**Genome-wide identification of genes involved in growth and fermentation activity
at low temperature in *Saccharomyces cerevisiae***

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ABSTRACT

Fermentation at low temperatures is one of the most popular current winemaking practices because of its reported positive impact on the aromatic profile of wines. However, low temperature is an additional hurdle to develop *Saccharomyces cerevisiae* wine yeasts, which are already stressed by high osmotic pressure, low pH and poor availability of nitrogen sources in grape must. Understanding the mechanisms of adaptation of *S. cerevisiae* to fermentation at low temperature would help to design strategies for process management, and to select and improve wine yeast strains specifically adapted to this winemaking practice. The problem has been addressed by several approaches in recent years, including transcriptomic and other high-throughput strategies. In this work we used a genome-wide screening of *S. cerevisiae* diploid mutant strain collections to identify genes that potentially contribute to adaptation to low temperature fermentation conditions. Candidate genes, impaired for growth at low temperatures (12°C and 18°C), but not at a permissive temperature (28°C), were deleted in an industrial homozygous genetic background, wine yeast strain FX10, in both heterozygosis and homozygosis. Some candidate genes were required for growth at low temperatures only in the laboratory yeast genetic background, but not in FX10 (namely the genes involved in aromatic amino acid biosynthesis). Other genes related to ribosome biosynthesis (*SNU66* and *PAP2*) were required for low-temperature fermentation of synthetic must (SM) in the industrial genetic background. This result coincides with our previous findings about translation efficiency with the fitness of different wine yeast strains at low temperature.

KEY WORDS: *S. cerevisiae*, genome-wide screening, psychrotolerance, industrial yeasts, ribosome biosynthesis

1. Introduction

Temperature is one of the main relevant environmental variables that microorganisms have to cope with. For the majority of microorganisms, including yeast species, the natural environment exhibits temporal fluctuations in temperature on scales that range from daily to seasonal. Temperature is also a key factor in some industrial processes that involve microorganisms. In the last few years, a winemaking trend has emerged that consists in lowering fermentation temperatures to improve the aromatic profile of wines (Beltran et al., 2008; Torija et al., 2003). However, lowering fermentation temperatures has its disadvantages, including prolonged process duration and a higher risk of stuck or sluggish fermentation (Bisson, 1999). These problems can be avoided by using yeasts that are better adapted to ferment at low temperature. Low temperature has several effects on the biochemical and physiological properties of yeast cells: poor protein translation efficiency, low membrane fluidity, changes in lipid composition, slow protein folding, stabilisation of mRNA secondary structures, or diminished enzymatic activities (Aguilera et al., 2007). Yet we are still far from fully understanding the molecular and physiological mechanisms of adaptation to low temperatures, or knowing what makes cells more psychrotolerant. From an industrial perspective, such knowledge is important to come up with better metabolic engineering strategies that consider the impact of novel genes and pathways on cold adaptation.

The mechanisms involved in adapting *Saccharomyces cerevisiae* to low temperature have been studied by both conventional and genome-wide technologies. Among the genome-scale approaches, transcriptome analyses are perhaps the most frequently employed method to analyse low temperature adaptation. Most of these studies have focused mainly on cold shock (Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004), but Tai et al. (2007) also analysed

transcriptional changes during long-term exposure of yeast to low temperature. Beltran et al. (2006) performed a transcriptomic study under conditions that were more closely related to industrial situations, and analysed the global transcription of a commercial wine yeast in different industrial wine fermentation stages at low temperature. At the gene expression level, transcription data have provided evidence for most of the adaptation strategies that have been previously described based on physiological studies and individual gene phenotype analyses (Beltran et al., 2006; Chiva et al., 2012; López-Malo et al., 2013; Tronchoni et al., 2014). For the same purpose, *i.e.* improving our understanding of mechanisms of adaptation and acclimatisation of yeast to low temperature, other so-called “omics” have been recently applied to analyse changes in proteins (García-Ríos et al., 2014; Salvadó et al., 2008) and metabolites (López-Malo et al., 2013) in response to growth temperature.

Despite many transcriptomic works, it has been shown that not all genes that are relevant for a biological process can be identified by their transcription profile and, conversely, a transcriptional response against a specific environmental condition does not always indicate the relevance of the cognate gene for adaptation to this condition (Birrell et al., 2002; Tai et al., 2007). Thus another high-throughput technique, which can complement and confirm previous results, is the genome-wide screening of *S. cerevisiae* mutant strain collections. Yeast knock-out (YKO) collections, which cover 96% of the yeast genome, are among the most useful tools that derive from international efforts made towards the genome sequencing and functional analysis of *S. cerevisiae* (Winzeler et al., 1999). Yeast deletion collections consist of ~21,000 *S. cerevisiae* strains, including haploid strains for both *MATa* and *MATα* mating types, and both heterozygous and homozygous diploid strains. Some winemaking-related stress factors have been studied using genome-scale yeast deletion collections by either competition

experiments or phenotypic analyses of individual strains. These included osmotic and ethanol stress (Auesukaree et al., 2009; Fujita et al., 2006; Teixeira et al., 2009; van Voorst et al., 2006; Yazawa et al., 2007; Yoshikawa et al., 2009), high-sucrose (Ando et al., 2006), high glucose (Teixeira et al., 2010), and growth on synthetic must (SM) (Delneri et al., 2008; Novo et al., 2013; Piggott et al., 2011), and high pressure and cold environments (Abe and Minegishi, 2008). Authors Abe and Minegishi used the haploid collection to determine essential genes for growth at low temperature. They identified 56 essential genes for growth at low temperature, involved mainly in amino acid biosynthesis, microautophagy, and in the sorting of amino acid permeases, mitochondrial functions, transcription, mRNA degradation and ribosome synthesis. As a result of this and similar studies, more than 222 mutant strains are now listed in the *Saccharomyces* genome database (SGD) as cold-sensitive.

In this work, we initially carried out the genome-wide screening of the *S. cerevisiae* genes required for growth at low temperature. To this end, the deletion strains of both the homozygous and heterozygous diploid collections were grown at 12°C in YPD. The diploid collections were chosen because it has been well documented that haploid yeast strains exhibit greater sensitivity than diploids to environmental stresses. Several studies have revealed deficiencies with haploid strains, *i.e.*, their lower tolerance to acids, ethanol and other fermentation inhibitors, which makes their use at the industrial level difficult (Garay-Arroyo et al., 2004; Martin and Jönsson 2003). After the genome-wide analysis, some of the genes required exclusively for growing at low temperature were deleted in a *S. cerevisiae* industrial wine yeast strain and these mutants were characterised for their growth capacity and fermentation activity at low temperature.

2. Material and methods

2.1 Yeast strains and media

The homozygous and heterozygous yeast deletion strains in the diploid BY4743 background were purchased from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis). The homozygous collection consisted of a set of approximately 4,800 diploid strains of *S. cerevisiae*, which harboured a deletion in non-essential genes. The heterozygous collection comprised a set of approximately 6,500 diploid strains of *S. cerevisiae*, in which one of the two copies of each gene was individually deleted. Commercial wine strain FX10 was used to construct the simple and double mutants of the selected genes in the genome-wide screening. Strains were cultured in SM (pH 3.3), as described by Riou et al. (1989), but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors. The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L. The following mineral salts were utilised: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, MgSO_4 250 mg/L, CaCl_2 155 mg/L, NaCl 200 mg/L, MnSO_4 4 mg/L, ZnSO_4 4 mg/L, CuSO_4 1 mg/L, KI 1 mg/L, CoCl_2 0.4 mg/L, H_3BO_3 1 mg/L and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ 1 mg/L. The vitamins that follow were employed: myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotin 0.003 mg/L. The assimilable nitrogen source was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in the amino acid form). The population inoculated in the synthetic grape must came from an overnight culture in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C.

2.2 Genome-wide screening for deletion mutants with a low temperature growth defect (LTGD)

To screen the homozygous and heterozygous mutant collections for growth defects at low temperature, the different strains were inoculated from stock cultures (96-well plates of liquid YPD stored at -80°C) onto the YPD-agar surface using a 96-pin in plates Nunc™ OmniTray™ (128 mm × 86 mm; Thermo Scientific). Each strain was spotted in quadruplicate. These plates were incubated for 7 days at 12°C until large colonies formed. Plates were revised visually and the detected strains with no visible growth were selected to be retested as indicated below.

The strains with no visible growth in the previous screening were pre-cultured in 96-well plate liquid cultures. After 48 h, strains were pinned onto the YPD-agar surface using a 96-pin tool, and were incubated at 12°C, 18°C and 28°C. Each strain was spotted in tetraplicate. The selected strains were monitored by means of Integrated Optical Density (IOD) measurements. The IOD quantification of each colony was obtained by an image analysis performed with the open source software COLONYZER, an image analysis tool that quantifies the cell density of the arrays of the independent micro-organism cultures grown on solid agar. A detailed description of COLONYZER, its algorithms, the motivation for its development, and some example analyses can be found in Lawless et al (2010). An example of how COLONYZER fits into the Quantitative Fitness Analysis workflow is seen in Banks et al (2012).

To quantitatively evaluate the growth capacity of the tested strains, the area under the IOD–time curve was employed as a measure of overall yeast growth performance. Use of this tool has been recently proved by Arroyo-López et al. (2009) because its relation with biological growth parameters. Area under IOD–time curve was inversely related to the lag phase, but linearly related to both the maximum population level and maximum specific growth rate of yeasts (Arroyo-López et al., 2009). In this step around 350 growth curves, including selected mutants, wild-type and positive

control strains, were registered and processed with the Excel software to obtain its area under IOD–time curve.

2.3 Construction of mutants in the background of a wine strain

A selection of genes positive for growth insufficiency at low temperature in the BY4743 laboratory background **was** deleted in FX10 (Laffort, S.A.), a homozygous and homothallic commercial wine yeast strain obtained by autodiploidisation of one ascospore. The heterozygous mutants were constructed with the short flanking homology (SFH) method (Güldener et al., 1996) by transforming FX10 using the lithium acetate procedure (Gietz et al., 2002) with a PCR fragment obtained by the amplification of the KanMX4 cassette and flanking regions (about 500-pb upstream and downstream) from the homozygous deleted strain in the BY4743 background. After transformation, strain selection was carried out using Geneticin (G418) added to YPD solid media at a concentration of 200 mg/L. In order to confirm genotypes, the total DNA from transformants resistant to G418 was analysed by PCR using the primers upstream and downstream of the deleted region combined with the primers inside KanMX.

The homozygous mutants were constructed by sporulating the heterozygous mutants and testing spores for G418 resistance. As expected, the geneticin resistance feature segregated 2:2. Since the original strain was homothallic, the strains recovered from the segregation analysis plates were spontaneous autodiploids (homozygous for the corresponding gene deletion), as verified by PCR.

2.3.1 Drop test

To analyse the growth phenotypes of mutant strains, the cells grown on YPD to the stationary phase (OD₆₀₀ ~ 4) were harvested by centrifugation, washed with sterile

water, resuspended in sterile water to an OD (600 nm) value of 0.5 followed by serial dilution. From each dilution, 3.5 µL were spotted onto YPD agar plates. Plates were incubated at 28°C and 12°C for 2 and 9 days, respectively.

2.3.2 Growth in YPD and SM

Growth of the FX10 mutant strains was also analysed in 96-well plate liquid cultures in YPD and SM at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12, 18 and 28 °C. At 28 °C, measurements were taken hourly for 3 days after a pre-shaking of 20 s. However for lower temperatures (12°C and 18°C), microplates had to be incubated outside the spectrophotometer and then transferred to the spectrophotometer to take measurements every 3 h for 10 days. The microplate wells were filled with 0.25 mL of YPD or SM medium and inoculated with 0.01 mL of preculture, which gave an initial OD of approximately 0.2 (inoculum level of ~ 6.0 log₁₀ CFU/mL). The uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All the experiments were carried out in triplicate.

Biological growth parameters were deduced from each growth curve by directly fitting OD measurements versus time to the reparameterised Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

$$y = D \cdot \exp\{-\exp[\frac{(\mu_{max} \cdot e)}{D} \cdot (\lambda - t)] + 1\}$$

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t ;

$D = \ln(OD_{\infty}/OD_0)$ is the OD value reached with OD_{∞} as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). OD/time data were fitted by a non-linear regression procedure to minimise the sum of squares of

the difference between the experimental data and the fitted model; i.e., loss of function (observed-predicted). This primary modelling was accomplished with the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option.

The area obtained under the curves (OD versus time) was used herein as a suitable procedure to estimate the effects of gene deletion on overall yeast growth because its relationship with biological growth parameters (Arroyo-López et al., 2009; García-Rios et al., 2014). This parameter was obtained by integration with the OriginPro 7.5 software (OriginLab Corporation, Northampton, USA).

2.3.3 Fermentations

Fermentations were carried out in laboratory-scale fermenters using 100-mL bottles filled with 60 mL of SM, which were fitted with closures that enabled carbon dioxide to escape and samples to be removed, at 28°C and 12°C, with continuous orbital shaking at 100 rpm. Yeast cell growth was determined by absorbance at 600 nm and by plating adequate dilutions on YPD agar by the end of fermentation. Plates were incubated for 2 days at 30°C. Fermentation was monitored by measuring the density of the media (g/L) in a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Fermentation was considered completed when density was below 998 g/L. The kinetics of these fermentations was estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of sugars in SM by representing density reduction vs. time and adjusting these values to the four-parameters logistic equation proposed by Speers et al. (2003). T5, T50 and T100 approximately matched the beginning (lag phase), middle (end of the exponential phase) and end of fermentation, respectively.

2.4 Statistical analysis

All the experiments were carried out at least in triplicate. Data were analysed with the Sigma Plot 13.0 software. The results are expressed as mean and standard deviation. To evaluate statistical significance, two-tailed *t*-student tests were run with a *p*-value of 0.05. Phenotypic data were fitted to the reparameterised Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0. GO term analysis was performed with STRING v.10 (<http://string-db.org>) (Franceschini et al., 2013). Enrichment was further done on the KEGG pathways. The *p*-values were corrected for multiple testing by the Bonferroni test for functional associations and GO analyses. The statistical level of significance was set at $P \leq 0.05$.

3. Results

3.1 Screen to identify the genes required for good growth at low temperature

In this study, a high throughput screen of both the homozygous and heterozygous diploid mutant collections was developed to identify those genes required for growth at low temperature. In the first step, all the mutant strains (4,828 homozygous + 6,513 heterozygous) were spotted in tetraplicate in microtiter format YPD agar plates and incubated for 7 days at 12°C. Thus 45,364 single colonies were cultivated and evaluated. Fifty-three heterozygous strains showed a visible growth defect in comparison with the parental strain BY4743. In addition, 81 homozygous YKO strains did not show visible growth after 7 days at 12°C. So they were selected as Low Temperature Growth Defect (LTGD) candidate strains to make a quantitative phenotype confirmation. These strains were regrown in solid media at 12°C, 18°C and 28°C to confirm their growth defect. The cell density of every single colony was quantified with the COLONIZER software. To quantitatively evaluate the overall

growth capacity of all the tested strains, the area under IOD–time curve (AUC) was used as a measure of overall yeast growth performance (Fig. 1A). We considered that a mutant strain had more severely affected growth at low temperature (12°C and 18°C) when its AUC was <10% than that obtained for the wild type (BY4743). The strains that obtained an AUC <50% at 28°C, compared to the wild type, were considered to show a growth defect at a non-restrictive temperature. Therefore, this phenotype was not considered specific for low temperature.

After an accurate evaluation, none of the heterozygous strains could be confirmed as showing LTGD. However, our results showed growth defect in 45, 32 and 25 of the 81 selected strains at 12°C, 18°C and 28°C, respectively (Fig. 1A). Seventeen mutant strains had a growth defect at whatever temperature considered, and five mutant strains presented a growth defect at both 12°C and 28°C. Thus all these strains were ruled out because their growth defect was not exclusive of low temperature. For further analyses, the nine mutant strains that showed a growth defect only at 12°C and the 14 strains with a growth defect both at 12°C and 18°C (23 strains in all) were selected (Table S1). A GO term analysis of these 23 genes that impaired growth at low temperature revealed four very significant functional categories (Fig. 1B). One of them was the most general “Metabolic pathways”, but more specific categories also appeared, like “Biosynthesis of secondary metabolites” and “Biosynthesis of amino acids”. Among the amino acids, the biosynthesis of phenylalanine, tyrosine and tryptophan was well-represented among the selected mutant strains.

3.2 Construction of heterozygous and homozygous LTSGD mutants in industrial wine strain FX10

Of the 23 genes whose deletion provoked a growth defect at low temperature in the BY4743 background, nine genes were selected as being representative to construct

heterozygous and homozygous mutant strains in the wine strain FX10 background (Table 1). This selection was intended to include the genes of the main metabolic pathways detected in the first screening (amino acids, phospholipids and ribosomes), as well as the genes that had not been previously related with low-temperature growth (Abe and Minegishi, 2008; Chiva et al., 2012; López-Malo et al., 2013).

The heterozygous mutants were constructed by deleting one of the two copies in the parental strain (diploid). The μ_{max} of these heterozygous mutants was calculated by growth in YPD and SM at 12°C and 28°C (Fig. S1). Similarly to what was previously observed in the lab strain, hemizyosity did not have a strong impact on the growth of the wine strain in YPD. Only strain $\Delta zuo1/ZUO1$ showed a slight, but significant, decrease in μ_{max} in comparison to the parental strain. Interestingly enough, most of these heterozygous strains showed significant growth improvements (higher μ_{max}) when grown in SM at 12°C. Only the hemizyosity in gene *URE2* resulted in a decrease in μ_{max} in SM at 28°C.

After the sporulation of the heterozygous strains, the homozygous mutants were obtained by autodiploidisation of one spore that harboured the deleted allele (geneticin resistant). We only constructed eight out of the nine previous selected genes because we never got the $\Delta\Delta aro7$ mutant, likely because this homozygous mutant may be inviable in the FX10 background. Once again, the homozygous strains were grown in YPD and SM at 12°C and 28 °C to calculate their μ_{max} (Fig. 2). In this case, the deletion of both copies of the selected LTGD genes impaired the growth of these homozygous mutants in both media and, on many occasions, at both temperatures. As mentioned above, we were unable to consider the genes whose deletion strongly affected growth at both 12°C and 28°C (i.e. *CDC50* and *ZUO1*). However, the strains deleted for genes *SNU66* and *PAP2* showed an important growth defect at low temperature and were

barely affected or not affected at optimum temperatures. A drop test of these mutants, which relates more with maximum growth capacity, is shown in Fig. 3, and confirmed the inability of mutants $\Delta\Delta snu66$ and $\Delta\Delta pap2$ to grow at low temperature. This drop test also revealed a growth defect of the $\Delta\Delta trp4$ mutant at low temperature. At 28°C, the growth of only the $\Delta\Delta pap2$ mutant seemed to be affected.

3.3 Fermentation activity of homozygous mutants

The most affected homozygous mutants ($\Delta\Delta snu66$ and $\Delta\Delta pap2$) and others that displayed an exclusive growth defect at low temperature in some of the growth tests ($\Delta\Delta drs2$, $\Delta\Delta trp4$ and $\Delta\Delta aure2$) were used as an inoculum in a SM. The fermentation kinetics for these mutants, compared with FX10, are shown in Figure 4 and Table 2. As expected, in accordance with previous tests, $\Delta\Delta snu66$ and $\Delta\Delta pap2$ had a major growth defect in terms of their growth rate and their maximum OD at 12°C. No significant differences were observed in the growth of these mutants compared with FX10 at 28°C (Table 2). Regarding fermentation activity, determined as the time needed to ferment 5%, 50% and 100% of the initial sugars of SM, only $\Delta\Delta snu66$ took almost twice the time to finish fermentation, and no differences were observed in the other mutants (Fig. 4C and Table 3). Mutant $\Delta\Delta pap2$ showed a delay in the T5 and T50, but no differences were observed at the end of fermentation (T100) (Table 3). Strikingly some of these mutants, such as $\Delta\Delta snu66$, took less time to finish fermentation at 28°C.

4. Discussion

Extensive ‘phenomic’ studies have been undertaken with laboratory yeast collections that comprised individual known single gene deletion mutants (deletants), whereby the phenotype of such deletants has been analysed to determine the genes associated with tolerance to a specific condition (Walker et al., 2014). In this study, the

homozygous and heterozygous diploid mutant collection of the lab strain BY4743 was screened to detect the genes involved in psychrotolerance. It is noteworthy that we did not detect any growth alteration in the heterozygous mutant strains. So even in this challenging low-temperature environment, retention of a single gene copy sufficed to maintain growth fitness unperturbed. This result supports and extends the observations made from *S. cerevisiae* lab strains in optimal environments that haploinsufficiency is remarkably rare (Deutschbauer et al., 2005; Gutiérrez et al., 2013).

Twenty-three cold-sensitivity genes were detected while screening the homozygous mutant strains. The identified genes are involved in diverse cellular functions. However, the GO term analysis clearly highlighted the significant enrichment in the proteins involved in amino acid biosynthesis, and more specifically in the synthesis of aromatic amino acids. In a similar study, Abe and Minegishi (2008) screened genes that were essential for growth under high-pressure and low-temperature conditions. They detected 56 genes responsible for low-temperature growth, and most were also needed for high-pressure resistance. Among the critically important cellular functions, Abe and Minegishi (2008) also found that the genes involved in amino acid biosynthesis were overrepresented, which they attributed to a direct correlation between cold-sensitivity and auxotrophy for tryptophan, phenylalanine and tyrosine. These results suggested that high pressure and low temperature have a similar impact on cellular membranes by decreasing their fluidity and impairing the uptake of these aromatic amino acids, probably due to reduced activity and/or degradation of their permeases.

We believe that this dependency of the aromatic amino acid biosynthesis and cold growth should be circumscribed to the background of the BY strains. Recently, we constructed mutant and overexpressing strains of some tryptophan metabolism genes

(López-Malo et al., 2014) in the background of an industrial wine yeast, and we observed no clear impairment in growth and fermentation activity during wine fermentation at low and optimum temperatures. On the contrary, deletion of tryptophan permease *TAT2* conferred this strain the highest nitrogen consumption capacity and the greatest fermentation activity at low temperature. In the present study, the construction of aromatic amino acid metabolism mutants ($\Delta\Delta\textit{aro2}$ and $\Delta\Delta\textit{trp4}$) in the FX10 background did not clearly impair growth or fermentation activity. Conversely, mutants $\Delta\Delta\textit{snu66}$ and $\Delta\Delta\textit{pap2}$ displayed a major growth defect and perturbed fermentation activity in this genetic background.

SNU66 is a component of the U4/U6/U5 small nuclear ribonucleoprotein (snRNP) complex that is involved in pre-mRNA splicing via spliceosome (Stevens and Albeson, 1999), and also required for pre-5S rRNA (Li et al., 2009). *PAP2*, also known as *TRF4*, is a non-canonical poly(A) polymerase involved in multiple tasks, such as nuclear RNA degradation, polyadenylation of hypomodified tRNAs, snoRNA and rRNA precursors, and serves as a link between RNA and DNA metabolism (Gavalda et al., 2013; Vanacova et al., 2005). A common feature of both genes is that they are required for efficient ribosome biogenesis, *PAP2*, presumably by facilitating the removal of aberrant pre-rRNA molecules (LaCava et al., 2005) and *SNU66* for its involvement in processing the 5S rRNA precursor (Li et al., 2009). The involvement of both genes in cold-sensitive phenotypes has been previously reported (Stevens et al., 2001; Edwards et al., 2003). These authors correlated the cold-sensitive phenotype with a severe pre-mRNA splicing defect. However, they did not explain why the deletion of these genes only resulted in strong growth defects at low temperature (Edwards et al., 2003), and further analyses will be required to elucidate this point. There are many examples of gene duplication and subfunctionalization in *S. cerevisiae* (Turunen et al.,

2009) and, likely, Snu66 and Pap2 are paralog genes required for translation at low temperature.

We previously identified translation efficiency as a crucial process during low temperature adaptation in different *Saccharomyces* species (Tronchoni et al., 2014). A transcriptome comparison made between *S. cerevisiae* and the psychrotolerant *S. kudriavzevii* at low temperature showed the common activation of the genes involved in translation (ribosome biogenesis, ribosomal proteins and protein synthesis). However, the *S. kudriavzevii* response was stronger, and showed an increased expression for dozens of the genes involved in protein synthesis. Low temperature causes the hyperstabilisation of RNA structures, which hampers the maturation of ribosomes and, consequently, translation initiation kinetics (Fortner et al., 1994; Hilliker et al., 2007; Li et al., 1996; Perriman et al., 2007; Staley et al., 1999; Zavanelli et al., 1994). The up-regulation of the genes and proteins involved in translation must be seen as a compensatory mechanism to overcome the blockage of this process at low temperature. The better fitness of different industrial strains under low temperature conditions might be related with enhanced translation efficiency, as formerly indicated by Tronchoni et al. (2014). However, the low temperature adaptation is a complex trait and we cannot rule out the involvement of other cellular processes as we did not test all the LTGD genes detected in the lab strain.

In conclusion, “phenomyc” studies of individual single gene deletion mutants are very useful for detecting essential genes for response and adaptation to a physiologically stressful environment. In this study, we observed the aromatic amino acid metabolism to be a limiting step in low-temperature adaptation in strain BY4743, although this response and adaptation are also strain-dependent. The genetic differences between lab and industrial strains are well-known, and to what extent the findings in lab

strains can be extrapolated to industrial yeasts remains unknown. In this study, gene deletions resulting in a strong growth defect in the lab strain barely affected the wine strain; that is, mainly those related with aromatic amino acid biosynthesis. Gutiérrez et al. (2013) previously highlighted the differences of nitrogen source preferences between wine and lab strains, and pointed out a different evolution in a nitrogen-poor substrate and with a different amino acid proportion to those available in synthetic lab media. Thus the present results evidenced translation efficiency as a much more limiting step during cold adaptation in wine strains. Given our interest in the different industrial applications of this study, translation efficiency could be an interesting target for selecting or improving strains to be used during low-temperature fermentations.

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Figure legends

Fig. 1. Screen of both the homozygous and heterozygous diploid mutant collections of BY4743. A) Growth of the 81 homozygous selected mutants and parental strain BY4743 at 12°C. Area under the curve (AUC) was calculated from each growth curve of these mutants grown at 12°C, 18°C and 28°C. B) Venn's diagram of the mutant strains that showed impaired growth at the different temperatures and the significant functional categories (GO term analysis) of the 23 genes that produced a low temperature growth defect (LTGD). *p*-values and number of genes of each functional category are provided in brackets. The statistical level of significance was set at $P \leq 0.05$.

Fig. 2. Growth of the homozygous mutant strains compared with control strain FX10. Relative maximum specific growth rate (μ_{max}) during growth in A) YPD and B) Synthetic Must (SM) at 12°C and 28°C in comparison with the parental strain (normalized as value 1). *Significant differences compared with the parental strain at the same temperature.

Fig. 3. Drop test of the homozygous mutant strains constructed in wine yeast FX10 grown in YPD at 12°C and 28°C.

Fig. 4. Growth capacity of the homozygous mutant strains during the fermentation of synthetic must (SM) at (A) 12°C and (B) 28°C. (C) Relative time to ferment 100% of the SM sugars (T100) by different mutant strains. *Results with statistically significant differences ($p\text{-value} \leq 0.05$).

Table 1. Genes selected from the Low Temperature Severe Growth Defect genes to construct deletion mutants in FX10

Gene Name	Function
<i>TRP4</i>	Anthranilate phosphoribosyl transferase; transferase of the tryptophan biosynthetic pathway; catalyses the phosphoribosylation of anthranilate
<i>URE2</i>	Nitrogen catabolite repression transcriptional regulator; inhibits <i>GLN3</i> transcription in a good nitrogen source; role in sequestering Gln3p and Gat1p to the cytoplasm; has glutathione peroxidase activity and can mutate to acquire GST activity
<i>PAP2</i>	Non-canonical poly(A) polymerase; involved in nuclear RNA degradation as a component of TRAMP; catalyses polyadenylation of hypomodified tRNAs, and snoRNA and rRNA precursors; required for mRNA surveillance and maintenance of genome integrity, serves as a link between RNA and DNA metabolism
<i>ARO7</i>	Chorismate mutase; catalyses the conversion of chorismate into prephenate to initiate the tyrosine/phenylalanine-specific branch of aromatic amino acid biosynthesis
<i>DRS2</i>	Aminophospholipid translocase (flippase) that maintains membrane lipid asymmetry in post-Golgi secretory vesicles
<i>CDC50</i>	Endosomal protein that interacts with phospholipid flippase Drs2p
<i>ARO2</i>	Bifunctional chorismate synthase and flavin reductase; catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to form chorismate, which is a precursor to aromatic amino acids
<i>ZUO1</i>	Ribosome-associated chaperone; functions in ribosome biogenesis
<i>SNU66</i>	Component of the U4/U6.U5 snRNP complex; involved in pre-mRNA splicing via spliceosome; also required for pre-5S rRNA processing

Table 2. Relative area under the curve (AUC) of the homozygous mutant strains in comparison with parental strain FX10 (normalised at 100%). *Significant differences compared with the control at the same temperature.

	12°C	28°C
Strain	Relative AUC	Relative AUC
$\Delta\Delta snu66$	14.89 $\pm$ 6.23*	101.58 $\pm$ 9.08
$\Delta\Delta ure2$	108.20 $\pm$ 13.14	98.77 $\pm$ 3.29
$\Delta\Delta trp4$	103.78 $\pm$ 0.23	91.58 $\pm$ 6.46
$\Delta\Delta drs2$	109.56 $\pm$ 23.29	94.42 $\pm$ 3.95
$\Delta\Delta pap2$	72.68 $\pm$ 15.08	91.45 $\pm$ 9.49

Table 3. Time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a synthetic must at 12°C and 28°C. *Significant differences compared with the control at the same temperature.

Strain	12°C			28°C		
	T5 (h)	T50 (h)	T100 (h)	T5 (h)	T50 (h)	T100 (h)
FX10	43.52 ± 2.45	94.09 ± 16.73	218.32 ± 16.74	7.00 ± 0.94	21.75 ± 0.38	63.50 ± 3.23
ΔΔ<i>snu66</i>	98.11 ± 2.32*	231.61 ± 30.60*	378.70 ± 24.81*	9.63 ± 1.52	22.00 ± 0.78	48.50 ± 3.68*
ΔΔ<i>ure2</i>	32.63 ± 7.95*	82.69 ± 2.39	194.63 ± 15.91	12.56 ± 0.27*	23.00 ± 0.43*	42.94 ± 2.39*
ΔΔ<i>trp4</i>	55.66 ± 3.52*	125.53 ± 0.00	223.03 ± 14.06	7.50 ± 0.53	21.00 ± 0.99	58.88 ± 0.53
ΔΔ<i>drs2</i>	45.70 ± 6.03	95.06 ± 6.89*	198.05 ± 4.31	7.25 ± 0.94	21.25 ± 0.87*	58.25 ± 3.12
ΔΔ<i>pap2</i>	85.92 ± 9.48*	151.73 ± 9.48*	227.30 ± 12.93	8.50 ± 0.57	21.88 ± 0.57	54.63 ± 2.91*

Figure 1

A Screening at 12, 18 and 28°C in YPDA of 81 genes affected at low temperature

B Screening at 12, 18 and 28°C in liquid-YPD of 81 genes affected at low temperature

Low Temperature Growth Defect genes (23 genes)

GO ID 1230 **Biosynthesis of amino acids** (2.59E-04) (6 genes)

GO ID 400 **Phenylalanine, tyrosine and tryptophan biosynthesis** (1.25E-03) (3 genes)

GO ID 1110 **Biosynthesis of secondary metabolites** (6.45E-03) (6 genes)

GO ID 1100 **Metabolic pathways** (6.45E-03) (9 genes)

Figure 2

A

B

Figure 3

Figure 4

Table S1. List of the 23 genes that produced growth defect at low temperature (LTGD)

Systematic name	Standard Name	Description
Growth defect at 12°C and 18°C		
YBR068C	<i>BAP2</i>	High-affinity leucine permease
YBR197C	-	Protein of unknown function
YCL007C	<i>CWH36</i>	Dubious open reading frame
YDR354W	<i>TRP4</i>	Anthranilate phosphoribosyl transferase of the tryptophan biosynthetic pathway
YDR359C	<i>EAF1</i>	Component of the NuA4 histone acetyltransferase complex
YEL045C	-	Dubious open reading frame; deletion gives MMS sensitivity, growth defect under alkaline conditions, less than optimal growth upon citric acid stress
YHL011C	<i>PRS3</i>	5-phospho-ribosyl-1(alpha)-pyrophosphate synthetase; synthesizes PRPP, which is required for nucleotide, histidine, and tryptophan biosynthesis
YLR056W	<i>ERG3</i>	C-5 sterol desaturase; glycoprotein that catalyzes the introduction of a C-5(6) double bond into episterol, a precursor in ergosterol biosynthesis
YNL229C	<i>URE2</i>	Nitrogen catabolite repression transcriptional regulator; acts by inhibition of GLN3 transcription in good nitrogen source
YNL248C	<i>RPA49</i>	RNA polymerase I subunit A49
YOL115W	<i>PAP2</i>	Non-canonical poly(A) polymerase; involved in nuclear RNA degradation as a component of TRAMP; catalyzes polyadenylation of hypomodified tRNAs, and snoRNA and rRNA precursors; required for mRNA surveillance and maintenance of genome integrity, serving as a link between RNA and DNA metabolism
YPR060C	<i>ARO7</i>	Chorismate mutase; catalyzes the conversion of chorismate to prephenate to initiate the tyrosine/phenylalanine-specific branch of aromatic amino acid biosynthesis
YLR087C	<i>CSF1</i>	Protein required for fermentation at low temperature
YPR153W	-	Putative protein of unknown function
Growth defect at 12°C		
YAL026C	<i>DRS2</i>	Aminophospholipid translocase (flippase) that maintains membrane lipid asymmetry in post-Golgi secretory vesicles
YCR094W	<i>CDC50</i>	Endosomal protein that interacts with phospholipid flippase Drs2p
YDR158W	<i>HOM2</i>	Aspartic beta semi-aldehyde dehydrogenase; catalyzes the second step in the common pathway for methionine and threonine biosynthesis
YGL148W	<i>ARO2</i>	Bifunctional chorismate synthase and flavin reductase; catalyzes the conversion of 5-enolpyruvylshikimate 3-phosphate (EPSP) to form chorismate, which is a precursor to aromatic amino acids
YGR285C	<i>ZUO1</i>	Ribosome-associated chaperone; functions in ribosome biogenesis
YHR039C-B	<i>VMA10</i>	Subunit G of the eight-subunit V1 peripheral membrane domain of the vacuolar H+-ATPase (V-ATPase)
YLR089C	<i>ALT1</i>	Alanine transaminase (glutamic pyruvic transaminase); involved in alanine biosynthesis and catabolism
YOR308C	<i>SNU66</i>	Component of the U4/U6.U5 snRNP complex; involved in pre-mRNA splicing via spliceosome; also required for pre-5S rRNA processing
YPL205C	-	Hypothetical protein; deletion of locus affects telomere length

A **B**

Fig. S1. Growth of the heterozygous mutants compared with the parental strain FX10. Relative maximum specific growth rate ($\mu_{\max}$) during growth in A) YPD and B) Synthetic Must (SM) at 12°C and 28°C in comparison with the parental strain (normalized as value 1).

*Significant differences compared with the parental strain at the same temperature.