4.3 ROS detoxification

Stresses such as osmotic and oxidative stress, commonly caused by non-optimal conditions during industrial fermentations, trigger a conserved physiological response initiated by ROS accumulation. In *K. marxianus* the mitogen-activated protein kinase (MAPK) Hog1 is involved in both osmotic and oxidative stress response (Qian et al.,

2011). Saini et al. (2017) reported that *K. marxianus* is tolerant to oxidative stress inducer H_2O_2 up to 50 μM. The robustness of *K. marxianus* to oxidative stress is thought to be due to the accumulation of glutathione (GSH) which acts as a scavenger for ROS. Various growth-inhibiting compounds released during the simultaneous saccharification and fermentation (SSF) process from cellulose biomasses were also reported to cause ROS accumulation in *K. marxianus*. In this case, the response involved differential expression of genes involved in maintaining the redox balance, NAD(P)+/NAD(P)H homeostasis or NAD+ synthesis, energy production, and iron transport or metabolism (Wang et al., 2018a).

5. Genome engineering of *K. marxianus*

Genome modification involving gene knockout, integration or point mutation, are essential ways to understand biological functions and to engineer a yeast into a cost-efficient cell factory (Yang & Blenner, 2020). Traditional genome engineering strategies involved random integration of an expression cassette or the use of episomal plasmids (Abdel-Banat et al., 2009; Antunes et al., 2000; Chen et al., 1989; Fonseca et al., 2008; Iborra, 1993; Meilhoc et al., 1990; Nonklang et al., 2008), but were limited in terms of possible modifications by the number of markers available. The advent of double stranded breakage (DSB) technologies such as transcription activator-like effector nucleases (TALENs) (Sun & Zhao, 2013), zinc finger nucleases (ZFN) (Carroll, 2011) and clustered regularly interspaced short palindromic repeats (CRISPR) (Stovicek et al., 2017) made it possible to achieve marker free gene disruption. Of these methods, the implementation of the CRISPR system in *K. marxianus* has achieved most use (Cernak et al., 2018; Juergens et al., 2018; Lee et al., 2018; Löbs et al., 2017).

5.1. Genome engineering of *K. marxianus*

Genome engineering tools take advantage of the two eukaryotic repair mechanisms of DNA double strand breaks (DSB): non-homologous end joining (NHEJ) and homology dependent repair (HDR) (Shrivastav et al., 2008). The preferred DSB repair mechanism in *Kluyveromyces spp* is NHEJ, which results in random integration of

heterologous DNA into the genome (Kooistra et al., 2004). Although NHEJ was initially exploited to perform random and targeted gene integration in *K. marxianus* (Abdel-Banat et al., 2009a), to perform promoter characterization based on fluorescent reporter (Suzuki et al., 2015), auxotrophic mutations identification (Yarimizu et al., 2013) and marker-based in vivo recombination (Hoshida et al., 2014), HDR is preferable when engineering a strain, because it allows for precise integration. For this reason, in order to increase *K. marxianus* HDR efficiency, *KU80* (Choo et al., 2014), *KU70* (Abdel-Banat et al., 2009; Chen et al., 2013) and *DNL4* (Rajkumar et al., 2019) genes, which are integral parts of the NHEJ mediated DSB repair mechanism, were deleted in *K. marxianus*, disabling the NHEJ mechanism and increasing the targeted integration efficiency. The advent of CRISPR technology further facilitated fine and efficient strain modification (Cai et al., 2019). The technology has a number of applications in *K. marxianus*'s genome editing. In this context, (Löbs et al., 2017) designed a novel RNA-polymerase III promoter for single guide RNA (sgRNA) expression, which enabled the disruption of genes for the characterization of ethanol biosynthesis in *K. marxianus*. Genome editing to perform targeted gene disruption has also been used to inactivate mating-type switch in *K. marxianus* (Cernak et al., 2018). Juergens et al., (2018) developed a Cas9 expressing plasmid utilizing ribozymes for gRNA expression in non-saccharomyces yeasts including *K. marxianus*, with a gene disruption efficiency of up to 100%.

Other than gene inactivation, a requirement for strain engineering is gene integration. Since marker availability is a limiting factor to perform targeted integration, a combination of base editing technology (target-AID) and CRISPR-Cas9 genome editing, allowed to first disrupt NHEJ and subsequently perform markerless integration using short homology flanking regions (Nambu-Nishida et al., 2017).

The provision of Cas9 endonuclease within the cell is also key in developing a functional CRISPR tool. This is mostly achieved in *K. marxianus* under the control of constitutive promoters from a plasmid, but genomic integration under the control of galactose-inducible *LAC4* promoter for genome editing by simply transforming gRNA cassettes has also been adopted (Lee et al., 2018). A Cas9-gRNA co-expression plasmid for molecular cloning of targeting sequences has also been described. This uses the Golden Gate assembly system for gene knock-down and knock-out in *K. marxianus* (Rajkumar and Morrissey, 2021). To that effect, a collection of biological

parts based on the MoClo/Yeast Tool Kit standard for rapid assembly of molecular modification constructs was previously made available (Rajkumar et al., 2019).

Finally, the implementation of the CRISPR tools in *K. marxianus* increased the strain's potential as a synthetic biology host and its industrial applicability via deletion of small number of genes (Hua et al., 2019; Löbs et al., 2017; Nonklang et al., 2008), or multiple deletion events (Donzella et al., 2021).

6. Biotechnological applications of *K. marxianus*

K. marxianus' exploitation in the biotechnology field has been studied for over 30 years (Dellomonaco et al., 2007; Duan et al., 2019; Gombert et al., 2016; Medeiros et al., 2001; Struyf et al., 2018). The yeast is a traditional food microbe and a natural component of many fermented dairy products such as cheese and kefir (Coloretti et al., 2017; Gethins et al., 2016; Lachance, 2011). It is also traditionally involved in non-dairy processes (Rao et al., 2008) such as natural fermentation of Agave-based alcoholic beverages (e.g. tequila or mezcal), is part of the natural flora of *Agaves* (Lappe-Oliveras et al., 2008; Verdugo Valdez et al., 2011) and is present in natural cocoa fermentation for the production of chocolate (Ho et al., 2014). Its GRAS/QPS status allows for food application, and its peculiar metabolic traits indicate the yeast would be a good candidate for production of various biochemicals. The growing concern over consumption of fossil fuels for chemical synthesis, has fuelled the interest in developing microbial bioprocesses for the production of these compounds in a sustainable fashion (Li et al., 2020; Liu et al., 2021). Metabolic engineering of yeast aims to develop efficient microbial cell factories that can produce a wide variety of valuable compounds at the highest yield and from various feedstocks (Guirimand et al., 2020). Below are listed *K. marxianus*' main studied biotechnological applications to date.

6.1 Enzymes production:

Traditional biotechnological applications of *K. marxianus* involve its fermentation for enzymes production, especially naturally secreted ones. One such enzyme is inulinase, naturally secreted from *K. marxianus* due to its ability to grow on plant fructans. The

interest of food industry for inulinases motivates several studies investigating the production of the enzyme from sustainable carbon sources (Hoshida et al., 2018; Jain et al., 2012; Santharam et al., 2017). Other enzymes naturally produced by *K. marxianus* with industrial applications include β-galactosidase, for lactose content reduction from cheese whey (Alves et al., 2019; Srivastava et al., 2015), pectinases for fruit juices clarification (Piemolini-Barreto et al., 2015) and β-glucanase (Lopes et al., 2014).

6.2 Bioethanol production

K. marxianus has been intensively studied for bioethanol production. Its ability to metabolize lactose meant that the yeast could be used to produce ethanol from lactose-rich milk whey, by-product of the dairy industry otherwise representing an environmental pollutant (Diniz et al., 2014; Silveira et al., 2005; Zafar & Owais, 2006). Although not feasible under anaerobic conditions due to cofactor imbalance, *K. marxianus* can effectively transport and metabolize the pentose xylose and arabinose, principal components of cellulosic material and crop waste (Knoshaug et al. 2015). For these reasons, bioethanol production from diverse cellulosic substrates such as sugar cane juice, an abundant wild crop in Brazil (Limtong et al., 2007; Madeira-Jr & Gombert, 2018a), inulin rich Jerusalem artichoke (Yuan et al., 2008) and pre-treated wheat straw (Saini et al., 2015) has been explored.

Moreover, *K. marxianus* thermotolerance and wide substrate range utilization ability (Madeira-Jr & Gombert, 2018b; Moreno et al., 2013; Yanase et al., 2010) allow for its application in simultaneous saccharification and fermentation (SSF) processes (Sivarathnakumar et al., 2019; Sukhang et al., 2020; Suryawati et al., 2009). Also for use during SSF, successful attempt have been made to engineer *K. marxianus* expressing and secreting heterologous lignocellulolytic enzymes to release the sugar monomers to be then converted in ethanol (Zhou et al., 2018, Yanase et al., 2010, Chang et al., 2013). Finally, in an attempt to improve CO_2 utilization and redox balance when growing on pentose and hexose sugars, the heterologous enzyme ribulose-1,5- biphosphate carboxylase/oxygenase (RubisCO) from the bacterium *Rhodopseudomonas palustris* has been expressed in a *K. marxianus* strain (Ha-Tran et

al., 2021). The engineered strain could be used in a dual microbial system to produce bioethanol from waste biomass.

6.3 Aromatic compounds production:

K. marxianus naturally produces high amounts of higher alcohols (Morrissey et al., 2015). These aromatic compounds are used as additives for food and fragrances, and are synthesized in yeast via the Ehrlich pathway (Hazelwood et al., 2008). The aldehyde dehydrogenases and alcohol dehydrogenases involved in various steps of this pathway are highly regulated in *K. marxianus*, depending on carbon and nitrogen sources (Gao et al. 2015; Gethins et al. 2015). Among the aroma compounds of industrial interest produced from *K. marxianus*, is 2-Phenylethanol (2-PE), a rose-like smelling molecule with a big global market in the food, beverage and cosmetics industry. 2-PE is synthesized in *K. marxianus*, through the Ehrlich pathway, from L-Phenylalanine (Etschmann et al., 2002; Wittmann et al., 2002). In recent years, *K. marxianus* has been engineered to produce high levels of 2-PE (de Lima et al., 2020; Kim et al., 2014; Rajkumar & Morrissey, 2020), also from diverse substrates (Adame-Soto et al., 2019; Martínez et al., 2018; Rodríguez-Romero et al., 2020). Phenylpyruvate decarboxylase (*ARO10*) and alcohol dehydrogenase (*ADH2*) genes of *S. cerevisiae* have also been overexpressed in *K. marxianus* to overproduce 2-PE, otherwise chemically synthesized from benzene (Kim et al., 2014). A combination of seven heterologous genes from yeast and bacteria was engineered into a *K. marxianus* strain, for accumulation of hexanoic acid, a chemical precursor with application in perfumes, medicine and food industry (Cheon et al., 2014). The successful development of cell factories relies on modularising DNA parts that can be rapidly cloned together for establishing gene expression in the host organism. The availability of such tools can considerably reduce the time for strain construction. For this purpose, a rational engineering approach was implemented to redirect *K. marxianus'* aromatic amino acid pathway (AAA) towards overproduction of phenylalanine (Rajkumar & Morrissey, 2020). The strain represents a promising platform for production of aromatic amino acid-based chemicals.

7. Future perspectives for *K. marxianus* in the bio-economy sector

The discussed natural traits such as thermotolerance and broad substrate specificity, make *K. marxianus* a valuable host for industrial applications. One key goal of the goals of the emerging bio based economy is to depart from traditional oil-based production of chemicals, biotechnology and microbial cell factories have become fundamental to achieve this goal (Lange et al., 2021). A synthetic biology approach can help enhancing *K. marxianus* naturally advantageous traits and satisfy the bioeconomy's demand for new bio-based chemicals. Rational engineering approaches and the emergence of genome editing tools have already allowed for the design of strains for the production of valuable compounds (Cernak et al., 2018; Cheon et al., 2014; Rajkumar & Morrissey, 2020). However, to achieve substantial strain modifications in a time efficient fashion, tools such as Cpf1 which allows for simultaneous targeting of more than one locus for DSB in a highly efficient manner (Swiat et al., 2017), is attractive although still not available in *K. marxianus*. Evolutionary guided strain design approaches such as Adaptive Laboratory Evolution (ALE), which takes advantage of the process of natural selection to optimize desirable strain properties, represent a valuable alternative to rational strain engineering (da Silveira et al., 2020; Saini et al., 2017; Sharma et al., 2017). Finally, a transcriptomic study showed the importance of species-specific genes for stress adaptation, since evolutionary young genes were the most responsive to stress in various budding yeast species, including *K. marxianus* (Doughty et al., 2020). Therefore, in order to better understand the stress tolerance phenomenon and improve a strain's robustness to the process, multi-OMIC studies and complete genome annotations (Fenton et al., 2022b) can contribute to exploit *K. marxianus*' full biotechnological potential.

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Chapter III

Physiological comparison of *K. marxianus* strains grown under industry-relevant conditions in bioreactors

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This chapter has been written in preparation for submission to a microbial physiology journal.

NM performed the experiments, interpreted the data and wrote the manuscript

CBM helped performing the bioreactor fermentations

JM designed the study, provided supervision and edited the manuscript

1. Abstract

Microbial cell factories are often faced with suboptimal growth conditions during industrial bioprocesses, such as elevated temperatures, resulting in stress on the cell and reduced productivity. *K. marxianus* is a thermotolerant yeast with promising traits for biotechnological applications; therefore understanding the physiological and transcriptomic basis of its innate stress adaptation mechanisms allows for engineering of robust industrial strains. However, a wide physiological variability is known between *K. marxianus* strains, and the physiological studies available focus on different strains and conditions, not allowing for comparison of the data. In this study, a systematic physiological comparison during batch cultivation is provided for three strains: the haploids CBS 6556 and NBRC 1777 and the diploid CBS 397. The comparison of growth parameters of the strains during batch cultivations at 30 °C versus 40 °C revealed that, while NBRC 1777 showed the fastest growth rate at 30 °C, both CBS 6556 and CBS 397 showed an almost doubled growth rate at 40 °C compared to 30 °C, indicating higher temperatures are more suitable for some *K. marxianus* strains. Decreased biomass yield on substrate and high oxygen uptake rate of NBRC 1777 at 40 °C suggest that these parameters are related to slower growth at higher temperature. Since adaptation mechanisms are observed upon long-term exposure to stress, the haploid strain CBS 6556 was then employed to investigate *K. marxianus* adaptation to diverse stresses during chemostat cultivation, namely: high temperature, low pH and high osmolarity. The analysis of the transcriptional changes revealed that, while there is an overlap of biological processes involved in the response to all tested stresses, there are no genes upregulated under all three stress conditions; which suggests a stress-specific response. In particular, the study of the response to high temperature stress revealed that, while the sugar uptake rate is higher in CBS 6556 under both high temperature and osmotic stress, likely to fulfil a higher energy demand, the respiratory activity is downregulated in order to reduce ROS accumulation. The opposite is observed during low pH stress. This study, in addition to laying out comparable physiological parameters for three *K. marxianus* strains during batch cultivation, also provides insights on some of the mechanisms responsible for *K. marxianus* stress adaptation.

2. Introduction

The emerging bioeconomy sector relies on the development of robust microbial cell factories to efficiently carry out sustainable bioprocesses (Lange et al., 2021). Thermotolerance is an important trait in industrial settings as maintaining high temperatures during fermentation reduces contamination risk and cooling cost (Abdel-Banat *et al.*, 2010). It is particularly valuable during simultaneous saccharification and fermentation (SSF) as the optimal temperature for enzymes employed in the saccharification of biomasses is usually over 50 °C (Jugwanth et al., 2020). Some non-saccharomyces yeasts are interesting microbial cell factories candidates, because of their innate robust traits including thermotolerance, acid tolerance and wide substrate range (Patra et al., 2021). *K. marxianus*, therefore, is a naturally strong candidate for bioethanol production, given its close to optimal growth temperature of 45°C (Lane *et al.*, 2011; Rouwenhorst *et al.*, 1990; Fonseca *et al.*, 2008) Moreover, *K. marxianus* presents additional advantageous traits such as: fast growth, low pH tolerance and capacity to metabolise lactose and pentose sugars (Karim et al., 2020).

A number of physiological studies are available that compare *K. marxianus* strains under well-defined conditions (Bellaver *et al.*, 2004; Fonseca *et al.*, 2007; Signori *et al.*, 2014); however, there is a considerable variability among these studies with regards to strains and growth conditions utilized. Moreover, the resulting growth parameters such as maximum growth rate and biomass yield are strongly dependent on the cultivation conditions even when comparing the same strain obtained from different collections (e.g. CBS 6556/ATCC 26548 in Rocha, Abrahão-Neto and Gombert, 2014; Hoekstra *et al.*, 1994; Bellaver *et al.*, 2004; Fonseca *et al.*, 2007). Therefore, considering the known physiological and genotypic variability among *K. marxianus* strains (Lane *et al.*, 2011; Lehnen et al., 20219; Ortiz-Merino *et al.*, 2018), when studying particular strains there is a need for a preliminary comparison under similar conditions in order to establish the baseline physiological parameters to use as a reference.

While initial studies mostly focused on the effect of aerobic batch cultivations on Crabtree effect and ethanol yield in different *K. marxianus* strains (Fleming *et al.*, 1993; Bellaver *et al.*, 2004; Fonseca *et al.*, 2007), more recent physiological studies report OMIC analysis of *K. marxianus* strains to evaluate their response to industrially

relevant conditions such as xylose fermentation or ethanol fermentation in presence of various inhibitors (Fu *et al.*, 2019; Wilkins *et al.*, 2008; Signori *et al.*, 2014; Wang *et al.*, 2018). However, these responses are mainly recorded after transiently applied stress conditions and are therefore representative of sudden stress response more than stress adaptation. In order to better understand *K. marxianus'* natural thermotolerance, a complex trait that most likely results from multiple concurring mechanisms (Lertwattanasakul *et al., 2015*), it is therefore helpful to compare the changes happening at a genomic level with the physiological parameters measured during a systematic comparison under high temperature.

Since *K. marxianus* strains used for dairy fermentations were found to be predominantly diploid or triploid, while natural isolates are haploid (Ortiz-Merino et al., 2018), we compared two haploid and a diploid strain under different conditions. The strains chosen in this study for comparison are: NBRC 1777, commonly used for genome engineering in *K. marxianus* but never physiologically characterized (Donzella et al., 2021; Kim et al., 2015; Rajkumar & Morrissey, 2020; R. Wang et al., 2013), CBS 6556, previously studied from both the CBS and ATCC collections (Hoekstra *et al.*, 1994; Bellaver *et al.*, 2004; Fonseca *et al.*, 2007), and the diploid strain CBS 397, previously characterized during a physiological comparative study (Rocha, Abrahão-Neto and Gombert, 2011).

In the present study, the growth of the three *K. marxianus* strains was first compared in batch at 30 °C and 40 °C and the physiological parameters were obtained for each strain. Since thermotolerance, unlike heat-shock response, is observed upon long-term exposure to high temperature (Shui et al., 2015), the haploid strain CBS 6556 was selected for further analysis in chemostat fermentations. The physiological and transcriptional changes resulting from adaptation to various stresses during chemostat cultivation were therefore analysed. In particular, we focused on highlighting *K. marxianus'* high temperature adaptation strategies in comparison to the low pH and osmotic stresses also tested. The study underlines the value of chemostat cultivations to study complex mechanisms such as stress tolerance because, unlike batch cultivation where cell physiological state is constantly changing (Tai et al., 2007), it reproduces the long-term stress condition similar to what the yeast would be exposed to in a challenging environment (Doughty et al., 2020). Comparison of the

transcriptomic responses, supported by physiological data, allowed us to explore the mechanisms of high temperature adaptation in *K. marxianus*.

3. Materials and methods

Strains and cultivation

K. marxianus strain NBRC1777 was obtained from the Biological Resource Centre, NITE (NBRC; Tokyo Japan). CBS 6556 (ATCC 26548; NCYC 2597; NRRL Y-7571) and CBS 397 strains were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). In preparation for batch, chemostat or plate assay growth, an overnight culture was prepared by inoculating a glycerol stock vial of the strain in 50mL of mineral medium plus glucose (MM-Glu) in a 250mL Erlenmeyer flask. The overnight culture was incubated at 30 °C in a shaking incubator (330 RPM) until use the following morning. Mineral medium (Verduyn et al., 1992) was prepared as per (Fonseca et al., 2007), was adjusted to pH 5.5 with KOH 1M and contained 2% w/v D-glucose as carbon source. For the drop tests, overnight cultures were prepared as described above. Following overnight growth, cells were washed once and diluted to A_{600nm} of 1, in sterile water. 5 µL of serially diluted culture (until 10^{-5}) of each strain were spotted on plates containing MM-Glu, pH 5.5, MM-Glu, pH 5.5 plus 0.6 M,1M and 1.5M KCl to test for osmotic stress tolerance, MM-Glu with pH buffered at 3.5 and 2.5 with addition of 1M HCl to test for acidic stress tolerance. The plates were incubated in a 30°C static incubator for 24h. For high temperature growth tests, plates were incubated in a 40°C and 45°C static incubator for 24h

Batch fermentations and sampling

Batch cultivations were performed in 4 L Applikon bioreactors, and monitored using EZ-Controllers (Applikon, the Netherlands). A calculated volume of the overnight culture was added, to achieve an initial A600nm of 0.1 in the bioreactor. Batch cultivation conditions were 30 °C (standard condition) or 40 °C (high temperature condition), 2 L working volume with pH maintained at 5.5 through addition of 0.1M KOH. The culture was sparged with air at a flow rate of 2 L min^{-1}, corresponding to 1 litre of air sparged per unit litre of growth medium per minute (VVM), and agitated at variable revolution per minute (RPM) in order to maintain a dissolved oxygen concentration above 60% saturation.

After inoculation (T0), the bioreactors were sampled every hour for 12 hours. A final sample was taken after 24h to confirm end of growth. Since the strain CBS 6556

displayed slower growth under the tested conditions, samples were taken every hour for 18 hours. Approximately 15 mL of sample was withdrawn, to allow for biomass dry weight calculation, A_{600nm} measurement and HPLC analysis of residual sugar and extracellular metabolites.

Chemostat fermentations, steady-state and RNA-seq sampling

Chemostat fermentations and sampling were performed as described in (Fonseca et al., 2007). Briefly, continuous cultivations were preceded by a 24hours batch cultivation at a working volume of 1L, under the same temperature, pH and stirring conditions as the chemostat fermentations. After glucose had been exhausted, which was verified online by the rapid increase of the dissolved oxygen concentration and offline by measurement of residual glucose via HPLC, the cultivations were moved to continuous mode. Growth in steady state was carbon limited with a glucose concentration in the feeding medium of 7.4 g/L. Aeration was maintained at a flow rate of 2 L min^{-1}. The reactor volume was maintained using a level probe activated effluent pump. Dilution rate (D) was kept at 0.1. Steady state was verified as described below, after at least five volume exchanges had taken place following the switch to continuous mode. Two samples were withdrawn at 24 hour intervals and assessed for constant biomass concentration via measuring the dry cell weight, and absence of residual glucose via HPLC, both methods are described below. After the steady state was confirmed, a third sample representing steady state culture (approximately 70 hours after entering continuous mode), was withdrawn for analysis. Samples (11 mL each) were collected from the reactor outlet in centrifuge tubes in an ice-water bath; 100 µL was used for OD600nm measurement, after appropriate dilution; 900 µL was centrifuged at 4 °C at 16,000 g for 1 min and the supernatant was stored at -20C for HPLC determination of extracellular metabolites and residual glucose. The remaining 10 mL were used for biomass dry-cell weight (DCW) determination. An additional 10 mL steady state culture sample was taken in a 50 mL ice-filled Falcon tube and immediately stored at -80 °C for RNA-seq analysis. All chemostat experiments were carried out in quadruplicate.

Gas measurement and analytical methods

CO_2 and O_2 outlet gasses was measured in continuous mode using pre-calibrated GXA – gas analysers (GasX, Ltd, UK). For CO_2 sensor calibration, defined gas mixes

containing defined concentrations of carbon dioxide (5%), and nitrogen (100%) were used for a two-point calibration. For O_2 sensor calibration, defined gas mixes containing defined concentrations of oxygen (20%) and nitrogen (100%) were used. Inlet CO_2 and O_2 concentrations were estimated at 0.04% and 21% respectively. Due to an unforeseen incorrect configuration of the gas analysers, it was not possible to use the gas reading to calculate the carbon recovery of the chemostat fermentations. For batch cultivations, oxygen uptake rate (OUR) and CO_2 emission rate (CER) were calculated as in Fonseca et al., (2007) from oxygen and carbon dioxide content in the inlet and exhaust gases, respectively. Nitrogen was taken as inert gas in the balance equation (Heinzle & Dunn, 1991). The absorbance at 600 nm (A_{600nm}) was measured in a spectrophotometer from 1mL cell-suspension, diluted as required.

Extracellular metabolites and residual glucose during batch and chemostat cultivations were measured using high performance liquid chromatography (HPLC). 900 µL of obtained sample was centrifuged and 800 µL of supernatant was injected into a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H_2SO_4, was run at 55°C at a flow rate of 0.6 ml min^{-1}. For measurement of biomass dry cell weight from batch cultures, a 10mL sample was collected in a pre-weighed falcon tube. The culture was centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded and the pellet was incubated at 46 °C for 24h in a static incubator until dry. The dried pellet was weighted to calculate the dry cell weight (DCW). For dry cell weight measurement from a chemostat, a 10mL sample was filtered through pre-weighed nitrocellulose filters with a pore size of 0.45 µm. Filters were washed with 10 mL demineralized water, dried in a microwave oven (20 min at 350 W) and reweighed to calculate DCW.

Calculation of physiological parameters

The physiological parameters were calculated as in Fonseca et al., (2007). For batch cultivation data, the exponential growth phase (EGP) was identified as the linear regression on an ln (DCW) vs. time plot. The maximum specific growth rate (μ_{max}) was determined as the slope of this linear region. The biomass yield on substrate ($Y_{X/S}$, where X is the grams of biomass and S is the grams of substrate per litre measured

by HPLC) was determined as the slope of the line on an X versus S plot, only including points belonging to the EGP.

The specific rate of substrate consumption (μ_s) was calculated according to the following equation:

$$\mu_s = \mu_{max} / Y_{X/S}.$$

For chemostat cultivations, specific growth rate (μ), specific rate of substrate consumption (μ_s) and biomass yield on glucose ($Y_{X/S}$) were calculated as follows:

$$\mu = D;$$

$$\mu_s = (1/X)\ D\ (S_F\text{-}S);$$

$$Y_{X/S} = X/(\ S_F\text{-}S).$$

Where S_F = substrate concentration in the feeding medium (g L^{-1}); S = substrate concentration in the bioreactor (g L^{-1}); D = dilution rate (L min^{-1}).

RNA-seq procedure and analysis

The RNA-seq dataset utilized for analysis was produced in our study (Doughty et al., 2020). RNAseq preparation, mapping and DE analysis was performed as previously described (Doughty et al., 2020). In brief, cells were mechanically lysed and extraction was performed using an RNeasy Kit from Qiagen. Libraries were prepared using the TruSeq mRNA Stranded HT kit. Sequencing was carried out using an Illumina NextSeq 500 High Output Kit v2 (75 bases), with a minimum of 8 million paired- end reads per replicate. RNA-seq dataset generated can be accessed using SRA accession PRJNA531619 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA531619/]. RNAseq read mapping was performed after analysis in FASTQC. RNAseq mapping for differential expression was mapped with STAR v2.7.049 and reads were assigned with featureCounts v1.6.050.

Differential expression results were generated using limma v3.40.651 and edgeR v3.26.852 R packages and tidyverse v1.3.053 was employed for various data rearrangements. Scripts used can be found at the OrthOmics page https://github.com/SysBioChalmers/OrthOmics. Filtering was used to remove lowly expressed genes/proteins. Differential expression was defined by a significance cutoff of absolute log2FC > 1 and P-Value < 0.05 for a stress condition compared to control.

An integrated toolbox for analysis of RNAseq and proteomics data that incorporates GO terms annotation and gene orthology information (from OrthoFinder) has been generated in R programming language. The genes detected from RNA-seq were annotated with gene ontology (GO) terms (Biological process) using the Blast2GO software (Gö Tz et al., 2008). Gene set analysis and network-based plots of overlapping gene sets and their significance were generated with the Bioconductor R package Piano.

4. Results

Comparison of *K. marxianus* strains during batch cultivation under different temperatures.

Given the known physiological and genomic variability among *K. marxianus* strains, we wanted to perform a comparison of different strains from our collection (NBRC 1777, CBS 6556 and CBS 397) under the same conditions, in order to physiologically characterize their growth. First, a preliminary screening of the three strains on solid media under stress conditions was carried out (Figure 1). CBS 397 showed equivalent tolerance to low pH and osmotic stress, but reduced thermotolerance, compared to the haploid strain NBRC 1777; while the haploid strain CBS 6556 appeared to be the least robust among the tested strains.

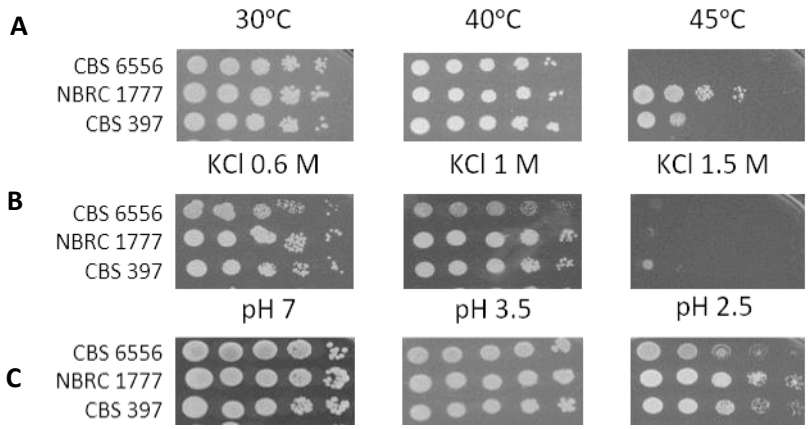

Figure 1 *K. marxianus* strains show diverse level of tolerance to several stresses. The *K. marxianus* strains were pre-grown in MM-Glu. Serial dilutions were plated onto agar plates with the appropriate stress, before being incubated at 30°C for 24 hours (other than high temperature plates, **a**). In each case increasing intensity of the stressor were tested as described in material and methods, and the figure shows the most relevant conditions. The *K. marxianus* strains were tested for high temperature (**a**), high osmolarity (**b**) and low pH growth (**c**).

All strains were then compared in batch under low (30°C) versus high temperature (40°C), in order to investigate the difference among the strains under these conditions. 40°C was chosen as high temperature for batch fermentations since it allows for growth of all the strains (Figure 1).The kinetics of cell growth, glucose consumption and accumulation of extracellular metabolites are reported in Figure 2. During the batch fermentation at 30 °C, NBRC 1777 presented the highest growth rate, with a μmax of 1.03 h^{-1} (DT=43 min), and the highest yield of biomass per gram of glucose

(0.55 gDW g^{-1}). Although decreased, NBRC 1777's growth rate is still high at 40 °C (0.82 h^{-1}). On the contrary, CBS 6556 strain, similarly to the diploid CBS 397, showed an approximately doubled growth rate at 40 °C (respectively 0.4 h^{-1} and 0.92 h^{-1}), compared to 30 °C (respectively 0.24 h^{-1} and 0.45 h^{-1}). The biomass yields on glucose at 30 °C (Table 1) are approximately the same between low and high temperature, with the exception of the NBRC 1777 strain that, concomitantly with the decreased growth rate, shows lower biomass yield at 40 °C.

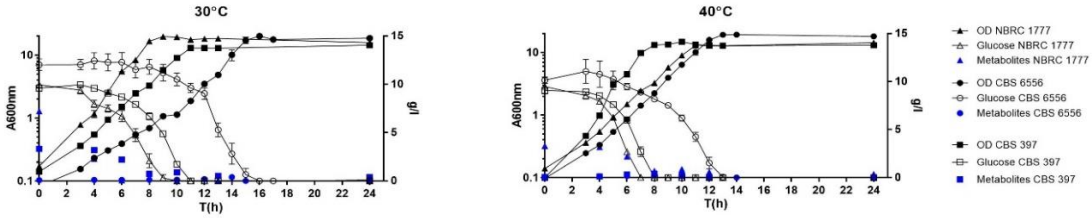

Figure 2 Comparison of aerobic batch growth of *K. marxianus* strains under different temperatures. *K. marxianus* strains were individually grown aerobically in batch under the temperatures indicated and sampled hourly for up to 24 hours. Resulting kinetics of growth, total metabolite formation and glucose consumption under low (30°C, **a**) and high temperature (40°C, **b**) conditions are plotted in the same graph for comparison.

The main physiological parameters obtained from the comparison are listed in Table 1. For CBS 6556 and CBS 397 strains glucose uptake rates (μs) are higher, but oxygen uptake rates (OUR) are lower at 40°C compared to 30°C, while NBRC 1777 shows a higher OUR under both temperatures (48.1 mmol h^{-1} and 58.8 mmol h^{-1}) compared to the other strains. High OUR in NBRC 1777, is coupled with a very high growth rate, but results in a decrease of biomass yield (Yx/s) at 40 °C (Table 1). Regarding the respiratory quotient (RQ), CBS 397 respiration rate demands exceeded the oxygen availability provided by constant aeration level of 1 VVM maintained across all the fermentations of this study and could not enable fully aerobic growth of the strain at 40 °C. This is indicated by the elevated RQ (2.79) measured under the mentioned condition, which suggests a respire-fermentative metabolism (Hagman, Sall and Piskur, 2014). For the haploid strains, the RQ close to 1 indicates fully respiratory metabolism at both temperatures.

The calculated carbon recovery ranged between 76 and 100%. CBS 6556 at 30 °C and NBRC 1777 at 40 °C, displayed the lowest carbon recovery (78% and 76 % respectively). In the case of CBS 397 strain growth at high temperature, an elevated

RQ was observed, accumulation of ethanol during fermentation was expected. However we only detected a negligible accumulation of ethanol and acetate (maximum of 0.39 g/l and 0.17 g/l respectively after 8 hours of fermentation) and no secreted pyruvate (Figure 2). Overall, the carbon recovery was lower at 40 °C compared to 30 °C for all strains, suggesting a loss of ethanol via evaporation (Agrawal, 2012), accentuated by the higher temperature.

Table 1 Physiological parameters from biorector cultivation of *K. marxianus* strains. The main growth parameters obtained in this study from K. marxianus strains grown in batch and chemostat mode under different conditions are listed in the table. In particular: specific growth rate (μmax), substrate consumption rate (μS), duplication time (DT), yield of biomass per gram of sugar (Yx/s), oxygen uptake rate (OUR), CO_2 emission rate (CER), respiratory quotient (RQ) and carbon recovery are reported, with relative units.

Condition	*μmax (h^{-1})*	*μS [g(gDWh^{-1})]*	*DT (h)*	*Yx/s (gDWg^{-1})*	*OUR (mmol h^{-1})*	*CER (mmol h^{-1})*	*RQ*	*carbon recovery (%)*
NBRC 1777 30C	1.03	1.83	0.71	0.55	48.1	40.7	0.85	100
CBS 397 30C	0.45	1.16	1.57	0.39	30	32.08	1.07	96.6
CBS 6656 30C	0.24	0.94	2.95	0.26	21.24	17.56	0.83	78.22
Batch cultivation 40C								
Nbrc 1777 40C	0.82	2.76	0.85	0.30	58.8	53.56	0.91	76
CBS 397 40C	0.92	2.43	0.75	0.38	2.85	7.95	2.79	82
CBS 6556 40C	0.4	1.48	1.73	0.27	7.12	6.44	0.90	85
Chemostat cultivation								
CBS 6556 Std	D = 0.1	0.29	6.93	0.34	-	-	-	-
CBS 6556 HiT	D = 0.1	0.35	6.93	0.28	-	-	-	-
CBS 6556 Osm	D = 0.1	0.29	6.93	0.35	-	-	-	-

CBS 6556 low pH	D = 0.1	0.27	6.93	0.37	-	-	-	-

Chemostat fermentation of CBS 6556 strain under different stress conditions.

Next, we wanted to assess the impact of different stressful growth conditions on *K. marxianus*. Therefore, the strain CBS 6556 was selected for chemostat fermentation, which reproduces long-term stressful conditions, and therefore allows study of the strain's adaptation response. Through maintenance of a constant growth rate, chemostat cultivation allows elimination of the growth stage-dependent variability of the response. The fermentation conditions of 40°C (for high temperature stress), pH 3.5 (for low pH stress) and KCl 1M (for osmotic stress) were selected for chemostat cultivation. Fermentation under standard conditions, consisting of 30 °C temperature and pH 5, was also run as control reference with no stress. The stress responses to the selected conditions are industrially-relevant as they reflect the suboptimal environment encountered during a industrial fermentations (Deprais et al, 2017). The fermentations under each condition were performed in triplicate. The chemostat dilution rate was set to 0.1, which represents a submaximal growth rate for *K. marxianus* under the selected conditions, in order to avoid wash-out (de Kock et al., 2000). Steady state growth was defined as less than 5% deviation in biomass dry weight over 72 hours of steady state growth conditions. In addition, other parameters, such as dissolved oxygen (dO_2) and percentage of CO_2 and O_2 outlet gasses were measured to be stable across the steady state culture (Figure 3). The main physiological parameters calculated from the chemostat fermentations are listed in Table 1. CBS 6556's biomass yield was lower under 40 °C compared to the standard condition (Table 1), but other stresses did not yield the same physiological effect. This suggests a specific physiological adjustment occurring in *K. marxianus* under high temperature condition, which may play a role in its innate thermotolerance.

Due to the incorrect configuration of the gas detectors, it was not possible to calculate respiratory quotient values or carbon balance for the chemostat fermentations. However a fully respirative metabolism can be assumed from previous batch fermentations, where CBS 6556 strain grew, with the same aeration level, at a faster growth rate whilst still maintaining an RQ close to 1. Close to 100% carbon recovery can be assumed from the fact that we did not observe accumulation of any metabolite in agreement with the batch fermentation.

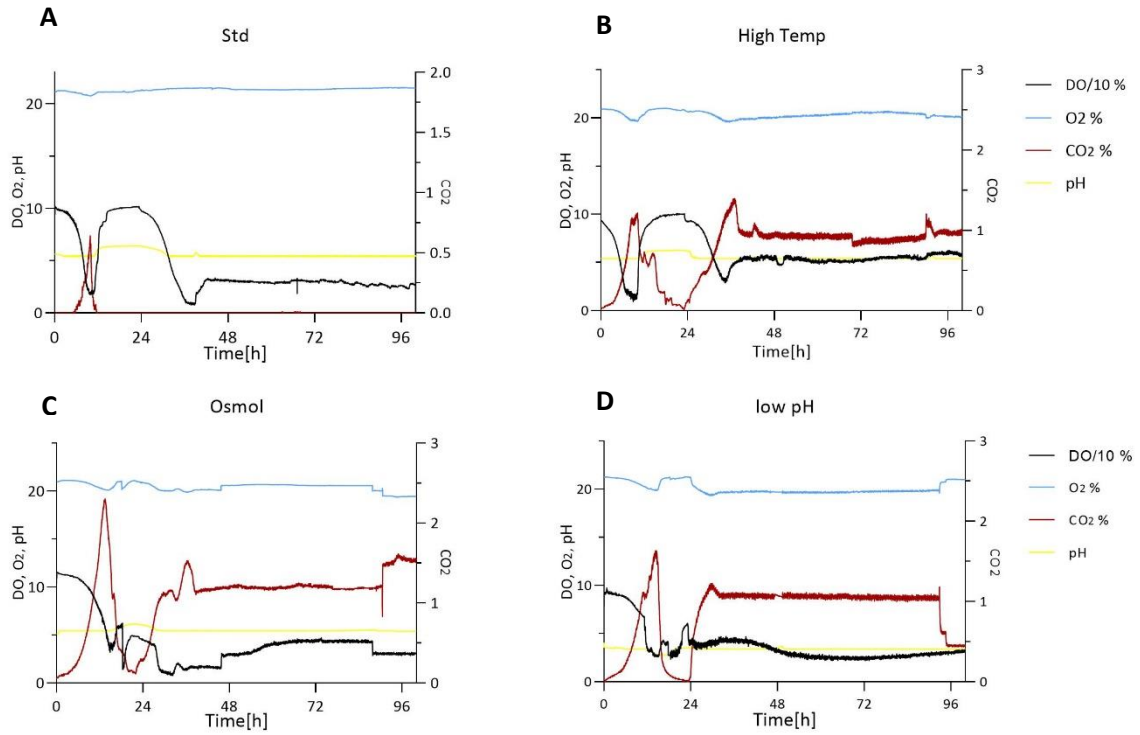

Figure 3 Growth profile of *K. marxianus* CBS 6556 strain chemostat culture under different stress conditions. The strain CBS 6556 was pregrown in MM-Glu in flask at 30C. Exponentially growing cells were inoculated in the fermenter under the indicated conditons (details listed in Table 1). After a first 24 hours phase of batch growth under each condition, wich allowed for full glucose consumption, the carbon-limited continous growth was mantained for 72 hours, to allow for 3 steady-state samplings, every 24 hours. The graphs report for each condition profiles of dissolved oxygen (DO/10%, red), off-gases composition in O2% (blue) and CO2% (black), and pH level (yellow), measured on-line with dedicated sensors. The dilution rate (D) was kept at 0.1 during the continous phase of growth.

K. marxianus' transcriptional response during adaptation to long-term stress is condition-specific

The transcriptomic response to each tested stress condition was compared to the standard condition and quantified via differential expression (DE) analysis. Gene-level statistics and DE values for each pairwise comparison are reported in Supplementary Table 1. For DE analysis purposes, we considered significantly regulated gene the ones with a -1> log2FC <1, and FDR<0.01. The number of genes found significantly up and downregulated under the three stress conditions tested is summarized in Table 3.

Table 2 Number of significantly DE genes under each stress condition. The table summarizes the number of DE genes when the trascriptome from cells growing under Standard condition was compared to the one under high temperature (Hit), osmotic strss (Osm) and Low pH conditions. Genes with a FRD <0.01 and -1>logFC >1 were considered differentially expressed.

Condition	*DE genes*	*Upregulated*	*Downregulated*
Hit	232	127	105
Osm	63	41	21
Low pH	191	61	130

Details of the pairwise DE analysis are reported in Figure 4. No gene was found significantly upregulated in all three stresses (Figure 4) while 3 genes were found significantly downregulated in all three conditions, and these are: KMXK_0C03250, KMXK_0H02920 and KMXK_0B01120, which upon BlastX analysis showed similarity for, respectively: Spo23p, a protein with unknown function associated with meiosis-specific Spo1p, KLTH0G19426p, an orthologous of an uncharacterized *K. lactis* protein with dimerization function and DNA-binding activity, and Ady2p acetate transporter. High temperature and low pH are the two stress conditions that show the higher number of common up and downregulated genes (14 and 35 respectively, Figure 4), and have the highest number of DE genes amongst the three stresses (Table 2), indicating that the osmotic stress condition is either triggered by a smaller number of genes compared to high temperature and osmotic stress, or it is not as challenging for strain growth. Overall, the low number of common DE genes amongst all three stress conditions, suggests that transcriptional rearrangements due to long-term stress adaptation are stress-specific.

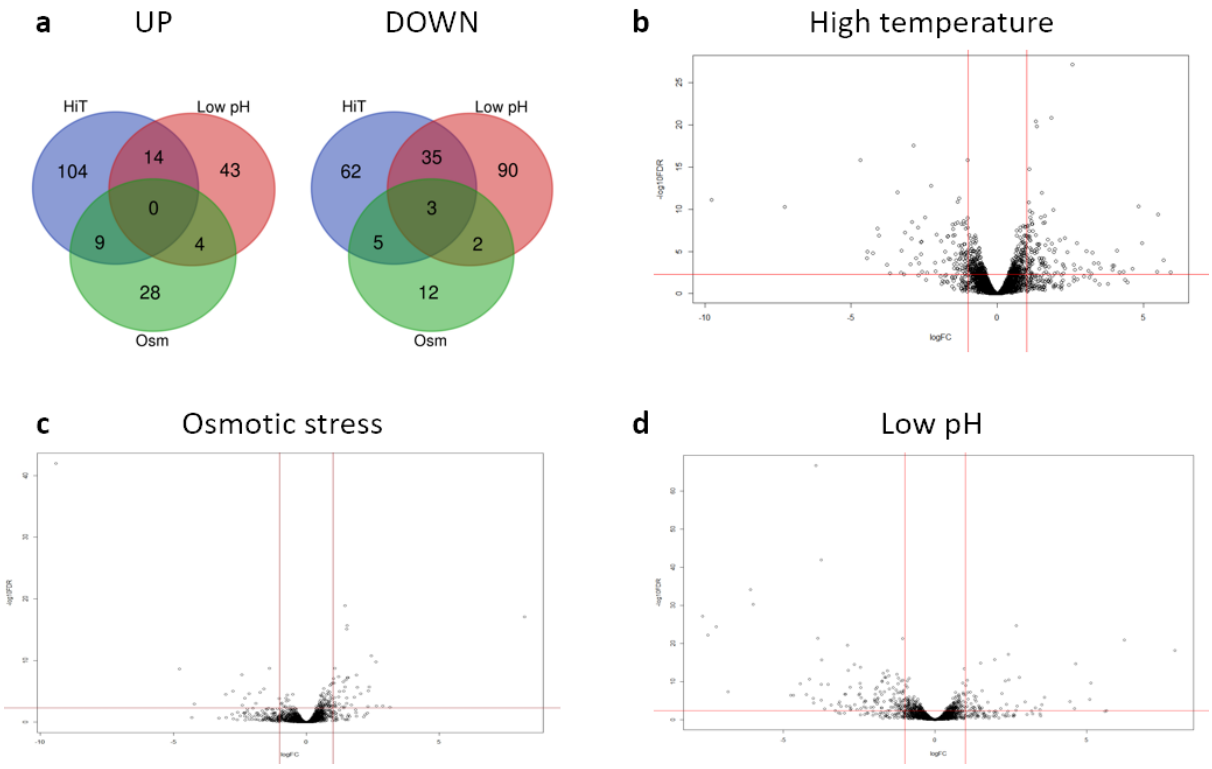

Figure 4 **DE analysis of *K. marxianus*' steady state growth under stress conditions**. a) Venn diagram showing common and not DE genes Up and downregulated among the three tested stress conditions. b,c and d) Volcano plots showing global level of DE of the genes under high temperature (b), osmotic (c) and low pH (d) stress. X-axis represents logFC, y-axes represents –logFDR.

GSA of chemostat growth under high temperature stress

In order to further understand the innate high temperature adaptation ability of *K. marxianus*, the transcriptomic response under the high temperature condition was studied by gene-set analysis (GSA). Biological process annotations for each CBS 6556 gene were inferred using BLAST2GO (Gö Tz et al., 2008); this process failed to annotate 20% of the genes measured by RNAseq because of lack of similarity of the protein sequences to the sequences on the Blast database. Result table of the GSA analysis is available as Supplementary Table 2.

Under high temperature stress, enriched upregulated biological processes included: ubiquitin dependent catabolic process (p value=0.003), cellular response to heat (p value=0.001), and response to stress (p-value=0.007). These last two are generic processes, and cluster together since they share common genes. They are linked to several upregulated other GO-terms, known to be involved in yeast temperature response (Figure 5A). These are: (1->6)-beta-D-glucan biosynthetic process, trehalose

biosynthetic process, protein refolding and de-novo protein folding (Table 3). These processes have been found upregulated in the yeast *S. cerevisiae* under high temperature stress before (Conlin & Nelson, 2007; Imazu & Sakurai, 2005; Leach et al., 2012; Lesage & Bussey, 2006; Magalhães et al., 2018; Mühlhofer et al., 2019). Among the significantly upregulated gene is *UTH1*, encoding for a mitochondrial protein known to have pleiotropic effects including cell wall biogenesis and mitochondrial stability (Ritch *et al.*, 2010), indicating its role in different stress-tolerance mechanisms. A group of genes known to be under the control of STRE elements are found highly upregulated in *S. cerevisiae*; these are: *SSA3* and *HSP26* (both heat shock response related genes, known to code for chaperone proteins (Lee *et al.*, 2001; Amoros et al., 2001)), *PGM2* and *ALD6*. The significantly upregulated genes for each mentioned GO-term are listed in Table 3. Notably, autophagy of mitochondrion was found to be significantly upregulated under the high temperature condition (p value=0.0017), while ATP synthesis-coupled proton transport (p value=$1.00E^{-04}$) and mitochondrial electron transport (p value=$1.00E^{-04}$) processes were found to be downregulated (Supplementary Table 2), suggesting a substantial rearrangement of the energetic status of *K. marxianus* under high temperature. The upregulated genes involved in autophagy of mitochondria are the homologues of the genes: *ATG3* (p value=7.8E-06), *ATG20* (p value=5.17E-08) and *UTH1* (p value=4.36E-11), each known to be involved in yeast mitophagy (Kanki et al., 2011). This indicates that mitochondrial abundance, as well as a change in activity under high temperature.

Among the processes downregulated at high temperature, we find translation (Figure 5B), including 89 genes belonging to the cytoplasmic translation GO-term (p value=$1.00E^{-04}$, Supplementary table 2). In addition, many chaperone-coding genes are upregulated to aid protein folding, including: *HSC82, ROT1, MDJ1, YDJ1 HSP60, HSP42* and *HSP10, SSA3*. Biosynthesis of amino acids lysine, cysteine, methionine and isoleucine is also downregulated. Downregulation of translation, together with the many chaperone genes found to be upregulated, indicates a considerable protein turnover at high temperature, probably to counteract the toxic accumulation of unfolded protein in the cell (Mühlhofer et al., 2019). Moreover, during steady-state cultivation of *S. cerevisiae*, a reduction in biomass protein content has been measured in concomitance with increasing temperatures (Lip et al., 2020). Therefore, we can

assume that the downregulation of some amino acid biosynthetic pathways, is the result of this rearrangement in biomass composition. Finally, the chromatin organization and histone acetylation processes, were found to be downregulated in this study (p-value=0.0003). Accordingly, chromatin remodelling and histone deacetylation allows for heat stress gene expression under stress (Alejandro-Osorio et al., 2009; Morano et al., 2012).

Table 3 Gene Sets enriched (p-value<0.01) in upregulated genes under high temperature stress growth in chemostat. Fold changes of comparative expression (in form of log2 FC) including gene-level p-values of differentially expressed genes for each functional category is provided. Only significantly upregulated genes are presented in the table (log2FC>0.5, gene-level p-value<0.05).

Biological process	Enrichment (p-value)	Genes*	Gene ID	Functional annotation	Gene-level p-value	Log2FC
Protein refolding	0.0005	12	KMXK0B02970	*HSP60*	7.920E-03	0.55
			KMXK0B07830	*MDJ1*	7.597E-04	0.59
			KMXK0E03480	*HSC82*	1.409E-04	0.67
			KMXK0F00530	*CPR6*	2.982E-13	1.12
			KMXK0F04730	*HSP78*	5.892E-06	1.17
			KMXK0G00960	*HSP10*	2.900E-04	0.61
Negative regulation of gluconeogenesis	1.00E-04	6	KMXK0C06920	*VID30*	1.275E-04	0.50
			KMXK0G02940	*VID24*	3.089E-05	0.68
Trehalose biosynthetic process	0.0002	5	KMXK0B00330	*TPS2*	1.013E-02	0.88
			KMXK0B00860	*TSL1*	8.229E-04	0.77
			KMXK0C01060	*PGM2*	1.897E-05	2.23
'de novo' protein folding	0.0006	5	KMXK0B02970	*SCS3*	7.920E-03	0.55
			KMXK0B04330	*ROT1*	1.060E-04	0.64
			KMXK0B07830	*MDJ1*	7.597E-04	0.59
			KMXK0E03480	*HSC82*	1.409E-04	0.67
(1->6)-beta-D-glucan biosynthetic process	0.0008	5	KMXK0A04840	*KRE9*	1.108E-08	1.71
Iron ion transmembrane transport	0.0008	5	KMXK_0C06160	*FTR1*	0.015	0.65
			KMXK_0E06210	*FET4*	0.049	2.1
			KMXK_0F03820	*FET3*	0.0091	0.74
Ubiquitin dependednt ERAD pathway	0.0011	28	KMXK_0F05730	*HUL5*	2.99E-05	0.70
			KMXK_0G03540	*SHP1*	4.28E-06	0.51
Proteasome-mediated ubiquitin-dependent protein catabolic process	0.0012	30	KMXK_0C06920	*NA*	1.77E-04	0.50
			KMXK_0D06320	*Ubx domain cont*	2.04E-04	0.55
			KMXK_0G02940	*VID24*	3.56E-05	0.70
Ubiquitin dependent	0.0012	30	KMXK_0A05550	*UBP8*	7.90E-05	0.62

catabolic process			KMXK_0A00310	*NPL4*	8.70E-05	0.54
			KMXK_0E00600	*UBX7*	2.35E-06	0.57
Autophagy of mitochondrion	0.0017	13	KMXK_0F01090	*ATG3*	7.80E-06	0.64
			KMXK_0F05620	*UTH1*	4.36E-11	1.17
			KMXK_0G01020	*ATG20*	5.17E-08	0.72
			KMXK_0A04590	*STL1*	2.10E-07	0.86
Nicotinate nucleotide salvage	0.0041	5	KMXK_0D02920	NA	4.74E-07	0.84
cAMP-mediated signalling	0.0047	5	KMXK_0A02830	*RHB1*	8.56E-07	0.75
			KMXK_0C02570	*STG1*	1.24E-08	0.86
mRNA catabolic process	0.005	5	KMXK_0A02030	*CNB1*	7E-04	0.55
			KMXK_0H03260	*SLT2*	7.5E-04	0.59
Biotin biosynthetic process	0.006	7	KMXK_0A05070	*BIO2*	2.66E-11	1.56
			KMXK_0E06300	*BIO3*	8.80E-04	1.30
Protein stabilization	0.007	7	KMXK_0B02970	*HSP60*	8.06E-03	0.56
			KMXK_0D04330	yer078c-like	1.8E-04	0.50
			KMXK_0E05300	*CDC37*	3.65E-04	0.58
			KMXK_0F04730	*HSP78*	3.5E-08	1.63
Protein targeting to vacuole	0.007	31	KMXK_0B05460	*SRN2*	5.80E-03	0.52
			KMXK_0G02940	*VID24*	3.65E-05	0.71
Response to stress	0.0079	32	KMXK_0A06710	mannosyltransferase	1.87E-08	0.82
			KMXK_0B02970	*HSP60*	0.00806	0.56
			KMXK_0E05410	*HSP42*	3.44E-08	1.41
			KMXK_0F00610	*PUG1*	3.21E-09	0.78
			KMXK_0F03300	*UTH1*	4.36E-11	1.17
			KMXK_0F05620	*HSP10*	0.000342	0.62
			KMXK_0G00960	*SSA3*	4.35E-12	1.47
			KMXK_0G03730			
Positive regulation of ATPase activity	0.0072	5	KMXK_0D00250	*ESF2*	0.000706	0.54
			KMXK_0E01320	*AHA1*	0.000366	0.84
Catabolic process	0.009	3	KMXK_0B07250	*ATG14*	1.43E-04	0.93

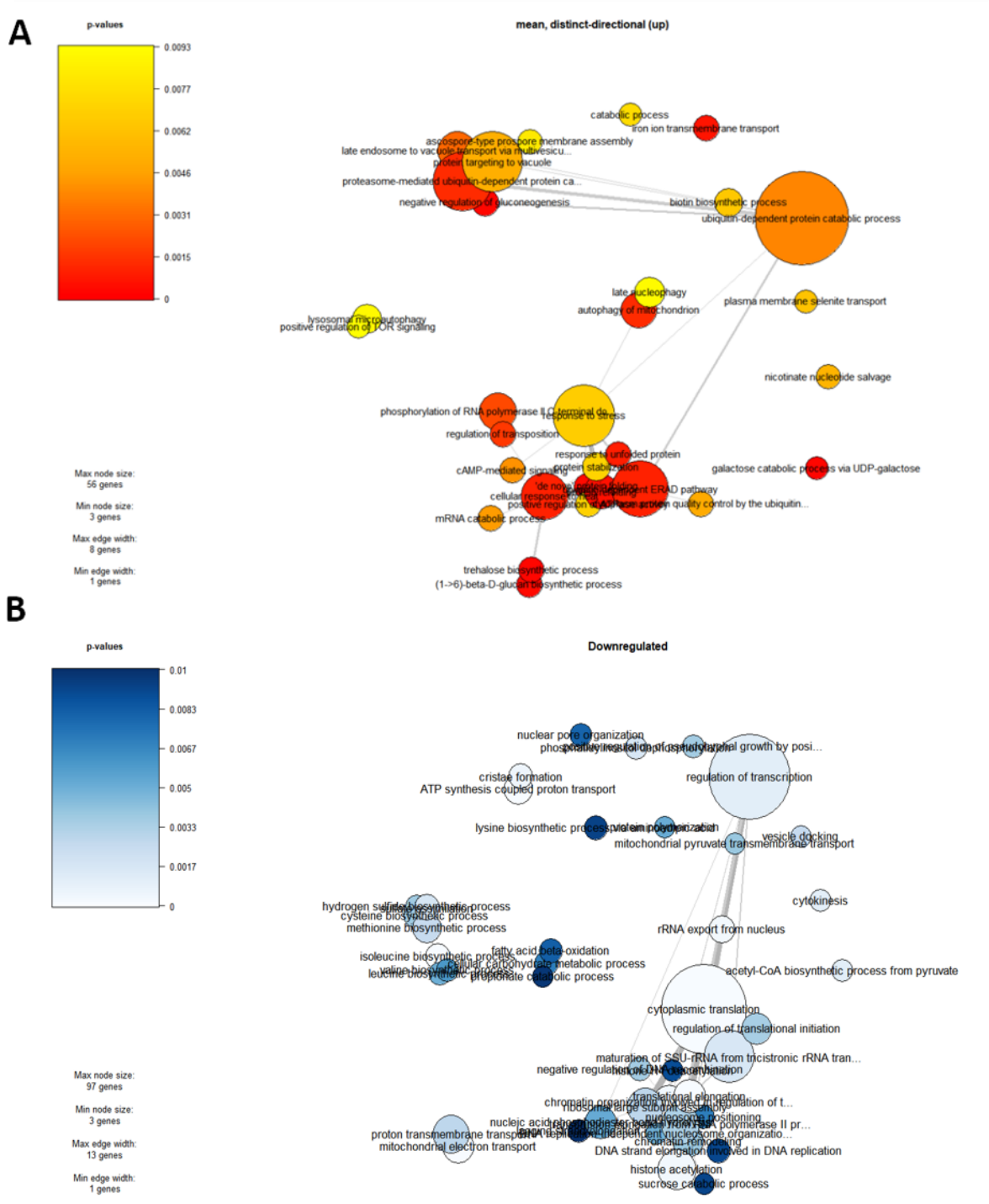

Figure 5 Gene-set analysis (GSA) of enriched biological processes during growth under high temperature condition in chemostat. Panel **A** shows the gene ontology (GO) terms corresponding to the biological processes found enriched in the upregulated genes, panel **B** the enriched GO terms for the downregulated genes. Gene sets were defined by GO terms that show significant (p-value <0.01) enrichment among the up or downregulated genes.

GSA of chemostat growth under osmotic stress

The response to the osmotic stress applied in this study triggered a mild transcriptional response compared to the other stress conditions, with logFC of DE genes between 3.14 and -4.77. Only *KMXK_0G04250*, a transmembrane gene encoding for a sexual differentiation protein was strongly overexpressed (logFC= 8.20). A graphic representation of the GSA results under the low pH condition is provided in Figure 6. The processes: protein transport, fungal type cell wall organization, protein targeting to vacuole, protein N-linked glycosylation, cellular response to drugs and glycerol transport were found significantly upregulated during growth under osmotic stress (Table 4). The main processes downregulated during growth under osmotic stress were: histone acetylation, protein import into mitochondrial matrix, mitochondrial translation, regulation of transcription and cytoplasmic translation. This indicates the osmotic stress triggers a reorganization of both transcriptional and translational activities, as well as a rearrangement in the mitochondrial apparatus, possibly for energy optimization purposes (Crawford & Pavitt, 2019).

Table 4 Gene Sets enriched (p-value<0.05) in upregulated genes under low pH growth in chemostat. Fold changes of comparative expression (in form of log2 FC) including gene-level p-values of differentially expressed genes for each functional category is provided. Only significantly upregulated genes are presented in the table (log2FC>0.5, gene-level p-value<0.05).

Biological process	Enrichment (p-value)	Genes	Gene ID	Functional annotation	Gene-level p-value	Log2FC
Siderophore transport	0.0007	6	KMXK_0A03220 KMXK_0A08370 KMXK_0A08390 KMXK_0D01050 KMXK_0D06600	*ARN1* *ARN2* *ARN2* *ARN2* *ARN1*	8.25E-03 1.74E-21 1.01E-07 2.8E-24 4.5E-05	1.6 7.9 5.09 6.23 3.3
Protein stabilisation	0.0009	7	KMXK_0D04330 KMXK_0F04730	*yer078c-like* *HSP78*	1.5E-08 4.3E-03	0.69 0.77
Ubiquinone biosynthetic process	0.0009	12	KMXK_0A06920 KMXK_0E03190	*COQ3* *COQ9*	2.7E-05	0.55 0.60

					1.23E-06	
Ubiquitin dependednt catabolic process	0.0018	56	KMXK_0D01310	*UBP14*	1.15E-04	0.65
Iron ion transport	0.0021	5	KMXK_0C06160 KMXK_0D03850 KMXK_0F03820 KMXK_0F05380	*FTR1* *FTH1* *FET3* *MRS4*	7.6E-10 5.6E-09 8.06E-04 1.09E-08	1.15 1 0.87 0.77
Protein folding in ER	0.0056	8	KMXK_0C01360	yer140w-like	0.002	0.56
Cellular Heat acclimatation	0.007	4	KMXK_0D01210	*HSP104*	0.039	0.51
Protein folding	0.008	59	KMXK_0G00760	*PDX1*	0.003	0.58
lysine biosynthetic process via aminoadipic acid	0.009	7	KMXK_0A00680	*LYS20*	7.74E-05	0.75
RNA polymerase II transcriptional preinitiation complex assembly	0.01	25	KMXK_0B06830 KMXK_0B08180 KMXK_0F01520	*TAF1* *TAF14* *HOT1*	2.43E-03 2.82E-05 5.24E-06	0.6 0.5 0.51
mitochondrial respiratory chain complex IV assembly	0.012	16	KMXK_0B03280 KMXK_0H01890	ymr244c-a-like *PET117*	2.04E-06 8.32E-05	0.74 0.56
Biosynthetic process	0.014	5	KMXK_0B04440 KMXK_0F01450	yer152c-lik *ARO*9	1.13E-09 4.25E-03	0.68 0.59
Proteasome assembly	0.02	11	KMXK_0C04640	*RPT4*	1.2E-05	0.54
Mitochondrial genome maintenance	0.025	30	KMXK_0B01500 KMXK_0D02580 KMXK_0E03710	PROB.MITO *MDV1* PROB. MITO	1.82E-06 2.8E-06 1.9E-03	0.61 0.70 0.70
Cellular metabolic process	0.032	9	KMXK_0C00200	ygl157w-like	0.003	0.83

Ubiquitin dependent ERAD pathway	0.033	28	KMXK_0C04640	*RPT4*	1.2E-05	0.54
proteolysis	0.04	75	KMXK_0A06510 KMXK_0B00950 KMXK_0D04330	yol073c-like *DAP2* yer078c-like	2.6E-06 2.5E-03 1.54E-08	0.57 0.67 0.69
Pantothenate biosynthetic process	0.046	11	KMXK_0H04340	*MT2649*	2E-04	1.5
Cell polarity	0.05	3	KMXK_0F00770	NA	7.1E-03	0.53

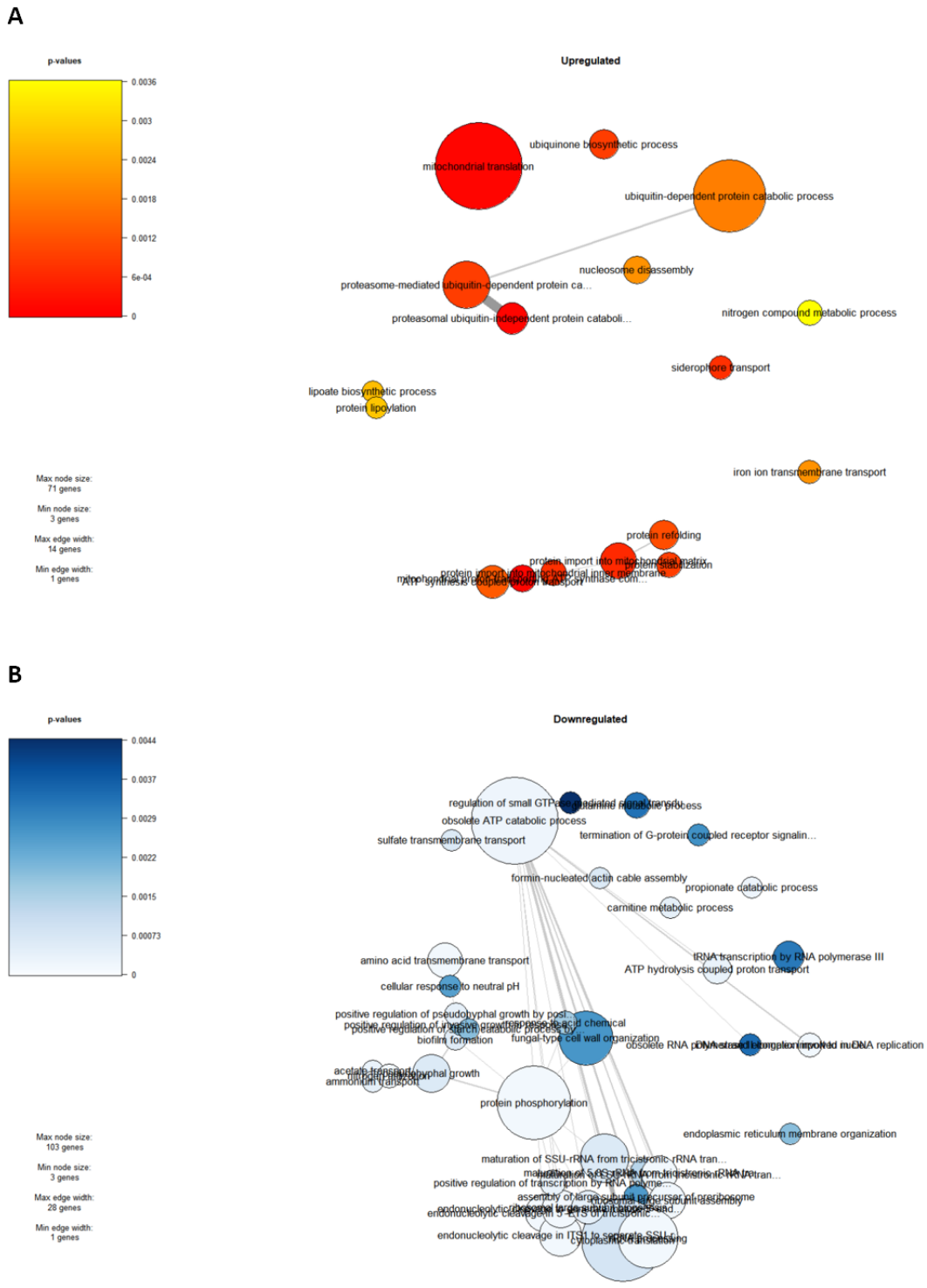

Figure 6 Gene-set analysis (GSA) of enriched biological processes during growth under low pH stress condition in chemostat. Panel **A** shows the gene ontology (GO) terms corresponding to the biological processes found enriched in the upregulated genes, panel **B** the enriched GO terms for the downregulated genes. Gene sets were defined by GO terms that show significant (p-value <0.05) enrichment among the up or downregulated genes.

GSA of chemostat growth under low pH stress

GSA analysis under low pH stress growth revealed upregulation of processes such as: mitochondrial proton-transporting ATP synthase complex assembly, mitochondrial translation and protein import into mitochondrial inner membrane and matrix (Figure 7), indicating that adaptation to such stress is energetically demanding. In fact, maintaining cell homeostasis in yeast cells under low pH stress is mainly maintained via ATP-coupled proton pumps (Fletcher et al., 2015). Accordingly, biosynthesis of ubiquinone, an essential component of the respiratory chain (Hsieh et al., 2007), is found upregulated (Table 5), with overexpression of the two ubiquinone biosynthetic enzymes Coq3 and Coq9 (logFC= 0.55 and 0.60 p-value=2.75e-=5 and 1.23e-06 respectively). The main processes found to be downregulated under low pH stress were: amino acid transmembrane transport, DNA strand elongation involved in DNA replication, nitrogen utilization, protein phosphorylation, ribosomal large subunit assembly, rRNA processing and obsolete ATP catabolic process (Figure 7), also indicative of an energy optimization strategy to cope with the stressful condition.

Table 5 Gene Sets enriched (p-value<0.05) in upregulated genes under osmotic stress growth in chemostat. Fold changes of comparative expression (in form of log2 FC) including gene-level p-values of differentially expressed genes for each functional category is provided. Only significantly upregulated genes are presented in the table (log2FC>0.5, gene-level p-value<0.05).

Biological process	Enrichment (p-value)	Genes	Gene ID	Functional annotation	Gene-level p-value	Log2FC
Protein transport	1.00E-04	84	KMXK_0B07250 KMXK_0B07560 KMXK_0E01900	*ATG14* *RHO5* *ATG23*	3.18e-05 2.17e-04 4.9e-0.4	0.93 0.51 0.58
Fungal type cell wall organization	1.00E-04	53	KMXK_0A02830 KMXK_0A04840 KMXK_0B04400 KMXK_0D05590 KMXK_0F04550	*RHB1* *KRE9* *SED1* ylr414c-like *ECM15* *GAS1*	1.73e-06 6.2e-05 9.73e-03 6e-05 1.1e-03 4.3e-02	0.67 1.09 0.87 1.44 0.57 0.75

			KMXK_0G02050			
Protein targeting to vacuole	1.00E-04	31	KMXK_0A05960 KMXK_0H00820	*MVP1* *VPS25*	3.50E-07 0.02	0.54 0.538
Protein N-linked glycosilation	0.0002	23	KMXK_0H04270	*KTR1*	4.57e-03	0.61
Cellular response to drug	0.0006	31	KMXK_0A02830 KMXK_0D02670 KMXK_0D03570	*RHB1* *PDR5* *PDK*	1.73e-06 8.9e-09 1.96e-08	0.67 1.45 0.66
Glycerol transport	0.0015	3	KMXK_0F03890	*STL1*	7.09E-08	1.25
Regulation of establishment or maintenance of cell polarity	0.0017	3	KMXK_0H03940	*PCL1*	3.2e-04	0.8
Vacuole fusion	0.0017	16	KMXK_0A01620	*BPT1*	2.76e-09	0.94
Establishment of cell polarity	0.0018	16	KMXK_0A06270	*MSB1*	4.7e-23	1.45
(1->6)-beta-D-glucan biosynthetic process	0.0021	5	KMXK_0A04840	*KRE9*	6.20E-05	1.09
Transmembrane transport	0.009	146	KMXK_0A00140 KMXK_0A01620 KMXK_0A01860 KMXK_0A06560 KMXK_0B04160 KMXK_0B04730 KMXK_0C06710 KMXK_0D00200 KMXK_0D01070	unch. Transp. BPT1 FUR4 UDP-GALT1 ybr241c-like FCY2 GIT1 TNA1 YKE4 PDR5 TPO4 HGT DAL5 DAL5	1.85e-03 2.76e-09 1.41e-03 1.52e-03 2.14e-02 5.09e-05 1.32e-06 5.4e-03 1.04e-03 2.96e-05 9.18e-03 4.27e-02 1.8e-07 8.01e-04	1.115 0.95 0.68 0.56 0.54 0.99 0.90 0.54 0.51 1.06 0.55 1.35 0.57 2.03

			KMXK_0D02680 KMXK_0D04460 KMXK_0E03660 KMXK_0F01550 KMXK_0F01570 KMXK_0F03890 KMXK_0G01990 KMXK_0H01420	STL1 YOR1 ITR1	7.08e-08 5.09e-05 7.04e-03	1.25 0.84 0.65
Late endosome to vacuole transport	0.0032	12	KMXK_0C04810	*SEC28*	3.07e-06	0.53
Potassium transmembrane transport	0.0033	5	KMXK_0A04140 KMXK_0E03840	yjr054w-like high affinity potassium transporter	0.0043 0.0024	0.57 2.87
ER to golgi vescicular transport	0.0042	64	KMXK_0C04810	*SEC28*	3.07e-06	0.53
Xenobiotic transport	0.0048	3	KMXK_0D02670 KMXK_0G01990	PDR5 YOR1	8.92e-09 5.09e-05	1.45 0.84
Negative regulation of transcription	0.005	5	KMXK_0F00190	*PST2*	0.0125	0.52
Protein glycosylation	0.008	22	KMXK_0G00720	*MNN1*	8.8e-05	3.15
Mitotic spindle orientation checkpoint	0.0096	4	KMXK_0F03810	*BUB2*	4.7e-06	0.88
Vacuolar transport	0.0099	12	KMXK_0B06970 KMXK_0E00660	*AVT1* carbopeptidase	1.44e-05 1.08e-05	0.53 0.56

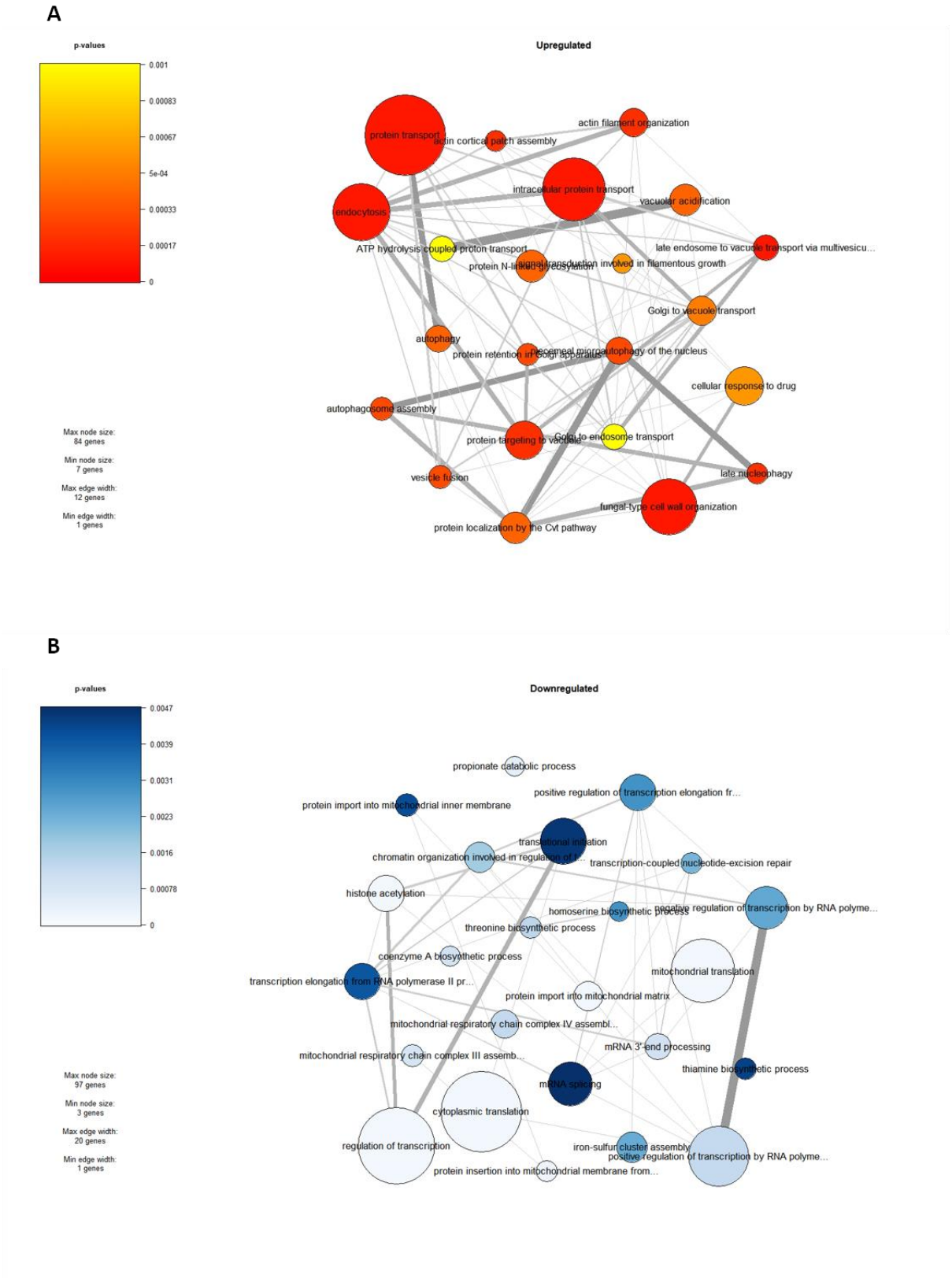

Figure 7 Gene-set analysis (GSA) of enriched biological processes during growth under osmotic stress condition in chemostat. Panel **A** shows the gene ontology (GO) terms corresponding to the biological processes found enriched in the upregulated genes, panel **B** the enriched GO terms for the downregulated genes. Gene sets were defined by GO terms that show significant (p-value <0.05) enrichment among the up or downregulated genes.

5. Discussion

A limitation of previous physiological studies on *K. marxianus*, is the variability of both the strains and conditions assessed, making it difficult to carry out comparative physiological studies with new strains. In order to have baseline physiological parameters for comparison, in particular under industrially relevant conditions such as high temperature, batch fermentation of three *K. marxianus* strains was performed at 30 °C and 40 °C during aerobic batch growth. The selected strains were a diploid (CBS 397) and two haploids (NBRC 1777 and CBS 6556). Since *K. marxianus* diploid and haploid strains are isolated in distinct environments, diploid and triploid strains being more common among industrial isolates (Ortiz-Merino et al., 2018), this suggests a peculiar robustness of polypoid strains to the industrial conditions. Overall, while NBRC 1777 showed the fastest growth rate at 30 °C, CBS 397 was the faster growing strain at 40°C, indicating that diploidy is possibly advantageous when it comes to thermotolerance; however, comparisons of more diploid strains is necessary to fully establish this. In this study, both the diploid CBS 397 and the haploid CBS 6556 showed an almost doubled growth rate at 40 °C compared to 30°C, indicating that this temperature is optimal for their growth. This preference of some *K. marxianus* strains for temperatures higher than 30 °C was reported before for CBS 6556 strain when grown in flasks at 30 °C and 37 °C (Rocha et al., 2011) and for the strains CBS 712 and ATCC 748 grown in batch fermentation (Lehnen, Ebert and Blank, 2019), and is unsurprising as *K. marxianus* is a naturally thermotolerant yeast (Fleming et al., 1993; D. Wang et al., 2018; Wilkins et al., 2008). On the contrary, both NBRC 1777's growth rate and biomass yield decreased at 40°C. These data indicate that the strain has higher maintenance costs associated with growth at high temperature (Zakhartsev et al., 2015) when compared to CBS 6556 and CBS 397 strains. The maximum growth rate measured for NBRC 1777 at 30 °C is only comparable to the 1.1 h^{-1} reported for the strain CBS 6556 growing in flask on glucose on rich medium (Hoekstra et al., 1994). A linear relationship between growth rate and respiration rate is noted in yeast (Devin et al., 2006); accordingly NBRC 1777 shows significantly higher oxygen uptake rates compared to the other strains. No significant extracellular metabolite accumulation was detected during the batch fermentations. As was previously observed during *K. marxianus* aerobic batch fermentations (Fonseca et al., 2007) where the concentration of excreted metabolites was very low, representing 6% of the

consumed carbon at most, it is possible that extracellular metabolites were produced under the detection limit during the batch fermentations. However, the low values of carbon recovery for some strains, especially under the 40 °C condition, suggest either a considerable accumulation of a metabolite which was not detected, or a high ethanol evaporation from the vessels at higher temperature. Chemostat growth allows for rigorous comparison of the same strain under different conditions while at the same physiological phase of exponential growth. Consequently, physiological parameters measured are independent from the cell growth stage. Under the conditions of our study, it also allows to reproduction of long-term stress, so that the observed physiological and transcriptional changes between the standard and the stress conditions, reflect the cell stress adaptation strategies. In this study, CBS 6556 was selected as model haploid strain to further study the stress adaptation mechanisms of *K. marxianus*, and its growth was compared during chemostat fermentations under a standard and three different stress conditions. Differently to what observed during the batch cultivation, where the biomass yield of the strain was unchanged at 40 °C compared to 30 °C, CBS 6556 showed lower biomass yields and higher glucose uptake rates at 40°C in chemostat fermentation. These physiological changes were only observed for the high temperature condition, suggesting different adaptation strategies to high temperature stress compared to the other tested stresses. Although reduction of biomass yield in chemostat at high temperature was originally reported for this strain (Verduyn, 1991), it is not fully explained and generally attributed to higher maintenance costs. We can speculate that the reduction in biomass yield is attributed to the high maintenance costs related to growth at high temperature, only visible in chemostat because it provides a true representation of the adaptation mechanisms that the cells undergo under long term temperature stress. In agreement with our findings, the biomass yield of *K. marxianus* at higher temperature was reported to be constant during batch fermentations (Lehnen, Ebert and Blank, 2019) as well as the increased oxygen uptake rate (Fu *et al.*, 2019), but no physiological studies are available in chemostat for comparison with what was determined in this study.

As well as the physiological variability, chemostat cultivation eliminates the transcriptional variability described for *K. marxianus* at different stages and rates of growth (Yu et al., 2021). Moreover, chemostat cultivation is considered an optimal way to study stress adaptation, since genes responsive to long term stress in yeast are

known to differ to the ones responsive to short term stress exposure (Richards *et al.*, 2014; Tai *et al.*, 2007). Therefore, the conditions set in this study allowed us to analyse the long-term stress adaptation of *K. marxianus* to multiple stresses, via pairwise DE analysis of the RNA-seq data obtained from the chemostat fermentations. Comparison of the DE genes identified under each stress, revealed zero upregulated and 3 downregulated genes common amongst all tested stresses, suggesting the transcriptional response to be stress-specific. This has been previously reported for other yeast species, including *Kluyveromyces lactis*, *Candida albicans*, *Yarrowia lipolytica* (Tirosh et al., 2011). Given the stress-specific nature of the *K. marxianus* transcriptomic response observed here, we focused on high temperature stress in order to provide a complete portrait of the natural thermotolerance of *K. marxianus*.

In order to identify the main biological processes involved in thermotolerance, we performed gene-set analysis (GSA) on the DE data comparing the standard and the three stress conditions. A comparison of the GSA results from all stress conditions revealed that, although several biological processes were enriched in response to two out of three stresses tested, indicating a certain functional overlap, a few common genes were differentially expressed within the same process, indicating the possibility that *K. marxianus* evolved stress-specific genes able to carry out the biological processes. An example is the protein target to vacuole process, upregulated under both high temperature and osmotic stress, but carried out by distinct genes (respectively *SNR2* and *VID24*, and *MUP1* and *VSP25*) under each condition. Therefore, while on a gene-level the transcriptomic response of *K. marxianus* seems to be highly stress-specific in agreement with what previously observed (Tirosh et al., 2011); GSA revealed how the enriched gene sets are somewhat conserved within several stresses.

Among the common responses (1->6)-beta-D-glucan biosynthetic process, autophagy of mitochondrion, endosome to vacuole transport, lysosome macro autophagy and protein targeting to vacuole were found upregulated under both osmotic and high temperature stress. In particular, the gene *KRE9*, involved in cell wall beta-glucan assembly, was found to be upregulated during growth under both the stresses. A common denominator among the upregulated processes could be the stress induced activation of the MAPK cascade, which directs the cell response to both osmotic and heat stress stimuli via promoting cell integrity, proliferation and import of osmolytes such as glycerol (Hohmann, 2002). The lysosome-like vacuole, responsible for protein

sorting-related activities in yeast, is known to have stress related functions such as protein sorting, organelle acidification, ion homeostasis and autophagy (Li & Kane, 2009). The response to high temperature and low pH exhibited the most extensive functional overlap among the tested stresses, with processes such as protein stabilization, protein refolding and ubiquitin-dependent protein catabolic upregulated in both, indicating that protein stability and turnover are needed for adaptation to growth under both stresses. Moreover, transmembrane transport of iron, ion involved in multiple processes including cellular respiration, lipid biosynthesis, translation and amino acid biogenesis, DNA replication and repair and oxygen transport (Martínez-Pastor et al., 2017) was found to be upregulated under both high temperature and low pH stress. In particular several high affinity iron transporters-encoding genes such as *FTR1*, *FET3*, MRS4 and *FTH1* and the siderophore transporters *ARN1* and *ARN2*, present in multiple copies in *K. marxianus* (Jia et al., 2021), were found upregulated under high temperature and low pH conditions in this study.

At 40°C, CBS 6556 displayed increased glucose uptake rate in both batch and chemostat fermentation. This indicates a higher maintenance energy demand to cope with growth under elevated temperature. In fact in carbon limited chemostat cultures on minimal medium, glucose is both a source of carbon and energy, while oxygen is the electron acceptor needed for ATP production during oxidative phosphorylation (Otterstedt et al., 2004). However, while an increase in ATP production seems an obvious strategy to provide energy for growth under stressful conditions, GSA analysis reveals that ATP synthesis-coupled proton transport and mitochondrial electron transport are downregulated under the 40 °C condition in the tested strain, while mitophagy was found to be upregulated. The same regulation pattern is also observed for the osmotic stress response, while the opposite was found under the low pH condition. Growth at high temperature is known to provoke oxidative stress and ROS accumulation in yeast (Davidson et al., 1996; Zhang et al., 2015), and leakage of electrons from the mitochondrial electron transport chain is the major intracellular source of ROS (Herrero *et al., 2008*). Since ROS accumulation under high temperature stress has been reported before in *K. marxianus* (Fu *et al.*, 2019), it can be speculated that tight regulation of mitochondrial biogenesis to reduce electron leakage is one of the strategies *K. marxianus* employs to cope with long-term temperature stress. Since ROS accumulation is also commonly reported following osmotic stress, this could

explain the similar pattern of modulation of mitochondria activity. On the contrary, ATP production becomes essential under low pH stress in order to keep the cellular homeostasis via ATPase proton pumps (Fletcher et al., 2015; Holyoak et al., 1996; Ndukwe et al., 2020), hence the upregulation of mitochondrial activity observed in this study. Finally, similarly to our findings, genes of the mitochondrial respiratory chain were previously found to be downregulated in *K marxianus* under high temperature stress, although respiration rate was found to be upregulated (Fu *et al.*, 2018). This could explain why NBRC 1777, showing the highest growth rate at 30 °C, showed a decreased growth rate at 40 °C. The very high oxygen uptake rate of the strain at 40 °C could be a disadvantage for its growth at high temperature because of the ROS accumulation resulting from it, hence showing a slower growth.

In conclusion, this study offers an example on how integration of physiological data and transcriptomic analysis can help characterize different strains under industrially relevant conditions. It also offers insights on stress adaptation mechanisms in *K. marxianus*, in particular thermotolerance.

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Chapter IV

Identification of a novel gene required for competitive growth at high temperature in the thermotolerant yeast *Kluyveromyces marxianus.*

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TD helped designing the experiment and performed the orthology analysis

ID performed the orthology and transcriptomic analysis on the high temperature condition

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VS and JN provided help with study design and planning.

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1. Abstract

It is important to understand the basis of thermotolerance in yeasts to broaden their application in industrial biotechnology. The capacity to run bioprocesses at temperatures above 40^{o}C is of great interest but this is beyond the growth range of most of the commonly used yeast species. In contrast, some industrial yeasts like *Kluyveromyces marxianus* can grow at temperatures of 44^{o}C or higher. Such species are valuable for direct use in industrial biotechnology and as a vehicle to study the genetic and physiological basis of yeast thermotolerance. In previous work, we reported that evolutionarily young genes disproportionately changed expression when yeast were growing under stressful conditions and postulated that such genes could be important for long-term adaptation to stress. Here, we tested this hypothesis in *K. marxianus* by identifying and studying species-specific genes that showed increased expression during high temperature growth. Twelve such genes were identified and eleven were successfully inactivated using CRIPSR-mediated mutagenesis. One gene, *KLMX_70384*, is required for competitive growth at high temperature supporting the hypothesis that evolutionary young genes could play roles in adaptation to harsh environments. *KLMX_70384* is predicted to encode a 68-83AA peptide and we used the GWIPS-viz genome browser to confirm its transcription and translation. The precise function of KLMX_70384 remains unknown but some features are suggestive of RNA-binding activity. The gene is located in what was previously considered an intergenic region of the genome, which lacks homologues in in other yeasts or in databases. Overall, the data support the hypothesis that genes that arose *de novo* in *K. marxianus* post the speciation event that separated *K. marxianus* and *K. lactis* contribute to some of its unique traits.

2. Introduction

A goal for a sustainable bioeconomy is to make the production of value added chemicals independent from petroleum as starting material, in favour of sustainably sourced biomass (Lange et al., 2021). Yeasts, used as microbial cell factories, represent a valuable resource to reach this goal, due to their ability to metabolise such biomasses, their robust physiology, fermentation properties, genetic tractability, and ability to tolerate harsh growth conditions (Patra et al., 2021). Whilst *Saccharomyces cerevisiae* is the most widely used cell factory yeast, in recent years, attention has also focused on non-Saccharomyces species ("non-conventional yeasts") because of their diverse and industrially-relevant traits (Rebello et al., 2018). As an example, due to its capacity to metabolise the sugar lactose as carbon source, *Kluyveromyces lactis* was proposed for lactose-rich dairy waste valorisation (Rodicio & Heinisch, 2013); the oleaginous yeast *Yarrowia lipolytica* can be exploited for lipid production (Zeng et al., 2018); thermotolerant yeasts such *Kluyveromyces marxianus, Ogataea polymorpha* and *Ogataea thermomethanolica* could facilitate high temperature processes such as simultaneous saccharification and fermentation (Cunha *et al.*, 2014; Phithakrotchanakoon *et al*., 2020); and finally, methylotrophic yeasts such as *Komagataella phaffii, O. polymorpha* and *O. thermomethanolica* have the capacity to successfully secrete proteins, complete with post translational modification such as glycosylation. The range of endogenous and heterologous promoters that are available in these yeasts make then very suitable for heterologous proteins production (Cankorur-Cetinkaya et al., 2018; Manfrão-Netto et al., 2019; Puseenam et al., 2018; Zhang et al., 2021). A major barrier that limited opportunities to exploit the intrinsic advantageous properties of non-conventional yeasts for industrial processes is being overcome by the development of molecular tools for rapid strain engineering (Juergens *et al.*, 2018; Cai, Gao and Zhou, 2019; Li *et al.*, 2020; Yang and Blenner, 2020; Patra *et al.*, 2021).

For example, the broad substrate range of *K. marxianus* conferred by a plethora of sugar transporters (Donzella *et al*., 2021) and its thermotolerance (Nurcholis *et al.*, 2019) make this yeast an attractive host for some applications. Furthermore, the availability of genome editing and synthetic biology tools mean that there are few limitations for strain construction (Juergens *et al.*, 2018; Karim, Gerliani and Aïder, 2020; Rajkumar and Morrissey, 2020). Nonetheless, further progress is required to

improve knowledge of the physiological response of *K. marxianus* under industrially relevant stresses to allow wider use in biotechnological processes (P. Li et al., 2019; Yu et al., 2021). One such response is thermotolerance, which is a desirable trait in strains used for industrial processes because it reduces cooling costs and allows fermentation at temperatures that mitigate bacterial contamination (Engineering Robustness of Microbial Cell Factories, 2017; Jiang et al., 2020). *K. marxianus* is capable of growth up to 45°C, and sometimes higher (Karim et al., 2020). For this reason it has been considered for application in processes requiring high temperature, such as lignocellulosic biomass fermentation for bioethanol production (Madeira-Jr & Gombert, 2018; Malairuang et al., 2020; Suzuki et al., 2019; Yanase et al., 2010). Although *S. cerevisiae* is not intrinsically thermotolerant, responses and adaptation to high temperature have been quite widely studied in this model species. Among the known heat stress responses in *S. cerevisiae* are increased expression of genes encoding protein folding chaperones, proteins involved in respiration and enzymes for utilisation of alternative carbon sources (Gasch et al., 2000); activation of Hog1 and MAPK-related pathways, including cell wall remodelling (Leach et al., 2012); changes in transcription rates and in mRNA stability (Castells-Roca et al., 2011); and triggering of calcineurin-activated gene expression (Kraus & Heitman, 2003). The integration of transcriptomic, proteomic and metabolomic data can help provide a complete picture of the stress response landscape in different yeasts (P. Li et al., 2019; Shui et al., 2015; Walther et al., 2010). Such omics studies indicate that there does not appear to be a single evolutionarily conserved thermotolerance mechanism; for example, a study of *K. marxianus* and *O. polymorpha* failed to find any similar patterns (Lehnen, *et al.*, 2019). Specifically in *K. marxianus*, other studies comparing response at 30°C versus 45°C, found that *K. marxianus* presents a multi-faceted response to high temperatures, including a reduction in central metabolic activity, increased protein turnover and DNA repair (Lertwattanasakul *et al.*, 2015), upregulation of the mitochondrial respiratory chain genes, downregulation of glycolytic genes (Fu *et al.*, 2019).

In a previous study, we carried out a large-scale comparative transcriptomic and proteomic analysis of *S. cerevisiae*, *K. marxianus* and *Y. lipolytica* growing under low pH, high temperature and high osmotic pressure conditions (Doughty et al., 2020). All experiments were carried out in chemostats at a constant growth rate, thereby assessing

long-term adaption to these stressful conditions rather than the short-term response to fluctuating stresses. The two major findings were first, there is little commonality in how each yeast responds to the same stress; and second, evolutionarily young genes were over-represented in the sets of genes changing expression under adverse conditions. These indicate that genus- or species-specific genes that change expression are likely to be important in the physiological changes necessary for growth in harsh environments and thus these genes could be very useful for modifying yeasts for biotechnological processes where the growth medium and conditions are often sub-optimal. In this study, we honed in on the evolutionary young genes that responded to elevated temperature in *K. marxianus* to determine their importance for the higher growth temperature of this yeast. To do this, we re-ran both the bioinformatic pipeline to identify this cohort of genes, and the transcriptomic analysis to identify differentially expressed genes, using an updated *K. marxianus* genome annotation (Fenton et al., 2021). We identified twelve *K. marxianus*-specific genes with increased expression at high temperature and successfully inactivated eleven of these. These mutants were assessed for growth at higher temperature, revealing that one of these genes, *KLMX_70834*, was specifically required for competitive growth at 45°C. The protein encoded by *KLMX_70834* is unique to *K. marxianus* but possesses structural features that may be suggestive of RNA binding activity. As well as identification of this novel protein that is required for high temperature growth in *K. marxianus*, our study validates the strategy of focusing on evolutionarily young genes to investigate niche adaptation in yeast.

3. Materials and methods

Strains and cultivation

All *K. marxianus* strains used in this study are listed in Table 1. Knock-out mutants ΔT1 to ΔT13 were constructed in wild-type *K. marxianus* NBRC 1777 and the Δ*KLMX_70384* strain was constructed in the NHEJ negative derivative, *K. marxianus* NBRC 1777 *dnl4*. Yeast strains were routinely grown on YPD medium (2% peptone, 1% yeast extract, 2% dextrose) at 30°C. Hygromycin B (200 µg mL^{-1}) and G418 (200 µg mL^{-1}) purchased from Sigma Aldrich, St. Louis, MI, United States were used for selection where required. For the drop tests, strains were grown overnight in 10 mL, harvested, washed and resuspended at A_{600nm} of 1 in sterile water. 5µL of 1:10 serial dilutions (until 10^{-5}) of each strain were spotted onto agar plates, which were incubated at the appropriate temperature for 24 h. For screening of growth of multiple mutant strains at high temperature in liquid medium, a BioLector© I (M2P-Labs) microfermentation system was used. For this, cells were grown overnight at 30°C in YPD medium; the following morning, the cells were diluted to an A_{600nm} of 0.1 in fresh medium and 800 µL of each strain were loaded in a BioLector 48-well flower plate. A program was run with the following settings: Temperature=46.5°C (setpoint: 47°C, actual measured temperature: 46.5°C), biomass filter=Ex620nm EM620nm, gain =20, shaking=1400RPM. *E. coli* DH5α strain was used for cloning purposes. The strain was maintained in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, 10 g L^{-1} NaCl) supplemented with 100 µg mL^{-1} ampicillin when required.

Cultivation temperatures

Three temperatures were employed for strains growth: 30°C, which represents the control condition; and 45°C and 47°C for the high temperature condition. On-plate screening for thermotolerance was performed at 47°C, which represents a challenging temperature for most *K. marxianus* strains on solid medium. In flask screening for thermotoleramce and competition experiments were performed at 45°C, as this is a challenging temperature for NBRC 1777 WT strain in liquid medium.**Table 1 Strains used in this study.**

Strain	Genotype	DMKU3-1042 gene ID	Mutation coordinates NBRC1777 genome	Source

NBRC 1777	WT			NITE Biological Research Centre, Japan
NBRC 1777 *dnl4*	*Δdnl4*			(Rajkumar et al., 2019)
Δhgt	Δkmar_10531, Δkmar_10530, Δkmar_10529, Δkmar_10528, Δkmar_10527		HGT: CHR I Δ1,131,464-1,145,439	(Varela et al., 2019)
ΔT1	Δkmar_10001	KLMX_10556	CHR I ΔA 1,146,757	This study
ΔT2		KLMX_10792	CHR I Δ1,624,363-1,624,367	This study
ΔT4		KLMX_50030	CHR V ΔG 59,582	This study
ΔT5		KLMX_60003	CHR VI ΔC 8,314	This study
ΔT6	Δkmar_60126	KLMX_60133	CHR VI ΔC 282,973	This study
ΔT7	Δkmar_60261	KLMX_60270	CHR VI ΔG 563,131	This study
ΔT8		KLMX_60369	CHR VI ΔA 778,443	This study
ΔT9		KLMX_70384	CHR VII ΔA 807,181	This study
ΔT10		KLMX_70441	CHR VII ΔA 931,070	This study
ΔT11		KLMX_80304	CHR VIII Δ676,409-676,411	This study
ΔT13		KLMX_10646	CHR I Δ1,311,008-1,311,010	This study
ΔKLMX_70384	*dnl4 ΔKLMX_70384*		CHR VII Δ806,975-807,369	This study
ΔT9 I5: *KLMX_70384*	ΔT9 ΔI5: KLMX_70384compl		insertion in I5 (chromosome IV: 240017…241741)	This study

Differential expression (DE) and orthology analyses

Differential gene expression (DE) analysis was performed on the previously published RNA-seq dataset of the strain CBS 6556 (Doughty et al., 2020). The RNA-Seq data are available at the SRA accession PRJNA531619. RNA-seq reads were aligned to the *K. marxianus* DMKU3-1042 genome (Lertwattanasakul *et al.*, 2015), then assigned to genes using an updated genome annotation of the DMKU3-1042 genome available on the GWIPs-viz genome browser (Riboseq.org). DE analysis between the standard (Std) and high temperature (HiT) condition, and orthologous inference to identify *K. marxianus*-specific genes, was performed using HISAT2 and Stringtie followed by the R-scripts contained within the OrthOmics package (Doughty et al., 2020). FASTA proteome files for orthology inference were obtained from uniprot.org. To check for potential roles of the target genes in different stress responses, a separate DE analysis was carried out on all *K. marxianus* RNA-seq datasets including low pH (lowpH) and osmotic stress (Osm) condition. Genome alignments and reads per gene calling was performed using Bowtie2 (Langmead & Salzberg, 2012), Samtools (Danecek et al., 2021) and featureCounts (Liao et al., 2014), followed by the edgeR scripts available at https://rnnh.github.io/bioinfo-notebook/docs/DE_analysis_edgeR_script.html. For DE analysis, genes exhibiting a FDR <0.01 and log2 fold change (FC) >1 were considered significantly regulated. Normalized count per million were calculated adjusting for library size according to the TMM normalization method. A table of equivalence for gene IDs used in this study to perform the DE analysis, and the gene IDs of the published annotation (Fenton *et al.*, 2021), is available in Supplementary table 1.

Molecular Techniques

Mutants were constructed in NBRC 1777 WT strain by non-homologous end joining (NHEJ) -mediated gene inactivation, using the CRISPR-Cas9 system previously described (Juergens et al., 2018; Varela et al., 2019). A detailed step by step protocol outlining these methods was recently published (Rajkumar *et al.*, 2021). In brief, targeting sequences (for gRNAs) were ordered as DNA primers and inserted into plasmid pUCC001 by Golden Gate assembly. All plasmids and primers used in this study are listed in tables 2 and 3, respectively. In the case of genes where an NBRC 1777 Gene ID was available, gRNA primers were designed using the sgRNA software (Xie *et al.*, 2014; Rajkumar and Morrissey, 2021). The remaining gRNAs were designed using the CRISPRdirect software (Naito et al., 2015), with the *K. marxianus*

NBRC 1777 genome as template. Plasmids were introduced into *K. marxianus* using the standard LiAc/SS carrier DNA/PEG method with selection for transformants on Hygromycin B (200 μg mL^{-1}). Transformants were screened for indel or frameshift mutations in the target gene using PCR amplification with diagnostic primers, followed by DNA sequencing. This method was successful in generating inactivating mutations in 11/12 target genes. The precise co-ordinates of deletion are listed in Table 1, using the genome co-ordinates from NBRC 1777 strain (Inokuma et al., 2016). To allow for CRISPR-Cas9 plasmid loss, the mutant strains were grown overnight in 10 mL of YPD without antibiotic selection. Single colonies were tested on YPD with Hygromycin B, to confirm lack of growth and hence loss of plasmid. Complementation of ΔT9 was achieved via chromosomal insertion of an expression cassette, containing the *KLMX_70384* CDS under the control of the constitutive promoter p*PDC1* and the t*INU1* terminator. The expression cassette contains a KanMX selection marker and 850-bp homology arms for integration into the I5 intergenic region in chromosome IV (Rajkumar et al., 2019). The pI5-KLMX_70384compl plasmid (Table 3) was constructed via Golden Gate assembly of the *KLMX_70384* CDS , with the regulatory parts PDC1pr/P2 and INU1t/T1 into the pI5-MTU-DO-G418 plasmid using previously described methods (Rajkumar et al., 2019). For transforming into *K. marxianus* ΔT9, 1 ug of pI5-KLMX_70384compl was digested with SgsI at 37°C for 1 hour. The digested product was transformed into the strain, with selection for transformants on G418. Colonies were screened via colony PCR with I5 locus-specific primers to identify positive transformants and integrity of the insert was confirmed by sequencing. A full open reading frame disruption of *KLMX_70384* was achieved in the *dnl4* derivative of *K. marxianus* NBRC 1777 using the same CRISPR-Cas9 system, this time taking advantage of the intrinsic homology dependent repair (HDR) apparatus. A 160-bp repair fragment was built by designing two primers Tar12_RF_F and Tar12_RF_R that shared 18 bp overlap and had 81 and 59 bp of homology to the 5' and 3' regions flanking *KLMX_70804*, respectively. The 5' and 3' homology arms were annealed and amplified via overlap extension PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Successful construction of the repair fragment was confirmed by gel electrophoresis and 1ug of repair fragment was co-transformed with the CRISPR-Cas9 plasmid carrying the *KLMX_70384*-specific gRNA, into NBRC 1777 *dnl4* strain. Mutants were screened for via colony PCR, using

Tar12_dia_F and Tar12_dia_R primers. *KLMX_70804* gene deletion was confirmed from the amplicon size of the PCR product (WT allele=798bp; deleted allele= 403bp).

Table 2 Plasmids used in this study.

Plasmid name	Relevant characteristics	Source
pUCC001	Modified from pUDP002 (Juergens et al., 2018) for easy cloning of new guide RNA (gRNA) targets by Golden Gate assembly	(Rajkumar et al., 2019)
pI5-MTU-DO-G418	Integrative plasmid modified from (Rajkumar et al., 2019) with GFP drop-out, kanMX, targeting integration site I5 (chromosome IV: 240017…241741)	(Rajkumar & Morrissey, 2020)
pI5-KLMX_70384compl	pPDC1-KLMX_70384-INU1t cloned into [pI5-MTU-DO-G418]	This study

Table 3 Primers used in this study.

Primer name	Sequence	Relevant genes:
gRNA primers		
Tar_1_gg_F	cgtcgcactcgaaccttataatag	KLMX_10556
Tar_1_gg_R	aaacctattataaggttcgagtgc	
Tar_2_gg_F	cgtcaacgtttttaaataattaaa	KLMX_10792
Tar_2_gg_R	aaactttaattatttaaaaacgtt	
Tar_4_gg_F	cgtcgtccttaattgtcactatgg	KLMX_50030
Tar_4_gg_R	aaaccaagagcagctgccgccaga	
Tar_5_gg_F	cgtctttcttctatctcacttcac	KLMX_60003
Tar_5_gg_R	aaacgtgaagtgagatagaagaaa	
Tar_6_gg_F	cgtctgtggtatacagcgatccac	KLMX_60133
Tar_6_gg_R	aaacgtggatcgctgtataccaca	
Tar_7_gg_F	cgtcacatgtagttcttgtgctcc	KLMX_60270
Tar_7_gg_R	aaacggagcacaagaactacatgt	
Tar_8_gg_F	cgtcgataaggtttcagcggatag	KLMX_60369
Tar_8_gg_R	aaacctatccgctgaaaccttatc	
Tar_9_gg_F	cgtctgctgaaacctctggtaaga	KLMX_70384
Tar_9_gg_R	aaactcttaccagaggtttcagca	
Tar_10_gg_F	cgtcgtgattagcgcttataactc	KLMX_70441
Tar_10_gg_R	aaacgagttataagcgctaatcac	
Tar_11_gg_F	cgtcgttctcttggggtaccccag	KLMX_80304
Tar_11_gg_R	aaacctggggtaccccaagagaac	
Tar_12_gg_F	cgtctcagggatgacaattattct	KLMX_10646
Tar_12_gg_R	aaacagaataattgtcatccctga	
Diagnostic primers to confirm mutations		
Tar_1_dia_F	tagaggaggtagatgtagcgg	KLMX_10556

Tar_1_dia_R	atgattccgtgaagccg	
Tar_2_dia_F	ggaaatgcgttagaaatgcttc	KLMX_10792
Tar_2_dia_R	caatgtactaacagggagca	
Tar_4_dia_F	ataaacggcagaatccgtt	KLMX_50030
Tar_4_dia_R	ggctgtgattaaaaagcact	
Tar_5_dia_F	catgtcattctcttacttaaccag	KLMX_60003
Tar_5_dia_R	aactttctccagatcaaatgaac	
Tar_6_dia_F	gcgtgtgttatattgtgttcg	KLMX_60133
Tar_6_dia_R	tcaccagaaagcagcatct	
Tar_7_dia_F	gtgtgcttacaatagcatagcac	KLMX_60270
Tar_7_dia_R	tccagtaaaaacaactacagagaa	
Tar_8_dia_F	atctgccaaattctccatg	KLMX_60369
Tar_8_dia_R	ctgagggttgatccttcac	
Tar_9_dia_F	cttctctaaactgctctgtct	KLMX_70384
Tar_9_dia_R	aagagcacagcggctaat	
Tar_10_dia_F	gaggaaatgaagaggtctttg	KLMX_70441
Tar_10_dia_R	ttcgtactttgtattctaggtttcc	
Tar_11_dia_F	ggtttggtttcccattc	KLMX_80304
Tar_11_dia_R	ctctacttcccaccattcc	
Tar_12_dia_F	attatgatatgaaagagaagcgc	KLMX_10646
Tar_12_dia_R	atctgtacgggaatgaaaa	

Complementation

Tar9_GG_F	gcatcgtctcatcggtctcatatgatgtctgacaaggtcgaaga
Tar9_GG_R	atgccgtctcaggtctcaggatttagttgatcaacttcttgaacttagca
I5US_F	agtagtgagtgacagacac
P2R	gcaattatttggtttgggtgtg

Repair fragment

Tar9_RF_F	gaaaactagttccatatagtatcccattattactcatttctctcttgttagctcgtattccagccaagcaaacgaaaagtccgtcgttacttaca
Tar9_RF_R	agatagattagattaattaattaattattaagtattatgggaattagaagactaaggatgtagtgtaagtaacgacg

Competition experiment

The *ΔKLMX_70384* and parental strains were grown individually overnight in 10 mL of YPD broth at 30°C. The following morning, they were mixed in a 50:50 ratio to a final A_{600nm} of 1, in a 50-mL falcon tube. The co-culture was then diluted to a final A_{600nm} of 0.1 into six 250-mL flasks, in YPD, to a final volume of 50 mL. Three of the flasks were placed in a shaking incubator at 30°C, the remaining three were placed in a shaking water bath, pre-warmed at 45°C. 1 mL of the co-culture was serially diluted and plated on YPD plates, for colony screening at time=0 (T0). The six flasks were sampled at a regular interval of 12 hours for 48 hours, and 1 mL of the co-culture was serially diluted and plated on YPD plates. The plates were incubated for 24 hours at 30°C, and 10 random colonies were picked from them for screening via colony PCR, using Tar12_dia_F and Tar12_dia_R primers (Table 3). Strains were distinguished by the size of the amplicon (WT allele=798 bp; deleted allele= 403 bp). The significance of the difference between the parental and *ΔKLMX_70384* strains in co-culture composition, between T0 and 24 hours, was calculated with a paired t-test, with $p<0.05$ considered to be significant.

4. Results

Identification of 12 novel genes with a possible role in thermotolerance in *K. marxianus*

Using an updated annotation of the *K. marxianus* DMKU3-1042 genome (Fenton *et al.*, 2021), we mapped the RNA-seq reads of the standard growth condition (Std) and high temperature (HiT) datasets to identify differentially expressed (DE) genes. A total of 216 genes with significant changes in expression were identified, and these are listed in Supplementary Table 2. As the updated annotation uses a different gene identifier (KLMX versus KMXK), the table also includes this information to facilitate comparison between the studies. We wished to know which of these were evolutionarily young and *K. marxianus* specific. To do this, single copy protein-encoding genes were identified and subjected to pairwise orthology inference using Orthofinder (Emms & Kelly, 2015) against the proteomes of *K. lactis*. *S. cerevisiae, Y. lipolytica*, and *Homo sapiens*. The yeast species were chosen because they span a wide range of the budding yeast subphylum (Saccharomycetaceae) (Shen *et al.*, 2018) and *H. sapiens* represents a distantly-related eukaryote.

Following the hierarchical approach described in Doughty et al., (2020), genes were segregated into five groups based on gene age, which was inferred by the presence/absence of orthologous proteins in the proteomes of the aforementioned species. Proteins only present in the final group, with no orthologues in any of the other proteomes, were considered to be encoded by evolutionarily young, K. marxianus-specific genes. Of the 55 genes in this group, 16 were found to be differentially expressed during high temperature growth, with 13 upregulated and 3 downregulated (Supplementary Figure 2). To be sure that these genes really were K. marxianus-specific, a Blastp search was individually performed with each protein sequence against the nr database which includes a wider range of species (Supplementary table 3). This led to exclusion of three candidate genes (1 upregulated and 2 downregulated) that potentially had orthologues in other yeast species. Of the remaining thirteen *K. marxianus*-specific genes one, *KLMX_70441* showed a significant hit (e-value: 9e-109, perc. identity: 71%, query coverage: 97%) with a NADPH-dependent oxidoreductase protein from the Acinetobacter spp., suggesting acquisition by horizontal gene transfer (Supplementary Figure 1). This gene was auto-

annotated as “YwnB” (Lertwattanasakul et al., 2015) on the GWIPs-viz browser (Michel et al., 2014, 2018, Fenton et al., 2021), reflecting homology to a B. subtilis gene of unknown function. Two others, KLMX_60133 (chitin synthesis regulation, E-value: 4e-122) and KLMX_60270 (lysine-rich arabinogalactan protein, E-value: 7e-168) were suggested to have particular functional domains but no clear orthologues were identified. The percentage of DE genes in the K. marxianus-specific group (24%) was higher than other orthogroups (2.4% to 13%), consistent with previous findings of enrichment in differential expression of evolutionary young genes under adverse conditions (Doughty et al., 2020). As we were most interested in genes that might have potential for biotechnology, we focused on the 12 upregulated K. marxianus-specific genes (Table 4). Six of these are from the set of newly annotated genes and so were absent from any previous analyses and, although all twelve genes are present in genomes of strains CBS6556 and NBRC1777, only two (NBRC1777) or three (CBS6556) are annotated with gene identifiers in those genomes, and so we use the DMKU3-1024 gene IDs for subsequent analyses and discussion for all strains. Since the CDS of most of the twelve genes are short (63 to 1713bp), there was the possibility that these genes could encode non-coding RNAs rather than proteins (Parker et al., 2018). To assess this, we examined each gene individually on the the GWIPs-viz browser (Fenton et al., 2021), which displays data from ribosome profiling and a range of other transcriptomic techniques (Supplementary Figure 2). Despite two of the genes showing low ribo-seq reads, we confirmed that all twelve genes are translated and are therefore confirmed protein-coding genes. To determine whether the identified genes are only responsive to high temperature stress, the DE analysis was repeated for the low pH and osmotic conditions used in the original study (Supplementary Table 4). The majority of genes were exclusively upregulated under high temperature growth,

though expression of one gene was also elevated under high osmotic pressure (Figure 1).

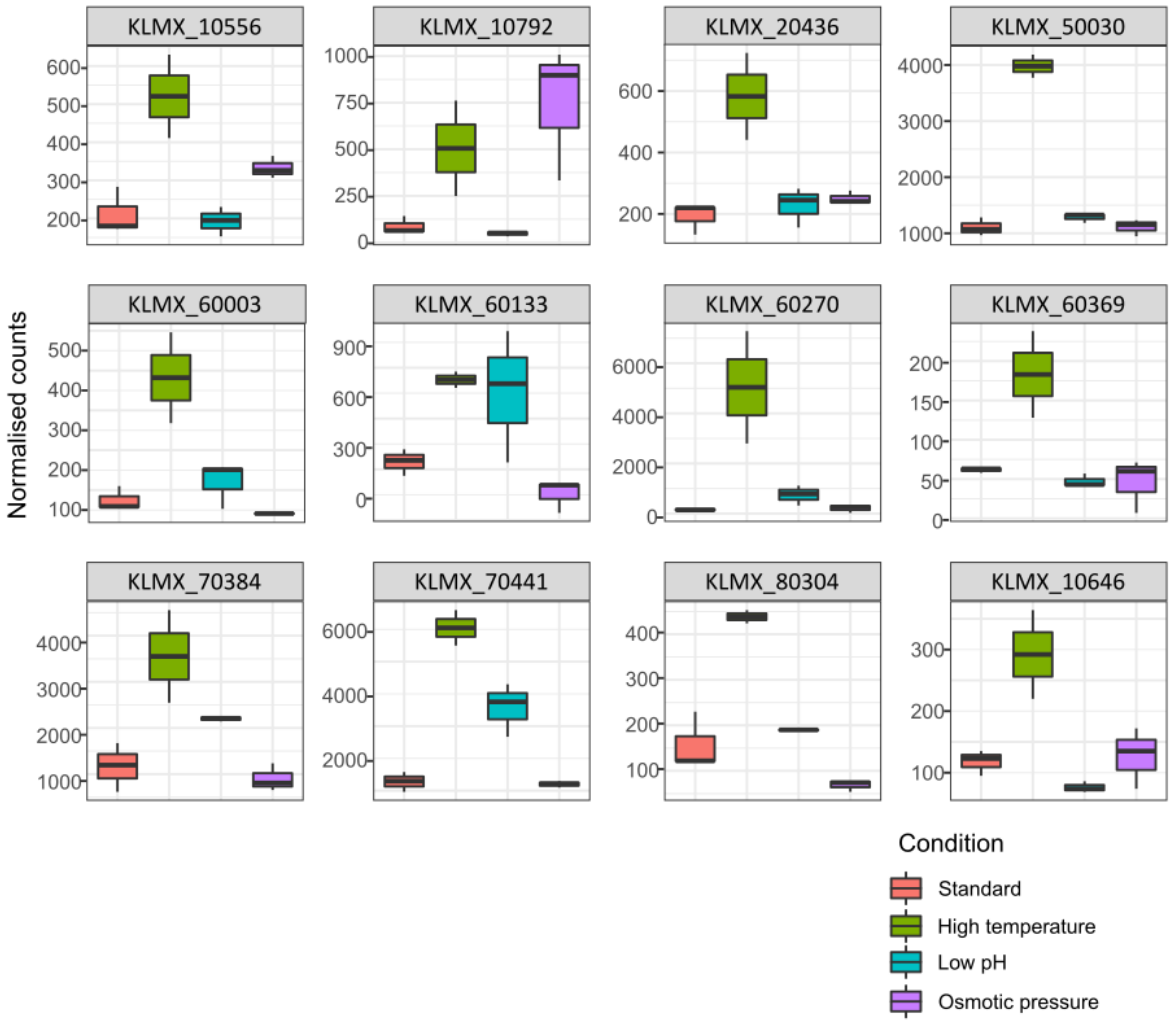

Figure 1. *K. marxianus*-specific genes are upregulated at higher temperature. Data show the expression of twelve *K. marxianus*-specific genes from chemostat cultures under different conditions. Boxplots showing normalized counts of target gene reads, representing relative abundance of transcripts at standard (30°C, pink) versus high temperature (40°C, green), low pH (3.5, blue) and high osmotic pressure (1M KCl, purple). Normalized counts for the genes were calculated using TMM normalization in edgeR. Boxplot was obtained using ggplot2.

Table 4 List of *K. marxianus*- unique genes upregulated at high temperature. List of genes unique to *K. marxianus* species, upregulated at high temperature. Where available, corresponding NBRC 1777 and CBS 6556 strains gene IDs are provided. Functional annotation deriving from Blast comparison is provided where available. Unknown = search on Blastx database shows no similarity to any other entry, but just a match with *K. marxianus* chromosomes or gene of unknown function.

* Previously unknown genes, identified mapping the Ribo-seq reads to DMKU3-1042 strain genome (Fenton et al., 2021).

Ψ Genes previously reported as upregulated under high temperature in Doughty et al., 2020.

Gene id DMKU3-1042	logFC	Gene id NBRC1777	Gene id CBS6556	Function
KLMX_10556*	1.44	NA	NA	Unknown
KLMX_10792*	2.59	NA	NA	Unknown
KLMX_20436*	1.56	NA	NA	Unknown
KLMX_50030*	1.82	NA	NA	Unknown
KLMX_60003*	2.30	NA	NA	Unknown
KLMX_60133Ψ	1.19	KMAR_60126	KMXK_0F04470	Chitin synthesis regulation
KLMX_60270Ψ	4.96	KMAR_60261	KMXK_0F03060	Lysine-rich arabinogalactan protein
KLMX_60369	1.55	NA	NA	Unknown
KLMX_70384*	1.53	NA	NA	Unknown
KLMX_70441	1.55	NA	NA	NADPH-dependent oxidoreductase
KLMX_80304	1.54	NA	NA	Unknown
KLMX_10646Ψ	1.81	NA	KMXK_0A02020	Unknown

Screening for the role of the unique upregulated genes in thermotolerance

To test whether these genes played a role in thermotolerance, eleven of the genes were individually inactivated using CRISPR-Cas9 and the endogenous NHEJ repair mechanism. Despite multiple efforts, we were unsuccessful in inactivating *KLMX_20436*, possibly because this gene may be essential. In each case, a deletion of

one to four bases occurred, resulting in frameshift mutations that inactivate the genes (Table 1). These strains, labelled ΔT1 through ΔT13, were assessed for growth on agar plates at 30°C and 47°C (Figure 2). For comparison, the thermosensitive *Δhgt* strain was used (Varela *et al.*, 2019). Out of the eleven mutants tested, only ΔT9, carrying a mutation in *KLMX_70384*, displays a growth-impaired phenotype after 24 hours growth at 47°C. For complementation tests, an expression cassette containing the *KLMX_70384* CDS under a strong *PDC1* promoter was also constructed and integrated into the genome of ΔT9 (Supplementary figure 3). Growth assays at 47°C showed that the complemented strain recovered the parental phenotype, confirming that the temperature sensitive phenotype in ΔT9 was due to inactivation of *KLMX_70384* (Figure 2). As ΔT9 has a single point mutation that could revert, for later studies, a *ΔKLMX_70384* mutant with a precise deletion of the entire CDS was constructed in a *dnl4* background (lacking NHEJ) using CRISPR-Cas9 and the HDR machinery of *K. marxianus*.

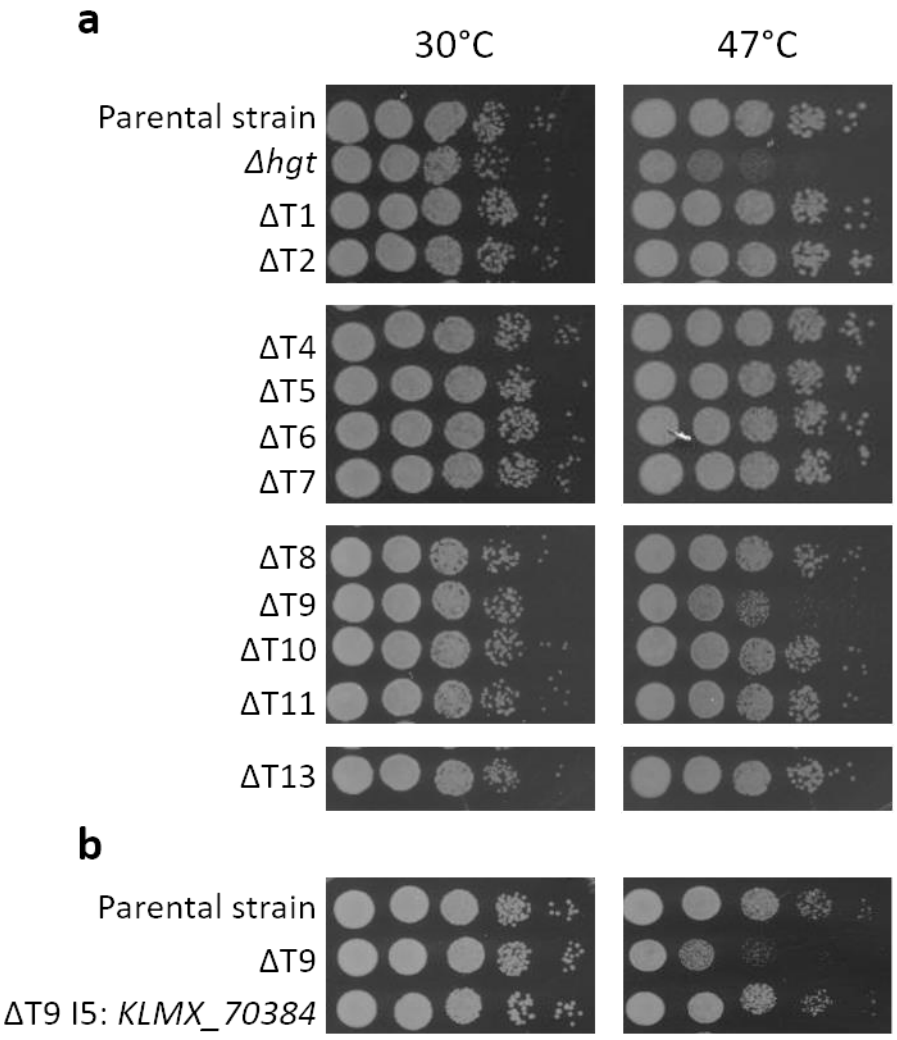

Figure 2. Assessment of growth of *K. marxianus* mutants at high temperature. a) The target genes identified in this study were inactivated by nucleotide deletion in the parental strain. The resultant knock-out mutants were plated in serial dilution on YPD plates and grown at 30°C and 47°C for 24 hours. **b)** Complementation of the ΔT9 mutant via insertion of a *KLMX_70384* expression cassette in the parental strain restores the phenotype.

Several experiments were also performed to assess growth in liquid culture (Figure 3). All eleven strains carrying the single point mutation were grown in a microtitre plate system again with only ΔT9 showing a ts phenotype, evident by an extended lag phase and lower A600nm after 26 h than the parental strain (Figure 3A, data just shown for ΔT9 and parental strain). A slower growth rate was also evident in flask culture, though the phenotype did not appear to be as pronounced as had been seen in the plate assay (Figure 3B). Next, to investigate the possible benefit conferred by *KLMX_70384* at higher temperature, a competition experiment was performed (Figure 3C). A co-culture containing equal cell number of the *ΔKLMX_70384* and parental strains was split and incubated at either 30°C or 45°C. The relative percentages of parental and

mutant were determined by differential PCR after 12, 24 and 48 h of growth. Whereas the ratio of strains remained constant at 30°C, the proportion of *ΔKLMX_70384* progressively decreased at 45°C, showing a statistically significant effect after 24 h (p-value=0.012), and complete exclusion of *ΔKLMX_70384* after 48 h.

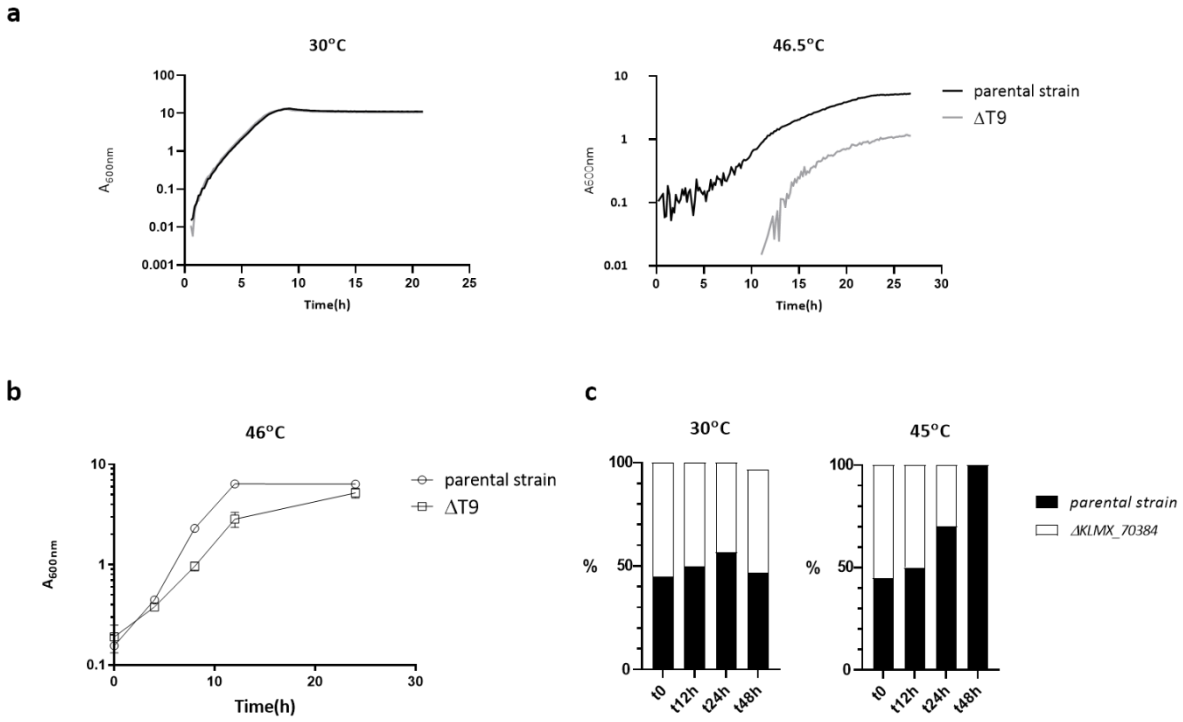

Figure 3 Deletion of *KLMX_70804* impairs high temperature adaptation of *K. marxianus*. a) Growth curves comparing growth of *K. marxianus* parental and ΔT9 strains in YPD medium at low (30°C) and high temperature (46.5°C) using a Biolector microtitre plate system. A600nm is reported in a log10 scale. The plot represents the average reads of four (30°C) and three replicates (46.5°C). **b)** Growth comparison of parental and ΔT9 strains at 46°C, individually grown in shake flask. The A600nm values represent the mean between 4 biological replicates and are plotted on a log10 scale. **c)** A competition experiment was performed, in which a 50:50 co-culture of the parental strain and *ΔKLMX_70384* strain was grown for 48 h at low (30°C) and high (45°C) temperature. The co-culture was sampled at the indicated times and serial dilutions were plated and incubated for 24 h. The proportion of parental and *ΔKLMX_70384* strains was determined using diagnostic PCR amplification of 30 randomly selected colonies at each time-point.

Computational analysis of *KLMX_70384*

KLMX_70384 is present in two versions among *K. marxianus* strains: a longer version (249 bp) coding for an 83 AA peptide, and a shorter version (225 bp) lacking a 23bp region which results in the loss of a region of the peptide between the amino-acids Ser30 and Lys39, resulting in a 68 AA peptide. An expression cassette containing both versions of the *KLMX_70384* ORF was integrated into the genome of ΔT9, and both

were able to complement the thermosensitive phenotype (data not shown), indicating that the lacking peptide region in the shorter version is not essential to the protein's function. In its longer version, *KLMX_70384* is predicted to encode an 83 AA peptide and its translation is confirmed by ribo-seq data (Figure 4A). A Blastp analysis does not find any orthologues in the database, and the only hit from a Pfam search (Mistry et al., 2021) was a low, non-significant similarity (e-value: 0.06) to a domain of human *SART-1*. *SART-1* is the orthologue of *S. cerevisiae SNU66*, which is encoded by *KLMX_20322* in *K. marxianus*. *Ab initio* modelling using Robetta (Song et al., 2013) predicts that KLMX_70384 forms a structure with three alpha-helices, oriented in a linear configuration (Figure 4B). *KLMX_70384* is located within a 2816-bp region previously annotated as intergenic on chromosome VII, between the snoRNA40 gene (*ENSRNA049515111)* and *CAF40* (*KLMX_70385*) (Figure 5). This entire region is conserved (90-100%) in sequenced strains of *K. marxianus* but bears no homology to the equivalent 2523-bp intergenic region in the closest relative, *K. lactis*, nor to any other sequences in databases. Comparison on the Saccharomyces Genome Database finds that synteny is conserved in *S. cerevisiae* but in this case the intergenic region (which lacks a *KLMX_70384* homologue) contains the autonomously replicating sequence ARS1407 (yeastgenome.org).

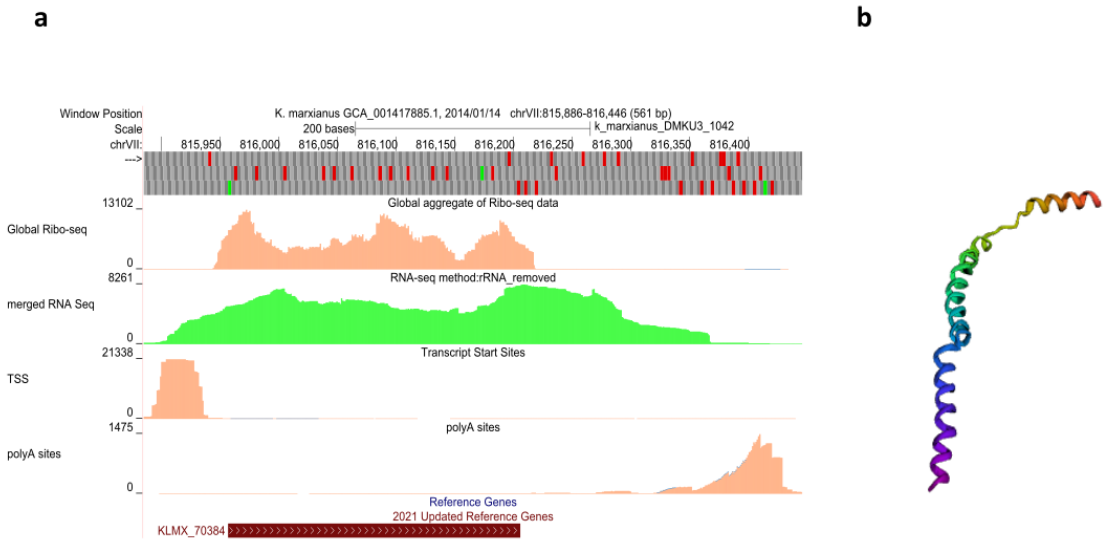

Figure 4.**Transcription, translation and structure of KLMX_70384 a)** GWIPS-viz) view of *KLMX_70384* showing, from top to bottom: Global Ribo-Seq reads (scale:0-13,102); merged RNA-seq

reads (scale:0-8261); transcription start sites (TSS, scale: 0-21338); polyA sites (scale: 0-1475). The CDS position is indicated by the red bar on the bottom track. **b)** *Ab initio* prediction of the tertiary structure of the KLMX_70384 protein with Robetta. Structure prediction is predominantly α-helical. Colour in figure from blue (N- terminus) to red (C-terminus).

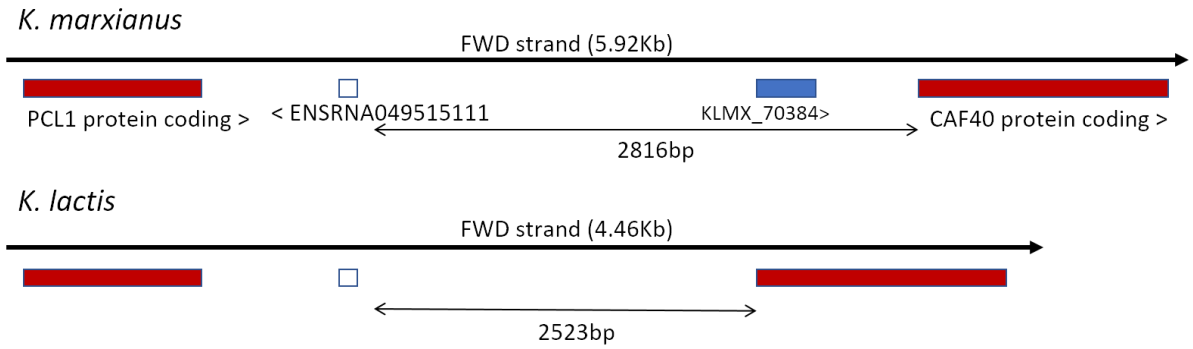

Figure 5 Genomic features of *KLMX_70384* Comparison of *KLMX_70384* genome locus between *K. marxianus* and *K. lactis*. Homologous flanking genes are reported in red, homologous snoRNA are represented by the white square, *KLMX_70384*'s CDS is represented by the blue square, and the non-homologous region between the *SNR40* and *CAF40* genes is indicated by the black arrow.

5. Discussion

The main aim of this study was to generate data to address the hypothesis put forward in a previous study that evolutionary young genes could be involved in adaption to growth under adverse conditions (Doughty *et al*., 2020). We did this by investigating genes that were upregulated during steady state growth at high temperature in *K. marxianus*. The availability of an improved annotation of *K. marxianus* that added ~170 genes and corrected mis-annotation of many others using ribo-seq data (Fenton *et al.*, 2021) was an advantage, but it also required us to perform a complete *de novo* bioinformatic analysis of the RNA-Seq data generated in our previous chemostat study (Doughty *et al.*, 2020). The outcome of this analysis was the identification of twelve genes that were unique in *K. marxianus* and upregulated at 40ºC. We successfully mutagenized eleven of the twelve candidate genes to assess their role in high temperature growth and found that inactivation of *KLMX_70384* displayed the predicted phenotype – normal growth at 30°C but compromised growth at 45 - 47°C. Interestingly, the mutant was completely outcompeted by its parent in a co-culture experiment indicating that the gene is required for competitive growth in high temperature niches. The other ten mutants did not exhibit decreased growth at higher temperatures, and future works might identify changes using competition assays or double or triple mutants to reveal new interactions between genes. Our finding that *KLMX_70384* is required for competitive growth at elevated temperature is consistent with the hypothesis that was being tested.

Evolutionarily young genes generally are short, non-essential genes and this was also true of the genes that we identified: ten of the open reading frames were between 28 and 225 amino acids, and *KLMX_20832* was the only gene that we failed to inactivate, possibly indicating that it is essential. With such short genes, there is always a concern that they are either not expressed or encode non-coding RNAs, but we confirmed using ribosome profiling data that all twelve are translated, and thus are genuine protein-encoding genes. This shows the utility of the *K. marxianus* ribosome profiling data in GWIPs-viz genome browser (Riboseq.org), a resource that is only available for few species amongst yeasts (Michel et al., 2014). Of the twelve genes, one shows evidence of having been acquired by HGT, and it was only possible to predict two putative functional protein domains in the others, illustrating the challenge of working with novel genes. The overall lack of functional annotation for the identified genes, reflects

the limitation of homology-based algorithms for genome annotation (Sinha et al., 2020), especially when it comes to species phylogenetically distant from the extensively annotated *S. cerevisiae* (Botstein & Fink, 2011). This matches the experience of previous studies with *K. marxianus, Y. lipolytica* and *Lachancea kluyveri (*Brion *et al.*, 2016; Doughty et al., 2020). *KLMX_70384* codes for a relatively short protein (83 aa) when compared with the average *K. marxianus* protein length of 500 amino acids (Lertwattanasakul et al., 2015). In yeast, proteins below 90 amino acids, are classified as small proteins (Hao et al., 2018) and are enriched for seven biochemical functions; namely, structural constituent of ribosomes, pre-mRNA splicing factor, ubiquitin-conjugating enzyme, cytochrome-C oxidase, thiol-disulphide exchange and tubulin binding. The low similarity to the splicing associated protein *SART-1*/*SNU66*, as well as its predicted linear three helix structure, encourage the speculation that KLMX_70384 may be an RNA-binding protein, possibly associated with the RNA processing or splicing machinery in some way (Hoang et al., 2016; Makarov et al., 2002). In this regard, it is notable that a group of deletion mutants for mRNA processing genes display a thermosensitive phenotype in *S. cerevisiae* (Hossain & Johnson, 2014).

The origins of species-specific genes remain opaque with different possible mechanisms postulated. The absence of any identified homologues apart from *KLMX_ 70441*, suggests that the main source is *de novo* evolution rather than acquisition by horizontal gene transfer, gene duplication, or recombination. The clue to the birth of *KLMX_70384* may lie with its location within a region of the genome previously annotated as intergenic. Notably, in *S. cerevisiae*, this intergenic region hosts an ARS which however shares no similarity with *KLMX_70384'*s sequence. Intergenic regions in yeast, are more prone to evolution and mutation, and is hence more likely for new coding sequences to originate from them (Vakirlis et al., 2018). A similar mechanism of evolution, although rare (Andersson et al., 2015), has been described before in yeast (D. Li et al., 2010). We previously suggested a scenario for evolution of genes that confer an advantage under harsh conditions (Doughty *et al.*, 2020). In that model, mutations regularly arise but those that occur in ancient genes, typically associated with core processes, are more likely to be detrimental and lost. Young genes, which are typically not required under standard growth conditions, can better tolerate mutations and so pools of mutants build up. When conditions become adverse, those

rare mutations that confer a growth advantage are selected. In this way, young genes become important for adaptation to new niches. Based on our data, we can speculate that the emergence of *KLMX_70384* played some role in helping *K. marxianus* adapt for growth at higher temperatures, a trait that is absent in all other *Kluyveromyces* species. It also helps explain why a conserved thermotolerance mechanism is not found in other yeasts, like *O. polymorpha* since it implies that different species-specific genes will be involved.

While understanding evolutionary processes is fascinating, part of the rationale for studying thermotolerance is to identify genes and processes that can be used to improve yeasts for biotechnology. One hope is that it may be possible to engineer thermotolerance into mesophilic yeasts by heterologous expression of single genes. We tested whether expression of *KLMX_70384* in *K. lactis* would improve the thermotolerance of this yeast, but it did not (data not shown). This is probably not surprising since it is generally considered that there are multiple requirements for higher temperature growth and thus overcoming one hurdle will not be enough. It is also possible that a protein such as KLMX_70384 will have specific interactions that only take place with other proteins that share an evolutionary history. Despite this, it is still valuable to try to understand what processes are required to function at higher temperature as this could identify alternative routes to strain improvement. While not definitive, the indications are that KLMX_70384 could be involved in RNA processing and is a further suggestion that this is an area worth further investigation.

6. Data availability

The RNA-seq dataset analysed in this study can be retrieved under SRA accession PRJNA531619 [https://www.ncbi.nlm.nih.gov/bioproject/PRJNA531619/]. DE analysis and OrthoFinder scripts used to analyse the standard and the high temperature RNA-seq datasets, are available at the OrthOmics page at https://github.com/SysBioChalmers/OrthOmics. Script used to compare all the stress datasets, can be found at https://rnnh.github.io/bioinfo-notebook/docs/DE_analysis_edgeR_script.html. DE analyses tables generated in this study can be found in Supplementary table 2 and 4. *K. marxianus* genome accession numbers are PRJDA65233 (DMKU3-1042) and SRX3541357 (NBRC 1777). The updated DMKU3-1042 annotation is publicly available on the GWIPs-viz genome browser (Riboseq.org).

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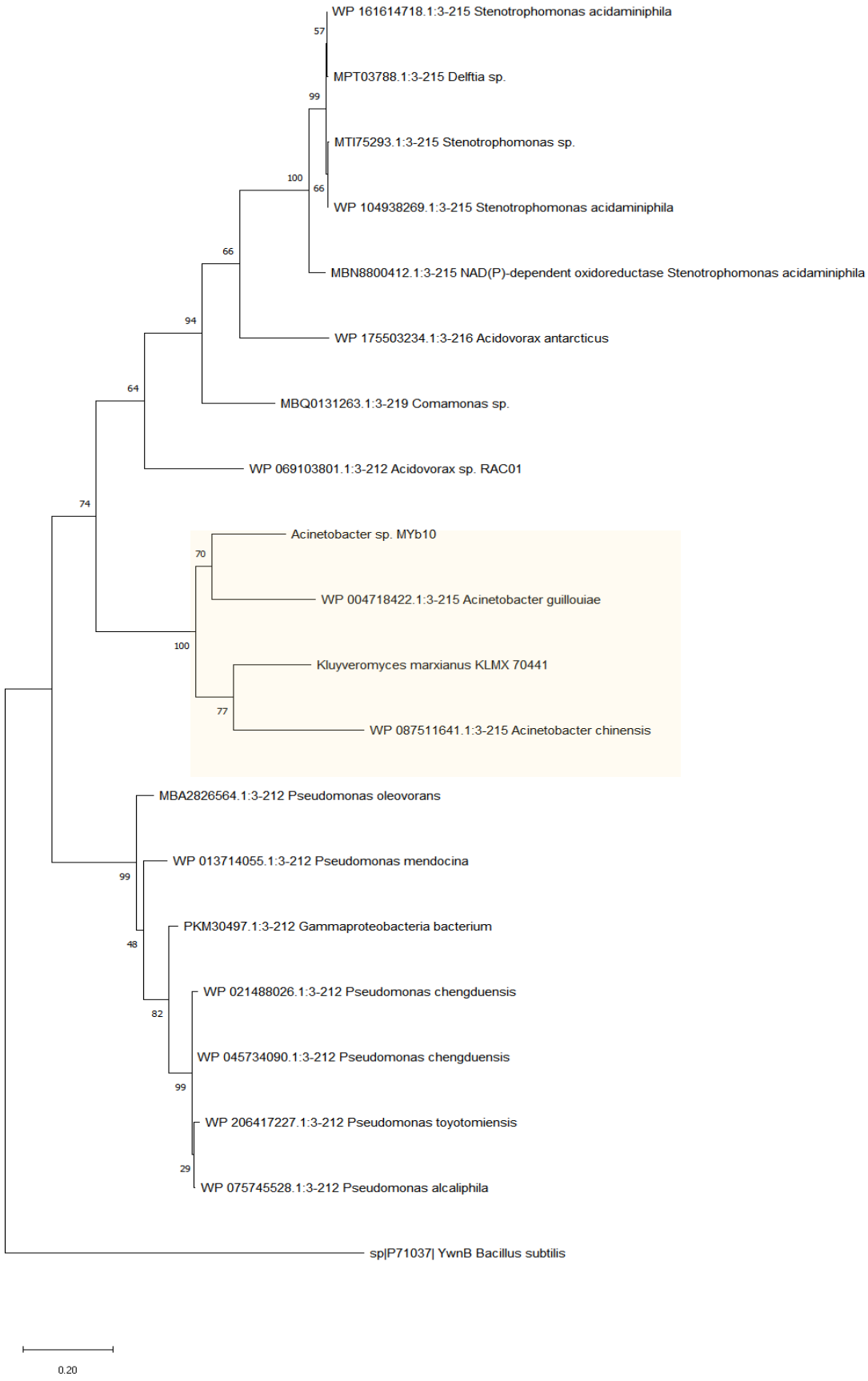
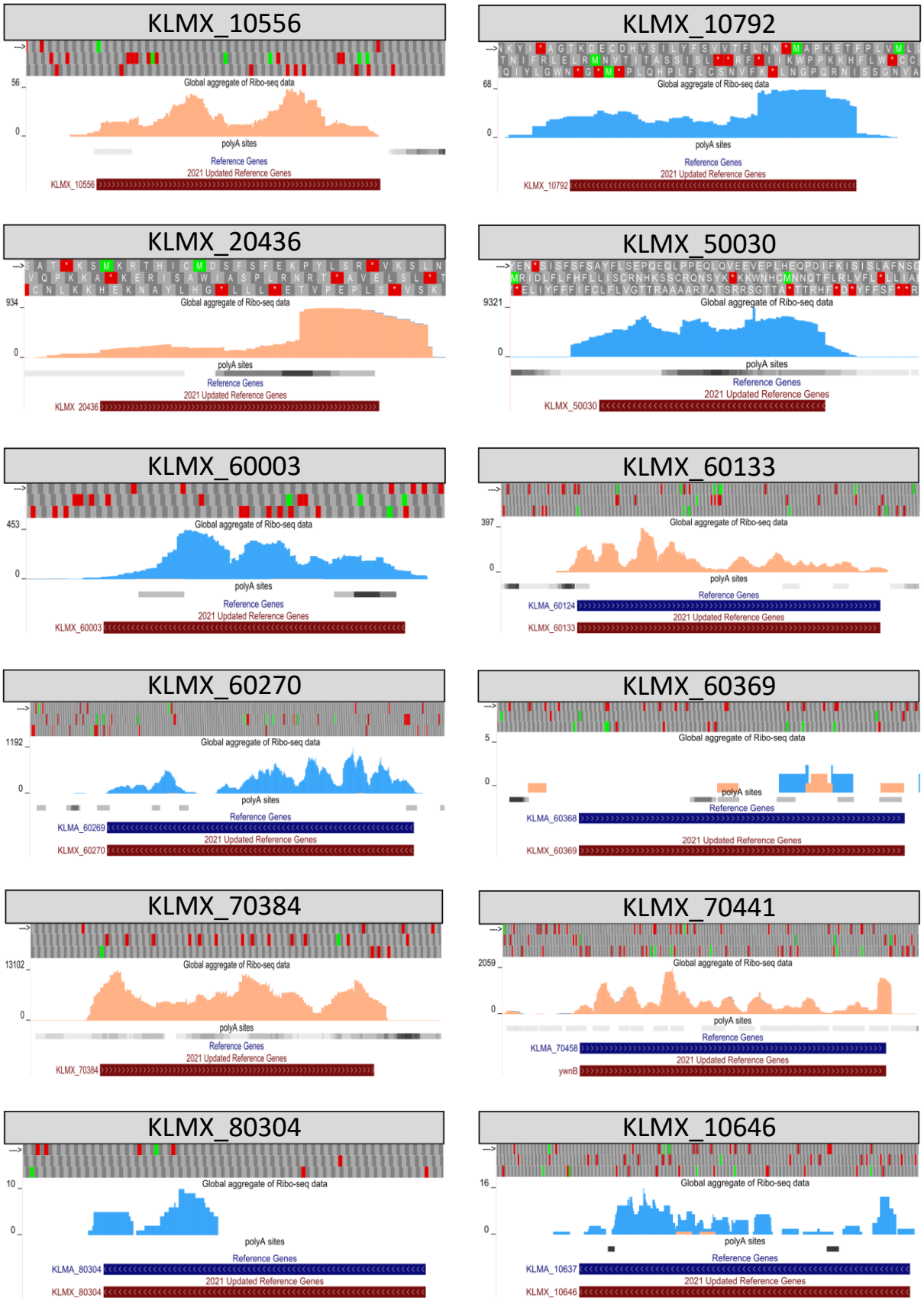
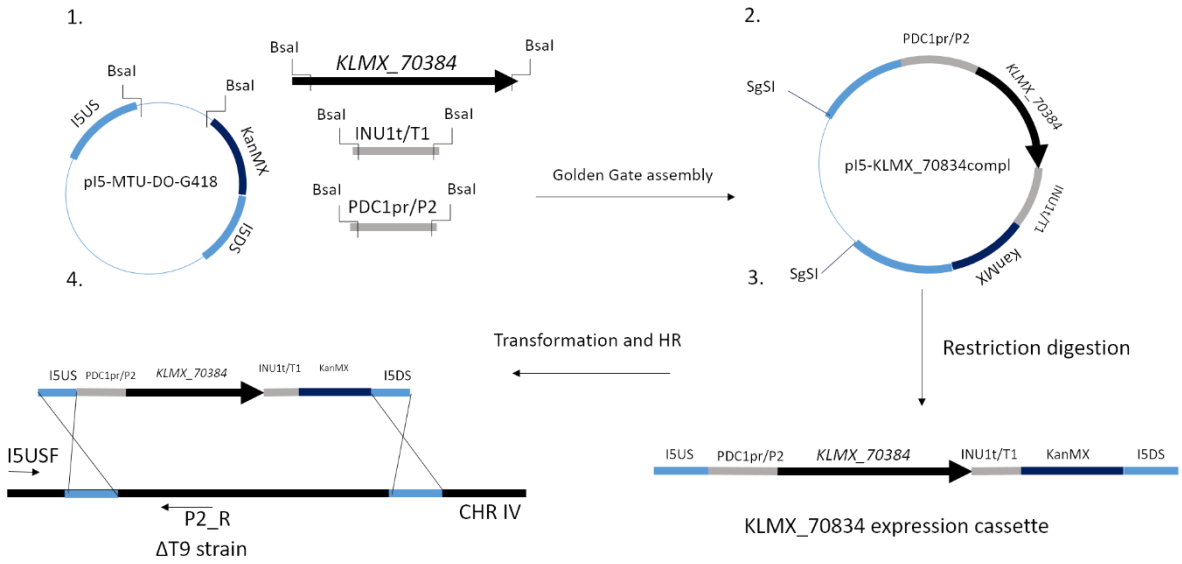
8. Supplementary figures



Supplementary figure 1. Phylogenetic comparison of *K. marxianus* KLMX_70441 with potential orthologues among the *Acinetobacter* sp. Phylogenetic analysis was carried out using MEGA6/MUSCLE. The tree is derived from maximum-likelihood analysis with 600 times bootstrapping. *K. marxianus'* KLMX_70441 clusters with a NADPH-dependent oxidoreductase protein from the *Acinetobacter* sp. (yellow). Orthologues sequences were retrieved via BlastP search, and represent the first 100 significant hits (e-value<10-5). KLMX_70441 shares 71% identity with a NADPH-dependent oxidoreductase protein from the *Acinetobacter* spp., with 97% of query coverage. The identity with *Bacillus subtilis'* YwnB is 33.4%, with 97% of query coverage.



Supplementary figure 2 GWIPS-viz data visualization for the target genes. CDS are displayed as blue and red bars (bottom track). The track contains Ribo-seq reads autoscaled and polyA sites (bottom, in black). Orange reads indicate mapping to the forward strand, blue reads to the reverse strand.



Supplementary figure 3. Schematic representation of the complementation of *KLMX_70834* knock-out mutant (ΔT9). 1) After amplification from genomic DNA, *KLMX_70384*'s CDS was inserted via Golden gate assembly into the integrative plasmid I5-MTU-DO-G418, with the regulatory parts PDC1pr/P2 and INU1t/T1 (Rajkumar et al., 2019). All parts had restriction sites for the enzyme BsaI. The plasmid pI5-KLMX_70834compl was hence obtained. **2)** Upon extraction and purification, pI5-KLMX_70834compl was digested with the enzyme SgSI. **3)** This released the *KLMX_70834* expression cassette between two 1-kb homology harms, for integration into the intergenic integration locus I4 on CHR IV. **4)** The restriction product was directly transformed into the ΔT9 strain with the LiAC/PEG method, where integration on CHR IV can happen via HR between the homology arms and the genomic locus I5, located on chromosome IV:240017…241741 (Downstream of *KmHSP104*). Single colonies of transformants were screened for locus-specific integration of the expression cassette with colony PCR and confirmed via sequencing.

Chapter V

Genomic and physiological characterization of a thermosensitive *K. marxianus* mutant

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NM designed and performed the experiments, performed the SNPs analysis and wrote the manuscript

DF performed the genome sequences assembly and SNPs calling

JM helped designing the experiments, provided supervision and edited the manuscript

1. Abstract

Kluyveromyces marxianus displays innate thermotolerance, a desirable trait for biotechnological application; however, the reasons underlying this trait are mostly unknown. In yeast, high temperatures trigger reactive oxygen species (ROS) accumulation, therefore the thermal stress response is tightly connected to the oxidative stress response due to a crosstalk between signalling mechanisms. In this study, we performed a physiological and genomic comparison between two *K. marxianus* strains, ATCC 26548 and UCC01, which is ultimately derived from *K. marxianus* ATCC 26548 but displays a thermosensitive phenotype. The physiological comparison in bioreactor outlined that UCC01's thermosensitive phenotype is linked to aeration and oxygen availability. Accordingly, UCC01 accumulates ROS at 45 °C and shows an oxidative stress sensitivity. The SNPs analysis found five SNPs-containing genes in UCC01, whose function is related to oxidative and general stress response. Although individual deletion of these genes in ATCC 26548 did not reproduce a thermosensitive phenotype, deletion of *CYC8* and *YAK1* resulted in an oxidative stress-sensitive phenotype. Finally, although the phenotype of UCC01 could not be fully explained from this study's approach, the importance of a crosstalk between oxidative and thermal stress response in *K. marxianus* was unveiled, suggesting that its innate thermotolerance could be linked to it. More studies are necessary to describe the details of *K. marxianus*' stress response's signalling.

2. Introduction

During industrial fermentations yeast strains are susceptible to multiple stresses, such as oxidative and high temperature stress (Matallana and Aranda 2017; Gibson et al. 2008; Saini et al. 2018; Burphan et al. 2018; Fu et al. 2019), which have the potential to impair the fermentation's performance. The necessary aerobic growth of the strains naturally causes the production of reactive oxygen species (ROS). ROS naturally originate in yeast from both the peroxisomes and the ER-reticulum, during lipid and protein oxidation (Perrone et al., 2008). However, the main source of ROS during aerobic growth is mitochondrial, from electrons escaping the respiratory chain during oxidative phosphorylation (Davidson and Schiestl 2001; Postmus *et al.*, 2011; Baez and Shiloach, 2014); this phenomenon is increased by higher oxygen availability during the fermentation (Baez & Shiloach, 2011). ROS accumulation, known as oxidative stress, can be detrimental to growth because it damages the main biological macromolecules, such as nucleic acids, proteins and lipids (Farrugia & Balzan, 2012) and even influence protein biosynthesis (Topf et al., 2018) and mitochondrial biogenesis (Bouchez & Devin, 2019).

Heat stress is also known to cause ROS accumulation in yeast (Fedoseeva *et al*., 2017; Zhang, Shi, and Jiang 2015b). Heat stress-caused oxidative stress derives by the decrease of the mitochondrial membrane's potential at high temperature, resulting in intracellular redox imbalance (Zhang et al., 2015a) which affects the entire cell's homeostasis. Evidence suggest that heat stress-generated oxidative stress mainly effects the cells in a metabolism-dependent fashion. In fact, respiration generates more oxidative stress than fermentation (Morano, Grant, and Moye-Rowley 2012; Davidson et al. 1996) and yeast cells cultured anaerobically are more resistant to heat shock than those grown in the presence of oxygen (John F Davidson & Schiestl, 2001). In addition, slower growing strains with impaired respiratory metabolism not only accumulate less ROS, but also show less sensitivity to high temperature (Lu et al., 2009).

A thermal and oxidative stress cross-tolerance is normally acquired by temperature adapted yeast strains (Lin et al. 2021; Kitichantaropas *et al*., 2016), indicating the responses to the two stresses overlap to a certain extent. Moreover, many stress responsive transcriptional factors are known to regulate genes involved in both heat

stress response (HSR) and oxidative stress response (OSR), further indicating a cross-talk between the two (Morano et al., 2012). The response to elevated ROS levels is a crucial step in preventing cell death from loss of redox balance, and depends on multiple transcriptional activators. As an example, in *S. cerevisiae* the transcription factor Yap1 has been described to counteract heat-induced oxidative stress by induction of antioxidant genes, including the glutathione biosynthesis genes *GSH1* and *GSH2* under aerobic conditions (Sugiyama et al., 2000), and its expression is boosted by H_2O_2-induced oxidative stress (Delaunay et al., 2000). Also the chromatin remodelling complex SWI/SNF (Du et al., 2020) is known to regulate genes involved in both heat-stress (Shivaswamy & Iyer, 2008) and oxidative stress response (Sahu et al., 2020). Finally, the main heat-response associated transcriptional factors Hsf1 and Msn2/Msn4 undergo respectively cellular relocation and change of conformation in response to superoxide and high temperature stress (Boisnard et al. 2009; Lee et al. 2000).

Thermotolerance is a relevant trait for the biotechnological application of *Kluyveromyces marxianus*, allowing to reduce cooling costs and contamination's risks during industrial fermentations (Karim et al., 2020). The reasons for *K. marxianus'* innate thermotolerance remain mostly unknown, although peculiar metabolic rearrangements were reported during the yeast's growth at high temperature (Lehnen, Ebert, and Blank 2019). In addition, the presence of an oxidative stress response was observed during growth of *K. marxianus* under high temperature (Lertwattanasakul et al. 2015). Moreover, increased generation of reactive oxygen species (ROS) and a lowered NADH/NAD+ ratio were indicated as responsible for the growth arrest of a *K. marxianus* strain at 45 °C during high temperature ethanol fermentation (Fu et al. 2019). These facts taken altogether suggest the oxidative stress response is paramount for *K. marxianus* thermotolerance. Regarding *K. marxianus'* ROS detoxification responses, an upregulation of glutathione and glycerol synthesis genes, and of expression of the superoxide dismutase (SOD) enzyme were reported (Saini, Beniwal, and Vij 2017; Pinheiro et al. 2003). Moreover, a comparison with *S. cerevisiae* found that an industrial thermotolerant *K. marxianus* strain showed good antioxidant capacity derived by higher catalase activity, lower level of lipid peroxidation and membrane enriched in saturated fatty acids (Mejía-Barajas et al., 2017). These findings suggests *K. marxianus'* ability to deal with oxidative stress is paramount for

its thermotolerance. However, the extent of the role of oxidative stress over thermotolerance is mostly uncharacterized from a physiological and genomic point of view, during aerobic growth and particularly in relation to varying levels of oxygen availability.

The starting point for this study was our observation that an in-house lab stock of a *K. marxianus* strain displayed a temperature-dependent phenotype, not reported in the original strain, and of course not typical of the species. This strain was originally isolated in Mexico, deposited in the Institute of Mexico's collection as NRRL Y-7571 (Herrera T, Ulloa M, 1973) and then in the ATCC culture collection as *K. marxianus* ATCC 26548. Subsequently, a stock went to the Westerdijk Fungal Biodiversity Institute collection, where it is named *K. marxianus* CBS 6556, and from there to the National Collection of Yeast Cultures in the UK as strain NCYC 2579. It is also in the Korean yeast collection as KCTC 17555 (Jeong et al., 2012). The strain has also been quite widely distributed between research laboratories and the precise ancestry of the strains used in every publication is not certain. Interestingly, previous authors noted some differences between different studies purporting to use the same strain *K. marxianus* CBS 6556 and suggested that lab stocks may have acquired mutations (Fonseca et al., 2008). In our lab, we had acquired both *K. marxianus* CBS 6556 and *K. marxianus* NCYC 2579 at different times and could not say with certainty which of these was the parent of the strain that we now noted to be temperature sensitive. We decided, however, that this isolate, which we named *K. marxianus* UCC01 for this study, offered an opportunity to identify traits and genetic loci that could be required for thermotolerance. To do this, we first carried out a series of phenotypic assays to compare the physiology of UCC01 with newly acquired stocks of *K. marxianus* ATCC 26548 and *K. marxianus* CBS 6556. We found that UCC01 was both temperature sensitive and sensitive to oxidative stress. Furthermore, when the strain was grown under low aeration conditions, the temperature sensitivity was alleviated, indicating the temperature sensitivity was due to the accumulation of reactive oxygen species. In an effort to identify the genetic basis of this, we carried out de novo whole genome sequencing (WGS) of *K. marxianus* ATCC 26548 and *K. marxianus* UCC01. A new WGS was also available for CBS6556 so we compared all three genomes. This identified candidate polymorphisms and some of these were tested by mutagenesis. While we were not able to explain all the phenotypes, we established that deletion of

SWI1 and *CYC8* lead to sensitivity to oxidative stress in *K. marxianus*, providing a partial explanation of the UCC01 phenotype.

3. Materials and methods

Strains and cultivations

A complete list of strains used in this study is reported in Table 1.Yeast strains were routinely grown on YPD medium (2% Peptone, 1% yeast extract, 2% Dextrose). Hygromycin B (200 µg ml^{-1}, SigmaAldrich, St. Louis, MI, United States) was used for selection where required. For drop tests plates and batch fermentation, the cells were cultured in mineral medium (Verduyn et al., 1992) containing a final concentration per litre of: $(NH_4)_2SO_4$, 5.0 g; KH_2PO_4, 3.0 g; $MgSO_4 * 7H_2O$, 0.5 g; a trace elements solution (EDTA, 15 mg; $ZnSO_4 * 7H_2O$, 4.5 mg; $MnCl_2 * 2H_2O$, 0.84 mg; $CoCl_2 * 6H_2O$, 0.3 mg; $CuSO_4 * 5H_2O$, 0.3 mg ; $Na_2MoO_4 * 2H_2O$, 0.4 ; $CaCl_2 * 2H_2O$, 4.5 mg; $FeSO_4 * 7H_2O$, 3.0 mg; H_3BO_3, 1.0 mg; KI, 0.1 mg), and a solution of vitamins (d-biotin, 0.05 mg; calcium pantothenate, 1.0 mg; nicotinic acid, 1.0 mg; myo-inositol, 25 mg; thiamine HCl, 1.0 mg; pyridoxin HCl, 1.0 mg; and para-aminobenzoic acid, 0.20 mg). The medium was buffered to pH 5.5 with KOH and supplemented with glucose at a final concentration of 20 gl^{-1} (for the drop test) or 10 gl^{-1} (for batch cultivations).

For the drop tests, strains were grown overnight in 10mL of YPD medium at 30°C, in a shaking incubator at 330 RPM. In the morning, cells were washed once and diluted to A_{600nm} of 1, in sterile water. 5uL of 1:10 serial dilutions (until 10^{-5}) of each strain was spotted on YPD or MM-Glu plates containing menadione (45mM and 75 mM) or H_2O_2 (5mM) to test for oxidative stress tolerance. The plates were incubated in a 30°C or 45°C static incubator for 24h. To test different oxygenation levels during the drop test, the plates were incubated in an air-tight cylinder with Oxoid ™ CampyGen ™ 2.5L sachets, which ensured partial oxygen deprivation (atmosphere reduced to 6% oxygen). *E. coli* DH5α strain was used for cloning purposes. The strain was maintained in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, 10 g L^{-1} NaCl) supplemented with 100 µg mL^{-1} ampicillin when required.

Table 1. List of strains used in this study.

Strain	*Genotype*	*Mutation coordinates NBRC 1777 genome*	*Source*
NBRC 1777	WT		Biological Resource Centre, NITE (NBRC; Tokyo Japan)
CBS 6556	WT		Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands)
ATCC 26548	WT		American Type Culture Collection (ATCC)
NCYC 2597	WT		National collection of yeast culutres (Norwich, UK)
UCC01			This study
ATCC 26548 dnl4	*ΔKMAR_80037*	CHR VIII: Δ99943-100181	This study
ATCC 26548 cyc8	ATCC 26548 *dnl4 ΔKMAR_80288*	CHR VIII: Δ649876-650427	This study
ATCC 26548 swi1	ATCC 26548 *dnl4 ΔKMAR_60368*	CHR VI: Δ796804-797461	This study
ATCC 26548 bnr1	ATCC 26548 *dnl4 ΔKMAR_20210*	CHR II: Δ472693-473199	This study
ATCC 26548 yak1	ATCC 26548 *dnl4 ΔKMAR_10318*	CHR I: Δ694820-695185	This study
ATCC 26548 ΔKLMX_30726	ATCC 26548 *dnl4 ΔKMAR_30702*	CHR III: Δ850751-851594	This study

Bioreactor fermentations and physiological parameters calculation

Bioreactor cultivations were performed in batch mode in 4 L Applikon vessels (Applikon, the Netherlands) individually connected to EZ-controllers (Applikon, the Netherlands). A calculated volume of the overnight culture was added, to achieve an initial A600nm of 0.1 in the bioreactor. Batch cultivation conditions were 30°C (standard condition) or 45°C (high temperature condition), 2 L working volume with pH maintained at 5.5 through addition of 1M KOH. The culture was sparged with air at a flow rate of 2 L min^{-1} (for high aeration condition) or 0.5 L min^{-1} (for low aeration condition) and agitated at variable RPM, in order to maintain a dissolved oxygen (DO) concentration above 20% of saturation.

After inoculation (T_0), the bioreactors were sampled bi-hourly for 12 hours. Approximately 1 mL of sample was withdrawn, to allow for A600nm measurement and HPLC analysis. A final sample was taken after 24h to confirm end of growth. Since the strain CBS 6556 displayed slower growth under 30°C 2 $Lmin^{-1}$ aeration condition, samples were taken every hour for 18 hours. The physiological parameters

were calculated as in Fonseca et al., (2007) during the exponential growth phase. The exponential growth phase (EGP) was identified as the linear regression on an ln (A_{600nm}) vs. time plot. The maximum specific growth rate (μ_{max}) was determined as the slope of this linear region. The glucose uptake rate (μ_s) was calculated as the slope of the linear regression on an ln (gl^{-1} of residual glucose) vs. time plot.

Gas measurement and analytical methods

CO_2 and O_2 outlet gasses was measured in continuous mode using pre-calibrated GXA – gas analysers (GasX, Ltd, UK). For CO_2 sensor calibration, two defined mixes of gas containing carbon dioxide (5%), and nitrogen (100%) were used for a two-point calibration. For O_2 sensor calibration, two defined mixes of gas containing oxygen (20%) and nitrogen (100%) were used. Atmospheric CO_2 and O_2 inlet concentrations were estimated at 0.04% and 21% respectively. Oxygen uptake rate (OUR) was calculated as in Fonseca et al., (2007) from oxygen and carbon dioxide content in the inlet and exhaust gases, respectively. Nitrogen was taken as inert gas in the balance equation (Heinzle & Dunn, 1991). Extracellular metabolites and residual glucose during bioreactor cultivations were measured using high performance liquid chromatography (HPLC). 900 µL of obtained sample was centrifuged and 800 µL of supernatant was injected into a Hewlett-Packard (HP) 1090 instrument using a Bio-Rad HPX-87H hydrogen ion resin column and an HP 1047A external refractive index detector. The mobile phase, 0.001 N H_2SO_4, was run at 55°C at a flow rate of 0.6 ml min^{-1}. The absorbance at 600 nm (A600nm) was measured in a spectrophotometer from 1mL cell-suspension, diluted as required.

ROS accumulation and viability measurement

Percentages of ROS-containing cells and viability were measured on exponentially growing cells during the bioreactor cultivation in batch mode via flow cytometric analysis, as described in (Signori et al., 2014). Dead or severely compromised cells were detected following Propidium Iodide (PI, Sigma-Aldrich CO., St. Louis, MO, USA) staining. Briefly, cell were washed twice with buffer (TrisHCl 50 mM, MgCl2 15 mM, pH 7.7), resuspended in a PI solution (0.23 mM), incubated in the dark on ice for 20 min and then analysed by flow-cytometry. Reactive oxygen species (ROS) were detected by Dihydrorhodamine-123 (DHR-123, Sigma-Aldrich CO., St. Louis, MO,

USA). Briefly, cells were incubated for 2 h with Dihydrorhodamine-123 (5 µg mL−1 from a 2.5-mg mL−1, stock solution in ethanol), washed twice with PBS buffer and then analysed by flow-cytometry. For both PI and DHR-123 staining positive and negative controls were performed. The PI positive control was obtained by killing cells with 70% ethanol; the ROS positive control was obtained by adding to the medium H_2O_2 to a final concentration of 10 mM. Samples were analysed using a FACScelesta (BD Biosciences) flow cytometer equipped with an Argon ion laser (excitation wavelength 488 nm). The fluorescence emissions were measured through detectors for 530/30 nm and 670nm band pass filters for DHR-123 and PI signals respectively. The sample flow rate during analysis did not exceeded 500 cells s−1. A total of 25000 cells were measured for each sample. The flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Life Sciences).

Genome sequencing and SNPs analysis.

Genomes of *K. marxianus* UCC01 and ATCC 26548 strains were sequenced and filtered from BGI (https://www.bgi.com/global) using the platform DNAbseq™, which generated 22,199,568 and 22,053,920 clean reads for ATCC 26548 and UCC01 strain respectively, with a Q20 of 97,29 and 97,70 respectively. Reads length was 100 bases, paired ends. Adapter sequences and low-quality reads were filtered using the SOAPnuke software developed by BGI, according to their in-house pipeline. *K. marxianus* DMKU3-1042 strain genome sequence (Fenton et al., 2021) was used as a reference, because contains the most updated annotation. The sequencing reads were assembled using SPAdes (v3.13.0). The scaffold level genome assemblies of these strains ("ATCC_scaffolds.fasta" and "UCC01_scaffolds.fasta") were annotated using Augustus (v3.3.3). Orthologous CDS between UCC01, ATCC 26548 and DMKU3-1024 strains were identified via Orthofinder. Orthologous tables are available in supplementary table 1.

A newly published genome sequence of CBS 6556 strain (Dekker *et al.*, 2021) was included in the genome comparison analysis. The complete pipeline for SNP analysis used in this study is reported in figure 1. In brief, ATCC 26548, CBS 6556 and UCC01's strains' genomes were compared in a pairwise comparison to the reference strain DMKU3-1024's genome via NucDiff v2.0 (Khelik et al., 2017). Using the same software, ATCC 26548's genome was also compared to CBS 6556 and UCC01's

genomes respectively. The pairwise comparisons of CBS 6556 and UCC01 strains were filtered to exclude variants in common with ATCC 26548's pairwise comparison, yielding a "CBS 6556 vs ATCC 26548" and a "UCC01 vs ATCC 26548" list of variants. The UCC01 vs ATCC 26548 list was further filtered to remove identical variants with the CBS 6556 vs ATCC 26548 list; obtaining UCC01's unique SNPs. The effect of the variants identified by the pairwise comparison on the protein coding sequences was then predicted using SnpEff software (Cingolani et al., 2012). The genomic and functional annotation of DMKU3-1042 strain was used as a template to predict the variants' effect; therefore, DMKU3-1042's genes ID were used (Fenton et al., 2022). Presence of the variants for the remaining target genes was confirmed via Sanger sequencing of the locus of the gene that is predicted to contain the variant. CBS 6556 strain's loci were sequenced too, in addition to ATCC 26548 and UCC01 strains.

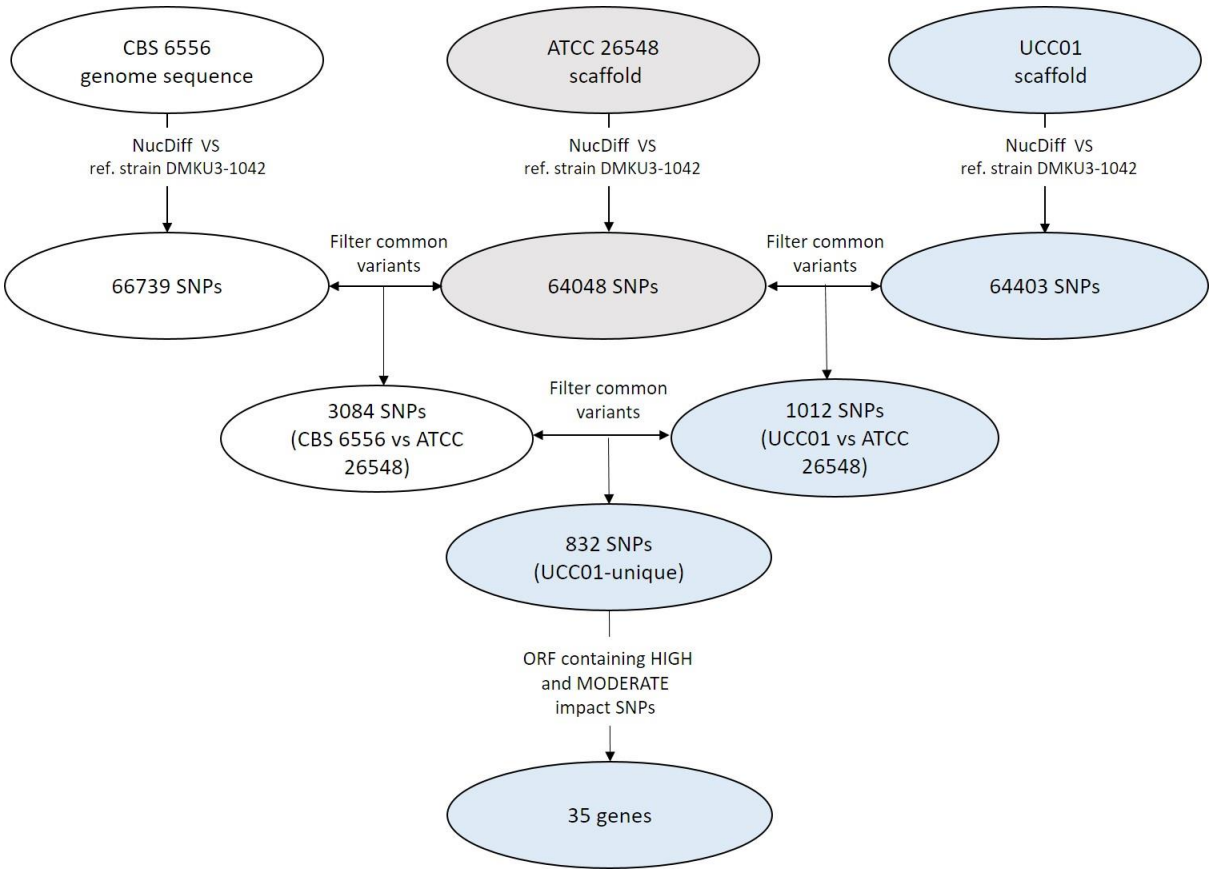

Figure 1. SNPs analysis of UCC01 genome identified variants in 35 genes of interest. In order to identify the genomic polymorphisms of UCC01 strain, a SNPs analysis was performed through comparison with DMKU3-1042 first and with ATCC 26548 and CBS 6556 WT strains, same strains from different collections, then. The pipeline, here represented, identified 35 SNPs-containing genes in UCC01. The genome annotation of DMKU3-1042 was used as a reference for the pairwise comparison of CBS 6556, ATCC 26548 and UCC01 strains. Common variants between CBS 6556 and ATCC 26548, and UCC01 and ATCC 26548 were filtered out, generating respectively CBS6556 vs ATCC26548 and UCC01 vs ATCC26548 lists of variants. CBS6556 vs ATCC26548 and UCC01 vs ATCC26548 lists were compared to eliminate common variants, identifying a UCC01-unique list of variants. The effect of such variants on the encoded protein was predicted using SnpEff, which identified 35 SNPs-containing ORF in UCC01 strain with high and moderate impact on the protein.

Molecular Techniques

The ATCC 2654 *dnl4* strain was obtained using the same CRISPR-Cas9 system and gRNA previously used to obtain NBRC 1777 *dnl4* strain (Rajkumar et al., 2019). The *cyc8*, *swi1*, *bnr1*, *yak1* and *ΔKLMX_30726* knock-out mutants (Table 1) were constructed in the ATCC 26548 *dnl4* strain by deletion of portion of the coding genes, using the CRISPR-Cas9 with repair fragment described in (Rajkumar and Morrissey 2021). In brief, targeting sequences (for gRNAs) were ordered as DNA primers and inserted into plasmid pUCC001 (Rajkumar et al., 2019) by Golden Gate assembly. As this construction relies on homology-dependent repair, the repair fragment was built by designing two primers which shared 20bp overlap and had approximately 80bp of homology to the 5' and 3' regions flanking the CRISPR-Cas9 targeting sequences. The 5' and 3' homology arms were annealed and amplified via overlap extension PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Successful construction of the repair fragment was confirmed by gel electrophoresis and 1ug of repair fragment was co-transformed with the CRISPR-Cas9 plasmid carrying target genes-specific gRNA, into ATCC 26548 *dnl4* strain using the standard LiAc/SS carrier DNA/PEG method with selection for transformants on Hygromycin B (200 µg mL^{-1}). gRNA primers were designed using the gRNA software (Xie et al., 2019; Rajkumar and Morrissey 2020). Target genes' deletion was confirmed from the amplicon size of the PCR product. A complete list of primers used in this study is reported in Table 2.

Note on gene IDs

Several genome annotations exist for different *K. marxianus* strains. In this study, the genomic and functional annotation of DMKU3-1042 strain was used as a template to predict the variants' effect; therefore, DMKU3-1042's genes IDs, with the "KLMX"

tags (Fenton et al., 2022), were predominantly used. However, the knock-out mutants were obtained in the strain NBRC 1777, for which an in-house annotation is available, presenting the "KMAR" gene tags. Therefore, the NBRC 1777's gene IDs (KMAR) equivalent to DMKU3-1042's, are reported for the molecular work in Table 2. For clarity, the relative functional annotation of the genes is provided. The equivalent gene IDs between the two strains' genome annotations was obtained from a previously performed orthology comparison between the two strains' protein sequences.

Table 2 List of primers and repair fragments used in this study.

Primer	***Sequence***	***NBRC 1777 Gene ID***	***Gene name***
gRNA primers:			
dnl4_hh_F	cgtcaaccatttaaactctggaga	*KMAR_80037*	*DNL4*
dnl4_hh_R	aaactctccagagtttaaatggtt		
cyc8_hh_F	cgtcggtatcctttacgaccggtacgg	*KMAR_80288*	*CYC8*
cyc8_hh_R	aaacccgtaccggtcgtaaaggatacc		
swi1_hh_F	cgtcccggaagagctcaaactgcttgg	*KMAR_60368*	*SWI1*
swi1_hh_R	aaacccaagcagtttgagctcttccgg		
bnr1_hh_F	cgtcttatcggcacttgcgcatca	*KMAR_20210*	*BNR1*
bnr1_hh_R	aaactgatgcgcaagtgccgataa		
yak1_hh_F	cgtcgatcagcatgtgaggagact	*KMAR_10318*	*YAK1*
yak1_hh_R	aaacagtctcctcacatgctgatc		
KLMX_30726_hh_F	cgtccttcttcgtccaagtcgcttagg	*KMAR_30702*	
KLMX_30726_hh_R	aaaccctaagcgacttggacgaagaag		
Diagnostic primers to confirm mutations:			
nl4_dia_F	ataaatccattcataaggcaact	*KMAR_80037*	*DNL4*
dnl4_dia_R	tgcggaaaccttctgtatataa		
cyc8_dia_F	tgaaacaaccagcaaaacca	*KMAR_80288*	*CYC8*
cyc8_dia_R	gtacaagacaccgatagagc		
swi1_dia_F	ttttggccaactcttcacc	*KMAR_60368*	*SWI1*
swi1_dia_R	cagtctatgttttctcctgct		
bnr1_dia_F	aattttgaaatacctatcggaccat	*KMAR_20210*	*BNR1*
bnr1_dia_R	tatccgccttagacttgtct		
yak1_dia_F	ctgtaacagaggccaaagt	*KMAR_10318*	*YAK1*
yak1_dia_R	atcgcctagttgtagcttgt		
KLMX_30726_dia_F	atgggcgagcatataccg	*KMAR_30702*	
KLMX_30726_dia_R	gacatcggtccatatcttgg		
Repair fragments:			
dnl4_RF	gtgtccgagttcttcggtaatctgagataatgacacaatgctttgattaaagtgttttcttttatattataaaccctcttctagctccaaca aagattgttgtgattctttctgttctttttctcatgaaccaagaaacactcattgttagaagttgaca		
cyc8_RF	ccgcaatggcctacgatgccactctacaacacaacccatcctcgacgaaagcccttacatcgttggcccaacgtttccttctacgat cctcaaaaggccctaaatctgctattgaagtcgttggagatagactctactga		
swi1_RF	ctctccaccgttaagtttttgaacatacatgtataaaataaagagttgtgccaattgttcctcgacaagttgtggtgatcccccggctat attatgtggagtacc		
bnr1_RF	aagaagaaagagattgaagacttgaaatccgaattacaattgcttagggatgagaaacaagtgtcaaaaggtgatttactgcagg aattatgtttagtatcatgaggaattttggaagctgcaaatggcacagaagtagattctgaaattcctgcagtaaatcaccttt		
yak1_RF	acctatatgaattgttaaagctaaatgaattccatggtctaagcatgactttaataaaaaggggaagaactcaaagaagttctaaatt gtactcgtctatcgttttcatcctaaacttttcagcactgttgtgttcatcttatggaaatacttctttgagttcttcccc		
KLMX_30726_RF	ttcaacaagtcgaagtacatcgcgttgcagcagcagaaacagatcagaaggatgaacctggactcgctggacaacgtcgtcata gagggcgacatacggctgatgggagagaacttccagttccagttcgattccatcg		

4. Results

The *K. marxianus* strain UCC01 is thermosensitive and displays increased oxidative stress sensitivity.

A stock of a *K. marxianus* strain, here called UCC01, displayed a temperature-sensitive phenotype not reported in the original strain CBS 6556 (nor NCYC 2597). In fact, having acquired both *K. marxianus* CBS 6556 and *K. marxianus* NCYC 2597 (same strain from different collections) at different times, we could not say with certainty which of these was the parent of UCC01 strain. This isolate however, because of its thermosensitive phenotype, offered an opportunity to identify traits and genetic loci that could be required for thermotolerance. To do this, we first carried out a series of phenotypic assays to compare UCC01 with newly acquired stocks of *K. marxianus* ATCC 26548 and *K. marxianus* CBS 6556. The strain NBRC 1777 was used since it is a strain routinely used from us for molecular work and known to be very robust. First, UCC01's growth at high temperature was compared to the other strains (Figure 2A). The strain was the only one showing growth impairment at 45°C among the tested strains.Since high temperature and oxidative stress are correlated in yeast, the strains' oxidative stress sensitivity was subsequently assessed on increasing concentrations of menadione (45 and 75 mM), a free-radical inducer (Kim et al. 2011) at 30°C and 45°C (Figure 2B), confirming that UCC01 is sensitive strain to both high temperature and oxidative stress.

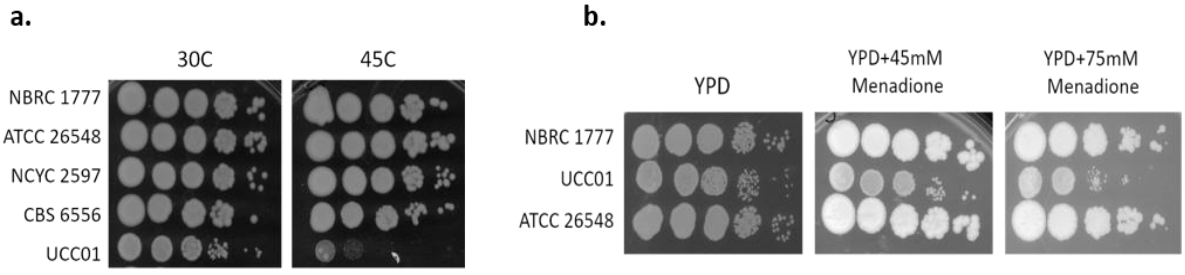

Figure 2. UCC01 strain is thermosensitive and shows increased sensitivity to oxidative stress. a. The strains ATCC 26548, NCYC 2597 and CBS 6556, same strain from different collections, and UCC01 were plated in serial dilution on YPD plates and grown at 30°C and 47°C for 24 hours. The strain NBRC 1777 was also plated as a control. **b.** The strains NBRC 1777, UCC01 and ATCC 26548 were plated in serial dilution on YPD plates, with the addition of menadione to a final concentration of 45 mM and 75 Mm, and grown at 30°C for 24 h.

ROS accumulation derived by a combination of high temperature and high oxygen availability, is responsible for UCC01strain's stress-sensitive phenotype.

UCC01 strain was compared to NBRC 1777 and ATCC 26548 control strains under fully aerated or limited aeration conditions, in presence of 5 mM of H_2O_2 and high temperature (45°C) on plates, to assess the role of oxygen, temperature and oxidative stress-sensitive phenotypes. The reduced aeration was achieved using microaerophilic sachets and an air-tight cylinder, which ensured partial oxygen deprivation, to a level of 6%.While the reduced oxygen availability yielded no appreciable phenotype on NBRC 1777 and ATCC 26548, it improved both UCC01's thermotolerance and oxyidative stress tolerance (Figure 3). *K. marxianus* strains were previously reported to be able to grow up to a concentration of 50 mM H_2O_2 (Arellano-Plaza et al., 2017; R. et al., 2003; Saini et al., 2017) but, while NBRC 1777 and ATCC 26548 strains did not show an appreciable growth impairment (Figure 3), UCC01 showed sensitivity to H_2O_2 at concentrations as low as 5 mM, indicating that the mutant strain is highly sensitive to oxidative stress.

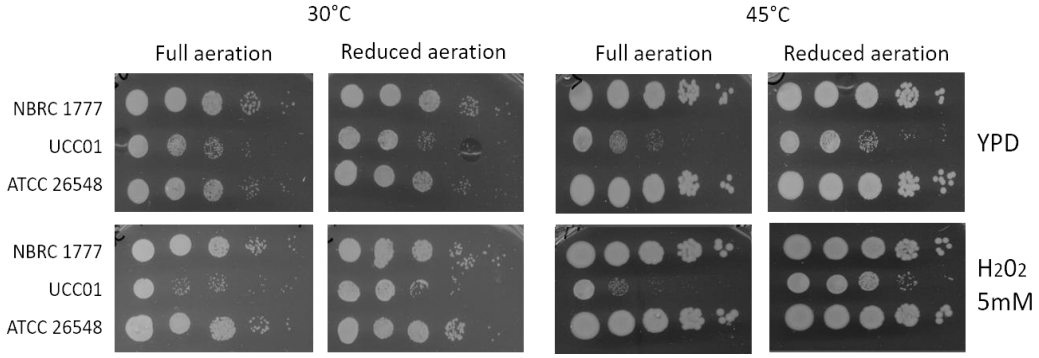

Figure 3. Higher oxygen availability exacerbates UCC01 strain's sensitivity to high temperature and oxidative stress. The strains NBRC 1777, UCC01 and ATCC 26548 were plated in serial dilution on YPD plates, with and without the addition of H_2O_2 to a final concentration of 5 mM, and incubated at 30°C or 45°C for 24 h. For the reduced aeration condition, the plates were incubated inside an air-tight cylinder containing micro-aerobic incubation sachets.

Subsequently, in order to study the physiological effect of oxygen availability on UCC01's growth and thermotolerance, this was compared with ATCC 26548 in bioreactors cultures in batch mode under low (30°C) and high temperature (45°C), and under two aeration conditions: 2 $Lmin^{-1}$ and 0.5 $Lmin^{-1}$. 45°C was chosen as high temperature for the batch fermentations because of the already visible phenotypical

difference between UCC01 and the WT strains on plates at that temperature (Figure 2). The growth and sugar consumption kinetics are shown in Figure 4. The growth parameters were calculated from exponentially growing cells and are reported in Table 4. On one hand ATCC 26548 strain shows a slower growth rate at 45 °C (0.7 h^{-1} and 0.93 h^{-1}) compared to 30 °C (1.2 h^{-1} and 1.3 h^{-1}) under both aeration conditions, indicating the temperature stress is affecting its growth. On the other hand its growth seems not to be influenced by the aeration levels, since the growth rates are comparable at 30 °C and 45 °C under both aeration conditions (Table 4). UCC01's growth rates resulted significantly slower than ATCC 26548 under all tested condition, ranging from 0.3 to 0.12 h^{-1}. Moreover, its growth was completely inhibited at 45°C under 2 $Lmin^{-1}$ aeration. Under 0.5 l/min aeration, UCC01's growth rate is slower at 45°C (0.12 h^{-1}) compared to 30 °C (0.3 h^{-1}). These data establish a link between slow or no growth at 45C and the effects of oxygen on UCC01.

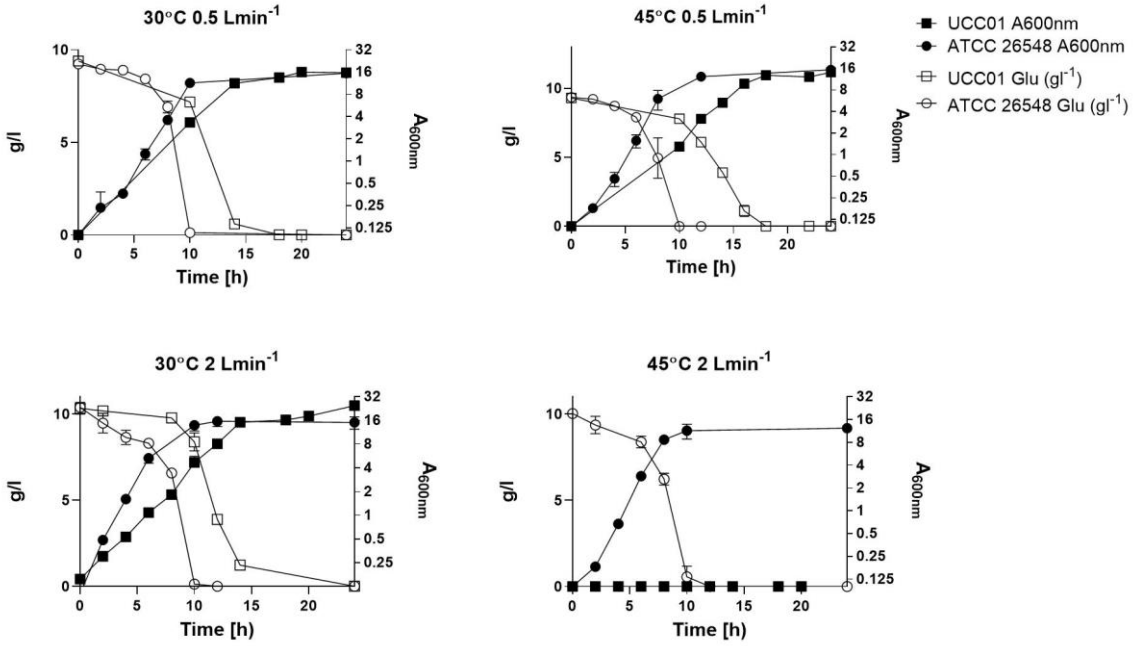

Figure 4 A combination of high aeration and elevated temperature inhibit UCC01 strain's growth during bioreactor fermentations. Growth and sugar consumption kinetics of ATCC 26548 and UCC01 strains during aerobic bioreractor cultivations under different temperature (30°C: **a**, **c** and 45°C: **b**, **d**) and aeration conditions (2 $Lmin^{-1}$: **a**, **b** and 0.5 $Lmin^{-1}$: **c**, **d**). Cells' growth was monitored by measuring the absorbance (A_{600nm}) every two hours for 24 hours. The glucose consumption was measured by HPLC analysis of the supernatant. Each fermentation was performed in duplicate.

The OUR of both tested strains decreased significantly under the 0.5 $Lmin^{-1}$ aeration condition compared to 2 $Lmin^{-1}$ (Table 4). Noticeably, for ATCC 26548, the OUR

was directly proportional to the maximum growth rate of the cells (Table 4), UCC01's OUR is not proportional to the growth rate. On the contrary, UCC01's OUR ranged between 1.6 and 0.06 $mmolh^{-1}$ independently from the growth rates (Table 4). This indicates a similar respiratory capacity between the two strains, but the slower growth of UCC01 already at 30 °C (compared to ATCC 26548) suggests that there is an impairment caused by oxygen availability. The very low OUR of UCC01 compared to ATCC 26548 under 0.5 $Lmin^{-1}$ aeration, suggests that the strain may be fermenting rather than purely respiring.

Table 3. Physiological comparison of growth parameters during bioreactor cultivation of UCC01 and ATCC 26548 strains. The maximum growth rate (µMAX), glucose consumption rate (µS) and oxygen consumption rates (OUR) of the two strains were calculated during the exponential growth phase of the bioreactor fermentations under different conditions. The reported values represent the average of two biological replicates.

Strain	***2 Lmin^{-1}***		***0.5 Lmin^{-1}***	
	30°C	**45°C**	**30°C**	**45°C**
		µMAX (h-1)		
ATCC 26548	1.2	0.7	1.3	0.93
UCC01	0.2	NA	0.3	0.12
		µS ((gl^{-1})/h)		
ATCC 26548	2	1.3	2	1.2
UCC01	1.8	NA	1.6	0.3
		OUR(mmolh^{-1})		
ATCC 26548	2.2	1.04	0.52	0.64
UCC01	1.6	NA	0.06	0.06

Notably, an extracellular accumulation of acetate (at 30°C) and acetate and ethanol (at 45°C) was detected under 0.5 $Lmin^{-1}$ aeration condition for UCC01 strain (Figure 5), but not in ATCC 26548; these metabolites were consumed after the depletion of glucose. The maximum extracellular concentration of acetate was reached after 14 hours at 30°C (0.52 gl^{-1}), and after 16 hours at 45°C (0.55 gl^{-1}). Ethanol was accumulated by UCC01 up to a concentration of 11 gl^{-1} at the end of the exponential phase at 45°C, and was depleted immediately after. The accumulation of ethanol and acetate, indicate the onset of fermentation of pyruvate to ethanol, which could be advantageous for UCC01's survival at 45°C.

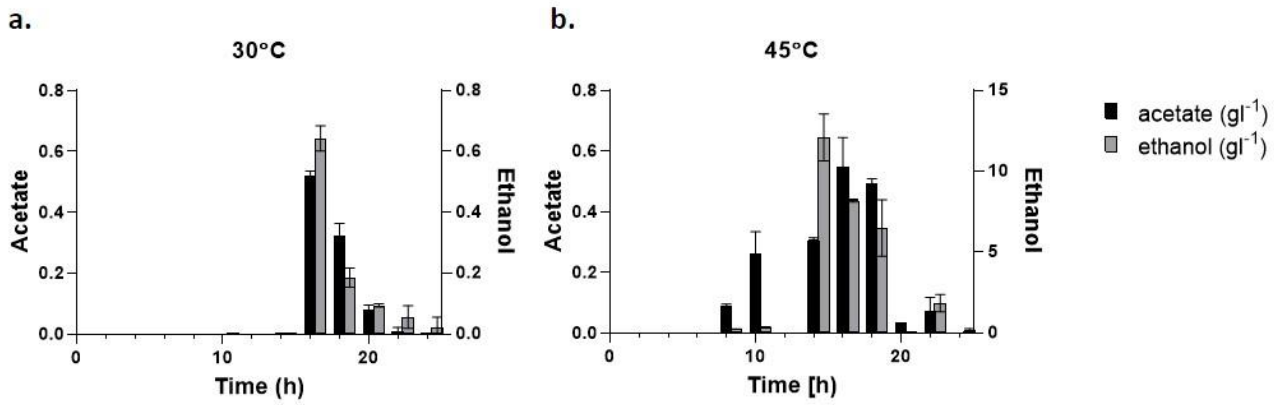

Figure 5. UCC01 strain accumulates ethanol and acetate during growth under 0.5 Lmin^{-1} aeration. Ethanol (grey) and acetate (black) accumulation during late stage of UCC01's fermentations at 30°C (**a**) and 45°C (**b**) measured by HPLC analysis of the supernatant.

The oxidative stress level of both strains at 30°C and 45°C under 0.5 Lmin^{-1} of aeration, was compared. Intracellular ROS content and cell viability was assessed via double treatment with the fluorescent probe DHR-123, and PI dye, and stained cells ratio was measured via flow-cytometry (Figure 6). Although UCC01's ROS content is 55% higher at 45°C compared to 30°C, the % of ROS-containing cells at 30°C was similar for both strains, indicating that high temperature is the main factor responsible for ROS hyper accumulation in UCC01. Notably, for ATCC 26548, the percentage of ROS-containing cells is 46% lower at 45°C compared to 30°C, indicating that the WT strain's metabolism is more suited to 45°C than 30°C, perhaps with a stronger ROS-detoxification response.

The strains' viability at high and low temperature, measured by simultaneously staining the exponentially growing cells with propidium iodide (PI), a DNA-binding fluorescent dye that only penetrates into cells with damaged membrane (Kwolek-Mirek and Zadrag-Tecza, 2014), was found high for both strains in all the tested conditions, with PI-positive cells not exceeding the 20% (Figure 6). Notably for UCC01, the PI-positive cells are significantly lower (unpaired t-test p-value: 0.0027) at 45°C compared to 30°C, while it remains unchanged at both temperatures for ATCC 26548 (Figure 6).

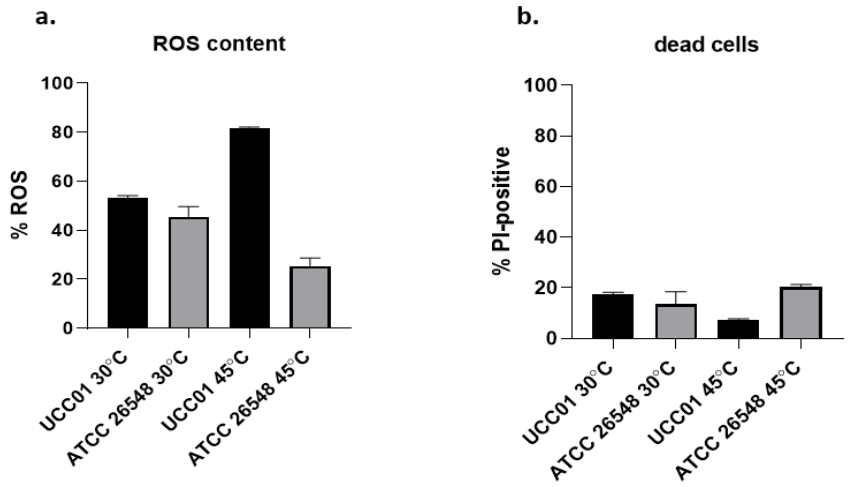

Figure 6. High temperature triggers ROS accumulation in UCC01 strain. Percentage of ROS-containing cells (**a**) and cell viability (**b**) was measured by flow-cytometry. UCC01 and CBS 6556 strains' cells were sampled from the bioreactor fermentations under the 0.5 $Lmin^{-1}$ aeration condition, during early exponential phase, and co-stained with 123-DHR and propidium iodide (PI).

A low genomic variability between the two strains, indicates that UCC01 is a spontaneous mutant derived from ATCC 26548

In an attempt to identify the genetic basis of UCC01's loss of thermotolerance, UCC01 and ATCC 26548' genomes were sequenced via short read sequencing and a single nucleotides polymorphisms (SNPs) analysis was performed on UCC01's genome. Since CBS 6556 strain's genome sequence was recently made available (Dekker *et al.*, 2021), it was included in the comparison to add extra depth to the analysis. The updated annotation of DMKU3-1042 strain (Fenton *et al.*, 2021) was used as genome reference for the SNPs analysis. The pipeline followed to perform the SNPs analysis in this study is described in the methods and summarized in Figure 1. ATCC 26548, CBS 6556 and UCC01's genomes contained an average of 65061 SNPs relative to the reference genome (Table 5), corresponding to an average of 0.6 % sequence divergence over the 10.9 Mb genome. 1012 SNPs were identified between UCC01 and ATCC 26548' genomes, corresponding to the 0.01% of nucleotide divergence (Table 5). Concurrently 3804 SNPs were identified between CBS 6556 and ATCC 26548' genomes, corresponding to the 0.03% of nucleotide sequence divergence (Table 5). The average measured inter-strains variability of the strains ATCC, CBS and UCC01with the reference strain DMKU, resulted three orders of magnitude bigger

(0.6%) when compared to intra-strains variability between UCC01 and ATCC 26548 strains (0.0092%), and CBS 6556 and ATCC 26548 strains (0.03%) (Table 5), allowing us to conclude that UCC01 was originally the same strain as NCYC 2597 (=CBS 6556=ATCC 26548=KCTC 17555). It is important to notice that, while UCC01 and ATCC 26548 were sequenced using a short-read sequencing platform as described in the methods, CBS 6556 was sequenced using a long-reads sequencing technology (C Dekker et al., 2021); therefore a different depth of coverage can be expected, and consequently a bigger number of identified variations.

Table 4. SNPs/Kb between UCC01 and different *K. marxianus* strains are one order of magnitude higher than between the UCC01/ATCC 26548/CBS 6556 strains. The table reports the number of SNPs identified between the compared genomes by the NucDiff analysis. The SNPs density (SNPs/Kb) was calculated as number of SNPs over the 10.9 Mb genome of the reference strain DMKU3-1042.

	Total		Coding		Intergenic	
Strains comparison:	**SNPs**	**SNPs/kb**	**SNPs**	**SNPs/kb**	**SNPs**	**SNPs/kb**
Inter-strains variability:						
ATCC 2666548 vs DMKU3-1042	64048	5.8	27638	2.52	36410	3.32
UCC01 vs DMKU3-1042	64403	5.87	27587	2.51	36816	3.36
CBS 6556 vs DMKU3-1042	66731	6.08	31430	2.86	35301	3.22
Average	65061	6	28885	2.63	36176	3.3
Intra-strain variability:						
UCC01 vs ATCC 26548	1012	0.092	827	0.075	185	0.02
CBS 6556 vs ATCC 26548	3804	0.34	968	0.09	2837	0.26
Average:	2408	0.216	897.5	0.0825	1511	0.14

Next UCC01's coding sequences were investigated for SNPs which potentially impaired the functionality of the encoded protein. Therefore, the effect of the SNPs identified in the "UCC01-exclusive" and "ATCC-exclusive" lists were predicted using the SnpEff software (Cingolani et al., 2012), which quantified and annotated the variants' occurrence in coding or intergenic genomes' regions based on DMKU3-1042 strain's genome's annotation (Table 5). The pairwise comparisons between the tested strains and the reference strain showed an average density of 2.63 coding SNPs/Kb and 3.3 intergenic SNPs/Kb (Table 5), while the comparison between UCC01 and ATCC 26548 genome (UCC01-exclusive) and CBS 6556 and ATCC 26548 (CBS-6556 exclusive) showed an average density of 0.082 coding SNPs/Kb and 0.14

intergenic SNPs/Kb (Table 5). The UCC01-exclusive variants located within the strain's coding regions are 827; of these, 51% were predicted to be of low impact (synonymous variants), 42 % of moderate impact (missense variations, conservative deletions or disruptive insertions), and 7 % were predicted to have high impact (frameshift and stop gained) on the protein sequence. The complete list of variants with predicted effect on the protein sequence is provided in Supplementary table 2. The UCC01-exclusive coding variants were manually filtered to eliminate identical variants with the CBS 6556-exclusive list, in order to further reduce false positive candidates. This process allowed us to identify 35 SNPs-containing genes in UCC01, which represent the strain's differences with both CBS 6556 and ATCC 26548 collection strains, and are predicted to have moderate and high impact on the coded protein sequences (Figure 1).

UCC01's SNPs-containing genes are involved in the general stress response, but are not individually responsible for the thermosensitive phenotype.

Of the 35 SNPs-containing genes identified in UCC01, 12 genes were determined to belong to a known multi-copy gene family in *K. marxianus* (Yu et al., 2021), and two were subtelomeric genes determined to be probable pseudogenes. These categories are known to have a higher tendency to accumulate loss of function mutations, but these are less likely to yield a negative effect, due to functional redundancy with paralog genes (Bergström et al., 2014), moreover a false positive is likely, due to the high similarity between the copies; therefore these genes were not considered further in the search for genomic mutations responsible for UCC01's phenotypes. The remaining medium and high impact SNPs (missense and frameshift variants), were validated with local sequencing of both UCC01 and the collection strains' genomic DNA' portion. This allowed to further exclude 12 genes, on the basis of either a gap in UCC01's assembly (for *KLMX_80195* and *KLMX_20724*), or because the same allele was present between UCC01 and at least one of the collection strains, excluding the possibility that the variation was responsible for UCC01's phenotype. *KLMX_80390* (*ARG8*) was excluded because the reported phenotype of *ARG8* deletion in *S. cerevisiae* (Janssen et al 1998) was not relevant to UCC01's observed phenotype. *KLMX_80360*, coding for *UBC6*, was also excluded from the list of possible candidates because the stop codon present in UCC01 is only within the last three

amino acids of the protein sequence, and therefore unlikely to disrupt the protein's functionality.

Finally, a subset of six genes were identified as possible candidates for UCC01's phenotype (Table 6), including a gene coding for a protein of unannotated function (*KLMX_60415*), and five genes whose functional annotation suggests a negative effect on oxidative stress and heat stress tolerance if the coded protein product is inactivated. These include orthologues of *YAK1 (KLMX_10341*), which regulates the two main stress-responsive transcriptional factors Hsf1 and Msn2 (P. Lee et al., 2008); *POL5 (KLMX_80024)* and *BNR1 (KLMX_20219)*, respectively essential ribosome biogenesis factor and protein involved in processes such as assembly of actin cables for transport of secretory vesicles and mitochondria (Gingras et al., 2020; Ramos-Sáenz et al., 2019), both reported to confer heat sensitivity upon reduction of function. Also *SWI1(KLMX_60377)* and *CYC8 (KLMX_80297)*, coding for a subunit of the SWI/SNF chromatin remodelling complex and a general transcriptional co-activator recruiting the SWI/SNF complex respectively, which is required for transcription of many stress-responsive genes (Du et al., 2020; Smith & Johnson, 2000) were also found to contain respectively a frame-shift and a missense variant. These five genes are reported to be directly or indirectly involved in *S. cerevisae* yeast's stress tolerance and could be responsible, individually or cooperatively, for the observed UCC01's phenotypes.

Table 5. UCC01 accumulated variations in five genes involved in the general stress response, with potential disruptive effect to the encoded protein's function. List of SNPs-containing genes identified by analysis using NucDiff and Snpeff softwares. Functional annotation is provided (where available) as well as the predicted amino-acid variations between UCC01 and DMKU3-1042 reference strain.

*after the gene-ID represents predicted frameshift (fs) variation.

Gene ID	***Functional annotation***	***Role***	***Variant***
KLMX_10341	*YAK1*	Protein kinase involved in glucose sensing and stress response	Asp532Val
KLMX_20219	*BNR1*	Actin assemble for budding and mitotic spindle	Arg574Lys
KLMX_80024	*POL5*	Involved in ribosomal 60S assembly	Ser213Tyr
KLMX_80297	*CYC8*	Co-repressor and co-activator, recruits SWI/SNF complex to promoter	Pro273Gln

KLMX_60377*	*SWI1*	Subunit of SWI/SNF chromatin remodelling complex. Regulates multiple processes.	Gln303fs

Pfam search to check for potentially affected protein domains showed that, apart from *SWI1* (which acquired a frame-shift mutation in Gln303) and *CYC8* (for which the missense variant does not fall within any recognized domain), the other three candidates had missense variants within one catalytic domain of the protein, which likely suggests loss of function in UCC01. In order to prove whether the SNPs contained in the five candidate genes were responsible for UCC01's phenotype, these genes were individually knocked-out in ATCC 26548 *dnl4* strain. The genes were inactivated via insertion of a repair fragment resulting in the disruption of a considerable portion of the reading frame. The disruption of the target genes was confirmed via amplicon size with specific primers (Table 2). *KLMX_80024*, coding for Pol5, could not be disrupted, probably because essential (Shimizu et al. 2002). Targeted mutagenesis to reproduce UCC01's *POL5* allele in ATCC 26548 *dnl4* was also attempted, but was unsuccessful. Finally, the knock-out mutants were compared via drop test to the parental strains under high temperature, oxidative and osmotic stress (Figure 7). Although none of the mutants reproduced UCC01's growth defect under high temperature, individual disruption of *CYC8* and *YAK1* resulted in increased oxidative stress sensitivity, indicating that these genes are involved in the general stress response of *K. marxianus,* activated by ROS accumulation.

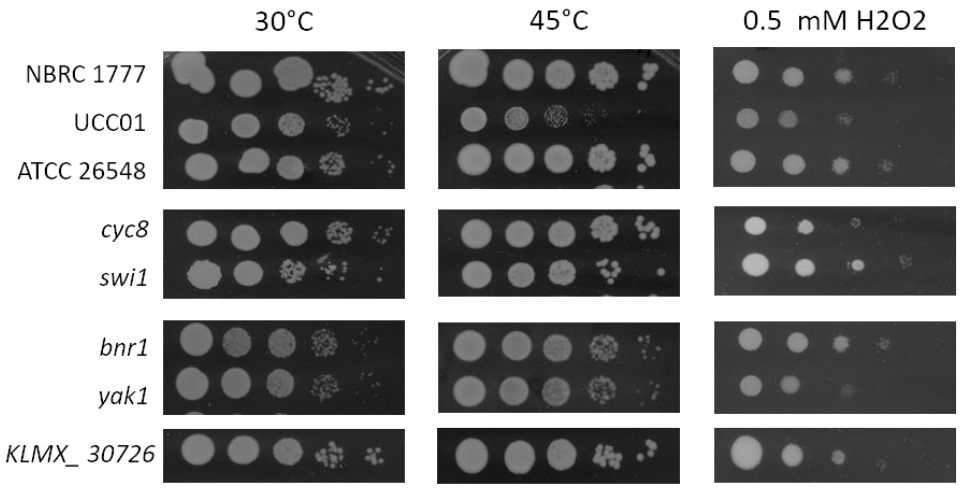

Figure 7. UCC01 acquired variants in genes involved in oxidative stress response. The target genes identified in this study were inactivated by insertion of a repair fragment in ATCC 26548 *dnl4* strain's genes' loci. The resultant knock-out mutants were plated in serial dilution on YPD plates and grown at 30°C or 45°C for 24 hours. To test for oxidative stress sensitivity, the serial dilutions were plated on YPD plates with the addition of 0.5 mM H_2O_2 and incubated at 30°C for 24h.The strains NBRC 1777, UCC01 and ATCC 26548 *dnl4* were used as control.

5. Discussion

It is well known in the microbiology community that laboratory strains acquire mutations and this can give rise to discordant results between groups (Arras et al., 2017). The starting point for our study here was both the reported phenotypic variation between *K. marxianus* CBS 6556 strains in studies from other groups (Fonseca et al., 2007) and our own observations of a similar phenomenon in one of our laboratory strains. Overall analysis of three versions of the same strain, ATCC 26548, CBS 6556 and UCC01, and comparison to a fourth strain, DMKU3-1042 yielded some fascinating data. First, the level of variation between the three strains though to be derived from the same original ATCC 26548 is sufficiently low to confirm that they are indeed the same strain, albeit with SNPs. This strain has about 60,000 SNPS relative to DMKU3-1042, which is about 6 SNPs per kb. There are about 1000 SNPs between UCCO1 and DMKU3-1042, which is lower than the approximately 3000 between CBS 6556 and DMKU3-1042. This seems odd since UCC01 is a descendant of CBS 6556 but it is most likely explained by the method of sequencing and filtering the sequence data, which was different for CBS 6556 since it came from a different study. We did not have whole genome sequence for the NCYC 2597 version of the strain but sequencing of individual genes to validate polymorphisms that came from the genome sequence analysis confirmed its close relationship to CBS 6556 and identified several polymorphisms that were shared with UCC01. Putting all this together, it was possible to establish the strain genealogy (Figure 8). Looking at this figure and knowing that only UCC01 has the temperature sensitive phenotype, it should be in principle relatively straightforward to identify the SNPs that could be responsible by comparing the UCC01 and NCYC 2597 sequences. Our main comparison was between UCC01 and ATCC 26548, where we identified over 1000 SNPs. We were able to reduce the number to analyse by taking advantage of the CBS 6556 sequence that became later available, but this was only partially helpful because of the different way the sequence was obtained and filtered. The ideal approach would be to sequence and analyse representatives of all the stocks in the same manner and this would clarify a lot of points.

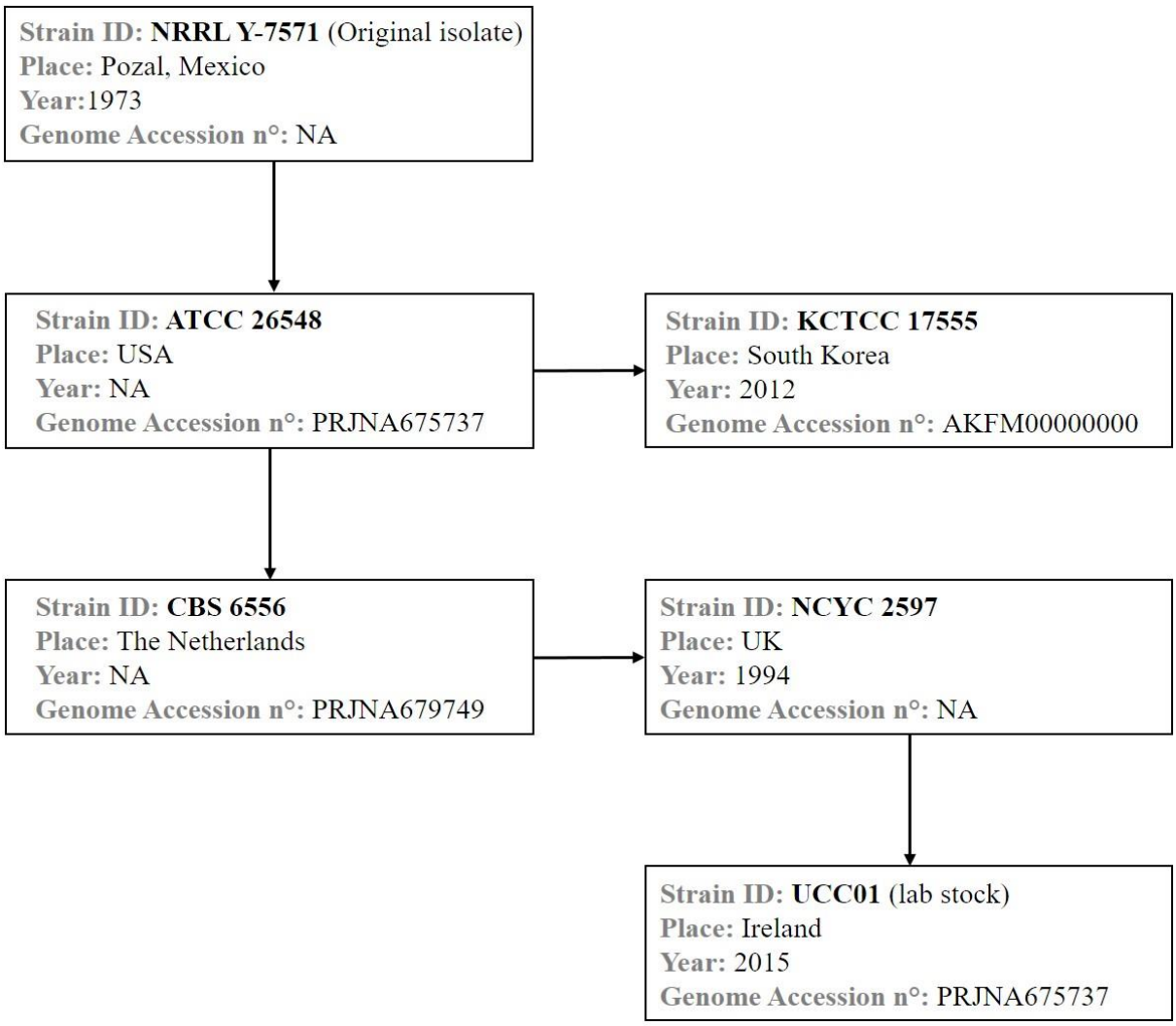

Figure 8 Chain of custody of *K. marxianus* UCC01 strain from its original isolate. Schematic representation of the chain of custody of UCC01 strain, from its original isolate through all the different collections to the working stock object of this study. Where available, year of acquisition and genome sequence accession numbers are reported.

Despite the challenges of the sequence comparison, it was possible to identify some very promising candidates. The prediction of the effect on the protein encoded by the SNPs-containing genes of UCC01, identified five candidate genes containing missense variants that are linked to general stress response activation or regulation in yeast. Although UCC01's sensitivity to high temperature could not be reproduced in ATCC 26548 strain by deleting individually each of these candidates, it is noticeable that knock-out of two out of five SNP-containing genes, *CYC8* and *YAK1*, resulted in a oxidative stress sensitive phenotype in ATCC 26548. *S. cerevisiae* early cellular response to ROS accumulation involves the transcription of several genes by transcriptional factors such as Msn2 and Msn4, regulated by *YAK1* (Farrugia &

Balzan, 2012); or the SWI/SNF transcription complex, of which *CYC8* is an activator (Smith & Johnson, 2000). Yeast strains adapted to grow under high temperature stress have been reported to acquire mutations in the SWI/SNF complex, confirming its role in thermotolerance (Huang et al., 2018). Therefore, seen the oxidative stress sensitive phenotype derived by inactivation of the two genes, and their relationship with thermotolerance, it is likely that the variants acquired within these transcriptional regulators play a role in UCC01's phenotypes. In fact, the SNPs analysis failed to identify unique variants within the coding regions of mitochondrial DNA (mtDNA) genes, genes belonging to the respiratory chain or genes coding for ROS detoxification enzymes (catalases and peroxidases), all of which are possible candidates for the oxidative stress-sensitive phenotype observed in UCC01 (O'Rourke et al. 2002; Zubko and Zubko 2014; Farrugia and Balzan 2012; Herrero et al.). We therefore speculate that UCC01 phenotypes are due to the lack of a proper oxidative stress response, which in yeast is normally initiated by transcriptional factors involved in the response to both high temperature and oxidative stress (Farrugia & Balzan, 2012).

A physiological comparison of ATCC 26548 and UCC01 strains on plate, showed that UCC01 is highly sensitive to both high temperature and oxidative stress compared to the collection strain, and that low oxygen levels improve UCC01's growth under both oxidative and high temperature stress. Moreover, the bioreactor cultivation showed a total loss of viability of UCC01 strain at 45°C under 2 $Lmin^{-1}$ of aeration. In contrast, under the lower level of aeration (0.5 $Lmin^{-1}$), UCC01 was still able to grow at 45°C. Altogether, this suggested that UCC01's oxidative stress sensitive phenotype is related to the thermosensitive phenotype, and is likely dependent on a higher availability of molecular oxygen, which has been previously reported to be responsible for ROS accumulation in yeast (Baez & Shiloach, 2014; John F Davidson & Schiestl, 2001).

UCC01 accumulated acetate and ethanol under 0.5 $lmin^{-1}$ of aeration, and showed a 55% increase of ROS at 45 °C compared to 30 °C. Acetate accumulation, previously measured during aerobic *K. marxianus* bioreactor fermentations (Fonseca et al. 2007; Lehnen, Ebert, and Blank 2019; Sakihama et al. 2019), has the function of regenerating reducing equivalents, such as NADPH, during the respire-fermentative metabolism (Blank et al., 2005). High temperature has been reported to generate ROS accumulation and a redox imbalance in *K. marxianus,* due to lowered ratio of reduced nicotinamide adenine dinucleotide (NADH)/oxidized nicotinamide adenine

dinucleotide (NAD+) (Fu et al. 2019). Therefore, acetate and ethanol accumulation could be a consequence of UCC01's elevated oxidative stress.

Contrary to UCC01, ATCC 26548 strain showed relatively elevated growth rates under both aeration levels. Noticeably, ATCC 26548's intracellular ROS content decreased by the 45% at 45°C compared to 30°C. This matches what reported before for a different *K. marxianus* WT strain, for which the level of ROS decreased under elevated temperature in the late stage of fermentations (Signori et al., 2014). Probably, the ROS accumulation is better managed in WT strains of *K. marxianus* at high temperatures, because of a strong antioxidant response induction as a result of both the oxidative stress and the thermal stress signalling, and the crosstalk between the two (Morano et al., 2012). In fact, heat and oxyidative stress responses involve the induction of an overlapping set of genes in yeast, initiated by general stress-responsive transcriptional regulators (Herrero et al. 2008; John F. Davidson and Schiestl 2001; Pyatrikas et al. 2015). Moreover, heat stress-responsive genes are induced by ROS accumulation (Fedoseeva et al. 2014). UCC01's oxidative and thermic stress-sensitive phenotype could be the result of variants affecting the strain's early stress response's transcriptional cascade, and allows to speculate that the same cascade is therefore responsible for the innate thermotolerance, as well as oxidative stress tolerance, of WT *K. marxianus* strains. However, more work is needed to confirm this hypothesis, and to investigate the details of *K. marxianus'* stress response' transcriptional cascade. Indeed, understanding the molecular mechanisms underlying the cross-talk between diverse stress responses is essential for strain improvement by genetic engineering. Finally, this study demonstrates the importance of controlling the oxygen flux during industrial fermentations, since the derived oxidative stress could affect important physiological traits of the strain, and its productivity.

6. Data availability

The scripts used for genome-scaffold assembly are publicly available with documentation on Bioinformatics Notebook v1.0.1 (http://doi.org/10.5281/zenodo.4266792). A full list of programs used by these scripts with version numbers can be found here: https://github.com/rnnh/bioinfo-notebook/blob/v1.0.1/envs/bioinfo-notebook.yml . The scripts used for NucDiff and SnpEff are available as supplementary material.

The FASTQ sequencing reads for *K. marxianus* ATCC 26548 and UCC01 are on NCBI's Sequence Read Archive under BioProject PRJNA675737. This BioProject will be made public at time of publication, or on 1/8/2021. Reviewer link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA675737?reviewer=k8dsv8racmp97l0oqeank0kjfm

Darren Fenton's *K. marxianus* DMKU3-1042 genome assembly and annotation are not publicly available at time of writing.

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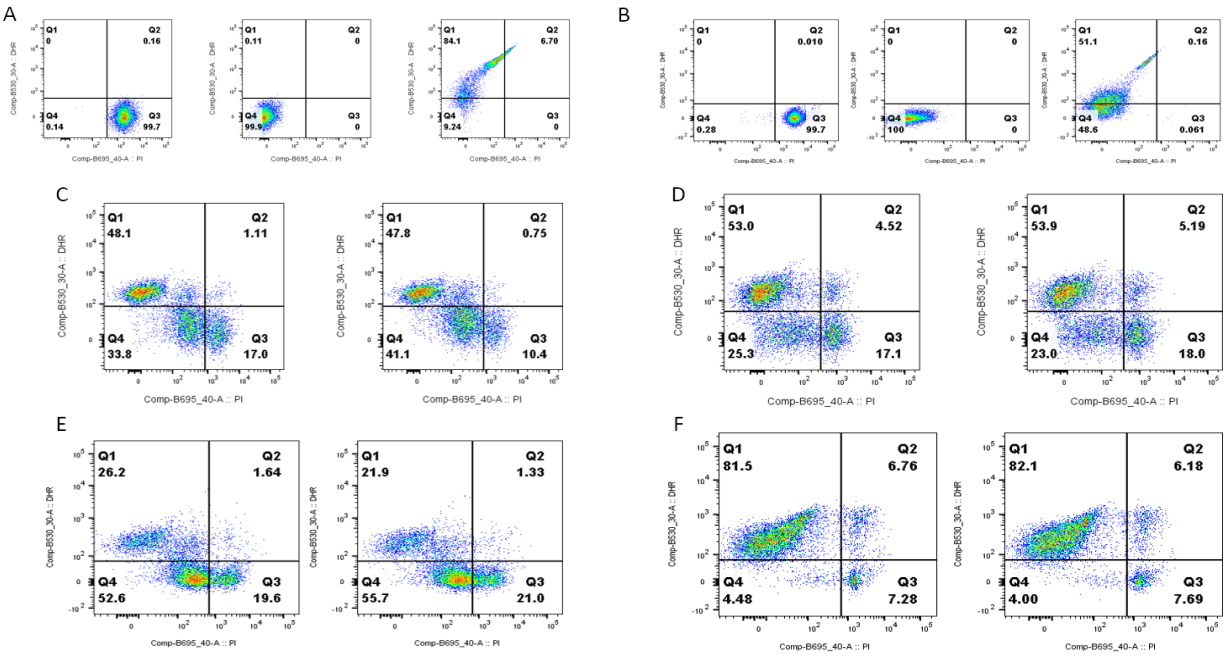

Supplementary Figure 1. Flow-cytometry graphs showing the percentage of 123-DHR or PI-positive cells during bioreactor fermentations under the 0.5 Lmin^{-1} aeration condition. The cells distribution in different quadrants indicates what portion of the culture was 123-DHR positive and PI-negative (Q1), 123-DHR positive and PI-positive (Q2), 123-DHR negative and PI-positive (Q3), or 123-DHR negative and PI-negative (Q4). Controls for ATCC 26548 (**A**) and UCC01 (**B**) strains were obtained as follows (from left to right): PI positive control was obtained by killing the cells with 60% ethanol; negative control was obtained from untreated cells, ROS positive control was obtained by adding H2O2 6 mM to the medium. The cells were sampled from ATCC 26548 and UCC01 strains' bioreactor fermentations at 30°C (**C** and **D** respectively) and 45°C (E and F respectively).

Chapter VI

Insights on the interaction between trehalose biosynthesis and galactose metabolism in *K. marxianus*.

Montini, N. and Morrissey, J.

This chapter is planned for submission to the journal FEMS Yeast Research)

NM designed and performed the experiments, interpreted the data and wrote the manuscript

JM helped designing the experiments, provided supervision and edited the manuscript

1. Abstract

Trehalose is considered a thermoprotectant which allows for yeast survival to heat stress. However, recent studies demonstrated a more complex role of trehalose metabolism, and of its intermediate T6P, in regulating the central carbon metabolism and even the homeostasis of the cell. Moreover, knock-out of different enzymatic subunits of the trehalose synthase complex display diverse phenotypes in various yeast species. Since several genes of trehalose metabolism were found upregulated under long term temperature stress by differential expression (DE) analysis, this study investigated the individual roles of *TPS1* and *TPS2* on *K. marxianus* thermotolerance by construction and analysis of knock-out mutants. First, deletion of *TPS1* was not found to be lethal in *K. marxianus*, unlike in *S. cerevisiae*, but yielded a thermosensitive phenotype. Moreover, deletion of *TPS2* yielded a thermosensitive phenotype exclusively on galactose. Further investigations revealed that the thermosensitive phenotype is due to the accumulation of T6P under high temperature, which deregulates the galactose metabolism genes. In fact, reduction of the intracellular levels of T6P via knock-down of *TPS1*, recovered the phenotype of the Δ*tps2* mutant. Furthermore, RT-qPCR revealed overexpression of the GAL genes at 47 °C, and of *GAL10*, *TPS1*, *TPS2* and *UGP1* genes at 30 °C in Δ*tps2*. The study shows evidence of T6P role in regulating central carbon metabolism in *K. marxianus*, revealing a new layer of metabolite repression mechanisms, linked to thermotolerance.

2. Introduction

Trehalose is a non-reducing disaccharide of glucose found in a variety of organisms such as plants, bacteria, insects, invertebrates and yeast (Thammahong *et al.*, 2017). In yeast, it is synthesized from glucose-6-phosphate (glucose-6P) and UDP-glucose, via the trehalose synthase (TPS) complex. The TPS complex includes three subunits, two catalytic enzymes (Tps1 and Tps2) and the regulatory *TSL1* subunit, necessary for complex function (Bell et al., 1998). The trehalose biosynthetic genes are upregulated in *S. cerevisiae* yeast upon heat stress, leading to trehalose accumulation (An et al., 2011; Herdeiro et al., 2006; Hottiger et al., 1987); hence the belief that trehalose acts as thermoprotectant against heat stress. In fact, trehalose acts as a protein stabilizer which helps counteract thermal denaturation in vitro (Kaushik & Bhat, 2003; Sola-Penna & Meyer-Fernandes, 1994), and has a protective effect on the yeast's membrane bilayer, avoiding lipid peroxidation following heat stress (Magalhães et al., 2018).

Single knock-out mutants in *S. cerevisiae* of the two TPS enzymatic subunits, *TPS1* and *TPS2,* display different phenotypes. For instance, the *tps1 m*utant shows a growth impairment on readily fermentable glucose (Luyten, *et al.*, 1993; Bell *et al.*, 1998), whereas the Δ*tps2* mutant has a thermosensitive phenotype, independently from the carbon source (Gibney et al., 2015). The thermosensitive phenotype of the *S. cerevisiae tps2* mutant can be rescued by expressing a heterologous phosphotrehalase from *Bacillus subtilis* (van Vaeck et al., 2001), indicating that T6P accumulation is responsible for the thermosensitive phenotype. Known consequences of *TPS1* deletion are accumulation of sugar phosphates and intracellular Pi depletion, both toxic for the cell (Fraenkel & Nielsen, 2016). The lethal *tps1* phenotype is generally ascribed to the absence of trehalose-6-phosphate (T6P), which plays a role in regulating the activity of the hexokinase Hxk2. This enzyme controls glycolytic flux and, in the absence of T6P, high Hxk2 activity leads to glucose-6P accumulation to toxic levels (Gancedo & Flores, 2004). T6P therefore plays a role in regulating glucose repression and gluconeogenesis in yeast (Deroover et al., 2016; van Vaeck et al., 2001). Reportedly, Hxk enzyme shows lower sensitivity to T6P in other yeast species, such as *Yarrowia lipolytica* , *Schizosaccharomyces pombe* and *Hansenula polymorpha* (Flores et al., 2011; Franco et al., 2000; Kramarenko et al., 2000), in which *Tps1* deletion does not result in a lethal phenotype on glucose. More recently, it was reported that a *tps1* mutant also showed hyper activation of Ras kinase, thus also linking trehalose

metabolism to proliferation and apoptosis (Peeters *et al.*, 2020). Indeed, T6P is also known to regulate important cellular processes such as development and proliferation in plants and insects (Matsushita and Nishimura, 2020; Zhang *et al.*, 2009; Thammahong *et al.*, 2017).

The yeast *K. marxianus* shows elevated thermotolerance, as well as peculiar carbon metabolism traits compared to other yeasts (Lehnen et al., 2019; Sakihama et al., 2019). While upregulation of *TPS2* and *TPS1* and trehalose accumulation have been reported for the yeast under osmotic stress (Saini et al., 2017), high temperature stress (Erdei et al., 2011) and ethanol stress (Alvim et al., 2019); the role of trehalose biosynthesis in thermotolerance or in central carbon metabolism has not yet been systematically characterized in this species. In this study, we first looked at the level of expression of the genes involved in the trehalose metabolism under high temperature stress, and identified three trehalose metabolism related genes upregulated under this condition. Moreover, since the role of each enzymatic subunit of the TPS complex was uncharacterized in *K. marxianus*, we obtained single knock-out mutants for *TPS1* and *TPS2*. The phenotype of the mutants was assessed at high temperature to establish the individual role of the subunits in thermotolerance. This revealed that the lack of trehalose synthesis, is not per-se responsible for a thermosensitive phenotype in *K. marxianus*. Moreover, deletion of the *TPS2* subunit caused a thermosensitive phenotype on galactose and deregulation of the galactose metabolism genes. Likely, T6P accumulation, which is only substantial at high temperature upon *TPS2* deletion, is responsible for the mutant's thermosensitive phenotype. The study provides new elements on *K. marxianus* thermotolerance mechanisms, and strengthen the link between trehalose metabolism and yeast central carbon metabolism.

3. Material and methods

Origin of differential expression and gene-set analysis data

The differential expression (DE) analysis results reported in this chapter was already described in detail in chapter 3. In brief, RNA was recovered from *K. marxianus* CBS 6556 growing at constant growth rate (chemostat) at either 30ºC or 40ºC and levels determined by RNA-Seq. Differential gene expression was defined by a significance cutoff of absolute log2FC > 0.5 and P-Value < 0.05 for the high temperature condition compared to the control condition. The complete table of results of the DE analysis is available in Supplementary table 1.

Strains and cultivation

All *K. marxianus* strains used in this study are listed in Table 1. Yeast strains were routinely grown on YPD medium (2% peptone, 1% yeast extract, 2% dextrose) at 30°C. Hygromycin B (200 µg mL^{-1}) purchased from Sigma Aldrich, St. Louis, MI, United States was used for selection where required. For test of growth on different carbon sources and for drop tests to check for glucose inhibition, 2% galactose (YPGal), 2% sucrose (YPSuc) or 2% Xylose (YPXyl) was added to peptone and yeast extract. For the drop tests, strains were grown overnight in 10 mL, harvested, washed and resuspended at A_{600nm} of 1 in sterile water. 5µL of 1:10 serial dilutions (until 10^{-5}) of each strain were spotted onto agar plates, which were incubated at the appropriate temperature for 24 h. For glucose inhibition tests, 2-Deoxiglucose (2-DOG) purchased from Sigma Aldrich, St. Louis, MI, United States was added to the YPGal, YPSuc or YPXyl plates, to a final concentration of 0.1% or 1% w/v. *E. coli* DH5α strain was used for cloning purposes. The strain was maintained in LB medium (5 g L^{-1} yeast extract, 10 g L^{-1} bactopeptone, 10 g L^{-1} NaCl) supplemented with 100 µg mL^{-1} ampicillin when required.

Table 1. **List of strains used in this study**. The knock out mutants contain base mutation inactivating the genes: *TPS1* (KMAR 80307), *TPS2* (KMAR 20021) and *MIG1* (KMAR 20054). The precise co-ordinates of deletion used the genome co-ordinates from NBRC 1777 strain (Inokuma et al., 2016).

STRAIN	GENOTYPE	MUTATION COORDINATE	SOURCE
NBRC 1777	WT		NITE Biological Research Centre, Japan
NBRC 1777 *DNL4*	*Δdnl4*		Rajkumar *et al.*, 2019
NBRC 1777_NM1	Δ*tps1*	CHR VIII Δ697,183-697,191	This study
NBRC 1777_NM2	Δ*tps2*	CHR II ΔC 49,762	This study
NBRC 1777_NM3	Δ*tps1* Δ*tps2*	CHR II ΔC 49,762 CHR VIII ΔC 697,191	This study
NBRC 1777_NM4	Δ*mig1*	CHR II ΔC 118,206	This study
NBRC 1777_NM5	Δ*tps2* Δ*mig1*	CHR II Δ49,308-49,542 CHR II Δ118,141-118,417	This study
NBRC 1777_NM6	*Δdnl4* *Δtps2* P_{10}-TPS1	CHR II Δ49,308-49,542 CHR VIII 697,996-699,003: P_{10} from pKmK.P10	This study
NBRC 1777_NM7	Δ*dnl4* Δ*tps1*	Δ*dnl4* CHR VIII Δ697,183-697,191	This study
NBRC 1777_NM8	Δ*dnl4* Δ*tps2*	Δ*dnl4* CHR II ΔC 49,762 insertion in I5	This study
NBRC 1777_NM9	Δ*dnl4* Δ*tps1* ΔI5: KMAR_80307 compl	CHR VIII Δ697,183-697,191 insertion in I5 (chromosome IV: 240017…241741)	This study
NBRC 1777_NM10	Δ*dnl4* Δ*tps2* ΔI5: KMAR_20021 compl	CHR II ΔC 49,762 insertion in I5 (chromosome IV: 240017…241741)	This study

Strain construction

Blast analysis of the NBRC 1777 genome sequence (accession number SRX3541357) using the DMKU3-1042 gene sequences was used to identify *KMAR_20054*, *KMAR_20021* and *KMAR_80307* as the *K. marxianus* NBRC 1777 orthlogues of *MIG1*, *TPS1* and *TPS2*, respectively. All primers and plasmids used in this study for strains construction are listed in Tables 2 and 3. Knock-out mutants NBRC 1777_NM1, NBRC 1777_NM2, NBRC 1777_NM3, NBRC 1777_NM4 and NBRC 1777_NM5 were constructed in the wild-type *K. marxianus* NBRC 1777. Mutants were constructed by non-homologous end joining (NHEJ)-mediated gene inactivation, using the CRISPR-Cas9 system previously described (Juergens *et al.*, 2018; Varela *et al.*, 2019; Rajkumar and Morrissey, 2021). In brief, targeting sequences (for gRNAs) were ordered as DNA primers and inserted into plasmid pUCC001 by Golden Gate assembly. gRNA primers were designed using the sgRNA software (Xie *et al.*, 2014; Rajkumar and Morrissey, 2021). Plasmids were introduced into *K. marxianus* using the standard LiAc/SS carrier DNA/PEG method with selection for transformants on Hygromycin B (200 µg mL^{-1}). Transformants were screened for indel or frameshift mutations in the target gene using PCR amplification with diagnostic primers, followed by DNA sequencing. To allow for CRISPR-Cas9 plasmid loss, the mutant strains were grown overnight in 10 mL of YPD without antibiotic selection. Single colonies were tested on YPD with Hygromycin B, to confirm lack of growth and hence loss of plasmid. NBRC 1777_NM7 and NBRC 1777_NM8 were obtained via inactivation of the *DNL4* gene in NBRC 1777_NM1 and NBRC 1777_NM2 strains respectively, as described in Rajkumar et al., 2019. Complementation mutants NBRC 1777_NM9 and NBRC 1777_NM10 were obtained in the NBRC 1777_NM7 and NBRC 1777_NM8 respectively via chromosomal insertion of expression cassettes containing the genes' CDS under the control of the constitutive promoter p*PDC1* and the t*INU1* terminator. The expression cassette contains a KanMX selection marker and 850-bp homology arms for integration into the I5 intergenic region in chromosome IV (Rajkumar et al., 2019). The pI5-KMAR_80307compl and pI5-KMAR_20021compl plasmids (Table 3) were constructed via Golden Gate assembly of the genes' CDS, with the regulatory parts PDC1pr/P2 and INU1t/T1 into the pI5-MTU-DO-G418 plasmid using previously described methods (Rajkumar et al., 2019). For

transforming into NBRC 1777_NM1 and NBRC 1777_NM2, 1 ug of the complementation plasmid was digested with SgsI at 37°C for 1 hour. The digested product was transformed into the strain, with selection for transformants on G418. Colonies were screened via colony PCR with I5 locus-specific primers to identify positive transformants and integrity of the insert was confirmed by sequencing. NBRC 1777_NM6 was obtained knocking down *TPS1* in the *tps2* mutant strain as described in (Rajkumar and Morrissey, 2021) with minor modifications. In brief, a target gRNA for promoter region targeting was manually identified using the CHOPCHOP tool (Labun et al., 2021) and, after checking against the *K. marxianus* genome for potential off-target, it was cloned into the pUCC001as described above to create pTPS1promHH plasmids. A repair fragment containing 60 bp of homology to the 5' and 3' regions flanking the promoter region (500bp) upstream of *KMAR_20021* (*TPS1*), and the promoter sequence from the plasmid pKmK.P10 (Addgene part number #125043) was PCR amplified. The primers used were designed with 60bp of homology to the *TPS1* promoter region and 20bp of homology to the weak promoter P_{ALD4} on the pKmK.P10 plasmid (Rajkumar et al., 2019). The Start codon of *TPS1* was contained in the 60bp overhang added by PCR, to ensure its correct functioning. The Δ*tps2* strain was co-transformed with the pTPS1promHH plasmid and the purified repair fragment containing the weak promoter as described above, and plated on selective plates. The promoter swap was confirmed via colony-PCR of individual colonies with diagnostic primers. The double mutant Δmig1tps2 was constructed in the dnl4 derivative of NBRC 1777 using the same CRISPR-Cas9 system, this time taking advantage of the intrinsic homology dependent repair (HDR) apparatus. 160-bp repair fragments were built by designing primers Tps2_RF_F/R and MIG1_RF_F/R that shared 18 bp overlap and had 60 bp of homology to the 5' and 3' regions flanking *TPS2 and MIG1* gRNA sequence, respectively. The 5' and 3' homology arms were annealed and amplified via overlap extension PCR using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). Successful construction of the repair fragment was confirmed by gel electrophoresis and 1ug of repair fragment was co-transformed with the CRISPR-Cas9 plasmid carrying the specific gRNA, into NBRC 1777 *dnl4* strain. Mutants were screened for via colony PCR, using Tps2_dia_F/R and Mig1_dia_F/R primers. Genes' deletion was confirmed from the amplicon size of the PCR product.

Table 2. List of primers used in this study for strain construction and verification.

PRIMER	SEQUENCE (5' – 3')
GRNA	
TPS2-HH-F	cgtccttatttccacgatacccca
TPS2-HH-R	aaactggggtatcgtggaaataag
TPS1-HH-F	cgtcaacgtgggtgcgttccctat
TPS1-HH-R	aaacatagggaacgcacccacgtt
MIG1-HH-F	cgtcaatcaagatttctgcgccaa
MIG1-HH-R	aaacttggcgcagaaatcttgatt
TPS1PROMSW_F	cgtcccaaaggcccccgattttag
TPS1PROMSW_R	aaacctaaaatcgggggcctttggg
FRAMESHIFT SCREENING AND SEQUENCING	
TPS2DIA-F	ccaatgcggtagtgttca
TPS2DIA-R	ctcaggaaggaccagtatcg
TPS1DIA-F	ccaagggttcacgatcaa
TPS1DIA-R	cggattccacacttacgattac
MIG1DIA-F	gatgagttgacgagacacag
MIG1DIA-R	gtctatcaatgaacttaaaggatggg
REPAIR FRAGMENTS	
TPS2RF_F	aactctaatagcaacaattctgatgagtaccggatcacatcatatggtggagatgttctt
TPS2RF_R	taaccaactcattaacctgggattccagtttaatgttgacatctgtaggagcagttggtc
MIG1RF_F	aagcccaaggggaagcggggtcggaagaagaagagcgagacgatagcgcgcgagaaggagttggagttgcagag acagagacagcagca
MIG1RF_R	agcagcagctccacggatccgttgttctgctgctgtgggtgtaccgagtggagcgtcgtggtattactggtttcctctga
COMPLEMENTATION	
TPS1COMPL_F	gcatcgtctcatcggtctcatatgatgacaatggataaagcgtttagc
TPS1COMPL_R	atgccgtctcaggtctcaggattcactggttagaggctccc
TPS2COMPL_F	gcatcgtctcatcggtctcatatgatggaaaacagtagtgcaagtaa
TPS2COMPL_R	atgccgtctcaggtctcaggattcaattagtcctagaagacactttct
PROMOTER SWAP	
TPS1PS_F	aagaatgcaaccaaaacctgctgcttcacatctaccgcactactgcccactagatctacaa tgtacaaattatggtattaggtaat
TPS1PS_R	gcctgttgcgctcgagctcttctctagcttcaactcgctaaacgctttatccattgtcat gttttgtgcacgtgtgat
	PROMOTER SWAP CHECK

P10_F	tgtacaaattatggtattaggtaat
TPS1PS_CHECK_R	caacgcagtgaccaacc

Batch fermentations and temperature-switch sampling

For the temperature switch experiment and sampling, the strains NBRC 1777 WT and *Δtps2* were cultivated in batch mode in 4 L Applikon bioreactors (Applikon, the Netherlands). A calculated volume of the overnight culture was added to achieve an initial A600nm of 0.1 in the bioreactor. Batch cultivation conditions were performed in YPGal medium at 30°C, in 2 L working volume with pH maintained at 5.5 through addition of 1M KOH. The culture was sparged with air at a flow rate of 2 L min^{-1} and agitated at variable RPM, in order to maintain a dissolved oxygen concentration above 60% saturation.

After inoculation (T0), the bioreactors were sampled every hour to check for cell growth until an A600nm of 1 was reached, indicating the beginning of the exponential growing phase. At this point, the temperature was quickly increased to 46°C with the help of a heated water pump, circulating hot water into the cooling finger of the bioreactor, in direct contact with the culture. The temperature was brought from 30°C to 46°C in less than 15 minutes. 10mL of cell culture was sampled from the vessel for RNA extraction and RT-qPCR analysis immediately before the temperature switch (30°C) and 30 minutes after the temperature switch (46°C). The cell pellet was immediately frozen with liquid nitrogen and stored at -80°C until RNA extraction. Each fermentation was performed in duplicate.

Table 3. List of plasmids used in this study.

PLASMID	PURPOSE	SOURCE
PUCC001	Contains CRISPR/CAS9 for cloning of guide RNA (gRNA) targets by Golden Gate assembly	(Rajkumar et al., 2019)
PI5-MTU-DO-G418	Integrative plasmid modified from (Rajkumar et al., 2019) for targeting integration in I5 (chromosome IV: 240017…241741)	(Rajkumar & Morrissey, 2020)
PI5-KMAR_80307compl	pPDC1-KMAR_80307-INU1t cloned into [pI5-MTU-DO-G418]	This study

PI5-KMAR_20021compl	pPDC1-KMAR_20021-INU1t cloned into [pI5-MTU-DO-G418]	This study
PKmK.P10	YTK #125043, contains Km*ALD4* promoter	(Rajkumar et al., 2019)
PTPS1HH	pUCC001 with guide RNA (gRNA) target for *TPS1*	This study
PTPS2HH	pUCC001 with guide RNA (gRNA) target for *TPS2*	This study
PMIG1HH	pUCC001 with guide RNA (gRNA) target for *MIG1*	This study
PTPS1PROMHH	pUCC001 with guide RNA (gRNA) target for *TPS1p*	This study

RNA extraction, cDNA preparation and RT-qPCR analysis

Total RNA was extracted from the thawed pellet using the hot acidic phenol protocol for yeast RNA extraction (Collart & Oliviero, 2001). The integrity of the RNA was checked on a RNA bleach gel as described in (Aranda, Lajoie and Jorcyk, 2012). The RNA was quantified and normalized using a Qubit fluorimeter (Invitrogen). Residual DNA contained in the sample was digested using an RNase free DNase I kit (New England Biolabs Inc.) according to manufacturer's instructions. Subsequently, the cDNA was obtained using a Protoscript First strand cDNA Synthesis kit (New England Biolabs Inc.) according to manufacturer instructions. The cDNA obtained was kept on ice and immediately used for the qPCR. The primers used for qPCR were designed and pre-tested with regular PCR on *K. marxianus* extracted genomic DNA, to test for efficiency to be within 90 and 110%, and a denaturation test was run to check for aspecific amplicons. The list of primers used is available in Table 4. A SYBR® Green RT-PCR Kit (Applied Biosystem) was used for qPCR according to manufacturer instructions. The qPCR was performed on a Light Cycler 480 (Roche) and a standard PCR program for 30 cycles was used: initial denaturation (98°C for 1 min), denaturation (98°C for 1 min) and final extension stage (72°C for 5 min). Annealing temperature was 50°C and extension time 20s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen et al., 2008) between each strain tested at each temperature. The housekeeping gene *ACT1* was used as reference genes. Each sample was tested in duplicate.

Table 4. List of primers used in this study for RT-qPCR of the galactose and trehalose metabolism genes.

PRIMERS	SEQUENCE
QACT1_F	ggctgaacgtggttactcct
QACT1_R	agaagcggtttgcatttctt
QTPS1_F	tatggcctttgttccactac
QTPS1_R	atcttcacgttcttcagctt
QTPS2_F	cagttaaatcgcaactgtcc
QTPS2_R	ctggactcaattttagggct
QUGP1_F	tgttcagaaggtatttgggg
QUGP1_R	tctcacctcgataacagact
QPGM2_F	ggaattgcttgatcaatggg
QPGM2_R	tcacaaacaagatctgggag
QGAL1_F	catcgaccaactcgatca
QGAL1_R	cgctaagaaactttatggacg
QGAL7_F	gtcttccacaaatgagtccaa
QGAL7_R	cgtgaggatgtaagttggaa
QGAL10_F	tatcgacgatggtggcac
QGAL10_R	tttggcaaaccagacgat

4. Results

Trehalose biosynthetic process is upregulated under high temperature.

Since trehalose metabolism has been associated with thermotolerance in yeast, we looked specifically at the level of expression of the genes involved in the trehalose metabolism process in *K. marxianus* during culture under high temperature condition in a chemostat. The data, available from the study in Chapter 3, showed upregulation of the genes encoding for the trehalose-6-phosphate phosphatase (Tps2, logFC:0.88), the regulatory subunit of the trehalose synthase (Tsl1, logFC:0.77) and the phosphoglucomutase (Pgm1, logFC:2.23). Other genes involved in trehalose biosynthesis, such as the first enzymatic subunit, Tps1, and Ugp1, necessary for the synthesis of precursors trehalose-6-phosphate and UDP-glucose, were not significantly upregulated. Tps2, Tsl1 and Pgm1 are responsible for redirecting the glycolytic flux from glucose to the biosynthesis of trehalose, via the formation of sugar phosphates: glucose-6P, glucose-1P, UDP-glucose and trehalose-6P (Figure 1).

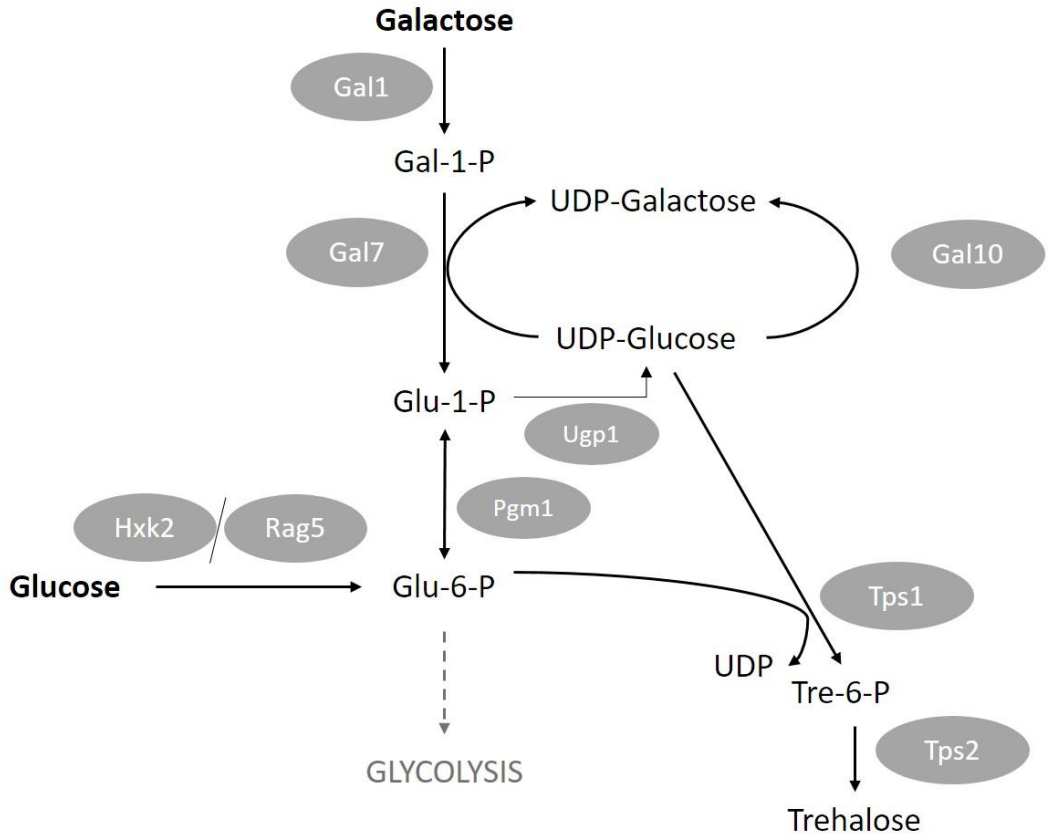

Figure 1 Trehalose biosynthetic pathway. Representation of the biosynthetic steps for trehalose biosynthesis, and overlap with the Leloir pathway for galactose metabolism. The first steps of glycolysis are represented too. Enzymes are represented in the grey circles. Abbreviations: Galactose-1-phosphate (Gal-1-P), Gluicose-1-phosphate (Glu-1-P), Glucose-6-phosphatre (Glu-6-P), Trehalose-6-phosphate (Tre-6-P).

It is important to notice that the enzyme Pgm1 is also involved in the Leloir pathway for the catabolism of galactose (Figure 1). Since the role of the two catalytic subunits of the TPS complex, known to also have a role in central carbon metabolism (Gibney et al., 2015) and cell apoptosis (Peeters *et al*., 2020), is mostly uncharacterized in *K. marxianus,* we performed further analysis to assess their individual role in thermotolerance.

Deletion of the two enzymatic subunits of the TPS complex causes a carbon-source dependent thermosensitive phenotype.

In order to determine the role of each enzymatic subunit of TPS, single knock-out mutants NBRC 1777_NM1 and NBRC 1777_NM2 and a double mutant NBRC 1777_NM3 in which both genes were disrupted were generated from *K. marxianus* NBRC 1777 by NHEJ-mediated gene inactivation which introduced short deletions and subsequent frameshifts (Table 1).

The growth of the three mutants and the WT strains was then compared on agar plates at 30 °C and 47 °C (Figure 2). This temperature was chosen as is at the upper end of the growth range for this particular strain of *K. marxianus*. The experiment was performed with both glucose and galactose as carbon source because of published data from *S. cerevisiae* indicating carbon source effects (Gibney et al., 2015). The mutants revealed carbon source dependent thermosensitive phenotypes (figure 2A). Namely, NBRC 1777_NM1 showed temperature sensitivity on both glucose and galactose; although the effect is far more pronounced on glucose with only modest impairment of growth evident on galactose. In contrast, Δ*tps2* is thermosensitive on galactose, showing no growth at 47 °C, but is quite thermotolerant on glucose, being able to grow with a mild impairment relative to the wild-type at 47 °C (figure 2A). NBRC 1777_NM3 double mutant, failed to grow at 47 °C on both carbon sources, exhibiting both single mutants' phenotype (figure 2). The temperature sensitivity of the Δ*tps2* strain on galactose was also seen during growth on liquid media (Figure 2B). In this case, at 30 °C, Δ*tps2* grows like the wild-type but no gorwth at all was observed at 45 ^{o}C. Complementation experiments were performed to verify the phenotypes of both NBRC 1777_NM1 and NBRC 1777_NM2 (Figure 2C). In order to facilitate targeted integration of the complementation cassette, the transformation was performed on NBRC 1777_NM7 and NBRC 1777_NM8 (derived by NBRC 1777_NM1 and NBRC

1777_NM2 after inactivation of NHEJ) by insertion of an expression cassette containing the ORF of *TPS1* or *TPS2* genes in an intergenic region of the respective mutants. NBRC 1777_NM7 and NBRC 1777_NM8 were used to facilitate locus specific integration of the complementation cassette. The complementation restored the phenotypes of the parental strains (Figure 2C).

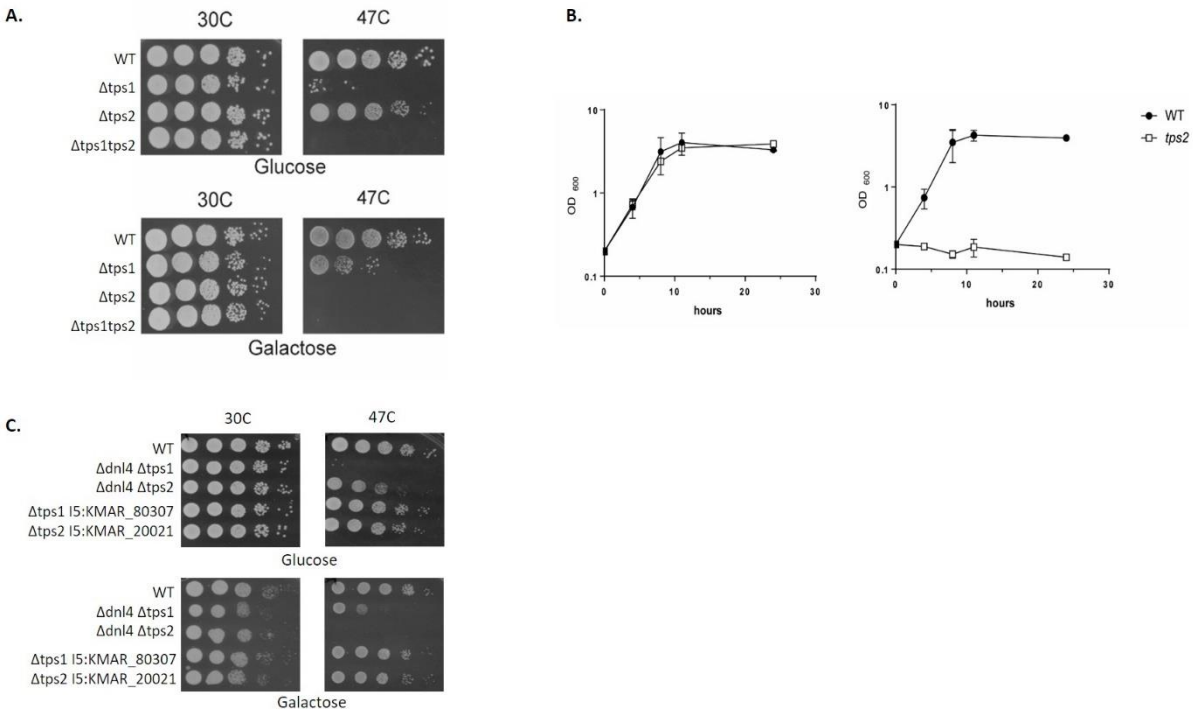

Figure 2. Single knock-out of the TPS subunits result in a carbon source-dependent thermosensitive phenotype. a) The subunits TPS1 and TPS2 of the TPS complex were inactivated by nucleotides deletion in the parental strain, individually and both in the same strain. The resultant knock-out mutants were plated in serial dilution on YPD and YPGal plates and grown at 30°C and 47°C for 24 hours. Strains: Δtps1 (NBRC 1777_NM1), Δtps2 (NBRC 1777_NM2), Δtps1Δtps2 (NBRC 1777_NM3) **b)** The tps2 mutant and the WT strain were compared during growth in liquid medium on YPGal in flask at 30C (left) and 4C (right). The A600 was measured periodically for 24hours. **c)** Complementation of NBRC 1777_NM1 and via insertion of *KMAR_80307 (TPS1)* and *KMAR_20021* (*TPS2*) expression cassettes in the parental strain restores the phenotype. Strains: Δdnl4Δtps1 (NBRC 1777_NM7), Δ*dnl4*Δ*tps2* (NBRC 1777_NM8), Δtps1 I5:KMAR_80307 (NBRC 1777_NM9), Δtps2 I5:KMAR_20021 (NBRC 1777_NM10).

T6P accumulation is toxic on galactose at high temperature

The peculiar phenotype of NBRC 1777_NM2, thermosensitive on galactose but not on glucose, suggests an exclusive role of Tps2 on galactose metabolism at high temperature. Based on the known enzymatic activities and data from analysis of mutants in *S. cerevisiae*, it is expected that a *tps1* mutant (or a double Δ*tps1*Δ*tps2* mutant) will accumulate Glucose 6-Phosphate (G6P), whereas a single Δ*tps2* mutant

accumulates trehalose 6-phosphate (T6P) (Figure 1). In a Δ*tps2* mutant, accumulation of T6P is greater at high temperature due to the increased expression of *TSL1*, *TPS1* and *TPS2*. We hypothesised that the galactose-specific temperature sensitivity is because T6P is toxic to an aspect of galactose metabolism. To address this, we performed an experiment to reduce the accumulation of T6P in NBRC 1777_NM2 without generating the phenotype of a *tps1* mutant. This was done by replacing the strong temperature-inducible native promoter of *TPS1* with the weak promoter from *ALD4*. This promoter swap at the *TPS1* locus was performed using the method described in (Rajkumar and Morrissey, 2021) and yielded strain NBRC 1777_NM6. Expression from P_{ALD4}-*TPS1* is likely to provide enough Tps1 activity to avoid toxic accumulation of G6P but insufficient to cause the strong accumulation of T6P at high temperatures since the gene is no longer temperature-inducible. The growth of NBRC 1777_NM2 and NBRC 1777_NM6 was then compared in glucose and galactose at 30°C and 47°C to determine whether knock-down of *TPS1* would have an effect (Figure 3A). Unlike NBRC 1777_NM2, NBRC 1777_NM6 was not temperature sensitive on galactose, supporting the hypothesis that NBRC 1777_NM2's phenotype is due to accumulation of T6P. The fact that NBRC 1777_NM6 is also not temperature sensitivity on glucose (unlike □*tps1*) indicates that a low level of constitutive expression of *TPS1* is sufficient to avoid this temperature-sensitivity. To further explore the galactose-specific nature of T6P toxicity at high temperature, we grew the WT and NBRC 1777_NM2 strains on concentrations of galactose ranging from 0.25% to 4% (Figure 3B). Temperature sensitivity showed a positive correlation with galactose concentration, suggesting that the T6P toxicity could be due to a temperature-dependent accumulation of an intermediate of the Leloir pathway in Δ*tps2*. Notably, NBRC 1777_NM1 is viable on glucose at 30°C in *K. marxianus*, but is thermosensitive (Figure 3). However, NBRC 1777_NM2's phenotype at 47°C on galactose is restored in the mutant NBRC 1777_NM6 (with *TPS1* knock-down), indicating that constitutive low expression of *TPS1* is sufficient to fulfil Tps1's role in the cell; while NBRC 1777_NM1's thermosensitive phenotype on glucose could be due to lack of regulation of glycolysis in addition to the thermal stress.

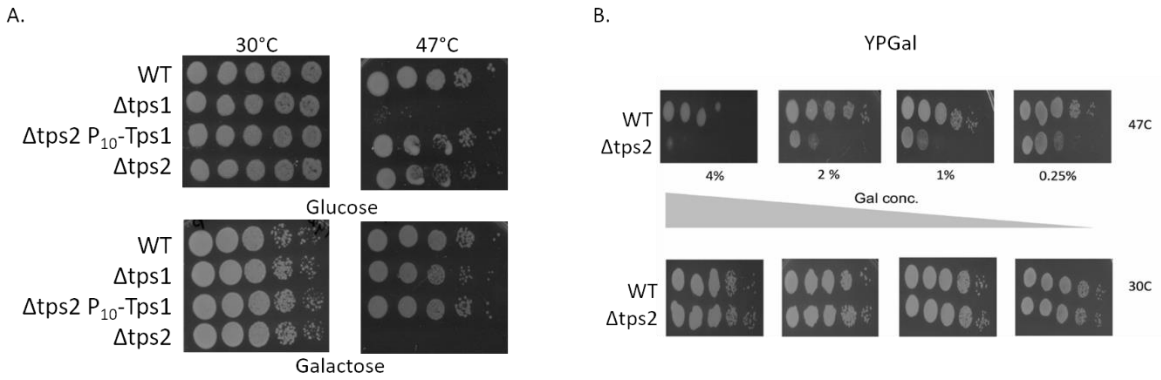

Figure 3. Accumulation of T6P results in growth impairment on galactose. a) *TPS1* expression was knocked down by promoter swap of the native promoter, with the weak KmP_{ALD4} promoter in the Δtps2 strain. The strains were plated in serial dilution on YPD and YPGal plates and grown at 30°C and 47°C for 24 hours. Strains: Δtps1 (NBRC 1777_NM1), Δtps2 (NBRC 1777_NM2), Δtps2 P_{10}-Tps1 (NBRC 1777_NM6)**b)** The WT and Δtps2 strains were plated in a serial dilution on decreasing galactose concentration, and grown at 30°C and 47°C for 24 hours.

Relationship between glucose repression and TPS' mutants

In *K. marxianus* galactose genes are subject to glucose repression via Mig1 transcriptional factor (Hua et al., 2019). The kinase Snf1 phosphorylates Mig1 in absence of glucose, sequestering it from the nucleus (Deroover et al., 2016; Treitel et al., 1998). Since there is evidence suggesting that T6P inhibits Snf1 in plants (Zhang et al., 2009), in a *tps2* mutant (where T6P accumulates under high temperature), Snf1 would be completely inhibited, the galactose genes would be consistently repressed, and growth on galactose would be impaired. On the contrary, by knocking out *MIG1* the galactose genes result constitutively expressed in *K. marxianus*, relieving glucose repression on galactose (Hua *et al.*, 2019). *MIG1* was therefore knocked out in the *tps2* mutant, in an attempt to remove the galactose metabolism genes from possible repression deriving from T6P-mediated inhibition of Snf1. Hence, the Δ*mig1*Δ*tps2* double mutant was obtained (NBRC 1777_NM5). Its growth was compared to the WT strain and Δ*tps2* on galactose at 47°C (Figure 4A). *MIG1* deletion did not restore Δ*tps2* phenotype, indicating that T6P does not act as transcriptional inhibitor of galactose genes via inhibition of *SNF1*.

In order to further explore whether there is any interaction between the glucose repression system and the phenotypes of the *tps* mutants in *K. marxianus*, NBRC 1777_NM1, NBRC 1777_NM2 and NBRC 1777_NM3, and the *mig1* mutants NBRC 1777_NM4 and NBRC 1777_NM5 were grown on plates containing galactose with

the addition of increasing concentrations of 2-deoxyriboglucose (2-DOG, figure 4B). NBRC 1777_NM1, which presents deletion of *TPS1* known to lead to excessive glucose phosphorylation (glucose-6-phosphate accumulation) in yeast (Fraenkel & Nielsen, 2016), resulted more sensitive to glucose repression than the WT strain. This is not surprising, since glucose repression seem to be directly correlated to high level of glucose phosphorylation in yeast (Lane et al., 2018). The *mig1* mutant (NBRC 1777_NM4) instead, was partially resistant to glucose repression on galactose, as previously reported (Hua et al., 2019). NBRC 1777_NM2, carrying *TPS2* deletion, showed a strongly non repressed phenotype. NBRC 1777_NM2 also displayed the same non repressed phenotype on sucrose (Figure 4C), suggesting that T6P has an effect on general glucose repression rather than specific galactose metabolism. Glucose repression on xylose metabolism genes does not operate via Mig1 (Hua et al., 2019), and this is confirmed from NBRC 1777_NM4 mutant's phenotype on xylose (Figure 4C).

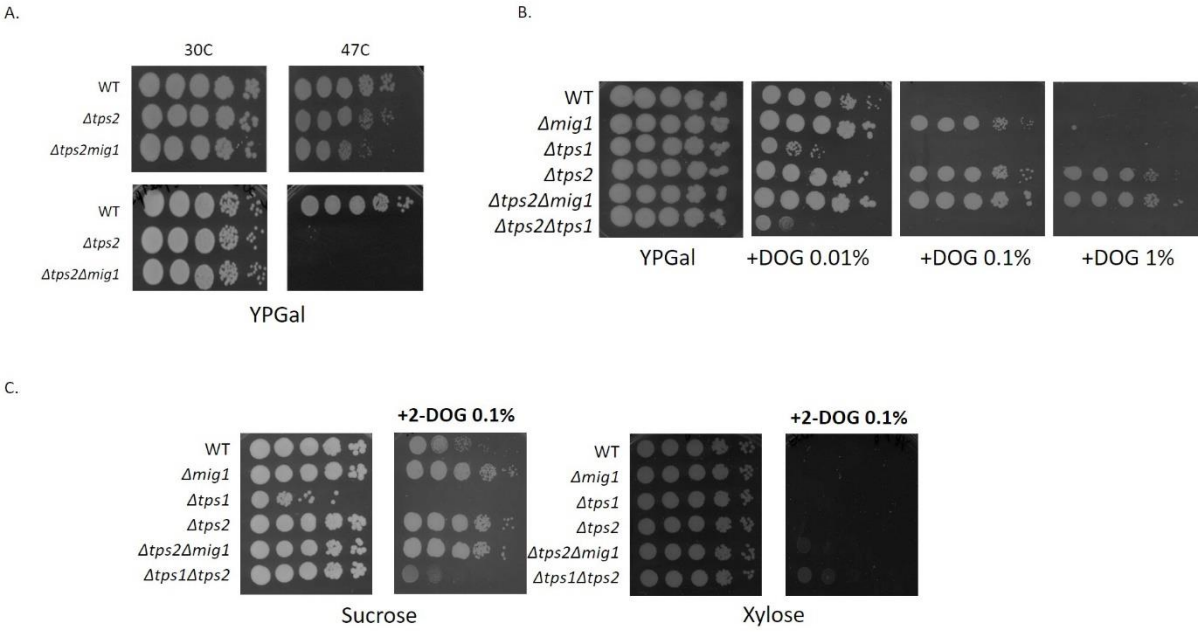

Figure 4 *TPS2* deletion deregulates galactose metabolism in a way indipendent of *MIG1*. A) *MIG1* was deleted in the *tps2* mutant. The resultant double mutant (Δ*tps2*Δ*mig1*), Δ*tps2* and the parental strain were plated in serial dilution on YPGal plates and grown at 30°C and 47°C for 24 hours. **b)** Δ*mig*1, Δ*tps1*, *Δtps2,* Δ*tps2*Δ*mig1* and Δ*tps1Δtps2* strains were compared to the parental strain via serial dilution on YPGal plates containinig increasing concentration of 2-DOG. The plates were incubated at 30 °C for 24 hours. **c)** Δ*mig1*, Δ*tps1*, Δ*tps2*, Δ*tps2*Δ*mig1* and Δ*tps1Δtps2* strains were compared to the parental strain's glucose repression was measured via spotting serial dilutions of the strains on sucrose and xylose with and without 2-DOG. The plates were incubated at 30 °C for 24 hours. Strains used: Δtps1 (NBRC 1777_NM1), Δtps2 (NBRC 1777_NM2), Δtps1Δtps2 (NBRC 1777_NM3), Δmig1 (NBRC 1777_NM4), Δtps2Δmig1 (NBRC 1777_NM5).

Regulation of GAL genes by T6P

In an attempt to shed some light on the reasons why NBRC 1777_NM2 has a temperature sensitive phenotype on galactose, and how *TPS2* deletion affects galactose genes regulation, gene expression from the Leloir pathway was measured via mRNA expression analysis of WT and Δ*tps2*, during a temperature switch experiment. The experiment was performed in bioreactor: the two strains were initially cultivated at 30°C on YPGal. When the strains reached an A600nm of 1, indicating the beginning of the exponential phase (Figure 5A), the temperature was rapidly increased to 47 °C by switching the bioreactor's temperature control set point (Figure 5B). Samples for mRNA extraction were collected right before the temperature control switch (30°C sample) and after 30 minutes from the temperature switch, when the culture had reached a temperature of 47 °C (Figure 5C). The cDNA relative mRNA abundance was quantified via RT-qPCR, to compare the galactose metabolism genes' expression between NBRC 1777_NM2 and the WT strains under both temperatures. The relative expression of *GAL1*, *GAL7*, *GAL10*, *TPS1*, *TPS2*, *PGM2* and *UGP1* genes was measured. The Ct values obtained are reported in Supplementary Table 1. First, the values were analysed to quantify the relative expression of the genes at 47 °C compared to 30 °C for the two strains (Figure 5C). Panel C shows that in the WT there is a strong temperature-dependent induction of *GAL10, TPS1, TPS2* and *UGP1*. This is expected and indicates induction of trehalose synthesis. However, a more modest induction of the same genes in Δ*tps2* was observed. Another way to analyse the same data is to compare genes expression between NBRC 1777_NM2 mutant and the WT strain under the two tempertures (Figure 5D). Panel D shows that, at 47 °C, expression of all the GAL genes is actually higher in Δ*tps2* than in the WT strain. In addition, at 30 °C the expression of *GAL10, TPS1, TPS2* and *UGP1* is much higher in the mutant strain than the WT. This explains the apparent weak induction of the genes at 47 °C seen in panel C: these genes are already highly expressed at 30 °C in Δ*tps2*. This indicates that accumulation of T6P (presumably only modest at 30 °C) leads to deregulated expression of the GAL genes at 30 °C, with even stronger effect at 47 °C.

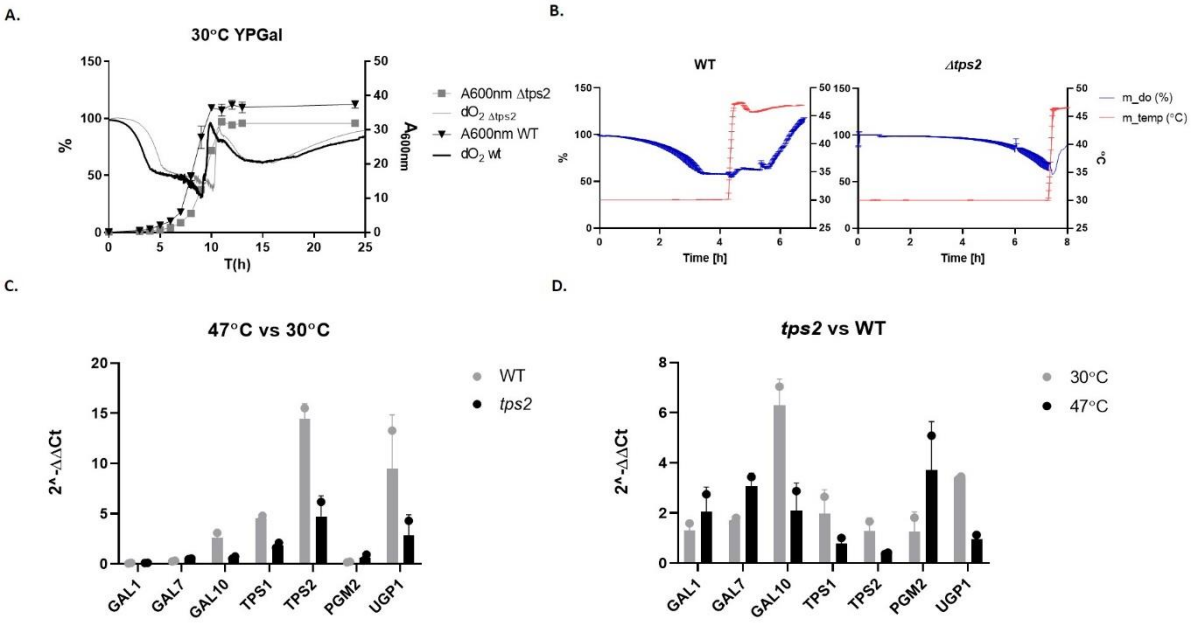

Figure 5. T6P accumulation causes different expression of galactose metabolism genes at high temperatures. a) Kinetic of growth (A_{600nm}) and dissolved oxygen (dO_2) consumption of Δ*tps2* and WT strains in bioreactor at 30°C on YPGal. The strains were individually grown in batch at 30°C for 24 hours to establish the time of beginning of exponential phase, represented by A600nm=1. The A600nm was measured regularly to monitor cells' growth. **b)** Temperature switch experiment. The WT and Δ*tps2* strains were grown individually in bioreactors at 30 °C on YPGal until reaching A600nm= 1. The blue line represents the kinetic of the dissolved oxygen (dO2, left y-axis), indicative of the strains' growth. When A600nm was reached, the temperature was switched from 30°C to 47°C (temperature represented by the red line, right y-axis). Samples were collected from the fermenter immediately before and 30 minutes after the temperature switch, for qPCR analysis. **c**) Relative expression of the galactose metabolism genes in Δ*tps2* and the WT strains, comparing the 47 °C versus the 30 °C conditions. Changes are reported as $2^{\wedge-\Delta\Delta Ct.}$ The gene *ACT1* was used as reference gene for ΔCt calculation. **d)** Relative expression of the galactose metabolism genes at 30 °C and 47 °C, comparing *Δtps2* versus WT strains. Changes are reported as $2^{\wedge-\Delta\Delta Ct.}$ The gene *ACT1* was used as reference gene for ΔCt calculation.

5. Discussion

Trehalose biosynthesis in yeast is traditionally associated with thermotolerance (De Virgilio *et al.*, 1994; Gancedo and Flores, 2004). In fac*t, TPS2* upregulation and accumulation of trehalose have been reported following heat stress (Argüelles, 1997; Hottiger et al., 1987), indicating that trehalose acts as thermoprotectant conferring cells thermotolerance. However, this assumption was challenged in more recent studies showing that the role of trehalose biosynthetic pathway is fundamentally metabolic (Gibney et al., 2015). Moreover, the two enzymatic subunits of the TPS complex (Tps1 and Tps2), as well as the intermediate trehalose-6-phosphate (T6P), have been found to be involved in central cellular processes such as sugar fermentation and glucose repression (Vicente *et al.*, 2018), regulation of gluconeogenesis (Deroover et al., 2016), and also autophagy and cell homeostasis (Kim et al., 2020).

Upregulation of *TPS1* and *TPS2* was previously reported in *K. marxianus* upon adaptation to osmotic stress (Saini et al., 2017) and under ethanol stress (Alvim et al., 2019).Moreover, trehalose accumulation was measured upon heat shock (Erdei et al., 2011). However, the role of the individual enzymatic subunits in the yeast's thermotolerance was not previously characterized in this yeast. Analysis of the transcriptome of *K. marxianus* grown in chemostat under high temperature, showed upregulation of the trehalose biosynthetic genes *TPS1*, *TSL1* and *PGM2*, indicating a possible role in thermotolerance. By constructing inactivating mutations, we confirmed that the TPS complex is involved thermotolerance but the differing phenotypes of *tps1* and *tps2* mutants indicates that the situation is more complex than simply the accumulation of trehalose as a thermoprotectant. Individual knock-out mutants for *TPS1* and *TPS2* are not equally thermosensitive on glucose or galactose, indicating that *K. marxianus*' innate thermotolerance is not due to the presence of trehalose itself, but is dependent on the presence of Tps1 or Tps2. This represents this study's first finding.

S. cerevisiae's *tps1* mutant is impaired because of the lack of T6P synthesis, which is needed to regulate the main hexokinase enzyme Hxk2. In its absence, the cell accumulates G6P and Fru-1,6biP, which are toxic to the cell (Fraenkel & Nielsen, 2016). In fact, *S. cerevisiae*'s *HXK2* is inhibited in vitro by T6P (Blázquez et al., 1993). Moreover, *TPS1* deletion has been linked to Ras kinase hyper-activation, due to the accumulation of one of the *HXK2* products, Fructose 1-6-BP. Ras hyper-activation

triggers apoptosis and results in a growth impairment of the *tps1* mutant on glucose (Peeters *et al.*, 2020). In contrast, *K. marxianus* NBRC 1777_NM1 is viable on glucose at 30°C, although thermosensitive. The thermosensitive phenotype of *tps1* mutants on glucose has also been reported in *Yarrowia lipolytica* , *Schizosaccharomyces pombe* and *Ogatea polymorpha* (Flores et al., 2011; Franco et al., 2000; Kramarenko et al., 2000), whose mutants are viable at 30°C, suggesting the lack of T6P is only detrimental at high temperatures. This is likely due to the combined effect of the accumulation of toxic glucose-6P and fructose 1-6-BP, and the temperature stress. The lack of growth impairment of *K. marxianus'* NBRC 1777_NM1 at 30°C indicates that its *HXK2* is perhaps insensitive, or not as sensitive, to T6P. Another possible explanation is that a different hexokinase is present in *K. marxianus*, which carries out the role of *HXK2*, without T6P influence, as previously suggested for *Y. lipolytica* and *O. polymorpha* (Flores et al., 2011; Kramarenko et al., 2000). However, a single hexokinase gene, also known as *RAG5*, is known in *K. marxianus* (Hua et al., 2019). This copy is highly similar (73% identity, e-value:0.00) to the one from *S. cerevisae*. Moreover, since the *tps2* mutant showed derepressed phenotype on sucrose, but repression on xylose, consistently with the hypothesis of an interplay between □□P accumulation and glucose repression, possibly via Hxk2/Rag5. NBRC 1777_NM2's glucose repressed phenotype on galactose, sucrose and xylose is consistent with what observed for mutants of *MIG1* in *K. marxianus* (Hua et al., 2019). However, since additional *MIG1* deletion in Δ*tps2* showed no recover of the phenotype at 47 °C, this indicates that T6P accumulation acts separately from the *SNF1/MIG1* mediated regulation of the galactose gene.

K. marxianus presents an innate strong redirection of the glycolytic flux toward the pentose phosphate pathway (PPP) (Bellaver *et al.*, 2004; Sakihama *et al.*, 2019; Jia *et al.*, 2021), which naturally metabolizes Glu-6P; therefore the negative effect of *TPS1* deletion could be attenuated by a better ability to metabolize increased quantity of sugar phosphates in *K. marxianus*. Accordingly, when NBRC 1777_NM1 was grown on galactose, similar *S. cerevisiae* (van Vaeck et al., 2001), the strain showed only a mild temperature sensitivity. On galactose in fact, glucose-6P is only synthesized from the Leloir pathway's enzyme Pgm1, bypassing Hxk/Ras5 and consequently avoiding sugar phosphates accumulation.

K. marxianus NBRC 1777_NM2 showed thermosensitivity when grown on galactose at 47 °C, but not on glucose, suggesting the effect of T6P accumulation on glucose repression, only evident at high temperature because it is when T6P is accumulated (De Virgilio' et al., 1993). Accordingly, a qPCR analysis on the Leloir pathway and trehalose metabolism genes showed overexpression of the GAL genes at 47 °C, and of *GAL10*, *TPS1*, *TPS2* and *UGP1* genes at 30 °C in Δ*tps2*. The deregulation of the GAL genes in *K. marxianus tps2* mutant, could cause a toxic accumulation of sugar phosphates intermediates such as Glu-6P and Gal-1P , resulting in growth impairment. Fittingly, *S. cerevisiae* mutant strains with increased flux toward the galactose metabolism showed upregulation of *PGM2* and accumulation of the sugar phosphate galactose-1P (Bro et al., 2005; De Jongh et al., 2008), found to be toxic (Gibney et al., 2018). Moreover, since NBRC 1777_NM2*'s* growth impairment on galactose is only observed under high temperature, this indicates that T6P accumulation is responsible for *K. marxianus* galactose metabolism's genes' deregulation.

In conclusion, this study showed the role of trehalose biosynthetic process in *K. marxianus*' central carbon metabolism's regulation, as evident by the different phenotypes generated by single disruption of the TPS subunits. These elements could be the cause of *K. marxianus*' peculiar traits, including its thermotolerance. In addition, the findings suggest an additional mechanism of glucose repression in *K. marxianus*, linked to TP6 accumulation but independent from *MIG1*. Although this was not identified, it should be further looked into, since improving lactose utilization is desirable for the valorisation of lactose-rich substrates or mixed-sugar biomasses of which galactose is a component, such as lignocellulose (Bro et al., 2005).

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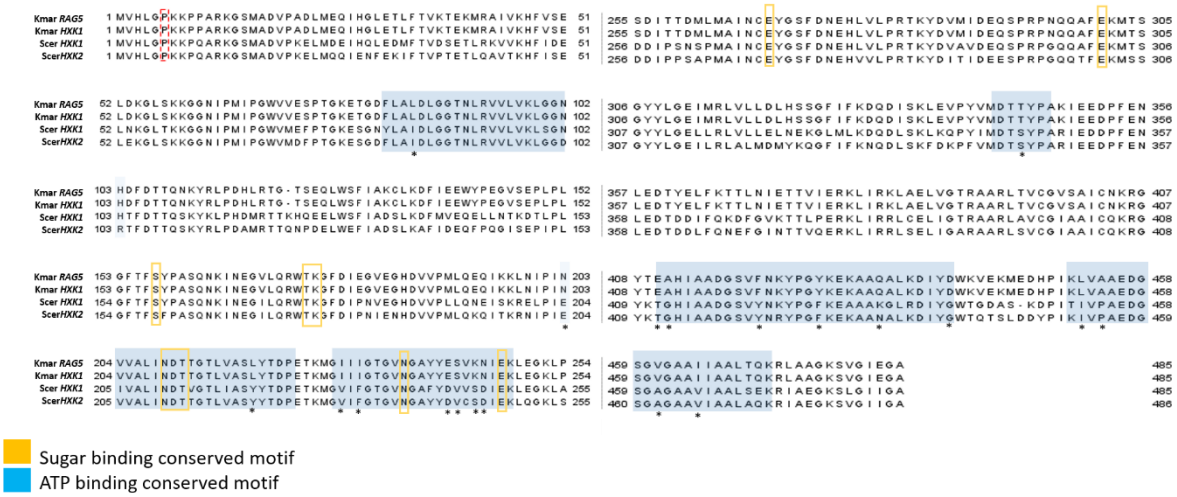
7. Supplementary figures



Supplementary figure 1. ATP binding sites are not conserved between *S. cerevisae* and *K. marxianus*' *HXK/RAG5* protein sequences. Protein sequences were retrieved from Blast NCBI database and aligned via Clustal. The sequence from *K. lactis* is included for comparison. The sugar (yellow) and ATP (blue) binding sites are indicated. The * represents differences between *S. cerevisiae*'s and *K. marxianus'* sequences.

Chapter VII

General conclusions

1. Summary of Results

The overall scope of this thesis was to characterize the thermotolerance phenomenon in *Kluyveromyces marxianus* from a physiological and genomic point of view. This topic was chosen due to two main reasons: although thermotolerance represents a desirable natural trait of this yeast, the subject has been poorly studied in *K. marxianus* and much of what is known can only be inferred from *S. cerevisiae* studies. In addition, obtaining a deeper understanding of stress response mechanisms could allow engineering of robustness in strains used for biotechnological applications, with beneficial effects on the process' sustainability. To study thermotolerance in this yeast, a combination of fermentation methods, genomics and molecular techniques was used.

Batch fermentations in bioreactor were carried out to obtain a reproducible portrait of *K. marxianus'* physiological parameters under high temperature (Chapter III). This revealed substantial differences between *K. marxianus* strains' metabolism at 30 °C versus 40 °C, including differences in growth rate and oxygen uptake rate. Chemostat mode fermentations were then used to reproduce long term stressful conditions and study the stress adaptation response of CBS 6556 strain under low pH, high temperature and high osmolarity stress (Chapter III). A transcriptomic analysis from this study revealed overlaps between the main biological processes induced or repressed across the three stresses, but showed a stress-specific induction of genes. In fact, no gene was upregulated in all three tested stresses.

A larger scale study (Doughty et al., 2020), including the transcriptomic data obtained in Chapter III as well as equivalent data from other budding yeast species, suggested that yeast's stress adaptation response is prevalently constituted by evolutionary young genes. This hypothesis was addressed in *K. marxianus* in Chapter IV, by focusing on genes that were upregulated during the steady state growth under high temperature and therefore involved in the yeast's adaptation to temperature stress. 11 out of the 12 upregulated genes identified as *K. marxianus'* evolutionary young genes were deleted using molecular techniques. Physiological characterization of the resulting mutants showed that *KLMX_70384* plays a role in adaptation to high temperature, providing more evidence supporting the initial hypothesis.

In Chapter V two strains: UCC01, a spontaneous mutant displaying uncharacteristic thermosensitivity, and ATCC 26548, a strain with innate thermotolerance were

compared. The genome comparison resulted in a list of approximately 1000 SNPs between the two strains' genomes. Upon filtration of the SNPs based on prediction of a disruptive effect on the encoded protein sequences, 5 variants were identified as potentially responsible for UCC01's phenotype. These variants were contained in genes predicted to have a role in the yeast's oxidative stress signalling response. Accordingly, a physiological characterization of UCC01 showed its oxidative stress sensitivity and ROS accumulation at high temperature compared to ATCC 26548. Deletion of two out of the 5 SNPs-containing genes in ATCC 26548, *CYC8* and *YAK1*, resulted in an oxidative stress sensitive phenotype, although did not result in thermosensitivity. Nonetheless, the findings allow us to speculate that the oxidative stress response is paramount for *K. marxianus*' WT strains' innate thermotolerance.

Finally, since trehalose accumulation is invariantly linked to thermotolerance in yeast, trehalose metabolism was studied in *K. marxianus*. Knock-out mutants of the two enzymatic subunit of the trehalose biosynthetic complex (TPS) were physiologically characterized at high temperature. The two mutants showed a carbon source-dependent thermosensistive phenotype which differs from what observed in other yeast species. This allowed to speculate a link between central carbon metabolism and trehalose biosynthesis and could be the cause of *K. marxianus*' peculiar traits, including its thermotolerance.

Overall, by undertaking different approaches, the studies carried out contributed to describing the many layers of *K. marxianus* thermotolerance response, which can be defined as the results of many concurring mechanisms as outlined below and summarized in Figure 1.

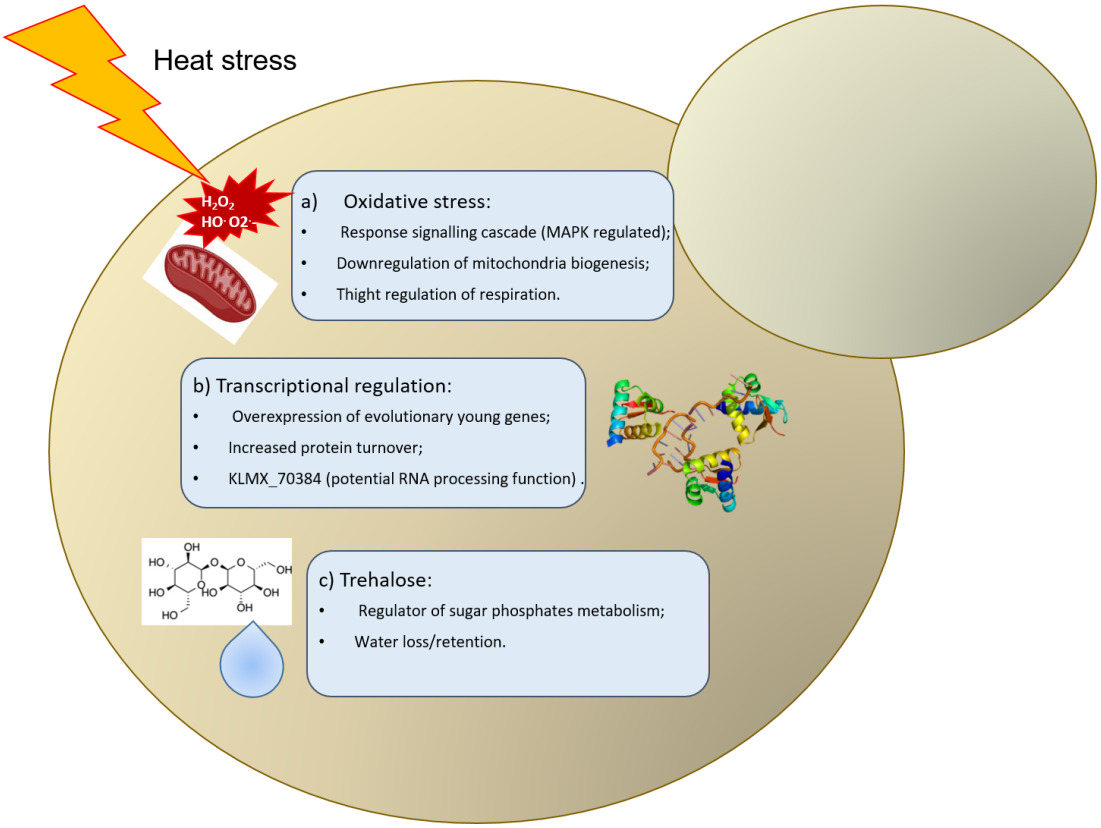


Figure 1 Thermotolerance in *K. marxianus*. This thesis work identified several concurring mechanisms contributing to *K. marxianus* thermotolerance. These, as listed in the below paragraphs, occur at several physiological levels and are here summarized in a schematic representation,

2. Physiological and genomic traits linked to *K. marxianus'* thermotolerance

A first approach to study *K. marxianus* thermotolerance involved a large scale investigation of its physiological and transcriptomic response to high temperature, using bioreactor fermentations and RNA-seq analysis, this allowed to identify the main biological processes involved. Firstly, considering the huge strain and conditions variability present across previous *K. marxianus* studies, a physiological comparison of three strains of interest was conducted during aerobic batch growth in bioreactors (Chapter III) in order to have baseline physiological parameter from which to start this investigation. The haploid strains NBRC 1777 and CBS 6556, and diploid CBS 397 were compared during growth under 30 °C and 40 °C. CBS 397 was the fastest growing strain at 40°C (μmax: 0.92 h^{-1}), indicating that diploidy is possibly advantageous when it comes to thermotolerance; however, comparisons of more diploid strains is necessary to fully establish this. Both the diploid CBS 397 and the haploid CBS 6556 showed an almost doubled growth rate at 40 °C compared to 30°C, indicating that this temperature is optimal for their growth and confirming *K.*

marxianus natural thermotolerance. Although NBRC 1777 proved to be the fastest growing strain at 30 °C (μmax: 1.03 h^{-1}), its biomass yield and growth rate both decreased at 40 °C, indicating a higher maintenance cost associated with growth under high temperature. The oxygen uptake rate was also identified as a parameter potentially influencing thermotolerance: NBRC 1777 showed significantly higher oxygen uptake rates compared to the other strains, but this seems not to favour the strain under high temperature, since its growth rate resulted diminished. Overall this comparison revealed that *K. marxianus* physiological responses to high temperature is highly variable and strain-dependent.

The analysis was continued in bioreactor fermentation in chemostat mode, which allows for rigorous comparison of the same strain under different conditions during steady state growth and it also allows reproduction of long-term stress conditions. CBS 6556 was selected for this comparison. A first finding was that the strain showed lower biomass yield and higher glucose uptake rate at 40°C in chemostat, differently to what observed during the batch cultivation for the same strain, where the biomass yield of the strain remained unchanged at 40 °C compared to 30 °C. We speculated that this reduction in biomass yield is due to the high maintenance costs related to growth under prolonged temperature stress. However, no physiological studies are available in chemostat for comparison with what observed in this study.

Chemostat cultivation also eliminates the transcriptional variability described for *K. marxianus* at different stages and rates of growth (Yu et al., 2021), and allows to study stress adaptation. CBS 6556 was therefore grown under three different long term stresses: high temperature, low pH and high osmolarity. The transcriptomic analysis performed on all three conditions allowed comparison of the biological processes involved in the response to different industrially relevant stresses, and to determine which aspects were specific to high temperature adaptation. The analysis revealed that (1->6)-beta-D-glucan biosynthetic process, autophagy of mitochondrion, endosome to vacuole transport, lysosome macro autophagy and protein targeting to vacuole are processes upregulated under both osmotic and high temperature stress. In particular the gene *KRE9*, involved in cell wall beta-glucan assembly, was found to be upregulated under both conditions. A common denominator among these processes could be the stress induced activation of the MAPK cascade, which directs the cell's response to both osmotic and heat stress stimuli, via promoting cell integrity,

proliferation and import of osmolytes such as glycerol (Chen & Thorner, 2007; Saito, 2010). Moreover, processes such as protein stabilization, protein refolding and ubiquitin-dependent protein catabolic were found upregulated in both high temperature and low pH, indicating that protein stability and turnover are needed for growth under both stresses. Transmembrane transport of iron, ion involved in multiple cellular processes (Martínez-Pastor et al., 2017), was also found to be upregulated under both high temperature and low pH stress. In particular several high affinity iron transporters-encoding genes such as *FTR1*, *FET3*, MRS4 and *FTH1* and the siderophore transporters *ARN1* and *ARN2*, present in multiple copies in *K. marxianus*, were found to be upregulated.

A peculiar aspect of thermotolerance emerging from this study is its relationship with oxidative phosphorylation. While an increase in ATP production seems an obvious strategy to provide energy for growth under stressful conditions, GSA analysis revealed that ATP synthesis-coupled proton transport and mitochondrial electron transport were downregulated during growth at 40 °C, while mitophagy was found upregulated. The same regulation pattern was also observed for the osmotic stress response, while the opposite was found under the low pH condition. Growth at high temperature is known to provoke oxidative stress and ROS accumulation in yeast (Davidson et al. 1996; Zhang, Shi, and Jiang 2015), and leakage of electrons from the mitochondrial electron transport chain is considered the major intracellular source of ROS. We therefore speculate that a tight regulation of mitochondrial biogenesis to reduce electron leakage is one of the strategies *K. marxianus* employs to limit ROS accumulation and cope with long-term temperature stress. Accordingly, genes of the mitochondrial respiratory chain were previously found downregulated in *K marxianus* under high temperature stress, although respiration rate was found upregulated (Fu et al. 2019). These findings altogether could explain why NBRC 1777 strain, showing the highest growth rate at 30 °C, also showed a decreased growth rate at 40 °C: the strain's very high oxygen uptake rate could be a disadvantage for its growth at high temperature, because of the ROS accumulation resulting from it.

Although many biological processes were enriched in response to two out of three stresses tested, indicating a certain functional overlap, there were no upregulated and only three downregulated genes in common amongst all stressful conditions, suggesting that at a gene level the transcriptional response is stress-specific in *K.*

marxianus. As an example, the protein target to vacuole process was upregulated under both high temperature and osmotic stress, but distinct genes were upregulated for this process (respectively *SNR2* and *VID24*, and *MUP1* and *VSP25*).

The data from the chemostat fermentations in Chapter III contributed to a larger study that hypothesised that evolutionary young genes could be involved in adaption growth under adverse conditions (Doughty et al., 2020). In order to investigate this, we assessed genes that were upregulated during steady state growth at high temperature in *K. marxianus*. Twelve genes were identified, these genes were unique in *K. marxianus* and upregulated at 40°C. We successfully mutagenized eleven of the twelve candidate genes to assess their role in high temperature growth and found that inactivation of *KLMX_70384* displayed the predicted phenotype – normal growth at 30°C but compromised growth at 45 - 47°C. The mutant was completely outcompeted by its parental strain in a co-culture experiment at 47 °C indicating, consistent with the hypothesis that was being tested, that the gene is required for competitive growth at high temperature.

Evolutionarily young genes are generally short and non-essential genes. This was also true of the candidate genes that we identified: ten of the open reading frames were between 28 and 225 amino acids, including *KLMX_70384*, which codes for a relatively short protein (83 aa). The low similarity to the splicing associated protein *SART-1*/*SNU66*, as well as its predicted linear three helix structure, allows to speculate that KLMX_70384 may be an RNA-binding protein, possibly associated with the RNA processing or splicing machinery (Hoang et al., 2016; Makarov et al., 2002). Accordingly, a group of deletion mutants for mRNA processing genes display a thermosensitive phenotype in *S. cerevisiae* (Hossain & Johnson, 2014).

The previously suggested scenario (Doughty et al., 2020) speculates that mutations that occur in ancient genes, typically associated with core processes, are more likely to be detrimental and lost. Young genes, which are typically not required under standard growth conditions, can better tolerate mutations and so pools of mutants build up. When conditions become adverse, those rare mutations that confer a growth advantage are selected. In this way, young genes become important for adaptation to new niches. Based on our data, we can speculate that the emergence of *KLMX_70384* played some role in helping *K. marxianus* adapt for growth at higher temperatures, a

trait that is absent in all other *Kluyveromyces* species. It also helps explain why a conserved thermotolerance mechanism is not found in other yeasts, since it implies that different species-specific genes will be involved.

2.1 Oxydative stress response and trehalose metabolism indirectly contribute to *K. marxianus* thermotolerance

Other factors were found to have a role, although indirectly, on *K. marxianus* thermotolerance; these are the integrity of a oxidative stress signalling cascade and early stress response (Chapter V) and the control of central carbon metabolism and sugar phosphates accumulation by intermediates of the trehalose biosynthetic pathway (Chapter VI).

In fact, the SNPs analysis carried out in Chapter V between a thermotolerant and oxidative sensitive mutant strain, UCC01, with the parental thermotolerant ATCC 26548, led to identification of five candidate genes containing missense variants that are linked to general stress response activation or regulation in yeast. Although UCC01's sensitivity to high temperature could not be reproduced in ATCC 26548 by deleting of any of these candidates, noticeably knock-out mutations of two out of five SNP-containing genes, *CYC8* and *YAK1*, resulted in an oxidative stress sensitive phenotype in ATCC 26548. We therefore speculate that UCC01 phenotypes are due to the lack of a proper oxidative stress response, which in yeast is normally initiated by transcriptional factors involved in the response to both high temperature and oxidative stress (Farrugia & Balzan, 2012). Accordingly, Yak1 activates the general stress transcriptional factors Msn2 and Msn4 in *S. cerevisiae* (Farrugia & Balzan, 2012); while Cyc8 activates the SWI/SNF chromatin remodelling complex, also involved in thermotolerance (Smith & Johnson, 2000). The importance of the oxidative stress response in *K. marxianus* thermotolerance, was further confirmed by physiological characterization of UCC01 which, contrary to ATCC 26548 strain, displayed ROS accumulation at high temperature, and a loss of viability at 45 °C dependent on high levels of oxygen availability. Therefore, although the SNPs analysis failed to identify unique variants responsible for the thermosensitive phenotype, UCC01's loss of thermotolerance could be the result of the accumulation of variants affecting the strain's early stress response's transcriptional cascade. Altogether these findings allows us to speculate that the same cascade is essential to

WT strains of *K. marxianus* to maintain their innate thermotolerance, as well as oxidative stress tolerance.

The other aspect found to play a role in *K. marxianus* thermotolerance was the regulation of central carbon metabolism by trehalose biosynthesis. In fact, unlike what traditionally believed for yeast (De Virgilio *et al.*, 1994; Gancedo and Flores, 2004), the accumulation of trehalose does not confer a thermotolerance phenotype, but more likely the regulation of sugar phosphates accumulation under high temperature deriving from the presence of the trehalose intermaediate T6P. Accordingly, individual deletion of the two enzymatic subunit of the TPS complex yielded differently thermosensitive phenotypes on glucose or galactose in yeast. In *S. cerevisiae* for instance, *tps1* mutant's growth is impaired because of the lack of T6P synthesis, which is needed to regulate the main hexokinase enzyme Hxk2. In its absence, the cell accumulates G6P and Fru-1,6biP, which are toxic to the cell (Fraenkel & Nielsen, 2016). Moreover, *TPS1* deletion has been associated with Ras kinase's hyper-activation, due to the accumulation of one of the *HXK2* products, Fructose 1-6-BP. Ras hyper-activation triggers apoptosis and results in a growth impairment of the *TPS1* mutant on glucose (Peeters *et al.*, 2020). In contrast, *K. marxianus* NBRC 1777_NM1 is viable on glucose at 30°C, although it displays thermosensitivity. The lack of growth impairment of *K. marxianus'* NBRC 1777_NM1 at 30°C indicates that its *HXK2* is perhaps insensitive, or not as sensitive, to T6P. Another possible explanation, already suggested for *Y. lipolytica* and *O. polymorpha* (Flores et al., 2011; Kramarenko et al., 2000), is that a different hexokinase is present in *K. marxianus*, which carries out the role of *HXK2* and irrespectively of T6P. However, a single hexokinase gene, also known as *RAG5*, is known in *K. marxianus* (Hua et al., 2019). More likely, the prevalent redirection of the glycolytic flux toward the pentose phosphate pathway (PPP) in *K. marxianus* (Bellaver *et al.*, 2004; Sakihama *et al.*, 2019; Jia *et al.*, 2021), which primarily metabolizes Glu-6P, allows for the attenuation of the negative effect deriving from *TPS1* deletion.

In addition, *K. marxianus* NBRC 1777_NM2, *tps2* mutant, showed thermosensitivity when grown on galactose at 47 °C, but not on glucose, suggesting an exclusive effect of T6P accumulation on galactose metabolism. A strong glucose de-repression of the galactose metabolism was shown after *TPS2* deletion, reproducing the phenotype of

the *mig1* mutant, but more accentuated. This was consistent with what observed for mutants of the transcriptional activators *SNF1/MIG1* (Hua et al., 2019). Since additional *MIG1* deletion in Δ*tps2* showed no recovery of the phenotype at 47 °C, this indicates that T6P accumulation acts separately from the *SNF1/MIG1* mediated regulation of the galactose gene. The deregulation of the galactose metabolism genes deriving from *TPS2* deletion was confirmed by RT-qPCR. In fact, the Leloir pathway genes were found overexpressed in NBRC 1777_NM2 compared to the WT strain on galactose at 30 °C. In addition *GAL1*, *GAL7* and *PGM2* were overexpressed in NBRC 1777_NM2 at 47°C compared to the WT. The overexpression of galactose metabolism genes in *K. marxianus tps2* mutant, could cause a toxic accumulation of sugar phosphate intermediates, such as Glu-6P and Gal-1P, resulting in growth impairment. Moreover, since NBRC 1777_NM2*'s* growth impairment is only observed under high temperature, we speculate that T6P accumulation is responsible for the galactose metabolism gene deregulation. This study revealed peculiar elements of *K. marxianus*' central carbon metabolism regulation by the trehalose biosynthetic process. These elements could be the cause of *K. marxianus*' peculiar traits, including its thermotolerance. In addition, the findings suggest an unknown additional mechanism of glucose repression of galactose genes in *K. marxianus*, linked to TP6 accumulation but independent from *MIG1*.

Noticeably, trehalose has been shown to protect the cell membrane from dehydration (Magalhaes *et al*., 2018), freeze-thaw (Chen et al., 2022) and osmotic stress (Iturriaga et al., 2009) in *S.cerevisiae*, as weel as in non-saccharomyces species (De Anchieta Camara *et al.*, 2019). In *K. marxianus*, trehalose accumulation has been observed under heat shock (Erdei et al., 2011) and osmotic stress (Saini et al., 2017). Fittingly the transcriptomic analysis under high temperature contained in this thesis, showed that trehalose metabolism genes are upregulated under both high temperature and osmotic stress conditions. This suggests that trehalose may play a role water content regulation and cell membrane protection in *K. marxianus*, both important players of thermotolerance (Matton et al., 2021).

3. Relevence and future perspectives

Bioreactor cultivations offer the advantage of providing highly comparable and reproducible growth conditions: in our case the comparison highlighted *K. marxianus*'

strain-dependent variability of the main physiological parameters under high temperature. This should be kept in mind when carrying out studies in different *K. marxianus* strains, when a pre-characterization of the basal physiological parameters might be needed for data comparability. The integration of physiological data and transcriptomic analysis in Chapter III helped characterize CBS 6556 strain under industrially relevant stressful conditions, and the use of chemostat cultivations also helped to gain insights on *K. marxianus*' stress adaptation mechanisms, in particular thermotolerance.

Part of the rationale for studying thermotolerance is to identify genes and processes that can be used to improve yeasts for biotechnology. One hope is that it may be possible to engineer thermotolerance into mesophilic yeasts by heterologous expression of single genes, and obtain a more robust strain. We tested whether expression of *KLMX_70384*, a young *K. marxianus* gene with a role in thermotolerance identified in Chapter IV, would improve the thermotolerance of *K. lactis*, but it did not. Regardless, the study contains a valuable pipeline which can be used to understand what processes are required to function at higher temperature, and could identify alternative routes to strain improvement. Moreover, while not definitive, the indication that *KLMX_70384* could be involved in RNA processing is a further suggestion that this is an area worth further investigation.

However, the overall lack of functional annotation for the identified *K. marxianus* young genes, reflects the limitation of homology-based algorithms for genome annotation (Sinha et al., 2020), especially when it comes to species phylogenetically distant from the extensively annotated *S. cerevisiae* (Botstein & Fink, 2011). In this respect, new techniques such as ribosome profiling, providing new potent genome annotations are a valuable resource to study *K. marxianus* traits (Fenton et al., 2022).

In Chapter V, a new aspect relating to thermotolerance in *K. marxianus* was unveiled: the relationship with the oxidative stress response and the early stress signalling cascade. Indeed, while the mode of action of several transcription factors is still not fully characterized in *K. marxianus* (Hua et al., 2019), understanding the molecular mechanisms underlying the cross-talk between diverse stress responses is essential for strain improvement by genetic engineering, and should be further explored. The study also demonstrates the importance of controlling the oxygen flux during industrial

fermentations, since the derived oxidative stress could affect important physiological traits of the strain, and its productivity.

Finally, the suspected role of trehalose in yeast thermotolerance was challenged by recent studies showing that its function is fundamentally metabolic (Gibney et al., 2015). In fact, the two enzymatic subunits of the TPS complex (Tps1 and Tps2), as well as the intermediate trehalose-6-phosphate (T6P), have been found to be involved in central cellular processes such as sugar fermentation and glucose repression (Vicente *et al.*, 2018), regulation of gluconeogenesis (Deroover et al., 2016), and also autophagy and cell homeostasis (Kim et al., 2020). This is consistent with the findings of Chapter VI, where an additional mechanism of glucose repression of *K. marxianus* galactose genes was hypothesized, linked to TP6 accumulation but independent from *MIG1*. Although this mechanism was not identified, it should be further studied, since improving lactose utilization is desirable for the valorisation of lactose-rich substrates or mixed-sugar biomasses of which galactose is a component, such as lignocellulose (Bro et al., 2005).

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Supplementary and supporting data:

The supplementary tables supporting chapters III, V and VI of this thesis' work are contained in the CHASSY_thesis_suppl_materialNM.xls file, which can be found at https://doi.org/10.5281/zenodo.6378732 . Chapter IV's supplementary material can be found in Montini et al. (2022). The supplementary tables contained in the file are listed below, divided by chapter. The name of the tables correspond to the name of the tabs in the CHASSY_thesis_suppl_materialNM.xls file.

Chapter III:

CHAPT3_Supp_Table_1. Gene-level statistics values of DE analysis deriving from RNA-seq between stressful (High osmolarity, low pH and high temperature) and standard conditions. The total RNA was extracted from samples collected during chemostat fermentations.

CHAPT3_Supp_Table_2. Gene set analysis (GSA) result tables deriving from RNA-seq between stressful (High osmolarity, low pH and high temperature) and standard conditions. The biological processes are reported, with relative statistical value for up or downregulation. The number of genes up or down-regulated for each process is also reported.

Chapter V:

CHAPT5_Supp_Table_1.Orthologous CDS between UCC01, ATCC 26548 and DMKU3-1024 strains, identified via Orthofinder.

CHAPT5_Supp_Table_2. List of UCC01 strain's exclusive variants. Nucleotide variants and coordinates of the variants are reported, as well as the predicted effect on the encoded protein. The prediction was made via SnpEff software.

Chapter VI:

CHAPT6_Supp_Table_1. Gene-level statistics values of DE analysis deriving from RNA-seq between high temperature and standard conditions**.** The total RNA was extracted from samples collected during the chemostat fermentations in chapter III.

CHAPT6_Supp_Table_2. Ct values from RT-qPCR analysis of galactose metabolism genes.