iTRAQ-based proteome profiling of *Saccharomyces cerevisiae* and
cryotolerant species *S. uvarum* and *S. kudriavzevii* during low-temperature
wine fermentation

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ABSTRACT

Temperature is one of the most important parameters to affect the duration and rate of alcoholic fermentation and final wine quality. Some species of the *Saccharomyces* genus have shown better adaptation at low temperature than *Saccharomyces cerevisiae*, which was the case of cryotolerant yeasts *Saccharomyces uvarum* and *Saccharomyces kudriavzevii*. In an attempt to detect inter-specific metabolic differences, we characterized the proteomic landscape of these cryotolerant species grown at 12°C and 28°C, which we compared with the proteome of *S. cerevisiae* (poorly adapted at low temperature). Our results showed that the main differences among the proteomic profiling of the three *Saccharomyces* strains grown at 12°C and 28°C lay in translation, glycolysis and amino acid metabolism. Our data corroborate previous transcriptomic results, which suggest that *S. kudriavzevii* is better adapted to grow at low temperature as a result of enhanced more efficient translation. Fitter amino acid biosynthetic pathways can also be mechanisms that better explain biomass yield in cryotolerant strains. Yet even at low temperature, *S. cerevisiae* is the most fermentative competitive species. A higher concentration of glycolytic and alcoholic fermentation enzymes in the *S. cerevisiae* strain might explain such greater fermentation activity.

Biological significance:

Temperature is one of the main relevant environmental variables that microorganisms have to cope with and it is also a key factor in some industrial processes that involve microorganisms. However, we are still far from understanding the molecular and physiological mechanisms of adaptation at low temperatures. The results obtained in this study provided a global atlas of the proteome changes triggered by temperature in three different species of the genus *Saccharomyces* with different degree of

cryptotolerance. These results would facilitate a better understanding of mechanisms for how yeast could adapt at the low temperature of growth.

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. For instance, low temperatures (10-15°C) are used in wine fermentations to enhance production and to retain flavor volatiles. In this way, white and rosé wines can be achieved with greater aromatic complexity [1-2]. However, lowering fermentation temperatures has its disadvantages, including prolonged process duration and a higher risk of halted or sluggish fermentation [3]. These problems can be avoided by providing better-adapted yeasts to ferment at low temperature.

Low temperature has several effects on biochemical and physiological properties in yeast cells: poorly efficient protein translation; low fluidity membrane; changes in lipid composition; slow protein folding; stabilization of mRNA secondary structures; reduced enzymatic activities [4-7]. However, we are still far from understanding the molecular and physiological mechanisms of adaptation at low temperatures, and from also knowing what makes them 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.

Despite the fact that *S. cerevisiae* is the predominant species responsible for alcoholic fermentation, other species of the genus *Saccharomyces*, such as the cryotolerant *S. uvarum* [8-9] or the hybrid strains of *S. cerevisiae* x *S. kudriavzevii* [10-11], have been

shown to better adapt to low-temperature fermentations during winemaking. *S. kudriavzevii* has been isolated only from natural environments [12-13]. However, previous physiological and enological works have indicated the huge advantage of fermenting *S. kudriavzevii* at low temperature [14-15]. The cryotolerant character of these two species, *S. uvarum* and *S. kudriavzevii*, in comparison to *S. cerevisiae*, has been well-established [16]. Therefore, using cryotolerant yeasts to study adaptation to low temperature can help us better understand this stress factor, and to discriminate if these adaptation strategies are species-specific or common to all the strains of the *Saccharomyces* genus. Recent studies by our group aimed to examine the cold adaptation of these cryotolerant species in-depth [17-18]. The metabolome comparison of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* grown at 12°C revealed that the main differences between the two cryotolerant species and *S. cerevisiae* lay in carbohydrate metabolism, mainly fructose metabolism. However, these two species have developed different strategies for cold resistance. *S. uvarum* presented strong shikimate pathway activity, while *S. kudriavzevii* displayed increased NAD⁺ synthesis [17]. Complementarily, the transcriptomic comparison of *S. cerevisiae* and *S. kudriavzevii* at low (12°C) and optimum (28°C) temperatures indicated an enhanced ability to initiate a quick efficient translation of crucial genes in cold adaptation as the main strategy for growing better at low temperature [18]. This study suggests that *S. kudriavzevii* has increased translation efficiency due to higher ribosome availability after adaptation to cold shock. What this implies is that translation efficiency may be an important target of adaptive evolution when cells face changing environments, as demonstrated for *S. kudriavzevii*.

In this study, we conducted a comparative proteomic analysis between a very well-known *S. cerevisiae* wine yeast (QA23) and a representative strain of species *S. uvarum*

and *S. kudriavzevii*. These strains were grown at the same growth rate in steady-state chemostat cultures at 12°C and 28°C. Although batch cultures are well-suited to study low-temperature adaptation dynamics, they are poorly adapted to study prolonged exposure to low temperature. In such cultures, the specific growth rate (μ) is strongly affected by temperature, which means that it is impossible to dissect temperature effects from specific growth rate effects. Two recent chemostat studies [19-20] have found that the growth rate itself has a strong effect on transcriptional activity. Chemostat cultures help accurately control the specific growth rate, and thus provide a good platform to study microbial physiology, proteome profiles and gene expression [7]. Differences in protein composition were assessed by the iTRAQ technique, which is a powerful proteomic method used to quantify relative protein levels [21]. The use of amine-specific isobaric tags for relative and absolute protein quantification (iTRAQ) has become a consolidated technique in quantitative proteomics since it allows large fold changes of protein expression within broad dynamic ranges of protein abundance to be measured quite accurately [22]. The aim of this study was to improve the feasibility of low-temperature wine fermentation by identifying key proteins in yeast adaptation to cold, and to compare it with a previous detection of induced genes [23-24] and metabolic adaptations [15, 17] at low temperature. It also aimed to detect the differential protein profiles that distinguish the two psychrotolerant species *S. uvarum* and *S. kudriavzevii* from *S. cerevisiae*. These differences may explain a more successful cold adaptation strategy.

2. Material and methods

2.1 Yeast strains and continuous culture conditions

A commercial *S. cerevisiae* (*Sc*) wine strain (QA23, Lallemand S.A., Canada), a *S. uvarum* (*Su*) strain (CECT 12600) and a *S. kudriavzevii* strain (*Sk*) (CR85) [13] were

used in this work. Cultures were grown in the synthetic grape must (SM) that derived from that described by Bely *et al.* [25] The SM composition included 200 g L⁻¹ of sugars (100 g L⁻¹ glucose + 100 g L⁻¹ fructose), 6 g L⁻¹ malic acid, 6 g L⁻¹ citric acid, 1.7 g L⁻¹ YNB without ammonium and amino acids, anaerobic factors (0.015g L⁻¹ ergosterol, 0.005 g L⁻¹ sodium oleate and 0.5 mL L⁻¹ Tween 80) and 0.060 g L⁻¹ potassium disulfite. The assimilable nitrogen source used was 0.3 g N L⁻¹ (0.12 g N L⁻¹ as ammonium and 0.18 g N L⁻¹ in an amino acid form).

Continuous cultures were performed at a dilution rate (D) of 0.04 h⁻¹ at 12°C and 28°C in a 0.5 L chemostat (MiniBio, Applikon Biotechnology) and with a working volume of 0.3 L. The dilution rate was chosen because was the maximum D for the less adapted strain at low temperature (*Sc*). A temperature probe connected to a cryostat controlled the cultures grown at 12°C. pH was measured online and kept constant at 3.3 by the automatic addition of 2 M NaOH and 1 M HCl. The stirrer was set at 100 rpm. Sampling was carried out during steady states, which was reached only after all continuous cultures had been running for at least five residence times and the concentration of biomass, nutrients and fermentative metabolites was constant.

2.2 HPLC analysis

Extracellular glucose, fructose, glycerol and ethanol were analyzed in all the supernatant samples during steady states. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, an autosampler and a UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min, and samples were diluted 10-fold and filtered through 0.22 µm pore size nylon filters (Micron Analitica, Spain). A total volume of 25 µL was injected into a HyperREZ XP Carbohydrate H+8 mm column

(Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H₂SO₄ with a flux of 0.6 mL min⁻¹ and a column temperature of 50°C. The concentration of each compound was calculated using external standards. Each sample was analyzed in duplicate.

2.3 Nitrogen content analysis

The ammonia concentration was measured with a kit following an enzymatic method (Roche Applied Science, Germany). The free amino acid nitrogen concentration was determined by the s-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure [26]. The results were expressed as mg N mL⁻¹.

2.4 Determination of biomass dry weight and physiological parameters during continuous cultures

Cell growth was monitored by measuring optical density at 600 nm (OD₆₀₀). Biomass dry weight (g DW L⁻¹) was determined by centrifuging a known volume of culture broth (approximately 30 units of OD₆₀₀) in pre-weighted tubes that were then washed with 2 volumes of distilled water and dried to constant weight at 70°C for 48 h.

Yield of hexoses represents the amount of sugars consumed for producing one gram of biomass and it is determined by dividing total produced biomass by total sugars consumed ($Y_{\text{hexoses}} = \text{DW} / \text{g L}^{-1} \text{ of consumed hexoses}$). The consumption rate (q) of sugars, amino acids and ammonium was calculated by dividing the consumption of these nutrients by biomass and multiplied by the dilution rate (D) ($q = (\text{g L}^{-1} \text{ of consumed nutrient} / \text{DW g L}^{-1}) \times D (\text{h}^{-1})$). The amount of sugar and nitrogen consumed was calculated by subtracting the residual amount present in the supernatant to the total amount included in the synthetic must. Similarly, the production rate of metabolites (q)

was calculated for ethanol and glycerol as described for the consumption rate of sugars and nitrogen.

2.5 Protein Extraction

When the steady state was reached, a volume of approximately 30 units of OD600 was centrifuged at 10000 g for 3 min at 4°C. After supernatant removal, the cell suspension was washed with PBS, transferred to a 1.5-2-mL microcentrifuge tube and was recentrifuged under the same conditions. The cell pellet was suspended in 150 µL of extraction buffer (25 mM TRIS buffer, pH 8; 8 M Urea and protease inhibitor cocktail (1/200) (Thermo Scientific)) and was broken by vortexing (4 to 6 times, 30 s) in the presence of glass beads (Sigma-G8772) (an equivalent volume to that of the cell pellet). Glass beads and insoluble material were eliminated by centrifugation (10000 rpm, 10 min) and then 150 µL of extraction buffer was added to the supernatant. Proteins were allowed to precipitate at -20 °C for 1 h and then the samples were centrifuged at 10000 g for 15 min. The pellet was washed with the 2-D Clean-Up kit (GE Healthcare), air-dried and solubilized in 25 µL of 7 M of urea, 4% (w/v) of CHAPS, 2 M of Thiourea, 20 mM of Tris and milliQ water. Insoluble material was removed by centrifugation (13000 rpm, 5 min) and finally, the protein concentration was determined by Bradford, with BSA as a standard.

2.6 Sample Preparation

Proteins were precipitated with 50 µL of TCA (Cf=10%) over night at 5°C. After centrifugation, the final pellets were washed with cold (-18°C) acetone with vigorous stirring, followed by centrifugation. Pellets were air-dried and dissolved with 75 µL of 8 M of urea in 500 mM of TEAB, pH 8 (Sigma), with sonication (5 min at 0°C). The final protein concentrations were determined by Qubit (Qubit™ Protein Assay Kits;

Invitrogen-Molecular Probes) and Lowry RC DC (Biorad) according to the manufacturer's instructions. Next 100 µg were taken from each sample and the solution was dried by rotatory evaporation. Samples were resuspended in Laemli buffer and loaded in 1D PAGE (12% Mini-PROTEAN® TGX™ Precast Protein Gels, 10-well, 50 µL – P/N 4561044 (BioRad) 12% precast polyacrylamide gel, 8.6 × 6.7 x 0.1 cm (W × L x thickness), resolving gel height (5.6 cm)). The gel was stopped after all the protein sample past the stacking region (4% T, 2.6% C, 1.1 cm) and the gel was colloidal Coomassie stained (QC colloidal Coomassie stain BioRad; P/N 161-0803) for determining the region of interest. Each slide was cut and digested [27]. This step was done to avoid the discrepancies in the quantitation methods due to interferences in the samples. The gel allowed us to clean and normalized the samples by densitometry.

The samples were reduced using 10 mM DTT in 50 mM Ammonium bicarbonate (20 min at 60°C). Free cysteines were blocked with 55 mM of iodoacetamide (IAM) during 30 min at room temperature. After that, the samples were subjected to trypsin digestion at 37°C o/n with 10 µg of Promega sequencing grade modified trypsin in 0.5 mM TEAB. Reaction mixtures were dried in a speed vacuum. Each sample was re-dissolved in 80 µL of TEAB + ethanol solution (3/7; v/v), with sonication for 10 min, added to the appropriate iTRAQ reagent vial and thoroughly vortexed (iTRAQ 1: Sc12.1-113, Sc12.2-114, Sc28.1-115, Sc28.2-116, Sk12.1-117, Sk12.2-118, Sk28.1-119, Sk28.2-121; iTRAQ 2: Sc12.1-113, Sc12.2-114, Sc28.1-115, Sc28.2-116, Su12.1-117, Su12.2-118, Su28.1-119, Su28.2-121). Immediately afterward, each sample vial was rinsed with an additional 20 µL measure of TEAB + ethanol solution and transferred to the correct iTRAQ reagent vials to be incubated at room temperature for 3 h. Then 300 µg of the peptide mixture were dissolved with 200 µL of 7M of urea/2M thiourea/1.6% ampholytes. One IPG strip (GE; 13 cm, 3-11 NL) was hydrated with the peptide

solution o/n. at room temperature and the peptides were isoelectrofocused with 5000 V to 30000 V h. After focusing, the strip was washed with milliQ water and cut into 15 equal pieces. Peptides were extracted with 100 μ L of the ACN solutions: A: 5% ACN 0.1% TFA, B: 50% ACN 0.1% TFA, C: ACN 0.1% TFA. The samples were cleaned and concentrated by POROS R2.

2.7 Mass spectrometry analysis

The clean peptide mixtures were dried by speed vacuum and resuspended to a concentration of ca. 0.3 μ g/ μ L in 2% ACN 0.1% TFA. 5 μ L of each sample was loaded into a trap column (Nano LC Column, 3 μ m C18-CL, 75 μ m x 15 cm; Eksigen) and desalted online with 0.1% TFA at 2 μ L/min for 10 min and the separated in an analytical column (LC Column, 3 μ m C18-CL, 75 μ m x 25 cm, Eksigen) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Peptides were eluted from the HPLC column by the application of a linear gradient from 5 buffer to 35% buffer B (B: ACN, 0.1% FA) in A (A: 0.1% FA) for 90 min at a flow rate of 300 nL/min. Peptides were analyzed in a mass spectrometer nanoESI qTOF (5600 TripleTOF, ABSCIEX). The tripleTOF was operated in the information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350–1250 m/z was performed, followed by 0.075-s product ion scans from 100–1500 m/z on the 25 most intense 2-5 charged ions. The collision energy was automatically selected by the instrument according to the following equation: $|CE|=(\text{slope}) \times (m/z) + (\text{intercept})$. The box ‘Adjust CE when using iTRAQ reagent’ was checked. This increased the calculated collision energies 9 volts in MS/MS experiments to increase the sensitivity in reporter ion region. For dynamic exclusion following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s.

2.8 Protein identification and data analysis

ProteinPilot default parameters were used to generate a peak list directly from 5600 TripleTof wiff files. The Paragon algorithm of ProteinPilot was used to search in the Expaty (Release-2014_01) protein database (515203 sequences; 181334896 residues). In detail, these parameters were as follows: iTRAQ Quantitation, trypsin specificity, cys-alkylation (IAM), no taxonomy restriction and the search effort set to throughout. Protein average ratios were calculated in the log space after background subtraction in order to buffer apparent changes. Final ratio displayed is the linear expression of the ratios obtained from the unshared peptides. To avoid using the same spectral evidence in more than one protein, the identified proteins were grouped based on the MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus the proteins that shared MS/MS spectra were grouped, regardless of the assigned peptide sequence. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed with different list number, usually toward the end of the protein list.

2.9 Paromomycin assays

For the halo assays, yeast cells were grown overnight in YPD and diluted the next morning. They were then grown until the mid-log phase (approximately 1×10^7 cells mL^{-1}) and then 175 μL were spread on each YPD plate. When the plate was absolutely dry, a filter imbibed with different amounts of paromomycin (10, 20 and 40 μg of drug) was placed on the surface and plates were incubated at 12°C and 28°C until the lawn was confluent. The measurement was taken from the point where the colonies were grown. Inside the halo, there were only single cells and clumps. The assays were carried out in triplicate.

2.10 Statistical Analysis

The metabolic data are the result of five replicates per fermentation (temperature and strains) and the proteomic data are the result of two culture replicates per condition. Significance was determined by analysis of variance (ANOVA) using the Statistica, version 7.0, software package. The statistical level of significance was set at a P value of ≤ 0.05 with a Tukey test. The data sets of the proteins with different concentrations at low temperature were treated with Venny (<http://bioinfogp.cnb.csic.es/tools/venny/index.html>) to select the common proteins from among the analyses. GO term Finder was used to group proteins into functional categories, which is found in the MIPS Functional Catalog (<http://mips.helmholtzmuemchen.de/funecatDB/>). Functional association studies were performed using the manually curated STRING database (the Search Tool for the Retrieval of Interacting Genes/Proteins) (<http://string-db.org>) [28]. Protein–protein interactions at a high level of confidence (score of >0.7) were considered. Enrichment was further done on the KEGG pathways. The p-values were corrected for multiple testing by the Bonferroni test.

3. Results

3.1 Metabolic profiles comparison among the three species

To investigate how the yeast proteome changed under a suboptimal growth temperature, we characterized the metabolic profile of the three *Saccharomyces* strains at optimum and low temperatures. Our experimental design was based on continuous-culture fermentations. This system offers a stable controlled environment for cells by maintaining constant biomass and the concentrations of nutrients and products [29], which make the comparison between fermentation conditions and strains more feasible. All the cultures were grown at the same dilution rate ($D = 0.04 \text{ h}^{-1}$), which corresponded

to the maximum D of the control condition (*Sc* at 12°C). When the steady state was reached (after five volume changes), sampling of supernatants and cells was done. Table 1 shows the physiological data of the three *Saccharomyces* strains during the steady state: the concentration of the main compounds in the supernatant, as well as the hexoses yield and the consumption/production rates of the main fermentative metabolites. According to Vázquez-Lima et al. [30] this dilution rate corresponded to the late exponential phase of a standard wine fermentation in the batch mode at 28°C. These authors defined this fermentation phase as the stage in which ammonium is depleted and growth is sustained solely on free amino acids. According to this definition, all our cultures had practically depleted all the ammonium and a percentage that ranged from 25-50% of the initial content of amino acids. Regarding sugar consumption, residual sugars were left in all the continuous cultures, which was expected for this fermentation phase. However, consumption was clearly determined by the temperature of the cultures. At 28°C, strains *Su* and *Sc* consumed 44% and 37% of the initial content in the SM, whereas this consumption dropped to 17% and 12%, respectively, at low temperature. *Sk* practically consumed the same amount of sugars at both temperatures, which did not represent more than 8% in any case. This result revealed that 28°C is also far away from the optimum growth temperature for this species [16]. Sugar consumption correlated quite well with biomass production, which was higher at 28°C. In spite of the cryotolerance of *Sk*, this species yielded the lowest biomass production at 12°C. However, in terms of sugar and nitrogen consumption rates and glycerol production rates, the *Sk* strain proved to be the most efficient species at low temperature, with greater nutrient consumption and higher metabolite production with a lower biomass concentration.

Differences in metabolite concentration, and in the consumption and production specific rates, were used to perform hierarchical clustering (Fig. 1), which grouped *Sc* 28°C and *Su* 28°C in a subcluster, and *Sc*, *Sk*, *Su* 12°C with *Sk* 28°C in another one. Intriguingly, the closest metabolic profiles were *Sc* 12°C and *Sk* 28°C. This resemblance of the *Sk* 28°C metabolic profile with a non-optimum or stress growth condition (*Sc* 12°C) again reveals the problems of *Sk* to grow at 28°C. Likewise, *Sc* and *Su* grown at 28°C presented a very similar metabolic profile.

3.2 Comparison of the proteomic profile at 28°C and 12°C

We carried out two 8-plex iTRAQ experiments comparing each cryotolerant strain with *Sc*, as control strain, at both temperatures. A total of 798 and 469 proteins were quantified for the comparisons *Sc/Sk* (iTRAQ 1) and *Sc/Su* (iTRAQ 2 experiment) respectively. When we compared the proteomes of the same strain but at different temperatures (12°C vs. 28°C), a total of 32, 129 and 226 proteins showed statistically significant differences in *Sc*, *Sk* and *Su* respectively. After filtering by applying the statistical criteria of a 95% up- or down-regulation likelihood ($[p > 1] < 0.05$ or $[p > 1] > 0.95$) and a fold-change higher than 30% (ratio of either < 0.70 or > 1.3) as the significantly altered relative levels, we were able to short-list to 12, 70 and 52 proteins with increased levels, and 17, 29 and 28 proteins with decreased levels, in *Sc*, *Sk* and *Su* at low temperature, respectively (Table S1). Figure 2 shows the relative abundance of the proteins that were over-represented at 12°C and 28°C in the three *Saccharomyces* strains and the more significant functional categories. The three strains showed the functional category “70.03 cytoplasm” very significantly in both the over- and under-represented proteins at low temperature. This result evidenced that low temperature adaptation mainly involves changes in proteins in this cellular location, chiefly related with protein synthesis and nitrogen metabolism.

Regarding *Sc* (Fig. 2A), the most strongly over-represented functional categories at 12°C were related to translation and proteins synthesis, together with glycolysis/gluconeogenesis, while at 28°C, the categories related more with purine nucleotide/nucleoside/nucleobase metabolism and amino acid metabolism. Likewise, *Sk* (Fig. 2B) at 12°C showed a huge number of categories that related with translation and protein synthesis, and also with glycolysis and alcoholic fermentation. Conversely at 28°C, an over-representation of categories that belonged to amino acid metabolism and G-protein-mediated signal transduction was observed. If we look at the *Su* (Fig. 2C) analysis, an increase in the proteins that belonged to the translation category and also to oxidative stress response occurred at 12°C, whereas at 28°C the amino acid metabolism category (especially aspartate and the aromatic family) was the most important one. The list of significant proteins in each *Saccharomyces* species was submitted individually to STRING 10 to elucidate the associations of these proteins (Fig. 3). An analysis was carried out at 0.7 of the confidence level. Figure 3A and 3B shows the network of the over-represented and under-represented proteins in *Sc* at low temperature, respectively. These associations are consistent with the enrichment in functional categories, being Ribosome and Metabolic pathways (amino acids biosynthesis) the categories with the highest *p*-values. Similarly in cryotolerant species *Sk* and *Su*, the common significant networks in both strains were ribosome, glycolysis/gluconeogenesis and biosynthesis of amino acids (Fig. 3C-3F). It is remarkable to note that the number of proteins that interconnected as a response to environmental temperature changes was much larger in *Sk* and *Su* than in *Sc*, which may reveal the cryotolerant character of these species.

3.3 Common over-represented proteins in the three *Saccharomyces* at low temperature

Regarding the number of common proteins (Fig. 4), *Sk* and *Su* shared a larger number than the comparison made with *Sc*, which suggests that the cryotolerant species gave a stronger proteomic response since they are better-adapted at low temperature. Most of these common proteins between *Sk* and *Su* are related with Translation (ribosomal proteins). Despite their small number, the common proteins between *Sc* and *Sk* also belonged to these functional categories and showed a common regulation in both strains (highlighted in red or green). Regarding the common proteins between *Sc* and *Su*, some nitrogen and vitamin metabolic pathways and proteins involved in nutrient sensing showed high significance. Finally, only three proteins were commonly over-represented in the three *Saccharomyces* strains, and two of them (*Adh1p* and *Rps5p*) had the same regulation. *ADH1* is the main alcohol dehydrogenase, and is involved in the synthesis of ethanol from acetaldehyde, while *RPS5* is a protein of the small (40S) ribosomal subunit.

3.4 Differential proteins in the three Saccharomyces at low temperature

As in the comparison made of each strain at both temperatures, the ANOVA analysis revealed 119 and 45 differential proteins between *Sc/Sk* and *Sc/Su* at 12°C, respectively, (Fig. 5). For *Sc/Sk* (Fig. 5A), major differences were observed in the metabolism of carbohydrates and amino acids in *Sc*, while the over-represented proteins in *Sk* once again related with translation, ribosome and proteins synthesis. In the *Sc/Su* (Fig. 5B) analysis, the strategy of *Sc* was the up-regulation of the proteins involved in the metabolism of carbohydrates and amino acids, in particular the biosynthesis of lysine and the proteins related with NAD/NADP and the oxidative stress response. As in *Sk*, *Su* showed an over-representation of the proteins that belonged to translation machinery. Figure 5C depicts the number of shared proteins and their regulation. There were nine shared proteins in both analyses, which mainly belonged to methionine

metabolism and translation. Recently, an important role of the sulfur assimilation pathway, including the biosynthesis of methionine and cysteine, has been shown during adaptation at low temperature in two *S. cerevisiae* wine strains [31].

3.5 Translation efficiency at low temperature

To demonstrate that *Sk* and *Su* adaptation to low temperature is related to enhanced translation efficiency; we tested its sensitivity to paromomycin, a potent translation inhibitor [32]. We studied the paromomycin (10, 20 and 40 µg) growth inhibition of yeast cells at either 28°C or 12°C for the three strains. The growth of a strain with powerful translation machinery is less affected by the protein synthesis blockage of this drug. A growth inhibition halo was observed under some conditions. Figure 6 indicates that *Sc* was severely affected by paromomycin at 12°C, whereas *Sk* and *Su* showed no growth inhibition at all. This result confirms the better translation performance of *Sk* and *Su* at 12°C. Conversely at 28°C, the *Sc* strain showed no growth inhibition, but *Sk* strain presented a mild negative effect at the maximum paromomycin concentration and *Su* was severely affected.

4. Discussion

The influence of temperature on microorganism growth has been widely studied by microbiologists, and different mathematical models have been developed to quantify and predict its effects. By applying mathematical-empirical approaches to estimate cardinal growth temperature, Salvadó et al. [16] considered *Sc* to be the most heat-tolerant strain within the genus *Saccharomyces*, with the highest optimum (32.3°C) growth temperature. *Su* and *Sk* were found to be the most cryotolerant species of this genus, with the lowest optimum (26.2 and 23.6°C) growth temperatures. Our data of specific growth, consumption and production specific rates (Table 1) reinforce that *Sk* is the most psychrotolerant, and has the highest sugar and nitrogen consumption rates at

low temperature. Clustering based on the metabolic profiles of these species also evidenced that *Sk* displayed a similar growth and metabolic pattern at both temperatures. Yet regardless of growth temperature, *Sc* had the highest biomass yield. In spite of the fitness decrease in *Sc* when the fermentation temperature dropped, it could be hypothesized that the superior competence of this species over other yeast species competitors mainly lies in its better growth efficiency within the whole range of temperatures used during winemaking.

The proteome analysis of the three *Saccharomyces* species revealed a similar strategy to help cope with low-temperature adaptation, but differences in the number of proteins involved and in the effectiveness of this response were encountered. A common response in the analyzed strains was the increase in the proteins involved in translation (ribosome biogenesis, ribosomal proteins and protein synthesis). We had already observed an up-regulation of many of the genes involved in translation in a transcriptomic comparison we previously made between *Sc* and *Sk*, grown at the same temperatures used herein [18]. Low temperature causes hyperstabilization of RNA structures, which prevents the maturation of ribosomes and, consequently, the kinetics of translation initiation [33-38]. 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. However, as mentioned earlier, this was not equally achieved by all the strains. The analysis of protein interaction and protein networks (STRING 10) clearly showed an increase in more ribosome proteins in psychrotolerant strains *Sk* and *Su*. This coordinated induction of proteins resulted in a higher translation efficiency of these species at low temperature compared with *Sc*, which was confirmed by the different susceptibility to paromomycin.

The other big group of proteins with differential concentrations in the three strains was related with the biosynthesis of amino acids. In fact, nitrogen metabolism is one of the most affected cellular processes at low temperature in *Sc*. Pizarro et al. [39] reported that the physiological and transcriptional response of laboratory and wine yeast strains to stress at low temperature was similar to growth under nitrogen-limiting conditions. Low temperature diminishes plasma membrane fluidity, which considerably reduces the molecular motion of phospholipids and membrane proteins [40]. This decrease in membrane fluidity might impair the activity of some permeases [41] by modifying the profile of the up-taken amino acids [42]. A paradigmatic example of these assimilation problems is the sensitivity of tryptophan uptake at low temperature as a result of dramatic conformational changes in high-affinity permease Tat2p [41]. It is interesting to note that “metabolism of tryptophan” is a common significant functional category between *Sc* and *Su*. Once again the increase in the proteins involved in one of the main amino acid pathways must be seen as a cellular response to surpass a metabolic bottleneck produced by a non-optimal growth temperature. As Tronchoni et al. [15] reported, a better adapted lipid membrane composition in *Sk* might enable better transport of nitrogen compounds and, therefore, greater amino acid metabolism activity.

Other significant functional categories with differential protein concentrations in both the comparison made between temperatures and among strains include “Glycolysis and gluconeogenesis” and “alcohol fermentation”. A recent global metabolic comparison made by our group [17] of the three species at low temperature revealed that the main differences between the two cryophilic species and *Sc* lay in carbohydrate metabolism. Strains *Sk* and *Su* had significantly higher intracellular glucose and fructose levels, and most intermediates were of the higher part of glycolysis (C6 sugars), the pentose phosphate pathway and trehalose metabolism. When considering residual sugars in the

supernatant of steady-state cultures (Table 1), on the one hand a higher sugar uptake by *Sk* and *Su* cannot explain this accumulation of higher glycolytic metabolites while, on the other hand, it may be related with a slower glycolytic flux in cryotolerant strains, mainly at the level of conversion of hexoses into trioses. Our current proteomic data evidence an induction of glycolytic and alcoholic fermentation enzymes at low temperature, and this increase is greater in *Sc*, which can partially explain the quicker glycolytic flux in this species. Another plausible explanation is the redox imbalance produced as a result of the slower kinetics of alcohol dehydrogenases at low temperature, which would result in NADH accumulation and glycolytic flux blockage. Yeast cells would respond to the slower conversion of acetaldehyde into ethanol by increasing the concentration of the main alcohol dehydrogenase, *ADH1*, which was one of the three common proteins to be induced at low temperature in the three species. Paget et al. [43] used a genome-scale metabolic reconstruction of yeast metabolism combined with a thermodynamic analysis to identify the metabolic genes associated with cold adaptation in *Sc* and *Sk*. Among the genes identified to have a strong effect on the temperature phenotype, those that related to the conversion of reduced NADH or NADPH into the oxidized form were overrepresented. Of them, the most representative was *ADH3*, which encodes the mitochondrial alcohol dehydrogenase isozyme. Compared with the parental strain, the *Δadh3* strain showed less fitness at cold temperatures, with *Sk* displaying the strongest effect.

5. Conclusions

Temperature is one of the leading factors that drives adaptation of organisms and ecosystems. Remarkably, many closely related species share the same habitat because of their different temporal or micro-spatial thermal adaptations [43]. In this study, we sought to find physiological and proteomic differences of closely related *Saccharomyces* species adapted to grow at low temperature. Although only one representative strain of each species was used and it is difficult to extend conclusions to the species level, our data corroborate previous transcriptomic results, which suggest that *Sk* is better adapted to grow at low temperature as a result of enhanced more efficient translation. Proteomic data also evidenced that translation efficiency can be an important target of adaptive evolution when cells face changing environments. Fitter amino acid biosynthetic pathways can also be mechanisms that better explain biomass yield in cryotolerant strains. Yet even at low temperature, *Sc* is the most fermentative competitive species. This fitness advantage has been related with quicker sugar uptake and speedier flux by the glycolysis pathway than its competitors [44], thus enabling better ethanol yields, which allow niche construction via ethanol production [45]. A higher concentration of glycolytic and alcoholic fermentation enzymes in the *Sc* strain might explain such greater fermentation activity.

Appendix A. Supplementary data

Table S1. Supplementary Table S1 is a single Excel file comprising five tables (sheets). Sheet 1, 2 and 3 are the list of proteins with different relative abundance between temperatures in *Sc*, *Sk* and *Su*, respectively. Sheet 4 and 5 are the list of proteins with different relative abundance comparing *Sc* at 12°C with *Sk* and *Su* at 12°C, respectively.

Accession Codes

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with dataset identifiers PXD002237 and DOI 10.6019/PXD002237.

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Author Contributions

EGR conducted the experiments, analyzed the data and wrote the manuscript. JMG and AQ conceived the study, participated in the study design and wrote the manuscript. All the authors have read and approved the final manuscript.

Conflict of interest

The authors have declared that no competing interests exist.

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- 682

Table 1. Physiological characteristics of *Saccharomyces* strains and extracellular metabolites in the steady state of continuous cultures.

	<i>Sc</i> 12°C	<i>Sc</i> 28°C	<i>Sk</i> 12°C	<i>Sk</i> 28°C	<i>Su</i> 12°C	<i>Su</i> 28°C
Extracellular metabolites						
Glucose (g L⁻¹)	88.30±3.12 ^a	57.39±2.12	91.69±1.03	88.69±1.99 ^c	81.09±3.73 ^a	48.58±0.01 ^c
Fructose (g L⁻¹)	88.04±3.09 ^a	67.89±1.75	94.58±0.90	94.22±0.99 ^c	83.10±3.76 ^a	61.93±0.02 ^c
Glycerol (g L⁻¹)	1.31±0.20 ^a	6.22±0.22	0.95±0.04 ^a	2.10±0.39 ^c	0.99±0.06 ^a	3.08±0.01 ^c
Ethanol (g L⁻¹)	10.30±0.22 ^a	36.99±0.41	2.65±0.05 ^{ab}	8.61±0.91 ^c	16.80±0.52 ^{ab}	38.13±0.11 ^c
Amino acids (mg NL⁻¹)	117.61±4.62	114.37±10.55	92.48±11.33	135.82±14.59	107.91±0.93 ^a	133.59±4.90
Ammonium (mg L⁻¹)	1.73±0.10	1.05±0.64	1.43±0.10	1.36±0.85	2.49±0.10 ^{ab}	0.75±0.01
Physiological data						
Biomass (g DW L⁻¹)	0.80±0.11 ^a	3.23±0.38	0.22±0.05 ^{ab}	0.54±0.05 ^c	0.51±0.00 ^a	1.34±0.06 ^c
Y_{hexoses} (g_{DW}·g_{hexoses}⁻¹)	0.034±0.004	0.043±0.002	0.016±0.006 ^b	0.031±0.002 ^c	0.014±0.002 ^b	0.015±0.00 ^c
q_{glucose} (g_{glucose}·DW⁻¹·h⁻¹)	- 0.57±0.07	- 0.52±0.03	- 1.56±0.59 ^b	- 0.83±0.05 ^c	- 1.46±0.27	- 1.52±0.07 ^c
q_{fructose} (g_{fructose}·DW⁻¹·h⁻¹)	- 0.58±0.07	- 0.39±0.02	- 1.02±0.43	- 0.42±0.02	- 1.31±0.28	- 1.12±0.05
q_{aa} (mg N) (mg_{Naa}·DW⁻¹·h⁻¹)	- 3.14±0.66 ^a	- 0.80±0.03	- 16.47±6.39 ^b	- 3.34±1.43	- 5.59±0.02 ^a	- 1.38±0.21 ^c
q_{NH4(mg)} (mg_{NH4}·DW⁻¹·h⁻¹)	- 5.93±0.82 ^a	- 1.48±0.18	- 21.94±5.79 ^b	- 8.82±0.88 ^c	- 9.12±0.06 ^a	- 3.53±0.17 ^c
q_{glycerol} (g_{glycerol}·DW⁻¹·h⁻¹)	0.06±0.01	0.07±0.00	0.17±0.05	0.15±0.01	0.07±0.00	0.09±0.00 ^c
q_{ethanol} (g_{ethanol}·DW⁻¹·h⁻¹)	0.51±0.06	0.46±0.05	0.49±0.14 ^b	0.63±0.00 ^c	1.30±0.05 ^a	1.12±0.04

^aSignificant differences (p value ≤ 0.05) in each strain compared with their control conditions (28°C)

^bSignificant differences (p value ≤ 0.05) in each strain at 12°C compared with the control condition (*Sc* 12°C)

^cSignificant differences (p value ≤ 0.05) in each strain at 28°C compared with the control condition (*Sc* 28°C)

Figure Legends

Figure 1. Global metabolic profile comparison of the three species. Hierarchical clustering of all the species at 12°C and 28°C.

Figure 2. Heat maps depicting the significant concentration differences of proteins in each strain when comparing 12°C and 28°C. The enriched functional categories in both temperatures are sorted by level of significance.

Figure 3. Network interactions of differentially expressed proteins at low temperature by a STRING analysis at the confidence level 0.7 in each strain. *Sc*, *Sk* and *Su* are represented respectively by A-B, C-D and E-F. A-C-E indicate the over-represented proteins at 12°C and B-D-F the ones at 28°C. The enriched functional categories are marked with colors circles.

Figure 4. Overlap in the proteins regulated by temperature in the three strains. These proteins that had a common regulation at low temperature in the compared strains are highlighted in red (over-represented) and green (under-represented). The enriched functional categories are sorted by level of significance.

Figure 5. Proteomic landscape of the cryotolerant strains (*Sk* and *Su*) in the comparison made with *Sc* at 12°C. (A) Heat map of the relative abundance of the proteins in *Sc* and *Sk* at 12°C. (B) Heat map of the relative abundance of the protein in *Sc* and *Su* at 12°C. The enriched functional categories are sorted by level of significance. (C) Overlap in the proteins regulated by low temperature in both experiments.

Figure 6. *Sk* and *Su* present increased translation efficiency at low temperature. The inhibitory effect of translation inhibitor paromomycin was evaluated by measuring the

720 halo diameter generated in the three species lawns grown in YPD plates at 12°C or
721 28°C. It is only shown the result for the maximum paromomycin concentration (40 µg).

CDC19, TEF1, VMA1, ADH1, TRP5, SSE1, RPS5, LYS2, SCP160, NPT1, RPL21A, URA5, YGP1, BNA1, PRC1

01.01.09.06 metabolism of tryptophan 2.34E-03
01.20.36 non-ribosomal peptide synthesis 2.54E-03
70.03 cytoplasm 2.86E-03
34.11.12 perception of nutrients and nutritional adaptation 7.12E-03
01.07.03 catabolism of vitamins, cofactors, and prosthetic groups 1.01E-02

CDC19, ADH1, RPS5

02.01 glycolysis and gluconeogenesis 7.74E-04
70.16.01 mitochondrial outer membrane 6.07E-03
02.16.01 alcohol fermentation 9.61E-03

CDC19, DED1, ADH1, RPS5, RPS16B, RPL31A, RPS18B, YML131W, ARG8

70.03 cytoplasm 1.46E-06
12.04 translation 1.79E-05
12.01.01 ribosomal proteins 3.64E-04
12 PROTEIN SYNTHESIS 7.40E-04
12.01 ribosome biogenesis 1.28E-03

MET6, EFT1, CDC19, PDC1, TKL1, SAM2, ENO1, YEF3, ADH1, HOM6, PDC5, PAB1, THR4, YHB1, TIF1, CYS3, RPS5, RPS3, EMI2, TDH3, RPS2, RPP0, RPS19B, RPL10, RPL12B, RPS20, SOD1, RPL28, BGL2, ARF1, YPT7, RPS24B, PEP4, RPL23B, POL30, SES1, RPS14B, TMA19, RPL25, ACO2, FBA1, PA41, PRB1, GPM1, RPL16B, SDS24, RPS21A, NOP12

12.04 translation 3.64E-14
70.03 cytoplasm 2.10E-13
12 PROTEIN SYNTHESIS 5.93E-10
12.01.01 ribosomal proteins 7.07E-10
12.01 ribosome biogenesis 8.14E-09

0.0 5.0

A

0.0 5.0

B

Sc 12

70.03 cytoplasm 9.00E-09
 01 METABOLISM 1.82E-08
 01.05 C-compound and carbohydrate metabolism 1.32E-07
 01.20 secondary metabolism 6.01E-07
 02.01 glycolysis and gluconeogenesis 1.05E-06
 01.03.01 purin nucleotide/nucleoside/nucleobase metabolism 8.97E-06
 01.05.02 sugar, glucoside, polyol and carboxylate metabolism 3.06E-05
 01.01 amino acid metabolism 4.46E-05
 12.04 translation 6.40E-5
 01.07.03 catabolism of vitamins, cofactors, and prosthetic groups 7.47E-05
 01.03.01.03 purine nucleotide/nucleoside/nucleobase anabolism 8.30E-05
 02 ENERGY 8.51E-5
 01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism 9.10E-05

Sk 12

70.03 cytoplasm 4.26E-12
 12.01 ribosome biogenesis 1.08E-11
 12 PROTEIN SYNTHESIS 2.41E-11
 12.04 translation 8.77E-09
 16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic) 1.42E-07
 70 SUBCELLULAR LOCALIZATION 2.26E-07
 12.01.01 ribosomal proteins 2.93E-07
 01.03.01 purin nucleotide/nucleoside/nucleobase metabolism 2.04E-06
 01.03 nucleotide/nucleoside/nucleobase metabolism 9.65E-05
 16.01 protein binding 1.31E-05
 11.04.01 rRNA processing 3.03E-04
 11.06.01 rRNA modification 1.69E-03

YGP1
 MET6
 TPI1
 PST2
 ADE13
 HO1M
 INO1
 CAR2
 TKL1
 PG11
 LYS2
 GRE3
 GLR1
 YNL134C
 BGL2
 RPP2A
 OYE2
 EFT1
 GSP1
 IMD2
 SOD2
 LEU2
 DCS1
 HXK2
 ERG13
 EPB1
 GPD1
 RPS4B
 PDC5
 RPL4B
 TRR1
 TDH2
 RPL9A
 RPL5
 RPS18B
 MET17
 GND2
 RPS12
 RPS5
 SDS23
 RPL14A
 SRP1
 ADH1
 UBA1
 RPL25
 PGK1
 SNU13

Sc 12

01 METABOLISM 6.12E-06
 01.20 secondary metabolism 1.55E-05
 02.01 glycolysis and gluconeogenesis 3.38E-05
 70.03 cytoplasm 3.89E-05
 01.05.02 sugar, glucoside, polyol and carboxylate metabolism 6.42E-05
 01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism 1.98E-04
 02.07 pentose-phosphate pathway 2.22E-04
 01.05 C-compound and carbohydrate metabolism 2.97E-04
 16.21 complex cofactor/cosubstrate/vitamins binding 1.03E-03
 01.01 amino acid metabolism 1.36E-03
 01.01.05.06.01 biosynthesis of lysine 1.39E-03
 01.20.36 non-ribosomal peptide synthesis 4.06E-03
 16.21.07 NAD/NADP binding 4.07E-03
 01.05.02.04 sugar, glucoside, polyol and carboxylate anabolism 4.36E-03
 01.01.05 metabolism of the aspartate family 4.66E-03

Su 12

70.03 cytoplasm 1.95E-10
 12.04 translation 1.68E-08
 12.01.01 ribosomal proteins 2.22E-07
 12.01 ribosome biogenesis 3.11E-07
 12 PROTEIN SYNTHESIS 2.78E-06
 70 SUBCELLULAR LOCALIZATION 8.50E-04
 16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic) 1.80E-03
 02.16.01 alcohol fermentation 2.38E-03

CR85 (Sk) 12600 (Su) QA23 (Sc)

28°C

12°C