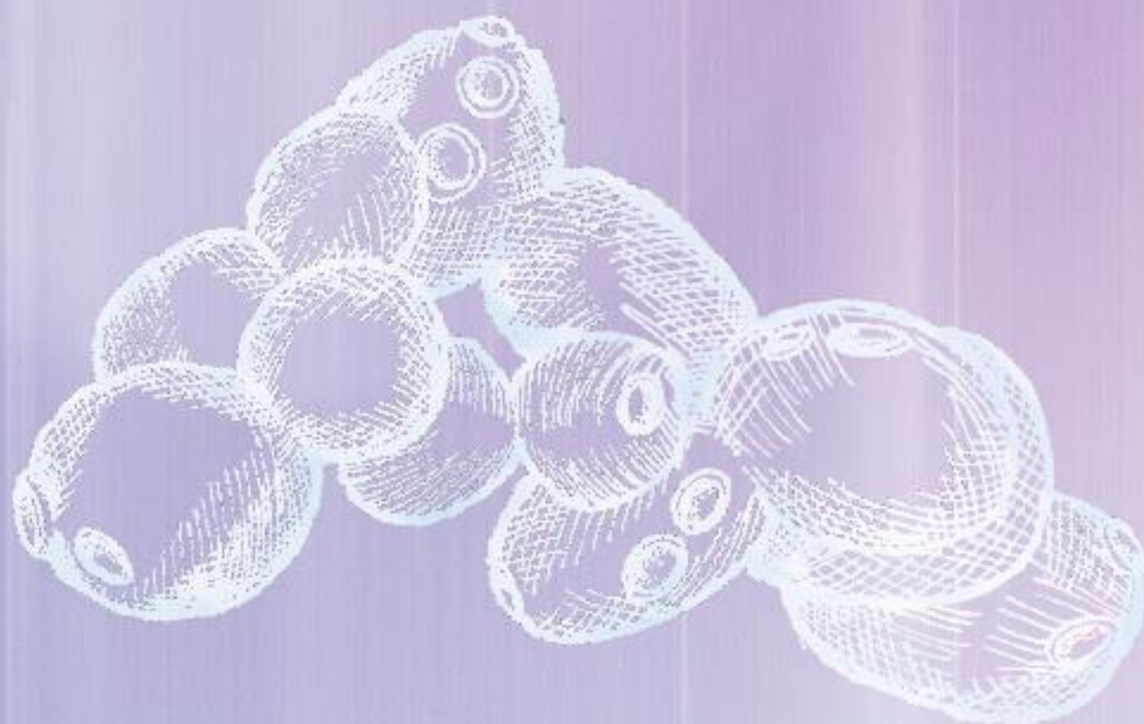
Unraveling the complex trait of low temperature adaptation in the wine yeast *Saccharomyces cerevisiae*

Estéfani García Ríos



Supervised by
Dr. José Manuel Guillamón Navarro
Valencia 2016

Unraveling the complex trait of low temperature adaptation in the wine yeast *Saccharomyces cerevisiae*

PhD thesis

by

Estéfani García Ríos

supervised by

Dr. José Manuel Guillamón Navarro

Dpt. de Bioquímica y Biología Molecular

Doctorado en Biotecnología

Estéfani García Ríos

El Dr. José Manuel Guillamón Navarro, Investigador Científico del Consejo Superior de Investigaciones Científicas (CSIC) en el Departamento de Biotecnología de Alimentos del Instituto de Agroquímica y Tecnología de Alimentos (IATA):

INFORMA

Que Dña. Estéfani García Ríos, Licenciada en Biotecnología por la Universidad de León, ha realizado bajo su dirección el trabajo titulado: **"Unraveling the complex trait of low temperature adaptation in the wine yeast *Saccharomyces cerevisiae*"**, que presenta para optar al grado de Doctor en el programa de Biotecnología por la Universitat de València. Asimismo, certifica haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia, a 11 de Octubre de 2016.

Dr. José Manuel Guillamón Navarro

El trabajo que aquí se expone se ha llevado a cabo en el Departamento de Biotecnología de los Alimentos del IATA (CSIC). Este trabajo ha sido posible gracias a la concesión de una ayuda predoctoral de formación de personal investigador FPI (BES-2011-044498) asociada al proyecto AGL2010-22001-C02-01 del Ministerio de Ciencia e Innovación. A su vez, he de agradecer la concesión de una ayuda a la movilidad que me permitió realizar una estancia breve en el Institute for Research on Cancer and Ageing (IRCAN, Niza, Francia), concedidas por el Ministerio de Economía y Competitividad.

Agradecimientos

Siempre que lees los agradecimientos de tesis anteriores y empezaban diciendo lo difícil que era escribir los agradecimientos, siempre pensaba que era la típica frase que había que poner pero ahora que me toca a mi enfrentarme a ello realmente me doy cuenta que no es una tarea fácil. Han sido muchos años, mucha gente y muchas experiencias vividas.

Me gustaría empezar agradeciéndole a la persona sin la cual esta tesis no habría sido posible, mi director de tesis José Manuel. Quiero agradecerte el haberme brindado tu confianza para llevar a cabo este proyecto y el apoyo y la comprensión durante todo el proceso. También quiero agradecerte el transmitirme el entusiasmo por este trabajo que aunque difícil, es my gratificante y sobre todo por ser optimista cuando yo no lo he sido. No he podido tener un mejor director.

También me gustaría agradecerle a Amparo Querol, Eladio Barrio y Sergi Puig porque siempre han estado dispuestos a ayudarme y aconsejarme cuando lo he necesitado. De la misma manera agradezco a todas las personas del IATA que de una u otra manera me han ayudado a lo largo de esta tesis: Carmela, Paloma, Roberto, Maite, Emilia, Lorena Perales, Jose Gimeno, Ana Cris, Vicky, etc.

En enero de 2011 llegué al lab 303 para realizar mi trabajo fin de máster. Ese lab 303 no tiene mucho que ver con el actual, pero a lo largo de toda su evolución me he llevado personas geniales. Quiero empezar agradeciéndole a Rosana por toda su ayuda y paciencia en mis inicios, a Ali y a Clara (ex 303) por las conversaciones, las risas y la comprensión, sois geniales y sin vosotras esto no hubiera sido lo mismo. A todas las personas que han pasado por aquí: Silvia, Víctor, Roberto, Sergio, Bruno, Mari Carmen, Lourdes, Cristina… tampoco se me pueden olvidar mis compis de la otra mitad del lab: Bea, Meri, Lucía Mendoza y especialmente Liliana, tu apoyo ha sido muy importante para mi. Por último de ese antiguo 303 quiero agradecerle de manera muy especial a María, por transmitir ese entusiasmo por la ciencia que tanto te caracteriza, por haberme ayudado tanto a nivel experimental cuando era tu alumnilla como a nivel personal cuando estaba de bajón. Por tener confianza en mi y en mis (nuestros) resultados, por todas las conversaciones intentando sacar cosas en claro sobre los QTLs (cuando aun no tenía muy claro de que iba el tema ☺), por echarme la bronca cuando me lo merecía y por ver siempre el vaso medio lleno. Por todo ello GRACIAS!!!

Voy a moverme a tiempos más actuales del 303…

Ying, gracias por tu sonrisa constante y por tu ayuda. N-Ric, gràcies per la teva ajuda amb el format de la tesi i sobretot amb la cerca de la llibreta suau. ;). A Antonio, nuestro miembro más reciente por su ayuda.

Aunque ya no seas del 303 en mi corazón seguirás estando en mi bancada, Lucía gracias por todo. Gracias por tu buen trabajo desde que viniste como alumna colaboradora hasta que expusiste tu fantástico TFM, por toda tu ayuda en el lab, por tus ánimos, tu confianza y tu amistad. Eres una persona fantástica, una parte de esta tesis es tuya y este camino sin ti no habría sido lo mismo. Por último, quiero darte las gracias enormemente a ti, Sara, porque personas como tu, quedan muy pocas y yo tengo la suerte de haberte conocido. Gracias por tu generosidad, tu ayuda (en tus tiempos de alumna y en el presente), tus ánimos y tu apoyo cuando he estado desquiciada (sobre todo en los últimos tiempos de escritura ☺), por tu confianza y por esos abrazos cuando los he necesitado (con rama y sin ella). Hay personas sin las cuales probablemente este trabajo no hubiera sido el mismo y tu eres una de ellas. GRACIAS POR TODO.

A todas las personas del lab 307 y 309, las que están y las que ya no, muchas gracias por vuestra ayuda. A David por tu generosidad y por intentar siempre ponernos las cosas fáciles a todos. A Laura, Jordi y a Peris por su ayuda y consejos. A Bruno por contagiarnos a todos tu alegría. Miguel, muchas gracias por tu ayuda en el análisis de los QTLs, creo que al final les has cogido cariño. Muchas gracias porque aguantarme, sobre todo cuando te hablaba de gamas cromáticas en R, no debio de ser fácil ☺. De manera más especial me gustaría agradecerle a a Jiří y a Javi. Jirito, empezamos juntos esta aventura y te has ido antes de poder ver como acaba la mía…gracias por todo (presecta y postsecta). Javier, compi de baja temperatura, tienes esa capacidad de unir, hemos vivido ya muchos momentos juntos (no me olvido que sacrificaste dormir en un hotel cómodo por estar conmigo en el camping de Levico terme, eso si que es amistad) y todavía nos quedan muchos más. Gracias porque contigo empezó todo. Ahora les toca el turno a las primas, las azúcar Romero. Quien me iba a decir que después de un año contigo haciendo el máster sin haber cruzado una sola palabra (ya sabemos que tus palabras no son baratas) iba a encontrar en ti una persona y una amiga tan genial, Anto, muchas gracias. Eres una persona imprescindible. Se que vas a seguir estando sea lo que sea lo que nos depare la vida. Adri, tu también has estado desde el principio, cuando todavía estabas en el Cavanilles, gracias por tu ayuda, tus consejos y tu apoyo. Pero sobre todo por amistad y por haber sacado de mi esa afición por la cultura egipcia. Tampoco puedo olvidarme de Andrea, mi argentina bella, 6 meses y me han parecido años.

Voy a moverme a los laboratorios pares, del lab 306 me gustaría agradecer a Patri, Montse y Gloria por su ayuda cuando llegué al IATA. A Walter, eres de esas personas que sin haber estado siempre ahí parece que lo has estado, se me hace complicado recordar cuando no habías llegado. Por último pero no menos importante a ti, Albis, por ser mi amiga. Ya se que crees que te excluímos del Yeast team, pero te prometo que algún día te llevaremos a un congreso de levaduras ☺. Muchas gracias por estar siempre ahí, por poder contar contigo y porque se me hace muy difícil encontrar buenos momentos en los que tu no hayas estado. Aurora, desde nuestros tiempos de TFM juntas en el tranvía hasta ahora, tu tesis y la mía siempre han ido juntas, gracias por compartir el camino. Isaac, sin ti mis inicios en la tercera no hubieran sido lo mismo especialmente por nuestras charlas y risas de pasillo.

Me gustaría agradecer al lab de Emilia y Agustín su ayuda con todo lo relacionado con el estrés oxidativo, especialmente cuando me iban surgiendo dudas en un camino desconocido para mi. Gracias a ti Ceci, por toda tu paciencia respondiéndome a cada pregunta que se me pasaba por la cabeza sobre un tema del que sabes mucho más que yo, por ayudarme con los experimentos, por animarme y escucharme cuando lo he necesitado, en definitiva por tu amistad. La ciencia necesita mas personas con tu entusiasmo que disfruten tanto de esta profesión tan bonita.

Al Dr. Gianni Liti y a todo su grupo del IRCAN en Niza, por haberme acogido en su grupo y haberme permitido aprender tantas cosas nuevas. En especial a Agnès, Merci!!

Quiero agradecer a mis amig@s y a mi familia por todo su apoyo y confianza desde el principio: Vero, Miriam, Christian, Rochy, Sara, etc. Isa y Natalia, gracias por vuestro apoyo constante. Marcos, gracias por ayudarme y escucharme, por estar a mi lado ☺. Especialmente a ti Lidia, como no podría resumirlo en 4 líneas y ni siquiera en todo un libro, simplemente gracias por estar ahí siempre, por ser mucho más que una amiga, por ser mi hermana.

Por último, a las personas más importantes de mi vida, mis padres, sin vosotros no estaría aquí. Gracias por haberme dejado volar, decidir y sobre todo equivocarme. Gracias.

To see a World in a Grain of Sand

And a Heaven in a Wild Flower,

Hold Infinity in the palm of your hand

And Eternity in an hour

William Blake

INTRODUCTION **15**

Section 1 General characteristics of the wine yeast *Saccharomyces cerevisiae* **18**

Section 2 *Saccharomyces cerevisiae* and the alcoholic fermentation **33**

Section 3 Low temperature fermentation **44**

Section 4 Yeast and post-genomic era **59**

BACKGROUND AND OBJECTIVES **75**

CHAPTER 1 **81**

Global phenotypic and genomic comparison of two *Saccharomyces cerevisiae* wine strains reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature fermentations

CHAPTER 2 **143**

Correlation between low temperature adaptation and oxidative stress in *Saccharomyces cerevisiae*

CHAPTER 3 **181**

The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*

CHAPTER 4 **231**

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

GENERAL DISCUSSION **271**

CONCLUSIONS **289**

SPANISH SUMMARY **295**

REFERENCES **321**

ANNEX 1: MATERIAL AND METHODS **361**

ANNEX 2: PUBLICATIONS **403**

INTRODUCTION

It is believed that grapes have been domesticated between the Black Sea and Iran during the 7000 – 4000 BC period. The first evidence of wine making comes from the presence of tartaric acid in an ancient jar dated from 5400 – 5000 BC in the Neolithic site of Tepe in Mesopotamia and from the remains for grape juice extraction from 5000 BC in the Neolithic site of Dikili Tash in Greece (Sicard and Legras, 2011). Colonization by the Romans spread winemaking all around the Mediterranean; by 500 BC, wine was being produced in Italy, France, Spain, Portugal and northern Africa. Cultivation of the vine also spread into the Balkan States, and the Romans took it into Germany and other parts of northern Europe, eventually reaching as far as Britain. European explorers in the sixteenth century introduced the vine into the New World. In 1530 the Spanish conquistadors planted *Vitis vinifera* in Mexico, Argentina, Peru and Chile. In 1655 Dutch settlers in South Africa planted French vine cuttings on the lower slopes of the Cape of Good Hope's majestic Table Mountain. Planting in California followed soon thereafter, and in Australia and New Zealand more than a century later, in 1813 (Pretorius, 2000a).

In the seventeenth century, Antonie van Leeuwenhoek developed high-quality lenses and was able to observe yeast for the first time. In the eighteenth and nineteenth centuries, chemists worked hard to decipher the nature of alcoholic fermentation through analytical chemistry and chemical

nomenclature. Our modern understanding of the fermentation process comes from the work of the chemist Louis Pasteur. Pasteur was the first to demonstrate experimentally that fermented beverages result from the action of living yeast transforming glucose into ethanol. Moreover, Pasteur demonstrated that only microorganisms are capable of converting sugars into alcohol from grape juice, and that the process occurs in the absence of oxygen (Barnett, 2000).

Later in 1890, Müller – Thurgau introduced the concept of inoculating wine fermentations with pure yeast culture (Pretorius, 2000a). At the present most of the wine production relies on the use of selected pure yeast culture as an oenological practice to produce wine with desirable characteristics and to guarantee the homogeneity of successive vintages.

1. GENERAL CHARACTERISTICS OF THE WINE YEAST *Saccharomyces cerevisiae*

1.1 Classification and taxonomy into the *Saccharomyces* genus

Together with the development of molecular methods of yeast characterization, the use of DNA sequencing enabled an improvement of yeast classification. Previously, the classification was based mainly on

morphological, reproductive and physiological characteristics, such as fermentation capacity or carbon and nitrogen source assimilation. The current advance of sequencing technologies, such as high-throughput sequencing and next-generation sequencing tools, led and still leads to a re-arrangement of the taxonomic classification.

Saccharomyces cerevisiae is a member of the genus *Saccharomyces* (previously included into the *Saccharomyces* sensu stricto group) (Borneman and Pretorius, 2015). The genus *Saccharomyces* belongs to the kingdom Fungi, the phylum *Ascomycota* (as the sexual reproduction is based on the formation of ascospores), the subphylum *Saccharomycotina*, the class *Saccharomycetes*, the order *Saccharomycetales* and the family *Saccharomycetaceae*. The taxonomy of the *Saccharomyces* genus currently involves seven species (Figure 1). Besides *S. cerevisiae*, there are *S. kudriavzevii*, *S. uvarum*, *S. paradoxus*, *S. mikatae*, *S. arboricolus*, and *S. eubayanus* (Borneman and Pretorius, 2015; Boynton and Greig, 2014; Hittinger, 2013). Moreover, these pure species has formed different hybrids, *S. cerevisiae*-*S. kudriavzevii* in wine and brewing (González et al., 2007; Peris et al., 2012), *S. cerevisiae* - *S. uvarum* hybrid in cider and brewing (Rainieri et al., 2006), and the most well-known hybrid, the lager yeast *S. pastorianus,* which is an interspecific hybrid between *S. cerevisiae* and the recently described *S. eubayanus* (Libkind et al., 2011; Peris et al., 2016a).

In *S. cerevisiae*, five "pure" populations have been described: North American, Sake, Malaysian, West African and Wine/European (Liti et al., 2009a). In *S. paradoxus* three populations depending on the geographic isolation were found: American (includes *S. cariocanus*), Far Eastern and European (Liti et al., 2006, 2009a).

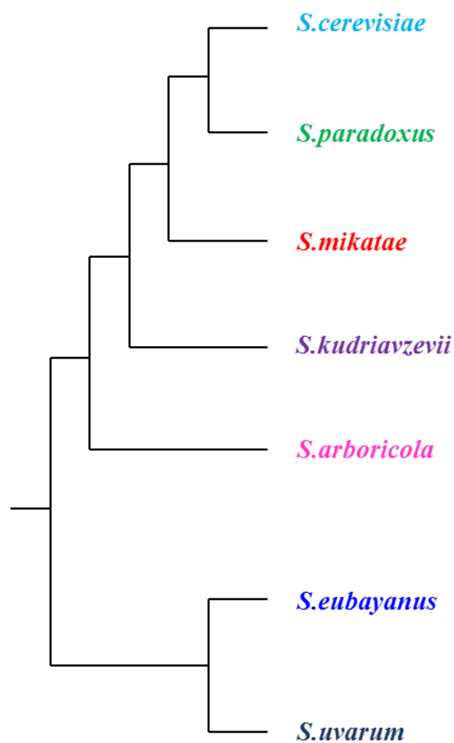


Figure 1. Phylogenic relationship among *Saccharomyces* species (Figure adapted from Hittinger et al., 2013).

1.2 Saccharomyces cerevisiae: Life cycle

Because of their abundance and dominance in many spontaneous fermentations, *Saccharomyces cerevisiae* strains were selected for the majority of controlled fermentation processes, including the traditional uses for production of beverages and baking, but also for production of recombinant antibodies, vitamins, and drugs, such as insulin (Bayne et al., 1988; Gerngross, 2004; Kazemi Seresht et al., 2013). Besides its employment in industrial applications, *S. cerevisiae* plays an important role in scientific research as a model organism. Many important biological findings have been obtained using model organisms, improving the understanding of human biology (Kaeberlein et al., 2005). *S. cerevisiae* is arguably the most intensely studied eukaryotic organism besides human beings. Its genetic tractability has made it a valuable model organism for genetics, genomics, cell biology and biochemistry. Several features make it ideal as a model: first, it is easy to cultivate quickly in large populations due to its short cell cycle; second, it is available as haploid, diploid or polyploid allowing meiotic and mitotic studies; third, rapid and easy gene deletions can be performed owing to a very efficient homologous recombination pathway and finally, heterologous gene expression from episomal plasmids is possible allowing functional gene comparisons in evolution studies

(Burke et al., 2014; Landry et al., 2006; Li et al., 2014; Long et al., 2015; Petranovic and Nielsen, 2008).

S. cerevisiae can multiply either asexually by vegetative multiplication or sexually by forming ascospores (Figure 2). Under optimal nutritional and cultural conditions, *S. cerevisiae* uses budding as vegetative growth, the most common and typical mode of reproduction of Ascomycetes yeasts. The cell division cycle in vegetative multiplication involves: G1 (period preceding DNA synthesis), S (DNA synthesis), G2 (period preceding the mitosis) and M (mitosis and cytokinesis). Once mitosis is complete and the bud nucleus and other organelles have migrated into the bud, cytokinesis commences and a septum is formed in the isthmus between mother and daughter.

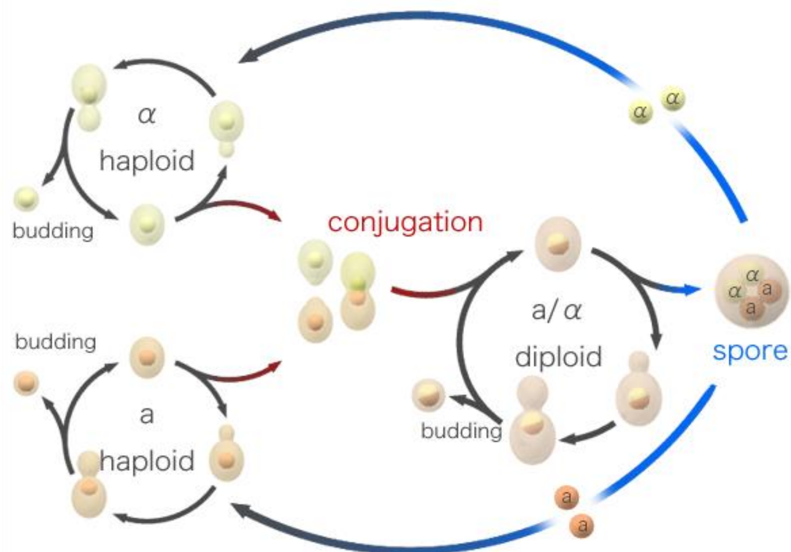


Figure 2. A schematic representation of the life cycle of yeasts.

Though vegetative growth is the major way of yeast reproduction, sexual reproduction is an alternative when nutrient supplies fall short. Sexual reproduction involves the formation of four haploid ascospores (two MATa and two MATα) within an ascus after meiosis and is induced during nutrient starvation, specifically nitrogen and fermentable carbon sources (Taxis et al., 2005). MATa spores can only mate with MATα and vice versa, resulting in the fusion of two cells to form a diploid cell (zygote) (Jackson and Hartwell, 1990). Spore released from the ascus can also continue asexual reproduction as haploids for many generations, and are termed heterotallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homotallic.

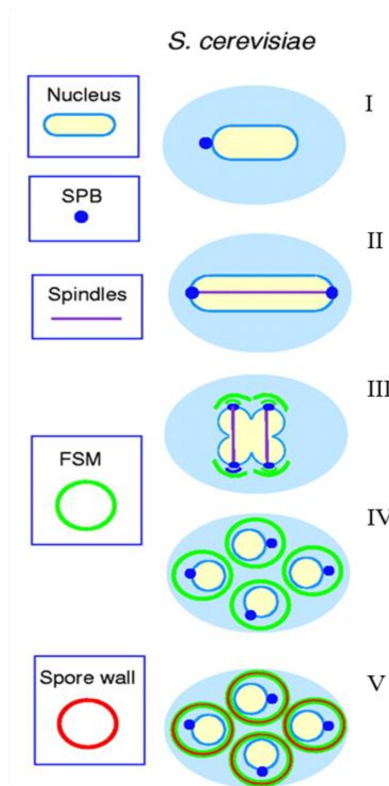


Figure 3. **Schematic representation of sporulation stages in the yeast *S. cerevisiae*.** Stage I, meiotic prophase I. Stage II, meiotic metaphase I/anaphase I. Stage III, meiotic metaphase II/anaphase II; formation of the Forespore membrane (FSM). Stage IV, completion of FSM assembly. Stage V, spore wall synthesis (Adapted from Shimoda, 2004).

1.3 Kinetics of yeast growth

Yeast population growth is the result of cell division and the progression through the cell cycle. Under optimal growth conditions, yeast

growth kinetic follows the typical microbial growth curve, comprising three main phases: lag phase, exponential phase and stationary phase (Figure 4). The lag phase reflects the time required for yeast cells to adapt to their new environment by synthesizing ribosomes and enzymes needed to establish growth at a higher rate. The duration of this phase depends on firstly the initial population size and secondly environmental conditions. Once the cell starts actively metabolizing, they begin DNA replication and shortly after the cells divide. This begins the second phase of growth called the exponential phase of growth. This is the period in which the cells reproduce at maximum specific growth rate (μ_{max}). The time it takes the culture to double is called generation time. Yeast strain, growth medium, and temperature are important factors in determining the generation time. Industrial fermentations aim to extend this phase for maximizing the output of biomass and metabolites production (López et al., 2004). The third phase in yeast growth is stationary phase; a period of no growth when metabolism slows and cell division is stopped. The factors that cause cells to enter stationary phase are related to change in the environment, such as nutrient deprivation, toxic metabolites and high temperatures. After prolonged periods in stationary phase, cells may die and autolysate.

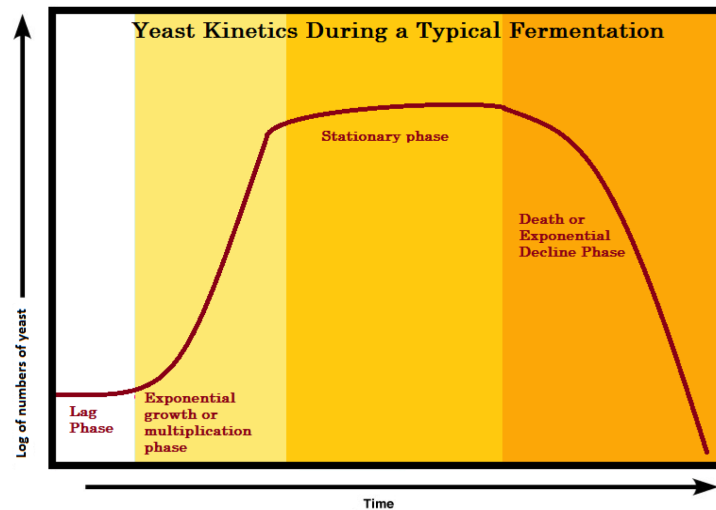


Figure 4. Typical yeast growth curve.

1.4 Genetic constitution of wine yeast: lab *vs* industrial strains

A number of *S. cerevisiae* strains have become important laboratory organisms for molecular and genetic research. The easy genetic manipulation and its small, compact genome has led to *S. cerevisiae* becoming the model organism of choice for studies on eukaryotic cells. Haploid strains contain 12 to 13 megabases (Mb) of nuclear DNA, distributed along 16 linear chromosomes. Each chromosome is a single DNA molecule with a length of about 200 to 2200 kilobases. In addition to the chromosomal DNA exist a non-Mendelian genetics elements e.g. Ty retrotransposons, 2µm plasmid DNA in the nucleus; 75 kb of mtDNA in the

mitocondria; and killer viral-like particles containing dsRNA and prion-like elements in the cytoplasm. The genome of the laboratory strain, S288c, was the first eukaryote for which a fully genome sequence was available. The complete genome sequence defines 5885 open reading frames (ORFs) that are likely to specify protein products in the yeast cell (Goffeau et al., 1996). Its primary annotation was updated recently in its first major update since 1996 (Engel et al., 2014).

Although most strains of *S. cerevisiae* are either haploid or diploid, some are aneuploid or polyploid with unbalanced chromosomes (Hauser et al., 2001). It has been proposed that an unbalanced chromosome set is an advantageous trait for industrial yeast, explaining the presence of aneuploid and polyploid strains. For example, polyploidy can increase the copy number and subsequent dosage and expression of beneficial genes, whilst decreasing the effects of deleterious mutations. These extra gene copies can diverge and take on new functions, increasing heterozygosity and allowing greater scope for adaptation to an ever-changing external environment. Many industrial yeast strains are also highly heterozygous, whereas lab strains are haploid, which makes breeding and genetic manipulation easier (Zara et al., 2005). Generally, wild type and industrial strains of *S. cerevisiae* are less easily accessible for genetic analysis and manipulation than laboratory strains. Low sporulation frequency and viability of spores,

instability of mating types and poor mating efficiencies, and the absence of easily tractable auxotrophic markers are some of the characteristics that compromise user friendliness of industrial yeast species (Winzeler et al., 2003).

The clear phenotypic differences between industrial and non-industrial strains combined with early indications from single gene studies, which suggested substantial novel nucleotide sequences and genomic rearrangements were present across strains of *S. cerevisiae*. Almost a decade passed before genome sequences for additional strains of *S. cerevisiae* became publicly available, RM11-1a and YJM789 (Wei et al., 2007). However, in the five years following the sequencing of RM11-1a and YJM789, there has been an exponentially increase in the number of *S. cerevisiae* strains for which whole-genome sequence are available. This was primarily owing to the development of "next generation" DNA sequencing (Borneman et al., 2013). The first industrial yeast genome to be assembled primarily from next-generation data was a haploid derivative of a wine yeast strain (Borneman et al., 2008) and there are other five, high quality commercial wine yeast genome assemblies currently available (Borneman et al., 2011; Novo et al., 2009). As more *S. cerevisiae* strains are sequenced, the suitability of S288c as a "reference" strain is becoming less clear, especially as it appears to lack a large number of ORFs found in many

others *S. cerevisiae* strains while containing an abnormally high number of Ty transposable elements. A major finding of *S. cerevisiae* comparative genomics was the discovery of numerous strain-specific ORFs in the wine strains. The genome of the wine yeast EC1118 contains at least two major telomeric insertions relative to the laboratory strain, one on the left arm of chromosome VI and one on the right of chromosome XV. These insertions are shared by subsets of others wine strains such as QA23. The region on chromosome XV has particular interest because has at least three genes that will potentially impact on wine-relevant traits. The first of these is a homolog of the *S. pastorianus* fructose/H+ symporter (Galeote et al., 2010). Two other genes of the genome encode oligopeptide transporters that allow for a greater variety of small peptide to be used as a nitrogen source. One of the most striking aspects of the wine yeast genome is the presence of a cluster of five genes that are postulated to have been horizontally transferred between *S. cerevisiae* and *Zygosaccharomyces spp* (Novo et al., 2009).

1.5 *Saccharomyces cerevisiae* ecology

Although much is known about yeast at the cellular and molecular level, little is known about their ecology or natural lifestyles in the environment. *S. cerevisiae* populations are genetically diverse and are found in numerous different environments, often inhabiting liquid or moist areas

(Landry et al., 2006). Common habitats include soil, plant exudates, animal tissues, and surfaces within vineyards and wineries. *S. cerevisiae* strains are found in diverse geographical and climatic environments, from tropical regions (fruit trees) to cold Northern climes (oak trees) (Fay and Benavides, 2005). Certain 'clinical isolates' are opportunistic pathogens in immuno-compromised patients (Llopis et al., 2012; Pérez-Torrado et al., 2012; Pérez-Torrado and Querol, 2016). The diversity and distribution of yeast in association with grapes and musts are influenced by the age, climate and geographical location of the winery or vineyard (Beltran et al., 2002; Schuller et al., 2005), grape varietals grown (Martini et al., 1980), presence of yeast starter cultures (Fleet, 2008; Valero et al., 2007), and the fermentation temperature used (Torija et al., 2003).

Previous studies on *S. cerevisiae* indicated that this domesticated yeast, chiefly adapted to man-made fermentations (wine, beer, sake, baker) and normally absent in natural ecosystems, might have evolved from wild *S. paradoxus* (Ciani et al., 2004). Natural wine fermentation involves a multitude of biochemical and ecological interactions between many microbial species, not just *S. cerevisiae* (Pretorius, 2000a). On the surface of ripe grape berries, non-*Saccharomyces* yeast dominate, such as those from the genera *Hanseniaspora* (*Kloeckera*), *Candida*, *Torulaspora* and *Metschnikowia*. Pre-harvest berries typically hold 10^4-10^6 colony forming

units (CFU) mL^{-1} yeast; however, only a small fraction of these populations are *S. cerevisiae* (approximately 10-100 CFU mL^{-1}) (Fleet, 2003). Fungi, yeast, lactic acid bacteria and acetic acid bacteria grow in grape juice before the initiation of fermentation (Rainieri and Pretorius, 2000). During this period, the non-*Saccharomyces* yeast proliferate, including species from *Candida*, *Hanseniaspora, Metschnikowia and Pichia* (Fleet, 2003; Masneuf-Pomarede et al., 2016). However, once the process of fermentation is initiated, anaerobic conditions are forced on the microbial populations, preventing the proliferation of organisms unsuited to anaerobic metabolism, namely certain fungi and acetic acid bacteria. During the fermentation progress, *S.cerevisiae* displaces the non-*Saccharomyces* species and occupies their fermentative niche. A strong selective pressure favors the *Saccharomyces*'s strategy of making life difficult for other microorganisms by rapidly converting the available sugars into ethanol, a toxic compound, which can be later respired by *Saccharomyces* (Piskur et al., 2006). Ethanol concentration creates strong pressure which limits growth of non-*Saccharomyces*, consequently, they cannot compete with the *Saccharomyces*, which could generally stand higher ethanol levels (10-15% v/v) (Fleet, 2003; Masneuf-Pomarede et al., 2016; Rainieri and Pretorius, 2000). Moreover, the temperature increase produced by *Saccharomyces*

during fermentation also provides these species with a clear advantage over non-*Saccharomyces* species (Salvadó et al., 2011b).

1.6 Cryotolerant *Saccharomyces* species

Saccharomyces kudriavzevii

S. kudriavzevii was first isolated only from decaying leaves in Japan and has not so far been found in industrial fermentation environments. So researchers made queries how this species formed hybrids that were found in wine (González et al., 2007) and brewing (Gonzalez et al., 2008) environments located in Europe (Naumov et al., 2000a, 2000b). The answers came recently with an isolation of several *S. kudriavzevii* strains from oak trees in Portugal and Spain (Lopes et al., 2010; Sampaio and Gonçalves, 2008). These isolation events were made possible due to the decrease in isolation temperatures, which confirmed cryotolerant character of *S. kudriavzevii*. Other interesting advantages of *S. kudriavzevii*, as compared to *S. cerevisiae*, were found in oenological properties. Although *S. kudriavzevii* does not appear to be present in wine environments, it is able to conduct fermentation of a sterile wine must (González et al., 2007). When compared to *S. cerevisiae*, *S. kudriavzevii* was found to produce higher amounts of glycerol and lower amounts of ethanol, which fulfill the new demands of the wine industry (González et al., 2007; Oliveira et al., 2014).

Saccharomyces uvarum

Although several strains have been sporadically isolated from insects, tree fluxes or mushroom (Naumov et al., 2003), the natural habitats of *S. uvarum* have not been identified so far. It has been found mainly in industrial environments, such as wine and cider fermentation processes performed at low temperatures (Demuyter et al., 2004; Naumov et al., 2000b, 2001). Like *S. kudriavzevii*, due to the good fermentation capacity at low temperature, *S. uvarum* is considered cryotolerant. From the oenological point of view, as compared to *S. cerevisiae*, *S. uvarum* has shown higher production of glycerol and lower production of ethanol and acetic acid (Bertolini et al., 1996; Giudici et al., 1995). Additionally, *S. uvarum* has been characterized by its higher capability to release desirable flavour components, such as 2-phenylethanol and 2-phenylethyl acetate (Gamero et al., 2013; Masneuf-Pomarède et al., 2010). The wines produced by *S. uvarum* strains have been described to have a stronger aromatic intensity than those produced by *S. cerevisiae*. This species is also able to form hybrid strains with *S. cerevisiae* as has been proved by Nguyen et al., (2000).

2. *Saccharomyces cerevisiae* AND THE ALCOHOLIC FERMENTATION

2.1 Kinetics of wine fermentation

Alcoholic fermentation is defined as the biotransformation of grape sugars, including glucose and fructose, into ethanol and carbon dioxide (CO_2) (Pretorius, 2000a). In the late 1850s, Louis Pasteur established that yeast are the organisms responsible for fermentation, that the process is nitrogen-dependent, and that ethanol and CO_2 are not the sole products of fermentation, as yeast must synthesize cell biomass. Yeast are strongly inclined to perform alcoholic fermentation under aerobic and anaerobic conditions (van Dijken et al., 1993). Fermentation is usually carried out anaerobically and generates energy in the form of adenosine triphosphate (ATP). Anaerobic metabolism generates only two ATPs per glucose molecule, compared to 36-38 ATPs during aerobic oxidation. In sugar concentrations above 20 g L^{-1} or even less, *S. cerevisiae* uses the fermentative pathway for sugar metabolism and aerobic respiration is blocked, even in the presence of oxygen (O_2). This is known as the Crabtree effect (van Dijken et al., 1993). During fermentation, yeast metabolizes the sugars in grape musts to pyruvate via glycolysis. Pyruvate is decarboxylated to acetaldehyde, which is reduced to ethanol. One glucose molecule yields approximately two molecules of ethanol and CO_2. Approximately 60-80% of the sugar is fermented during the first half of fermentation. The

fermentation rate slows and by the end of fermentation, approximately 95% of the sugars have been converted into ethanol and CO_2 (Roustan and Sablayrolles, 2002). The remaining sugars are used by the yeast to produce cellular material (1%) and other secondary metabolites (remaining 4%).

2.2 Causes of stuck and sluggish fermentations

Despite considerable improvements in our ability to monitor and control fermentation, stuck and sluggish fermentations remain major challenges for the international wine industry. Bisson (1999) defined incomplete or "stuck" fermentations as those having a higher than desired residual sugar content at the end of alcoholic fermentation (>2-4 g L^{-1}), while slow or "sluggish" fermentations are characterized by a low rate of sugar consumption by the yeast (Figure 5).

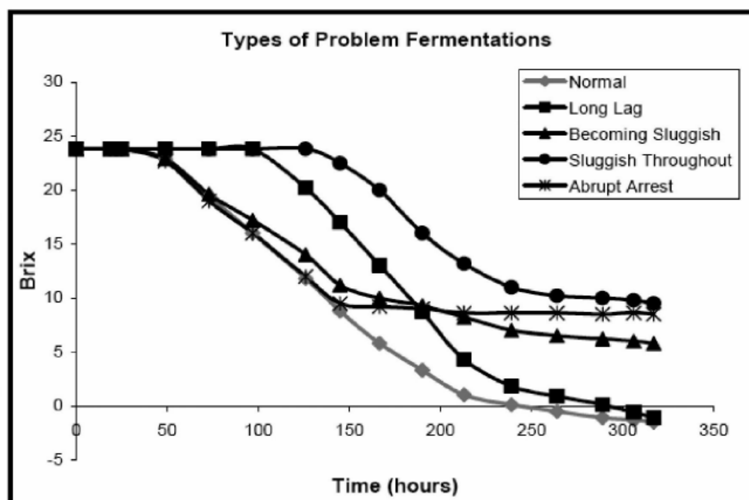


Figure 5. Types of problem fermentations (Bisson, 1999).

Stuck wines with residual sugars are more susceptible to spoilage by acetic acid and lactic acid bacteria, which metabolize the sugars into volatile acids and form abnormal ester compounds. Stuck and sluggish fermentations lead to a loss of tank capacity in a winery, due to the increased fermentation period, and are also vulnerable to oxidation, as the protective CO_2 layer is lost from the top of the must (Pizarro et al., 2007). The economic and logistical consequences of sluggish and stuck wine fermentations in industrial cellars demand significant investigation into the causes and the determination of methods to avoid this problem.

Factors accounting for stuck and sluggish fermentations include nutrient deficiencies or imbalances (vitamins, minerals and nitrogen), high

ethanol levels, high acidity, high sugar concentrations (> 300 g L^{-1}), fructose accumulation, presence of inhibitory wild yeast and the accumulation of toxic by-products (fatty acids, peptides, acetic acid or sulfites) (Alexandre and Charpentier, 1998; Charoenchai et al., 1998). Furthermore, extremes in fermentation temperature (too high or too low) act in combination with must-associated factors, especially nitrogen deficiency (Alexandre and Charpentier, 1998; Coleman et al., 2007). These factors often place additional stress on the yeast, resulting in interference with cell morphology (*i.e.* plasma membrane or cell wall alterations) and enzyme inhibition (Pizarro et al., 2007).

Stuck fermentations are extremely difficult to restart, and the best time to correct a problematic fermentation is before the uptake of sugars becomes too slow or has stopped altogether (Pizarro et al., 2007). Solutions include resuspending the yeast using a pump, adding extra grape solids, adding nitrogen and nutrients for the yeast, warming the ferment to a higher temperature, adding oxygen, or reinoculating the ferment with a more ethanol-tolerant and fructose-fermenting 'finishing' strain (Quirós et al., 2014; Santos et al., 2008).

2.3 Stresses encountered during wine fermentation: oxidative stress

Fermentation exposes yeast to multiple environmental stresses including high osmotic pressure, low pH, low O_2, high sugar, high ethanol, nutrient starvation and temperature fluctuations (Bauer and Pretorius, 2000; Marks et al., 2008). Initial studies on the yeast response to environmental fluctuations were performed in non-fermentable media, and termed the environmental stress response (ESR), also known as the common environmental response (CER) or general stress response (GSR). DNA microarray studies have determined that approximately 10-15 % of the yeast genome is either induced or repressed during the ESR as a common signature (Causton et al., 2001; Gasch, 2003; Gasch and Werner-Washburne, 2002).

The ESR appears to represent a generalized response in which cells adjust their growth rate in response to a range of stresses. Components of the ESR may be used to control the rate of cell cycle progression, re-establish the energy supply balance to account for these fluctuations, as well as provide a protective role against stress (Giaever et al., 2002; Lai et al., 2005). Marks et al., (2008) analyzed the specific response of yeast to environmental fluctuations in fermentable media and coined the term fermentation stress response (FSR). Throughout fermentation, 40% of the genome displayed differential expression. Of these differentially expressed genes, 223 were upregulated and designated as FSR genes. The FSR is a

novel stress response, as 62% of the genes had not previously been implicated in stress response and 28% of the genes have no functional annotation. Downregulated genes were excluded from the FSR, as they were primarily involved in protein synthesis and ribosome processing, a well-characterized stress response, which is linked to growth arrest (Marks et al., 2008; Rossignol et al., 2003).

The FSR includes a component of the ESR, with 20% of the FSR genes showing overlap. Genes shared between the FSR and ESR include chaperone-associated proteins and heat shock proteins (HSPs), that repair the damage caused by environmental stress and prevent any further damage from occurring. Chaperones and HSPs are also the most highly expressed genes at late-stationary phase and represent a priority for protein synthesis (Rossignol et al., 2009; Zuzuarregui et al., 2006). FSR genes also include those involved in counteracting hyper-osmotic stress, such as glycerol-synthesizing genes. Fifty genes which are known to be induced by sugar stress are induced during the FSR. Other FSR genes are involved in ethanol toxicity, nitrogen depletion and oxidative stress. Although the FSR and ESR represent a common response to environmental fluctuation, there are also strain-specific stress responses, which are responsible for controlling the stress resistance of individual yeast strains (Zuzuarregui et al., 2005).

Oxidative stress response

In general, all eukaryotic organisms have an oxygen dependent metabolism as along evolution this molecule has been selected as a final electron acceptor during the respiration process. The consequence is that all aerobic organisms are subject to the call "oxygen paradox" (Davies, 1995) because they depend on it for their survival during cellular respiration but at the same time, the oxidizing power give as a result toxic compounds, called reactive oxygen species (ROS). An oxidative stress is said to occur when ROS overwhelm the antioxidant defenses, resulting in genetic degeneration and physiological dysfunction, leading eventually to cell death (Halliwell and Gutteridge, 1986).

ROS representing different oxidation states of molecular oxygen (O_2) and include superoxide anion ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). ROS can be generated by exogenous sources, such as air contaminants, oxidizing chemicals (menadione, diamide, hydrogen peroxide, tert-butyl hydroperoxide), UV radiation or heavy metals, whose main action is DNA damage and indirectly oxidative stress, phototoxicity and even photosensitivity (Zagarese et al., 2001). In *S. cerevisiae* there are also endogenous sources of ROS that are closely related to the transition of fermentative metabolism, favored by high concentration of glucose in the medium, to a respiratory metabolism when sugars are depleted (Carlson,

1987; Gancedo, 1998; Gasmi et al., 2014; Roberts and Hudson, 2006). This fact promotes a gene expression changes that allows the synthesis of enzymes that are necessary for respiration and induction of the cellular antioxidant defenses to cope with the increased activity of the electron transport chain (Costa and Moradas-Ferreira, 2001; DeRisi et al., 1997; Verghese J, Abrams J, Wang Y, 2012).

Antioxidant defenses include a number of protective enzymes that are present in different subcellular compartments and can be upregulated in response to ROS exposure (Figure 6). Non-enzymic defenses typically consist of small molecules that can act as free radical scavengers; to date, only ascorbic acid and glutathione have been extensively characterized in yeast (Farrugia et al., 2012; Morano et al., 2012). The main antioxidant systems are:

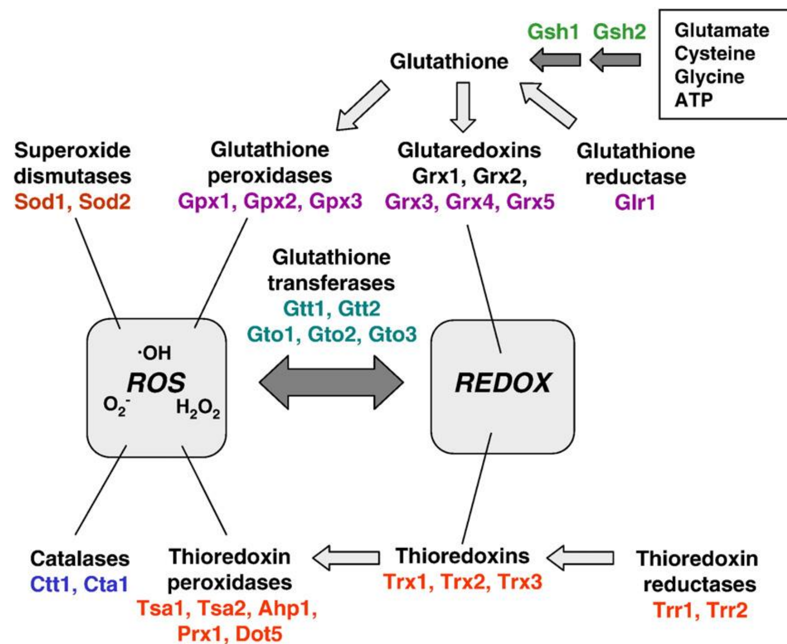


Figure 6: Enzymatic systems involved in ROS detoxification and in control of the redox state of protein sulphydryl groups in *S. cerevisiae*, with their interrelationships (Herrero et al., 2008).

Catalases: Catalases are ubiquitous heme-containing enzymes that catalyze the dismutation of H_2O_2 into H_2O and O_2. Yeast has two such enzymes: the peroxisosomal catalase A, encoded by *CTA1*, and the cytosolic catalase T, encoded by *CTT1* (Hiltunen et al., 2003; Martínez-Pastor et al., 1996).

Superoxide dismutases: Superoxide dismutases (SODs) convert the superoxide anion to hydrogen peroxide, which can then be reduced to water by catalases or peroxidases. SODs are ubiquitous antioxidants, which differ

in their intracellular location and metal cofactor requirements between different organisms. Yeast contains a cytoplasmic Cu, Zn-SOD (*SOD1*) and a mitochondrial matrix Mn-SOD (*SOD2*) (Culotta, 2000; Culotta et al., 2006).

Methionine sulfoxide reductase: Amino acids are susceptible to oxidation by ROS (Stadtman et al., 2003, 2005). Methionine residues are particularly susceptible, forming a racemic mixture of methionine-S-sulfoxide (Met-S-SO) and methionine- R-sulfoxide (Met-R-SO) in cells (Dean et al., 1997). Most organisms contain methionine sulfoxide reductases (MSRs), which protect against methionine oxidation by catalyzing thiol-dependent reduction of oxidized Met residues. This is particularly important because it means that methionine oxidation is readily reversible and can play an antioxidant role in scavenging ROS (Campbell et al., 2016).

Thioredoxins: *S. cerevisiae*, like most eukaryotes, contains a cytoplasmic thioredoxin system, which functions in protection against oxidative stress (Pedrajas et al., 2015). This comprises two thioredoxins (*TRX1* and *TRX2*) and a thioredoxin reductase (*TRR1*) (Gan, 1991). As in most organisms, yeast thioredoxins are active as antioxidants and play key roles in protection against oxidative stress induced by various ROS (Izawa et al., 1999; Kuge and Jones, 1994).

Peroxiredoxins: Peroxiredoxins (Prx) have multiple roles in stress protection, acting as antioxidants, molecular chaperones, and in the regulation of signal transduction (Wood et al., 2003). They use redox-active Cys residues to reduce peroxides and have been divided into two classes, the 1-Cys and 2-Cys Prx's, on the basis of the number of Cys residues directly involved in catalysis. During catalysis, the peroxidatic cysteine residue of one subunit is oxidized to a sulfenic acid, which condenses with the resolving cysteine from the other subunit to form a disulfide that is reduced by thioredoxin.

The glutathione system: The oxidation of sulfhydryl groups is one of the earliest observable events during ROS- mediated damage. This underlies the importance of GSH (g-glutamylcysteinylglycine) which is typically found as the most abundant low molecular-weight sulfhydryl compound (mM concentrations) in most organisms. Many roles have been proposed for GSH in a variety of cellular processes including amino acid transport; synthesis of nucleic acids and proteins; modulation of enzyme activity; and metabolism of carcinogens, xenobiotics, and ROS (Schafer and Buettner, 2001).

Glutaredoxins: Glutaredoxins (Grx) are small heat-stable oxidoreductases, which were first discovered in *E. coli* as GSH-dependent hydrogen donors for ribonucleotide reductase (Holmgren 1989). Classical

cellular glutaredoxins contain a conserved dithiol active site (Cys-Pro-Tyr-Cys) and form part of the glutaredoxin system, in which glutathione reductase transfers electrons from NADPH to glutaredoxins via GSH. It has been proposed roles in many cellular processes including protein folding and regulation, reduction of dehydroascorbate, and protection against ROS and sulfur metabolism (Holmgren, 1989).

Glutathione peroxidases: Eukaryotic glutathione peroxidases (Gpx's) are thought to provide the major enzymatic defense against oxidative stress caused by hydroperoxides. They reduce hydrogen peroxide and other organic hydroperoxides, such as fatty acid hydroperoxides, to the corresponding alcohol, using reducing power provided by GSH (Michiels et al., 1994).

Glutathione transferases: Glutathione transferases (GSTs) are a major family of proteins, which are involved in the detoxification of many xenobiotic compounds (Sheehan et al., 2001). They catalyze the conjugation of electrophillic substrates to GSH prior to their removal from cells via glutathione conjugate pumps.

3. LOW TEMPERATURE FERMENTATION

Many factors such as must composition, juice clarification, the temperature of fermentation or the yeast strain inoculated strongly affect alcoholic fermentation and aromatic profile of wine (Ribéreau-Gayon et al., 2005). With the effective control of fermentation temperature by the wine industry, low temperature fermentation (10-15 ºC) are becoming more frequent due to the aim of producing white and "rosé" wines with more pronounce aromatic profile (Beltran et al., 2006; Molina et al., 2007; Torija et al., 2003). This practice is limited to fermentation of white and "rosé" wine since in red winemaking higher temperatures (22-28 ºC) are needed for extracting phenolic compounds from the skin of the grape. Low temperatures increase not only the retention but also the production of some volatiles compounds (Killian and Ough, 1979). In these conditions greater concentration of aroma compounds are produced, such as esters that impart sweet and fruity aromas and lesser amounts of unpleasant compounds are produced, such as certain higher alcohols and acetic acid (Beltran et al., 2006). Another interesting aspect is that low temperatures notably reduce the growth of acetic and lactic acid bacteria, facilitating the control of alcoholic fermentation (Ribéreau-Gayon et al., 2005).

Despite fermentations at low temperature have interesting improvements; this practice also has some disadvantages. The optimal growth temperature of the wine yeast *S. cerevisiae* is around 32 ºC (Salvadó

et al., 2011b). Restrictive low temperature increases the lag phase and reduces the growth rate, producing sluggish and stuck fermentations (Bisson, 1999).

3.1 Effect of low temperature on yeast growth

Temperature of fermentation directly affects the microbial ecology of the grape must and the biochemical reactions of the yeast (Fleet, 2003). Several authors have suggested that some species of non-*Saccharomyces* have a better chance of growing at low temperature than *Saccharomyces* (Fleet, 1998; Sharf and Margalith, 1983) because they can increase their ethanol tolerance (Gao and Fleet, 1988). Also the number of different species, as well as their endurance during alcoholic fermentation is conditioned by both the temperature of the must and the temperature during fermentation. These changes determine the chemical and organoleptic qualities of the wine (Fleet, 1998).

On the other hand, while the toxic effects of ethanol serve to poison other competing yeast in the community, it also appears that *S. cerevisiae* is better adapted to other stresses imposed by the juice environment at higher temperatures. An increase of temperature from 16 to 23 ºC, as a consequence of the highly vigorous fermentative consumption of sugars, favoured the rapid growth of *S. cerevisiae* cells and final imposition

(Goddard, 2008). Similar results showed Salvadó et al., (2011) that found higher increases of the μ_{max} of the *S. cerevisiae* wine strains than the μ_{max} of non-*Saccharomyces* at temperatures above 20 ºC.

In spite of this higher tolerance of *S. cerevisiae* to high temperatures, this parameter also impact on yeast viability, which decreases as the temperature increases (Torija et al., 2003). The synergic effect between high temperature and ethanol toxicity explains that fermentations at low temperature show higher viability of the cell population throughout the alcoholic fermentation.

3.2 Effect of low temperature on yeast physiology

Low temperatures greatly affect the physiology and metabolism of yeast, requiring the cell to respond and adapt rapidly. This response is accompanied by extensive changes in gene expression and enzyme activity, allowing yeast to maintain growth and survival at low temperatures (Sahara et al., 2002; Schade et al., 2004; Tai et al., 2007). There are differences between *S. cerevisiae* strains in their physiological and transcriptional response to cold temperatures, but in general, yeast are prone to maintaining metabolic functions during cold temperature stress. A study by Tai et al., (2007) demonstrated that although the activity of glycolytic enzymes was ~7.5-fold lower at 12 °C compared to 30 °C, yeast were able to maintain the

same level of glycolytic flux in chemostat cultures (D = 0.03 h^{-1}). Wine yeast are typically better at adapting to cold temperatures than laboratory strains (Pizarro et al., 2008).

Cold temperature has a massive effect on cell physiology. These effects include decreased membrane fluidity, increased stabilization of DNA and RNA secondary structure, reduced efficiency of protein translation and protein folding, increased protein denaturation, clustering of integral membrane proteins and decreased enzyme activity (Al-Fageeh and Smales, 2006; Sahara et al., 2002). The cell membrane, which contains a large proportion of lipid molecules, is considered to be the primary target of low temperature trauma (López-Malo et al., 2013a, 2014a; Redón et al., 2011; Tronchoni et al., 2012b). Temperature directly affects the organization of these membrane lipids by causing them to solidify, decreasing membrane fluidity and reducing intra- and extracellular transport and diffusion rates of compounds and ions (Inouye and Phadtare, 2004). The cell counteracts this effect by increasing fluidity through altered unsaturated fatty acid production (homeoviscous adaptation) (Al-Fageeh and Smales, 2006; López-Malo et al., 2013a, 2014a).

The stabilization of nucleic acid secondary structure is considered to be a unique consequence of low temperatures and reduces the rates of mRNA elongation during transcription and mRNA movement on the

ribosome during translation (Inouye and Phadtare, 2004). The mRNA 5' untranslated region (5'UTR) tends to form stable secondary structures at low temperatures, becoming less accessible to the ribosome (Al-Fageeh and Smales, 2006). Low temperature also produces a higher stabilization of proteins and folding problems. To counteract problems associated with protein misfolding and denaturation at low temperature, yeast synthesize HSPs, which act as protein chaperones and help restore enzyme activity at low temperatures (Murata et al., 2006).

Some authors have reported that, during cold stress, yeast cells synthesize large amounts of protectant compounds, such as the reserve carbohydrates trehalose and glycerol, to preserve and defend internal cellular components (Sahara et al., 2002). The disaccharide trehalose, which acts as a chemical chaperone for membrane and protein stabilization, accumulates after cells are incubated for approximately 12 h at 10, 4 or 0 °C. The production of glycogen is also induced after this period; however, its role in the cold response has not yet been elucidated (Aguilera et al., 2007). Glycerol, an osmoprotectant solute, accumulates to higher levels at 4 °C than 12 °C and the rate of accumulation is dependent on the Stl1p glycerol active transporter (Aguilera et al., 2007). Glycerol protects the cell by counteracting the effects of osmotic shrinkage during freeze and thaw cycles (Panadero et al., 2006). The antioxidant response is also elicited at

low temperatures to protect the cell from reactive oxygen species (ROS) and free radicals which are formed under environmental stress (Ballester-Tomás et al., 2015; Paget et al., 2014; Salvadó et al., 2008; Zhang et al., 2003). Antioxidant compounds and enzymes, including glutathione, catalase and superoxide dismutase, are all induced at low temperatures and detoxify ROS for the maintenance of viability (Ballester-Tomás et al., 2015; Murata et al., 2006; Paget et al., 2014; Zhang et al., 2003).

3.3 Low temperature on fermentation kinetic

Although cold stress has been widely studied, very little is understood with regards to how cells adapt to cold temperatures during processes such as winemaking, where multiple stresses are present (Aguilera et al., 2007; Beltran et al., 2006; López-Malo et al., 2013a; Panadero et al., 2006; Redón et al., 2011). The effect of temperature on fermentation efficiency varies markedly for different *S. cerevisiae* strains and the ability of a strain to ferment well at cold temperatures depends on its cold tolerance and the rate of adaptation (Torija et al., 2003). Cold fermentation temperatures significantly decrease the speed of fermentation and the maximal fermentation rate (V_{max}), whilst increasing the lag period and the overall duration (Beltran et al., 2008; Erten, 2002) (Figure 7). For example, fermentations conducted by Erten et al. (2002) using non-

Saccharomyces and *Saccharomyces* yeast were completed with less than 2 g L^{-1} sugar at 15, 20 and 25 °C, but still contained 9.5 g L^{-1} sugar at 10 °C after 33 days. Sugar uptake and consumption is also decreased at low temperatures and cells consume less nitrogen, most likely due to impaired nitrogen transport caused by a loss of plasma membrane fluidity at cold temperatures (Beltran et al., 2007; D'Amato et al., 2006). These factors greatly increase the chances of stuck and sluggish fermentations.

The diversion of carbon into various sinks is altered at low temperatures, resulting in higher ethanol yield and modified production of secondary metabolites. The increase of trehalose during cold stress influences ethanol yield, as high trehalose concentrations allow the cell to produce higher ethanol and to protect against protein oxidation (Trevisol et al., 2011). The decrease in production of acetic acid and possibly acetaldehyde allows more carbon to be diverted towards the formation of ethanol (Llauradó et al., 2002).

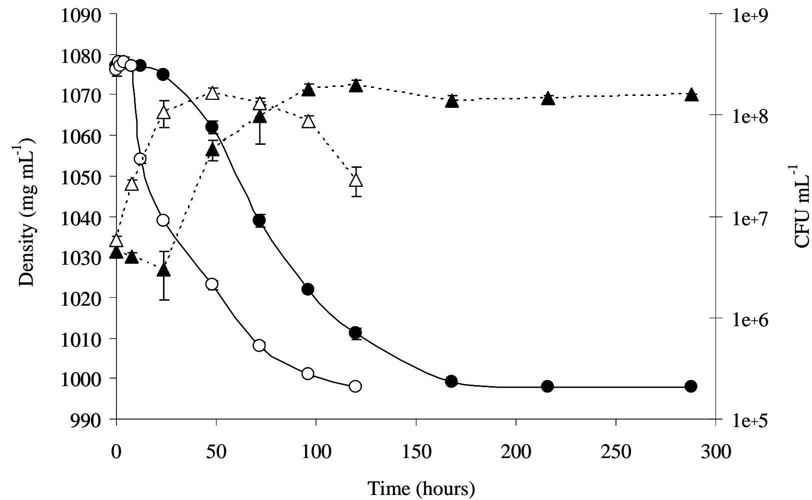


Figure 7. Fermentation kinetics (density reduction) and growth (CFU mL^{-1}). Filled symbols indicated fermentation at 13 ºC, and open symbols indicated fermentation at 25 ºC (Chiva et al., 2012).

3.4 Low temperature and stress response

In the past years many attempts have been made to elucidate the cold response in *S. cerevisiae* using the DNA microarray technology (Homma et al., 2003; López-Malo et al., 2015; Murata et al., 2006; Sahara et al., 2002; Tai et al., 2007). Low temperature has several effects on biochemical and physiological properties in yeast cell and therefore modifying a large set of genes. There are several aspects that need to be taken into account in order to interpret the effects of low temperature. Primarily, the response to low temperature can be categorized into two different groups based on the severity of the temperature. Cold shock is defined as the response of cells

when exposed to temperatures of 10-15 ºC, whereas the near-freezing response occurs at temperatures of <10 ºC (Al-Fageeh and Smales, 2006).

Examination of different microarray-based studies in *S. cerevisiae* after a decrease in temperature reveals that part of the genetic response to low temperature seems to be time dependent. Sahara *et al*. (2002) found three different phases according to the expression profile: (1) in the early phase genes involved in RNA polymerase I and rRNA processing are up-regulating; (2) in the middle phase genes involved in cytosolic ribosomal proteins are up-regulated; (3) in the late phase genes involved in general stress response are up-regulated. On the other hand similar results were shown by Schade *et al*. (2004) who identified two distinct responses during cold adaptation: early cold response (ECR) during ≤ 2 h and are induced genes implicated in RNA metabolism and lipid metabolism; and late cold response (LCR) during ≥ 12 h and are induced genes which encode proteins involved in protecting the cell against variety of stresses. Thus, in both studies the late response is overlapped with the environmental stress response (ESR). Genes induced during ESR are involved in a variety of cellular functions such as protein folding and degradation, transport and carbohydrate metabolism. Repressed ESR genes generally are involved in cell growth-related process, including RNA metabolism, nucleotide

biosynthesis, secretion and ribosomal performance. The regulation of LCR (ESR) is determined by the function of two transcription factors, Msn2p and Msn4p that bind to stress response elements (STREs) in the promoters on their target genes (Görner et al., 1998; Martínez-Pastor et al., 1996; Schmitt and McEntee, 1996). In contrast, the transcriptional response of the ECR genes was Msn2p/Msn4p independent and seems to be cold specific response.

Some genes involved in lipid metabolism are induced during ECR, in order to control the membrane fluidity. The fatty acid desaturase gene *OLE1* is induced to counteract the decrease in membrane fluidity (López-Malo et al., 2013a; Nakagawa et al., 2002). Furthermore other lipid genes are induced at low temperature such as *INO1* and *OPI3* (Murata et al., 2006). Tai *et al*. (2007) also showed that lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature in batch and chemostat cultures. Other cold-induced genes during ECR were those encoding RNA helicase, RNA-binding proteins and RNA-processing proteins, e.g. *NSR1*, required for normal pre-rRNA processing) (Chiva et al., 2012; Kondo and Inouye, 1991) or *DBP2*, a RNA helicase (Barta and Iggo, 1995).

One of the major ECR genes groups includes the family of temperature inducible proteins (*TIP*). The *TIP* family has been renamed the *DAN/TIR* family to incorporate the *TIP*-related (*TIR*) genes and Delayed Anaerobic (*DAN*) genes (Abramova *et al.*, 2001). The nine members of *DAN/TIR* family are serine- and alanine-rich cell wall mannoproteins which function as "low temperature growth genes" by maintaining cell wall integrity and aiding the adaptation to extreme temperature shifts (Abramova et al., 2001; Homma et al., 2003; Kondo and Inouye, 1991; Schade et al., 2004). It has been shown that the *DAN/TIR* genes are induced under cold temperature stress particularly when there is a lack of oxygen (Abramova et al., 2001). Seripauperin (*PAU*) family genes (*PAU1*, *PAU2*, *PAU3*, *PAU4*, *PAU5*, *PAU6* and *PAU 7*) were also induced at low temperature, which have been shown to display phospholipid interacting activity (Zhu et al., 2001).

During LCR a variety of *HSP* genes (*HSP12*, *HSP26*, *HSP42*, *HSP104*, *YRO2* and *SSE2*) were also found induced, suggesting a requirement for nuclear chaperones for protein folding and maintaining protein conformation in the cold (Schade et al., 2004). In addition genes belonging to the glutathione/glutaredoxine system (*GTT2*, *HYR*, *GPX1*, *TTR1* and *PRX1*) were induced in the LCR (Murata et al., 2006; Schade et al., 2004). These genes were previously shown to be induced by oxidative

stress and they were also implicated in detoxification processes (Gasch et al., 2000).

Despite the plethora of low-temperature transcriptome datasets, major questions still have to be addressed. There are major discrepancies in the low-temperature transcriptome data already published (Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). For instance, the observed expression of ribosomal protein (RP) genes has some inconsistencies. Although Sahara *et al*. (2002) reported an increase of many RP genes during a temperature downshift to 10 ºC, a similar temperature downshift resulted in a totally different transcriptional response in the study by Schade *et al*. (2004). Second, although the induction of genes involved in reserve carbohydrates seems to be a consistent feature of cold shock, trehalose is only indispensable for survival in near-freezing conditions (Kandror et al., 2004). In fact, above 10 ºC *Δtps1Δtps2* double mutant showed no growth defects or viability loss (Panadero et al., 2006).

Most of these studies have mainly been focused on the genome-wide transcriptional responses to cold-shock (Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). In interpreting effects of low temperature and other environmental parameters on microbial physiology, the time scale of exposure is essential. Sudden exposure to environmental changes (e.g. cold shock) is likely to trigger rapid, highly dynamic stress

response phenomena (adaptation). While acclimation is a prolonged exposure of an organism to nonlethal stimuli in which its regulatory mechanisms have resulted in full adaptation of genome expression to the environmental conditions. On an even longer time scale, mutational changes lead to evolutionary adaptation of the genome itself (Brown et al., 1998).

In batch cultures the specific growth rate (μ) is strongly affected by temperature, which makes it impossible to dissect temperature effects on transcription from effects of specific growth rate. This is relevant because specific growth rate as such has a strong impact on genome-wide transcript profiles (Castrillo et al., 2007; Regenberg et al., 2006). Furthermore all culture variables (*i.e.* intra- and extracellular metabolites) evolve in time and result in complex data patterns and make the identification of temperature-specific responses very difficult (Tai et al., 2007). In contrast to batch cultures, chemostat cultures enable accurate control of specific growth rate, independent of other culture conditions (Vázquez-Lima et al., 2014). In chemostat cultures, the dilution rate (D) is defined as the ratio of the flow rate of the ingoing medium (f, $L \cdot h^{-1}$) and the culture volume (V, L). When the culture volume is kept constant by continuous removal of culture broth, a steady-state will be reached in which the specific growth rate (μ, h^{-1}) is

equal to the dilution rate (D). In steady-state chemostat culture the concentrations of all metabolites and substrates are constant in time.

In a decisive study, Tai et al., (2007) compared their transcriptomic results obtained in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature. This comparison revealed large differences between transcriptional response during long-term low temperature acclimation and the transcriptional response to rapid transition to low temperature. In contrast to observations in cold-shock and batch culture studies, transcript levels of environmental stress response genes and trehalose biosynthesis genes were reduced at 12 ºC. Interestingly, lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature.

4. YEAST AND POST-GENOMIC ERA

4.1 Systems biology perspective

The first complete sequence of a eukaryotic genome (Goffeau et al., 1996) produced a dramatic transformation of yeast research. The transformation began with technical improvements that greatly accelerated research, especially any research involving identification of pieces of DNA cloned, and technologies unimaginable before, such as DNA microarrays

containing each and every yeast gene, became common place. The availability of the entire genome sequence has made possible the asking of new kinds of research questions, questions that can be answered only when one has truly comprehensive information about an organism.

The current ‘genomic revolution’ is generating large amounts of valuable information in the form of genome sequences. This new knowledge has to be accompanied by post-genomic studies based on new advanced methods, strategies and technologies which have to be continuously developed and improved. The most advanced post-genomic strategies are directed to the elucidation of new genes, their function and mechanisms of regulation, using new techniques at different levels of study: genome, transcriptome, proteome and metabolome, in an integrative or systems biology perspective (Delneri et al., 2001; Kitano, 2002; Oliver, 2002; Oliver et al., 2002; Paget et al., 2014; Petranovic and Nielsen, 2008).

Functional genomics aims to identify the roles that play the different genes in the biology of organisms with sequenced genomes. The field encompasses diverse techniques that allow biological study at multiple levels. The transcriptome, analysis of mRNA molecules with the use of full-genome microarrays, is not a direct measure of functionality, but rather a measure of translational potential. The proteome is a “snapshot” of total cellular protein, currently utilizing mainly two-dimensional gel

electrophoresis, and the subsequent analysis of gel spots by mass spectrometry techniques. Global protein profile (proteomic) is a true measure of cellular functionality. The metabolome, aiming to analyze the metabolite profile at a given point within cell, is genome independent. Multiple genes may be involved in the synthesis and degradation of a single metabolite and, as such, the exploitation of known genes on metabolic profiles can elucidate functions of unknown genes (Delneri et al., 2001). It is the use of these approaches that will allow the formation of an integrated biology of organism whose genome is fully sequenced (Hoskisson and Hobbs, 2005).

Although most of these functional analyses have been carried out with lab strains, it is important to carry out global functional analysis in industrial strains and in experimental context that approximates industrial conditions. Such studies are not only valuable in terms of basic research but also beneficial for application to the productive sector. This is the case of some studies which dealt with stress conditions frequently found in alcoholic fermentation, such as nutrient limitation (Boer et al., 2003), anaerobic conditions (Kwast et al., 2002), ethanol stress (Alexandre et al., 2001; Navarro-Tapia et al., 2016), osmotic stress (Munna et al., 2015; Yale and Bohnert, 2001) or cold stress (Beltran et al., 2006; López-Malo et al., 2014b; Salvadó et al., 2012; Tronchoni et al., 2014). The identification of

key genes that are useful for particular aspects of the biotechnological industry might improve the process either through the selection of proper strains or through the genetic modification of current industrial yeasts (Chiva et al., 2012).

4.2 Complex traits and QTL mapping

S. cerevisiae strains vary at the genotypic and phenotypic level exhibiting a wide diversity within the species. This diversity can be explained by many genetic interactions and polymorphisms which generate a continuity of values for a specific phenotype (Cubillos, 2016). Complex traits typically exhibit a quantitative phenotype, in contrast to monogenic Mendelian traits. Thus, determining the genetics behind phenotypic variation is essential to understand natural population diversity (Borevitz and Chory, 2004; Mackay et al., 2009; Wilkening et al., 2014). In complex traits, the genetic and phenotypic variation is not collinear, due to the polygenic nature of traits with many loci contributing to the variation through small direct effects as well as interactions with other loci and the environment (Abiola et al., 2003; Glazier, 2002; Warringer et al., 2011). Phenotypic variation can also be caused by environmental factors independent of the genotype (Abiola et al., 2003). Determination of the genes that contribute to complex traits, also denoted Quantitative Trait Loci (QTLs), is challenging since genetic interactions such as epistasis and gene-

environment along with pleiotropy or expression variability are common, making these loci difficult to analyze. QTLs are genes, or DNA regions, that contribute to complex traits at different levels (Borevitz and Chory, 2004; Glazier, 2002; Wilkening et al., 2014). There are three main advantages of QTL mapping: a) no *a priori* hypothesis on gene function and sequence variation is required, b) often the technique is capable of detecting multiple genes that affect the value of a single trait and c) is able to identify essential genes (Marullo et al., 2007). The distinction between Mendelian loci and QTLs is ambiguous since both can be mapped using similar techniques. However, genes that contribute to complex traits generate a continuous phenotypic effect, whilst in the Mendelian loci only up to three distinct phenotypes are governed by a single gene. Within a trait with many QTLs, each QTL may show different quantitative effects, being traits with many small effect QTLs the most difficult to map (Glazier, 2002). QTL mapping can be performed by their linkage to molecular markers with classifiable genotypes, this is, if a QTL is linked to a marker, then divergent genotypes for the marker will show phenotypic differences for a trait (Bhatia et al., 2014; Mackay et al., 2009). The current availability of genome sequences allows known polymorphisms, such as SNPs or microsatellites to be used as markers. QTL analysis can be separated into two stages: 1) from phenotype and genotype to QTL (detection) and 2) from QTL to gene (localization).

QTL detection depends on the effect of the loci and the phenotypic variance explained. A QTL that explains a large proportion of the trait will be easier to detect (Darvasi and Pisanté-Shalom, 2002). The main approaches to identify QTLs are through linkage analysis and association mapping, both aided by population structure knowledge (Figure 8). Linkage analysis uses either designed crosses between individuals with diverged phenotypes or sibling family studies, where recombination disrupts marker linkage predicting that causative loci will segregate together with the nearest genetic marker. The number of markers depends on the linkage disequilibrium (LD) in the population: lower LD increases the mapping resolution (Mackay et al., 2009). Once the QTL is detected, the next step is to reduce the critical interval size as much as possible. The identification of candidate genes is not an easy task. Some QTLs result from variation within a single gene or closely linked genes (Steinmetz et al., 2002) which can be confirmed by reciprocal hemizygosity, allele swapping approaches or mutational analysis (Cubillos et al., 2011; Darvasi and Pisanté-Shalom, 2002; Glazier, 2002; Kessi-Pérez et al., 2016; Parts et al., 2011). Reciprocal hemizigosity consists of the independent deletion of both allelic variants in a hybrid between two haplotypes with diverged phenotypes, which are then phenotypically compared (Steinmetz et al., 2002). Similarly, allele swapping consists on the

exchange of allelic or nucelotide variants between founder strains (Glazier, 2002), both only easily done in yeast.

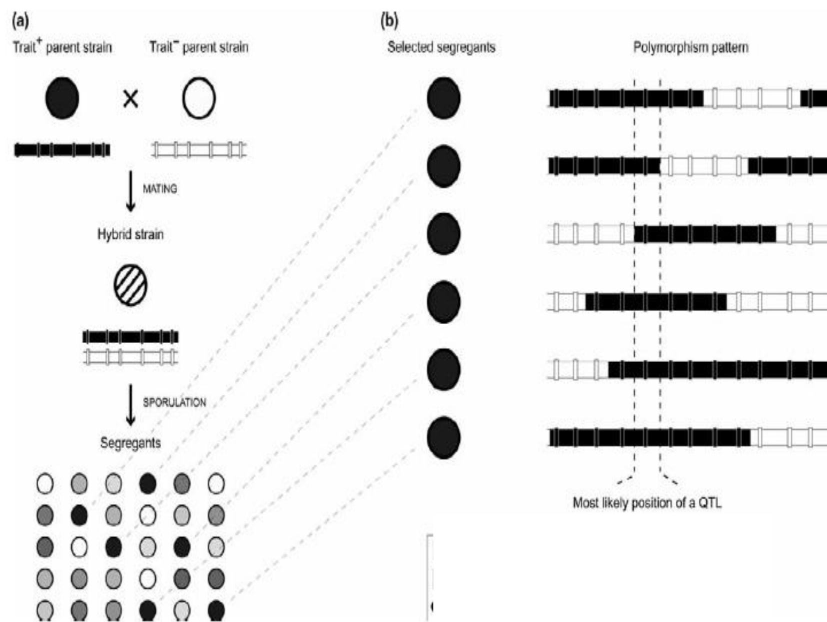


Figure 8. Schematic overview of QTL mapping in *S.cerevisiae.* The parental haploid strains with divergent phenotype are crossed and a hybrid between them is generated. After an sporulation step, a segregant population with different phenotypes is obtained. The segregants selected for their extreme expression of the quantitative trait can be used to infer the location of the unknown QTL (Figure adapted from Swinnen et al., (2012)).

4.3 Adaptation mechanism to industrial environments

Man has selected strains of *Saccharomyces* to the development of different fermented products such as bread, wine, and beer, among others. In the case of winemaking, *Saccharomyces* strains growing in these substrates are, in fact, well adapted to the oenological environment and can therefore ferment grape juice very efficiently (Pretorius, 2000a). In a fermentative environment like wine production and the production of other alcoholic beverages, there are a number of stresses for yeast cells (Attfield, 1997; Bauer and Pretorius, 2000; Querol et al., 2003b) that significantly affect the viability of the cells. At the beginning of the alcoholic fermentation, the major stresses are the osmotic caused by a high concentration of sugars in the grape must and the low pH of the medium. At the end of fermentation, ethanol production and depletion of some nutrients, such as nitrogen source, carbon and vitamins, are the main factors causing stress to the cells. The temperature can also be considered as another stressor (Cardona et al., 2007) since, in processes where the temperature is not controlled, it can rise to values above 30 °C. Some works have been directed to study the molecular mechanisms involved in the adaptation of yeasts to industrial processes, and the genomic changes of yeast that have been selected through billions of generations (Querol et al., 2003a, 2003b).

Genome renewal

In 1994 the pioneering yeast geneticist Robert Mortimer proposed the “Genome Renewal Hypothesis” to explain patterns of genetic variation observed in the budding yeast *S. cerevisiae* (Magwene, 2014; Mortimer, 2000; Mortimer et al., 1994). Mortimer and colleagues observed that most yeast strains isolated from vineyards were diploid and heterozygous at one or more loci. The vast majority were also homothallic, meaning that haploid cells produced from these strains were capable of undergoing mating-type switching followed by mother-daughter mating. This process, known as autodiplodization or haploselfing, leads to diploid cells that are homozygous at all but the mating type locus. Mortimer documented a negative correlation between the number of detectable heterozygosities in vineyard isolates and the percentage of viable spores produced; homozygous isolates had nearly 100% spore viability while heterozygous isolates showed clear evidence for deleterious or sometimes lethal alleles. Finally, isolates that were homozygous were inferred to have been derived from heterozygous backgrounds via autodiploidization. Mortimer and colleagues proposed that these observations could be explained by an evolutionary scenario involving long periods of clonal reproduction in which diploid strains accumulated recessive, primarily deleterious alleles in a heterozygous state. They posited that rare sexual cycles involving meiosis followed by mating type switching

and autodiploidization would facilitate the loss of deleterious alleles and fix beneficial alleles, thus leading to “Genome Renewal” (Magwene, 2014).

Duplications

Duplication is the most important source for the generation of new genes. This can occur in a single gene or groups of adjacent genes (Puig et al., 2000), in chromosomes, causing aneuploidy (Hughes et al., 2000), or in the whole genome (Marcet-Houben et al., 2015; Wolfe, 2015; Wolfe and Shields, 1997), generating polyploidy (Selmecki et al., 2015).

Gene redundancy is maintained if it provides an evolutionary advantage, such as an increase in gene dosage, or if one of the duplicate genes maintains its function and the other acquires mutations that generate an improved function (neo-functionalization) (Lynch et al., 2000; Merhej et al., 2015; Voordeckers et al., 2012). This fact is very unlikely, since the accumulation of mutations may lead to a loss of function becoming a pseudogene (Wagner, 1998, 2000). However, sometimes to maintain the two copies of a pair of genes, it has been proposed an alternative process, the subfunctionalization, whereby both members of a pair acquire complementary degenerative mutations in independent subfunctions, originally present in the ancestral gene. In this way, both duplicates are required to produce the full patterns of activity of the single ancestral gene,

and subsequent adaptive evolution promotes their subfunctional specialization.

The *GAL1* and *GAL3* paralogous genes of the *Saccharomyces* species provide an example of subfunctionalization in yeasts (Hittinger and Carroll, 2007). The galactose-inducible *GAL1* gene encodes a galactokinase that catalyzes galactose-1-phosphate production from galactose and ATP, whereas the galactose-inducible *GAL3* gene encodes a regulatory protein involved in the activation of both *GAL1* and *GAL3* genes in the presence of galactose and ATP. *Kluyveromyces lactis* contains one single *GAL1* gene that encodes a protein with both regulatory and structural functions. The phylogenetic analysis of these genes indicates that *K. lactis GAL1* diverged from the *Saccharomyces GAL1-GAL3* genes before the gene duplication event, which indicates that each paralog is specialized by subfunctionalization. Most duplication of a gene and multigenic families correspond to tandem repeats; in general, multigene families are located in subtelomeric regions, but there are some scattered throughout the genome. Telomeres protect the ends of the linear eukaryotic chromosomes from end-to-end fusions and serve as buffer zones against sequence loss due to incomplete replication. Subtelomeric regions span an average of 25 kb on each chromosome end immediately adjacent to the telomere repeats TG1-3 (chromosomes in *S.cerevisiae* end in 300 bp of a heterogeneous sequence C

$_{1\text{-}3}$ A(CA) $_{1\text{-}6}$/(TG) $_{1\text{-}6}$ TG $_{2\text{-}3}$, commonly abbreviated C $_{1\text{-}3}$ A/ TG $_{1\text{-}3}$ (Shampay et al.,1984; Wang and Zakian,1990)).

Subtelomeres consist of a mosaic of repetitive elements exhibiting a high degree of polymorphism, among strains and different chromosome ends (Figure 9). All subtelomeric regions have a minimal core X containing an ARS (autonomously replicating sequence, part of the origin of replication) consensus and 0-4 tandem copies of the Y' element containing two large ORF, both of which are transcribed in specific conditions (Duina et al., 2014; Förstemann and Lingner, 2001; Louis and Borts, 1995; Wellinger and Zakian, 2012; Yamada et al., 1998). Between these two elements, short subtelomeric repeats (STR) also exist. Subtelomeric regions are dynamic, undergoing frequent recombination (Horowitzet al. 1984; Louis and Haber 1990). The complex mixture of repeats and the dynamic nature of this region has made the mapping and study of its functional components difficult (Lydall, 2003; Pryde and Louis, 1997). Many gene families are either solely or predominantly located and spread around different chromosome ends. Among the mostly studied are the *PAU*, *DUP*, *MAL*, *SUC*, *MEL* and *ERR* families, highly variable in location and copy number, making them efficient for strain fingerprinting and identification (Carlson et al., 1985; Louis and Borts, 1995).

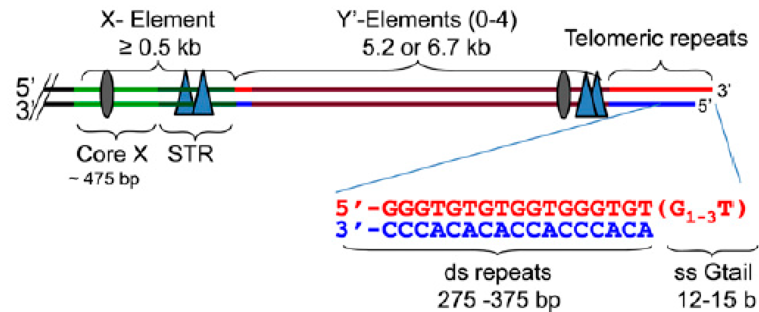


Figure 9. Chromosome ends in *S. cerevisiae*. The structure of chromosome ends in *S. cerevisiae* consists of dispersed middle repetitive and tandem repeats (adapted from Wellinger and Zakian, 2012).

Chromosomal rearrangements

The analysis of chromosomal DNA by pulse field gel electrophoresis (PFGE) has revealed important chromosome length polymorphisms in yeasts (Bidenne et al., 1992; Nadal et al., 1999; Querol et al., 1992). These polymorphisms are due to gross chromosomal rearrangements (GCR), such as translocations, inversions, duplications and deletions of large chromosomal regions.

The chromosomal rearrangements occur due to recombination between short sequences which are present in non-homologous genes. This process can generate new chimeric genes with a different function or may change the pattern of expression of these genes (Khrameeva et al., 2016; Naseeb et al., 2016). They may be involved in the mechanisms of adaptation

of the yeast to industrial environments, especially in the relocation of these genes under stronger promoters during the industrial process.

For example, in the wine strain T73, there is an increased expression of *SSU1* gene, transporter of sulfur dioxide (antiseptic widely used in enology). This phenomenon is due to a translocation between chromosomes VIII and XVI which has placed the coding region of *SSU1* under the control of a strong promoter of *ECM34* gene, leading to increased expression and therefore providing greater resistance to sulfur in the yeast that hold this translocation (Pérez-Ortín et al., 2002). In a recent QTL analysis study (Zimmer et al., 2014), another translocation between chromosomes XV and XVI has been related with a higher *SSU1* expression. This translocation involves the promoter regions of *ADH1* and *SSU1*, and confers increased *SSU1* gene expression during the first hours of alcoholic fermentation.

Hybridization

For the genus *Saccharomyces*, one of the most interesting mechanisms in the adaptation to industrial processes is the formation of interspecific hybrids (Libkind et al., 2011; Lopandic et al., 2016; Morales and Dujon, 2012; Peris et al., 2016a). *Saccharomyces* species are present in the same ecological niche and they may be involved in hybrid formation. Haploid cells or spores of these species are capable of interbreeding together

to form viable but sterile hybrids, which are maintained by asexual reproduction (Greig et al., 2002a; Naumov et al., 2000c).

Hybrids are less competitive than their parental in specific environmental conditions, but can be better adapted to intermediate fluctuating conditions, which provides to hybrids a selective advantage (Greig et al., 2002b). On the other hand, hybrid acquire physiological properties of both parents, for example, tolerance to alcohol and glucose of *S. cerevisiae*, low temperature tolerance of *S. kudriavzevii* or an increased production of aromatic compounds of *S. uvarum* (Gamero et al., 2013, 2015; Mertens et al., 2015; Peris et al., 2016b).

BACKGROUND AND OBJECTIVES

The selection of suitable microorganisms for use in industrial processes is a key issue in food technology. Due to the demanding nature of the modern winemaking practice and sophisticated wine markets, there is an ever-growing quest for specialized wine yeast strains that possess a wide range of optimized, improved or novel oenological properties.

This PhD has been carried out in the Systems biology in biotechnological yeasts group located at the Institute of Agrochemistry and Food Technology (IATA) of the Spanish Scientific Research Council (CSIC), Valencia (Spain) (https://www.iata.csic.es/es) . The general topic of this group aims yeasts with industrially relevant (especially wine-industry-relevant) properties. Our group has a wide trajectory in the study of low temperature adaptation in wine yeast. When I arrived to the lab, I joined to the project that aimed shed light into the molecular and physiological mechanisms that determine greater tolerance at low temperature.

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. However, lowering fermentation temperatures has its disadvantages, including prolonged process duration and a higher risk of halted or sluggish fermentation. Our working hypothesis was that these industrial processes can be optimized by improving our knowledge about the mechanisms that determine a higher capacity to deal with this stress and by providing better-adapted yeasts to ferment at low temperature.

In this context, the main objective of this thesis work was to study the molecular and physiological mechanisms involved in the adaptation of wine yeast to low temperatures during the fermentation process. This general objective has been dealt in the following partial objectives:

1. **To elucidate the main transcriptomic, proteomic and genomic changes between two commercial wine strains, which showed clear differences in their growth and fermentation capacity at low temperature.**

   To detect these differences, we followed a global approach by comparing the transcriptome, proteome and the whole-genome between these two strains, selected on the basis of a significant

divergent phenotype growing at low temperature among a collection of 27 commercial *S. cerevisiae* strains.

The results are shown in chapter 1: Global Phenotypic and genomic comparison of two *Saccharomyces cerevisiae* wine strains reveals a novel role of the sulfur assimilation pathways in adaptation at low temperature. BMC Genomics (2014) 15: 1059.

2. **To determine physiological and molecular correlation between low temperature and other cellular stresses described during wine fermentation.**

   Previous data pointed out to a common response between low temperature adaptation and oxidative stress in *Saccharomyces cerevisiae*. We aimed to quantify the correlation between recovery after shock with different oxidants and cold, and then to detect the key genes related with this response involved also in cold adaptation. The results are shown in chapter 2: Correlation between low temperature adaptation and oxidative stress in *Saccharomyces cerevisiae.* Frontiers in Microbiology (2016) 7:1199.

3. **To unravel the genetic determinants (genes and genomic regions) of low-temperature fermentation.**

We mapped quantitative trait loci (QTLs) by bulk segregant analyses in the F13 offspring of the two *Saccharomyces cerevisiae* industrial strains with divergent performance at low temperature.

The results are shown in chapter 3: The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae.* BMC Genomics (Submitted).

4. **To compare the molecular and physiological mechanisms of adaptation at low temperature detected in *S. cerevisiae* with two cryotolerant species of the genus *Saccharomyces*: *S. uvarum* and *S. kudriavzevii*.**

   In an attempt to detect inter-specific metabolic differences, we characterized the proteomic landscape of these cryotolerant species grown at 12 ºC and 28 ºC, which we compared with the proteome of *S. cerevisiae* (poorly adapted at low temperature).

   The results are shown in chapter 4: iTRAQ-based proteome profiling of *Saccharomyces cerevisiae* and cryotolerant species *Saccharomyces uvarum* and *Saccharomyces kudriavzevii* during low-temperature wine fermentation. Journal of Proteomics (2016) 146: 70–79.

CHAPTER 1

Global phenotypic and genomic comparison of two *Saccharomyces cerevisiae* wine strains reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature fermentations

Estéfani García-Ríos, María López-Malo and José M. Guillamón

BMC Genomics 2014, 15:1059

ABSTRACT

Background

The wine industry needs better-adapted yeasts to grow at low temperature because it is interested in fermenting at low temperature to improve wine aroma. Elucidating the response to cold in Saccharomyces cerevisiae is of paramount importance for the selection or genetic improvement of wine strains.

Results

We followed a global approach by comparing transcriptomic, proteomic and genomic changes in two commercial wine strains, which showed clear differences in their growth and fermentation capacity at low temperature. These strains were selected according to the maximum growth rate in a synthetic grape must during miniaturized batch cultures at different temperatures. The fitness differences of the selected strains were corroborated by directly competing during fermentations at optimum and low temperatures. The up-regulation of the genes of the sulfur assimilation pathway and glutathione biosynthesis suggested a crucial role in better performance at low temperature. The presence of some metabolites of these pathways, such as S-Adenosilmethionine (SAM) and glutathione, counteracted the differences in growth rate at low temperature in both strains. Generally, the proteomic and genomic changes observed in both strains also supported the importance of these metabolic pathways in adaptation at low temperature.

Conclusions

This work reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature. We propose that a greater activation of this metabolic route enhances the synthesis of key metabolites, such as glutathione, whose protective effects can contribute to improve the fermentation process.

Keywords: Wine yeast, Cold adaptation, Transcriptomics, Proteomics, Genomics, Oxidative stress, Glutathione biosynthesis, Genotype-phenotype association.

1. Introduction

In natural environments with diurnal and/or seasonal temperature changes, temperature is one of the main relevant environmental variables that microorganisms, including yeast species, have to deal with. Temperature is also a key factor in some industrial processes involving microorganisms. Low temperatures (10-15 ºC) are used in wine fermentations to enhance production and to retain flavor volatiles. In this way, white and “rosé” wines of greater aromatic complexity can be achieved (Beltran et al., 2002; Torija et al., 2003). Yeast undergoes considerable stress during wine fermentation due to the high concentrations of sugars in grape must, which leads to high osmotic pressure at the beginning of the process. Then as fermentation proceeds, ethanol accumulation, limiting nitrogen concentration, or even presence of SO_2, imposes further pressure on wine yeast. Therefore to these difficulties, which are inherent to the process, we should add a sub-optimal temperature for the primary fermentation agent. Temperatures below its optimum range for growth, around 32 ºC (Salvadó et al., 2011a), affect both the yeast growth and fermentation rates, and give rise to not only a prolonged lag phase, but also to the production of stuck and sluggish fermentations (Bisson, 1999). 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 (Aguilera et al., 2007; Sahara et al., 2002; Schade et al., 2004; Tai et al., 2007). These problems can be avoided by providing better-adapted yeasts to ferment at low temperature. In past years, some attempts have been made to elucidate the response to cold in *Saccharomyces cerevisiae* through a variety of high-throughput methodologies. Some studies have analyzed the genome-wide transcriptional response of *S. cerevisiae* to low temperatures. These studies have focused mainly on cold shock (Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). Schade et al. (2004) identified two distinct phases in the cold shock response: 1) an early cold response (ECR) occurring within the first 12 h after exposure to low temperature; 2) a late cold response (LCR) taking place beyond 12 h after exposure to low temperature. An ECR induces the genes implicated in RNA and lipid metabolism, whereas the genes induced during an LCR encode mainly the proteins involved in protecting the cell against a variety of stresses. In fact, the LCR response is very similar to the general stress response mediated by the transcription factors Msn2p/Msn4p. However, the response type depends on the duration of exposure to stressful conditions. Sudden exposure to environmental changes (e.g., cold shock) is likely to

trigger a rapid, highly dynamic stress response (adaptation). Prolonged exposure to nonlethal stimuli leads to acclimation; i.e., establishment of a physiological state in which regulatory mechanisms, like gene expression, fully adapt to suboptimal environmental conditions (Tai et al., 2007). Tai et al., (2007) compared their transcriptomic results obtained during cold acclimation in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature, and found major discrepancies among low-temperature transcriptome datasets. These authors partially explained these major differences by the cultivation method used in different transcriptome experiments. 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 makes it impossible to dissect temperature effects on transcription from specific growth rate effects. Two recent chemostat studies (Castrillo et al., 2007; Regenberg et al., 2006) also found that the growth rate itself has a strong effect on transcriptional activity. Furthermore, chemostat cultures help to accurately control the specific growth rate, so the concentration of all the metabolites is constant over time, thus providing a good platform to study microbial physiology, proteome profiles and gene expression (Tai et al., 2007).

Other recent studies of our group analyzed the changes in the proteomic profile (Salvadó et al., 2008, 2012)and in the metabolome (López-Malo et al., 2013b) due to low temperature. Nine proteins were identified as representing the most significant changes in proteomic maps during the first 24 h of fermentation at low (13 ºC) and standard (25 ºC) temperatures. These proteins were involved mainly in oxidative stress response and glucose and nitrogen metabolism. In the global metabolic comparison, the main differences in the *S. cerevisiae* strain growing at low temperature were metabolites related with lipid metabolism and redox homeostasis.

So far, none of these previous studies have tackled adaptation at low temperature using a global approach, which involves differences at the genomic, transcriptomic and proteomic levels of two commercial wine strains, selected on the basis of a significant divergent phenotype growing at low temperature. In the first stage of the work, these strains were selected from a collection of 27 commercial *S. cerevisiae,* which were grown at temperatures ranging from 4 to 45 ºC in both minimal media (SD) and synthetic must (SM). The fitness differences at low temperature of these selected strains were confirmed in a competition experiment during wine fermentation. In a second stage, the aim was to decipher the molecular basis underlying this divergent phenotype by analyzing the genomic, proteomic

and transcriptomic differences between both strains at low temperature. The up-regulation of the genes of the sulfur assimilation pathway and glutathione biosynthesis suggested a crucial role in better performance at low temperature. The presence of some metabolites of these pathways, such as S-adenosilmethionine (SAM) and glutathione, counteracted the differences in growth rate at low temperature in both strains. Generally, the proteomic and genomic changes observed in both strains also supported the importance of these metabolic pathways in adaptation at low temperature.

2. Materials and methods

Yeast strains

In this study, lab strain BY4743 and a collection of 27 *Saccharomyces cerevisiae* commercial wine strains were used. The industrial strains were kindly provided by Lallemand Inc. (France). These strains were typing by their interdelta sequences (Legras and Karst, 2003), and were thus named according to their delta pattern (from P1 to P27). Their corresponding commercial names are shown in Table S1 and their enological features can be obtained from the company's website (http://www.lallemandwine.com). Inocula were prepared by introducing one single colony from pure cultures of each strain into 5 ml of YPD medium (1% yeast extract, 2% peptone and

2% glucose). After 24 h of incubation at 30 °C, the volume required to obtain a concentration of about 2 x 10^6 cells mL^{-1} in the different media was used. The correct inoculation size was always confirmed by surface spread on YPD agar plates. These yeast suspensions were used to inoculate the different experiments as described below.

We also constructed a derivative P5 strain, which was labeled with the Green Fluorescence Protein (P5-GFP). The induction of this fluorescence protein allowed this reporter strain to be monitored by flow cytometry. One copy of the open reading frame (ORF) of gene *GAL1* was replaced with the deletion cassette GFP-*KanMX4* by the short flanking homology (SFH) method (Güldener et al., 1996). Plasmid pKT127 (Sheff and Thorn, 2004) was used as a template to obtain this deletion cassette. *S. cerevisiae* transformation was carried out by the lithium acetate method (Daniel Gietz and Woods, 2002). Transformants were selected by resistance to geneticin. Correct integration of the deletion cassette was confirmed by PCR using the primers upstream and downstream of the cloning site. Moreover, the fluorescence emission of the transformants was also tested after a 3-hour culture in YP-Gal medium (galactose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}).

Media and growth conditions

The growth media selected for the experiments were SD (Yeast Nitrogen Base (YNB, Difco) supplemented with 20 g L^{-1} of glucose as the carbon source) and synthetic grape must (SM), which was derived from that described by Riou et al., (2007). 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). For the assays, the SD and SM media were inoculated as described above and were incubated at different temperatures (°C: 4, 8, 12, 15, 22, 28, 33, 37, 40, 42, and 45) in order to obtain the whole temperature range within which yeasts can grow.

To test the influence of some key metabolites of the sulfur assimilation and glutathione biosynthesis pathways on growth, SM was supplemented with one of these compounds: 0.2 mM of S-Adenosyl methionine (SAM, Sigma-Aldrich), 0.5 mM of Glutathione oxidized (GSSG, Sigma-Aldrich) and 1 mM of Glutathione (GSH, Sigma-Aldrich). To test differential stress oxidative resistance, yeast cells were incubated in PBS with 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM of peroxide of hydrogen for 1 h. After this stress

oxidative shock, cells were centrifuged at 10000 rpm for 3 min at 4 ºC and inoculated in SD and SM as previously described.

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 4 days after a 20-second pre-shaking for all the experiments. At low temperatures (4-15 ºC) however, microplates had to be incubated outside the spectrophotometer to be then placed inside before being measured (every 8 h for 14 days). Microplate wells were filled with the required volume of inoculum and 0.25 ml of SD or SM medium to always ensure an initial OD of approximately 0.2 (inoculum level of about 2 x 10^6 cells mL^{-1}). Uninoculated wells for each experimental series were also included in the microplate to determine, and to therefore subtract, the noise signal. All the experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al., (1990):

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

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific

growth rate (h^{-1}), and λ the lag phase period (h) (Aguilera et al., 2007). GT was calculated using the equation GT =ln2/ μ_{max}.

Competition tests under microvinification conditions

Fermentations were performed at 28 and 15 ºC with continuous orbital shaking at 100 rpm. Fermentations were done in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM and fitted with closures that enabled carbon dioxide to escape and samples to be removed. Fermentations were monitored by the density of the media (g L^{-1}) using a densitometer (Densito 30PX, Mettler Toledo, Switzerland). Fermentations were considered complete when density reached 995 g L^{-1}. Yeast cell growth was determined by absorbance at 600 nm and by plating on YPD. The percentage of each strain competing throughout fermentation was monitored by both the replica plating from YPD to YPD-geneticin (G-418, Formedium) and by flow cytometry. The percentage of fluorescent cells was determined in a flow cytometer (Beckman Coulter Epics XL Flow Cytometer, Minnesota, USA) after GFP induction in YP-Gal (1% yeast extract, 2% peptone and 2% galactose) for 4 h at 25 ºC (no changes in population size were detected during this incubation). In all, 20000 cells of the sample were measured at a voltage of 700 V in FL1 FITC, which revealed the number and percentage of fluorescent cells and fluorescence intensity. The EXPO 32 ADC software was used for these measurements.

The parameters measured with the cytometer were the number of fluorescent cells and average fluorescence intensity (Gutiérrez et al., 2012).

Chemostat cultures and sampling

Continuous cultures were performed at 28 and 15 ºC in an 0.5 L reactor (MiniBio, Applikon Biotechnology) with a working volume of 0.35 L. The dilution rate (D) of cultures was 0.028 h^{-1} at both temperatures. A temperature probe connected to a cryostat controlled temperature cultures. pH was measured online and kept constant at 3.3 by the automatic addition of 2M NaOH. The stirrer was set at 100 rpm. The population inoculated in the chemostat was approximately OD = 0.2. Prior to starting the continuous culture, cells were allowed to grow at the same temperature as the continuous culture to achieve enough biomass in a batch phase. When the batch culture entered the stationary phase, the continuous culture was connected. Steady states were sampled only after all the continuous cultures had been running for at least five residence times and biomass values were constant. A volume of approximately 30 units of OD600 was centrifuged at 10000 g for 3 min at 4 ºC. After supernatant removal, cell suspension was washed with PBS, transferred to a 1.5-2 mL microcentrifuge tube and centrifuged again under the same conditions. The pellet was flash-frozen with liquid nitrogen and stored at -80 ºC until analyzed.

Transcriptome analysis.

RNA was isolated using the RNeasy Mini Kit (Quiagen) according to the manufacturer's instructions. RNA was quantified spectrophotometrically with a NanoDrop ND-1000 (ThermoFisher Scientific) and integrity was determined by electrophoresis in 1% agarose gel. Next 2.5 µg of total RNA from each sample were linearly amplified and chemically modified with Amino-Allyl-UTP using the SuperScript RNA Amplification System (Invitrogen, Life Technologies). Then 5 µg of each amplified amino-allylRNA were indirectly labeled with Cy3 or Cy5 mono-reactive dyes (Amersham GE Healthcare™, Amersham UK) and were later purified with the RNeasy Mini Kit to remove nonincorporated dyes. Dye incorporation was monitored by NanoDrop ND1000. A mixture of 350-400 pmol of the two labeled samples was concentrated in a Concentrator Plus (Eppendorf™, Hamburg, Germany). Competitive hybridization was performed on a Yeast Array (Viladevall et al., 2004) (PCR-amplified ORFs of yeast S288c strain, Servei Genomica, Universitat Autonoma Barcelona, Spain) in AHC hybridization chambers (ArrayIt Corporation, CA, USA) at 42 °C overnight (17 h). The prehybridization solution contained 3X SSC, 0.1% SDS and 0.1 mg/ml BSA; the hybridization solution contained 50% deionized formamide, 5X SSC, 0.1% SDS and 0.1 mg mL^{-1} of salmon DNA. Microarrays were washed manually with solutions containing decreasing concentrations of filter sterilized SSC 20x and SDS 10% (Sol.1: 1x SSC-

0.2% SDS; Sol.2: 0.1x SSC-0.2% SDS; Sol.3: 0.1x SSC; Sol. 4: double deionized water). The signal intensities of Cy3 and Cy5 were acquired with a GenePix 4100A scanner (AXON, Molecular Devices, CA, USA) using the GenePix Pro v.6.1 software at a resolution of 10 μm.

The microarray data were derived from three independent experiments for RNA hybridization. The raw data with a global background subtraction were generated with GenePix pro 7.0. Analyses were done using the Acuity 4.0 software (Molecular Devices, CA, USA). The individual data sets were normalized to a log2 ratio value of 1. After normalization, data were filtered to remove spots flagged as not found. Only the spots with at least three replicates were considered. Finally, replicates were combined and their medians were calculated. Genes with 2-fold differences in the log2 ratio values were considered to have a significant differential expression if the p-values of the Student's t-test were ≤0.05 after applying the Benjamini and Hochberg (BH) method to adjust for a false discovery rate (FDR) (Benjamini and Hochberg, 1995). GO term Finder was used to group genes into functional categories, and is found in the MIPS Functional Catalog (http://mips.helmholtz-muenchen.de/proj/funcatDB/).

Proteomic analysis

Protein Extraction

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). To the supernatant, 150 µL of extraction buffer were added. Proteins were allowed to precipitate at -20 °C for 1 h, and the precipitate was recovered after centrifugation at 10000 g for15 min. The pellet was washed with the 2-D Clean-Up kit (GE Healthcare). The final pellet was air-dried and solubilized in 25 µL of 7 M urea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 2 M Tiourea, 20 mM Tris and milliQ water. Insoluble material was removed by centrifugation (13000 rpm, 5 min). Protein concentration was determined by Bradford, with BSA used as a standard.

Two-dimensional electrophoresis (2DE)

Soluble proteins were run in the first dimension using a commercial horizontal electrophoresis system (MultiphorII; Amersham Pharmacia Biotech). Then 100 µg of the protein sample were mixed with Destreak Rehydration Solution (GE Healthcare), dithiothreitol (DTT) (20 mM) and IPG buffer, pH 3-10 NL (GE Healthcare), and loaded onto Immobiline™

DryStrip pH 3-10 NL, 24 cm (GE Healthcare). IPG strips were allowed to rehydrate overnight. Samples were run at 50 mA per strip. In the first step, voltage was ramped to 500 V during a 5-hour period, maintained at 500 V for another 5-hour period, re-ramped to 3500 V during a 9.5-hour period and was finally maintained at 3500 V for 5 h. After the first dimension, IPG strips were then equilibrated twice for 15 min in equilibration solution (0.05 M Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol and 2% w/v SDS), first with 65 mM dithiothreitol (reduction step) and finally with 135 mM iodoacetamide (alkylation). The second dimension was done in a vertical electrophoresis system (Ettan DALTsix; Amersham Pharmacia Biotech) in a 12.5% (26 cm_20 cm_1 mm) polyacrylamide gel, where proteins were separated according to molecular size. The electrophoresis conditions were 1 W per gel until the dye front reached the bottom of the gel. Sets of three gels were used for each sampling time.

Staining and image analyses

The staining protocol was performed as described by Blomberg. (1997). Gels were scanned using an Image Scanner UMAX, Amersham (300 dpi, 12-bit image), which allowed us to obtain spot intensities in pixel units. Images were analyzed using the PDQUEST software (Bio-Rad). Normalization was performed by the aforementioned software based on the total required in gel density to compensate the image differences caused by

variations under the experimental conditions (e.g., protein loading or staining). Spot detection was implemented using the PDQUEST automated spot detection algorithm. The gel image showing the largest number of spots and the best protein pattern was chosen as a reference template of the image analysis, and the spots in the standard gel were then matched across all the gels. Matching software features were used to relate and compare sets of gels. Finally, in order to achieve maximum reliability and robustness of the results, a Student's t-test was performed. This test allowed us to identify those sets of proteins that showed a statistically significant difference with the confidence level set at 95%.

MS analysis and protein identification

Protein spots were excised manually and samples were digested with sequencing grade trypsin (Promega) (Shevchenko et al., 1996). The digestion mixture was dried in a vacuum centrifuge and re-suspended in 4 μL of 2% ACN, 0.1% TFA. A BSA plug was analyzed in the same way to control the digestion process. Next 1 μL of the digestion mixture was spotted onto the MALDI target plate. After droplets were air-dried at room temperature, 1 μL of matrix (5 mg mL^{-1} CHCA (Bruker) in 0.1% TFA-ACN/H_2O (1:1, v/v)) was added and allowed to air dry at room temperature. The resulting mixtures were analyzed in a 5800 MALDI TOFTOF (ABSciex) in the positive reflectron mode (3000 shots per position). Five of

the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for each position for the MSMS analysis. The MS/MS data were acquired using the default 1kV MS/MS method. Previously, the plate and acquisition methods were calibrated with 0.5 μL of CM5. The MS and MS/MS information was sent to MASCOT via Protein Pilot (ABSciex). A database search was done on Expasy. Searches were done with tryptic specificity to allow one missed cleavage and tolerance on the mass measurement of 100 ppm in the MS mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification, while oxidation of Met and deamidation of Asn and Gln were employed as variable modifications.

Then 3 μL of each sample were diluted to 6 μL with 0.1% TFA, 2% ACN. Next 5 μL of each final solution were loaded onto a trap column (NanoLC Column, 3 μ C18-CL, 75 umx 15cm; Eksigen) and desalted with 0.1% TFA at 2 μL min^{-1} for10 min. Peptides were loaded into an analytical column (LC Column, 3 μ C18-CL, 75 umx 12 cm, Nikkyo)equilibrated in 5% acetonitrile 0.1% FA. Peptide elution was carried out with a linear gradient of 5-40% B in 30 min (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL min^{-1}. Peptides were analyzed in a nanoESIqQTOF mass spectrometer (5600 TripleTOF, ABSCIEX). TripleTOF was operated in the

information-dependent acquisition mode, in which a 0.25-s TOFMS scan from 350-1250 m/z was performed, followed by 0.05-s product ion scans from 100-1500 m/z on the 20 most intense 2-5 charged ions. The MS/MS information was sent to search the database with the PARAGON algorism using the ProteinPilot software, v. 4.5 (ABSciex).

SOLiD sequencing.

Genome sequencing of the selected strains was performed by 5500xl SOLiD sequencing. Genomic libraries were prepared following the manufacturer's standard instructions. Emulsion PCRs were performed using the SOLiD™ EZ Bead™ Systems. Sequencing was carried out by 75nt single read exact call chemistry (ECC) and following the manufacturer’s standard protocols. The LifeScope software (v2.5.1, Life Technologies) was used to map color space reads, including the Accuracy Enhancement Tool (SAET) along the EF.4 Ensembl reference *S.cerevisiae* S288c genome assembly. Then SNPs and small-sized InDels were identified using the LifeScope software. The DiBayes algorithm, with highest-stringency calling, was used for single-nucleotide variant calling. The SNPs and InDels specific of strains P5 and P24 were identified by comparing the list in the “vcf” format. CNV was identified by mapping the reads of P5 to P24. The average depth of read coverage was computed in nonoverlapping windows of size 69 bp, and was normalized by the genome-wide median coverage for each strain. The log2

values of these ratios were then calculated using CNVseq (http://tiger.dbs.nus.edu.sg/CNV-seq). Finally, only the CNVs with a length longer than 1000 bp were considered. The whole-genome sequences have been published in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/) and are available with access number SRP048919.

Statistical analysis

Data were analyzed with the Sigma Plot 12.5 software and the results are expressed as mean and standard deviation. To evaluate statistical significance, tailed t-student tests were applied with a p-value of <0.05. Benjamini and Hochberg (BH) correction was used for the transcriptomic and MIPS analyses. Phenotypic data were fitted to the reparameterized Gompertz model by nonlinear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0.

3. Results

Effect of temperature on wine yeast growth and selection of two strains with different growth behavior at low temperature

In order to select two strains with different growth behavior at low temperature, we tested growth capacity at different temperatures with a collection of commercial strains (Table S1). Figure 1 shows the global distribution of μ_{max} for all 27 strains at different temperatures in SD and SM. This representation follows a normal (or Gaussian) distribution in which the μ_{max} values were lower and variance was wider in SM (Figure 1B) than in SD (Figure 1A). In addition, the higher temperature was, the wider variance became. For the temperatures assayed, the average optimum temperature of these strains can be settled at around 33 ºC for SD and somewhat lower for SM.

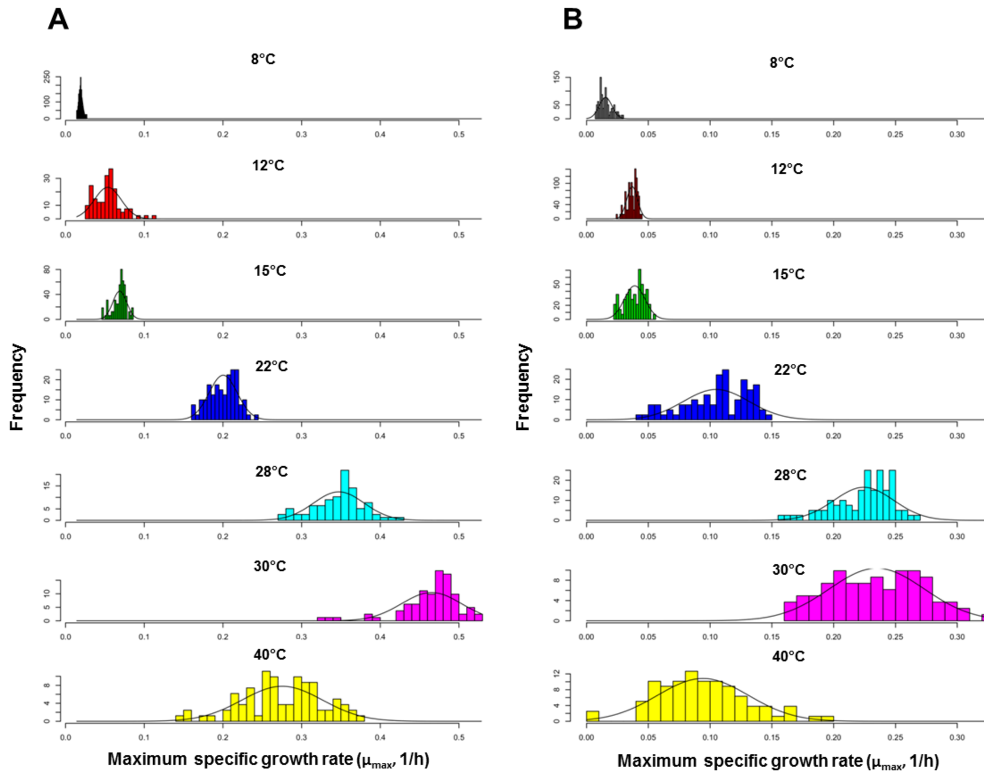


Figure 1. Histogram of the distribution of the maximum specific growth rate μ_{max} (h^{-1}) according to the temperature in two media: SD (A) and SM (B). Bars represent the frequency of the individuals with an μ_{max} value within the same range of variation. The superimposed bell-shaped line shows the growth data at different temperatures following a Gaussian distribution. Due to differences in the growth rate, different scales were used for μ_{max} distribution in SD and SM.

Similar conclusions were drawn from the boxplot representation of each individual strain if compared to the average μ_{max} of the 27 strains within the whole temperature range assayed (Figure S1). Dispersion in these boxplots

was much greater for each strain growing in SM than in SD. This representation also revealed the general fitness of each strain in comparison to the average fitness of all the strains and temperatures.

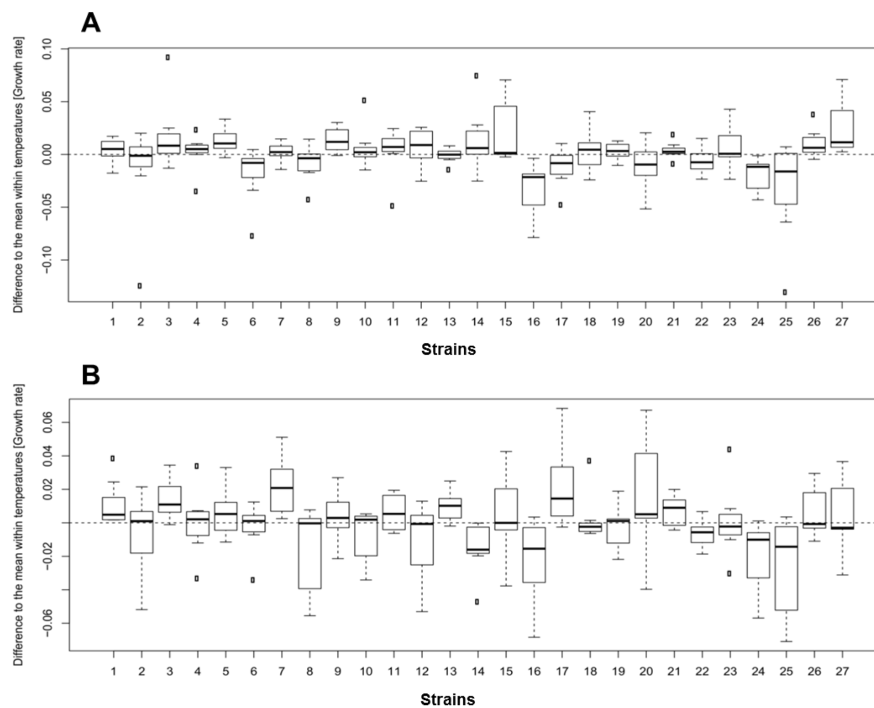


Figure S1: Box plot representation of the μ_{max} distribution in each strain within the complete temperature range assayed. Growth was performed in SD (A) and synthetic must (B). Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution, and the circle represents extreme data points. Dashed line denotes the median value of μ_{max} of the 27 strains within the whole temperature range assayed.

Considering the whole data set at different temperatures, a decision was made to take 28 °C as the optimum reference temperature and 15 °C as the reference temperature for cold. These temperatures showed the biggest differences between strains and media. The μ_{max} values were used to select two strains with evident different growth behavior at low temperature, regardless of the growth medium, but with no significant differences at the optimum temperature. Following these selection criteria, P5 and P24 were chosen as the candidate strains with good and bad growth behavior at low temperature, respectively (Figure 2). P5 showed the best growth performance at low temperature in SD and one of the best ranked in SM. Conversely, P24 was ranked among the strains with a lower μ_{max} value in both media. P5 corresponds to commercial strain Lalvin®ICVGRE, which is recommended for temperature fermentations ranging from 15 to 30 ºC by the marketer.

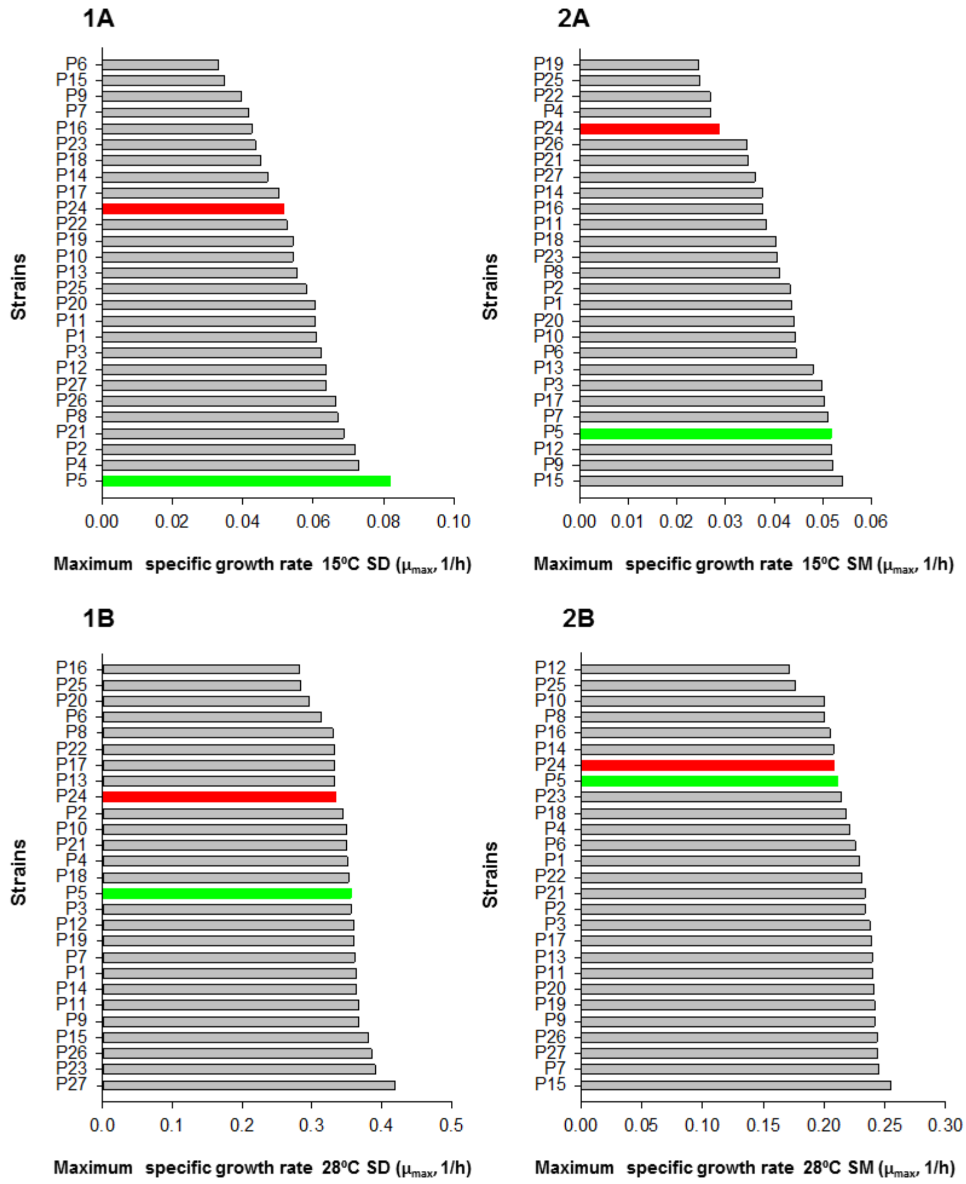


Figure 2. Selection of strains with a divergent phenotype at low temperature. Strains P5 (green) and P24 (red) were selected on the basis of the μ_{max} (h^{-1}) in SD (1) and SM (2) at 15 ºC (A) and 28 ºC (B). Due to differences in the growth rate, different scales were used for μ_{max} distribution in SD and SM.

Fermentation kinetics and competition analysis of the selected strains

In order to evaluate whether the higher μ_{max} values growing in a miniaturized system correlated well with higher fitness during alcoholic fermentation, a competition experiment was performed under microvinification conditions between the “good” and “bad” strains at the low and optimum temperatures. In order to make monitoring competitive capacity during fermentation easier, a P5 reporter strain was constructed by deleting one copy of the open reading frame (ORF) of gene *GAL1* and replacing it with the deletion cassette GFP-*KanMX4.* This system was based on the expression of the green fluorescent protein (GFP) under the control of the *GAL1* promoter, a gene which is absolutely repressed during wine fermentation and activated in the presence of galactose. The cells from the fermentation culture were incubated directly in YPGal to determine the percentage of fluorescent cells by flow cytometry. The SM fermentations were inoculated with either a pure culture of each strain or a mixture of both strains. In order to verify that the deletion of one copy of the *GAL1* gene did not affect the fitness of strain P5, the fermentations inoculated with either the reporter P5-GFP strain or a mixture of the parental P5 and the reporter P5-GFP strains were also carried out. 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 (Table 1). T5, T50 and T100

approximately matched the beginning (lag phase), middle (end of the exponential phase) and end of fermentation, respectively. As expected, the replacement of *GAL1* gene with a GFP cassette did not modify the fermentation fitness of strain P5. Surprisingly, P5-GFP finished its fermentation a few hours earlier than its wild-type P5 (Table 1). Moreover, the percentage of each strain was kept at around 50% throughout the mixed fermentation (P5 GFP/P5) (data not shown).

Strain P24 underwent significant delays in all the low-temperature fermentation stages if compared to the P5 fermentations, whereas only the beginning of fermentation (T5) was delayed at 28 ºC. It is noteworthy that the interaction of both strains in the mixed fermentation (P5 GPF/P24) significantly affected fermentation lengths at both temperatures, with long delays noted in the fermentation ends if compared to P5 fermenting as a pure culture. This result was even more surprising when the percentage of each strain was monitored during these mixed fermentations (Figure 3). Strain P5 gradually took over the fermentation process at low temperature and obtained percentages of around 85% of the total population at the end of the process. Conversely no strain dominated the fermentation process at 28 ºC, with populations of around 50% for each strain. The greater competition capacity of strain P5 at low temperature was also corroborated by repeating this experiment in SD medium (Figure S2).

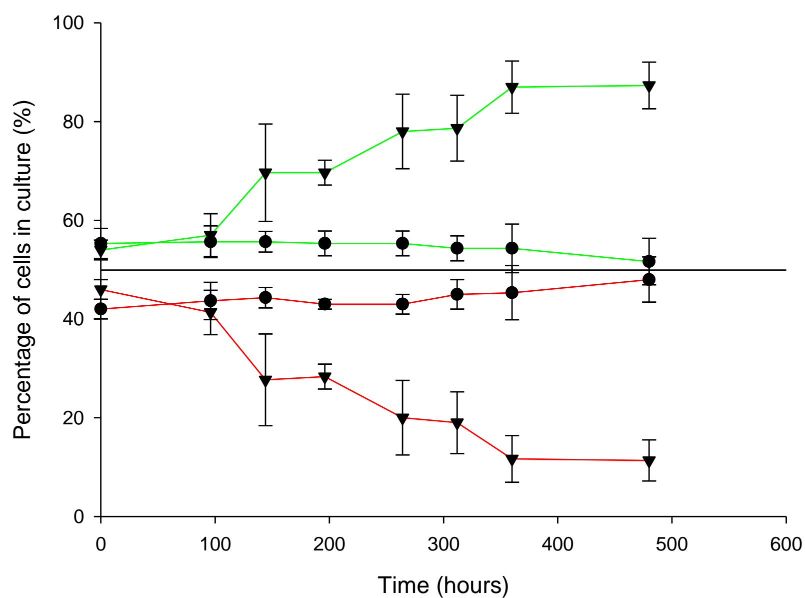


Figure 3. Population dynamics of a mixed culture between strains P5 (green lines) and P24 (red lines) growing in synthetic must (SM). The percentage of each strain was determined by flow cytometry during fermentation (0, 24, 48, 72, 96,144, 240 and 480 h) at 15 ºC (▲) and 28 ºC (●).

Table 1.The time, expressed in hours, required for the selected yeast strains to consume 5% (T5), 50% (T50) and 100% (T100) of the available sugars in the synthetic grape must. These values are the mean ± SD of three independent experiments.

Yeast strain	Temperature	T5	T50	T100
P5	15 °C	23.06±3.84	221.65±8.00	889.18±19.34
	28 °C	12.93±0.28	44.53±0.32	118.87±2.59
P24	15 °C	30.75±0.00[a]	395.90±58.03[a]	930.15±0.00[a]
	28 °C	21.28±2.61[a]	47.53±1.97	118.66±3.53
P5GFP	15 °C	25.18±1.10	188.98±9.67[a]	869.96±2.21[a]
	28 °C	13.78±2.93	47.53±0.56	87.93±1.62
P5 GFP/P5	15 °C	28.18±4.43	210.51±2.93	869.96±2.21[a]
	28 °C	14.90±0.97[a]	46.21±2.27	121.12±6.32
P5 GFP/P24	15 °C	50.67±0.00[a]	270.98±6.23[a]	1125.79±0.00[a]
	28 °C	21.84±0.58[a]	53.71±4.60[a]	126.37±2.12[a]

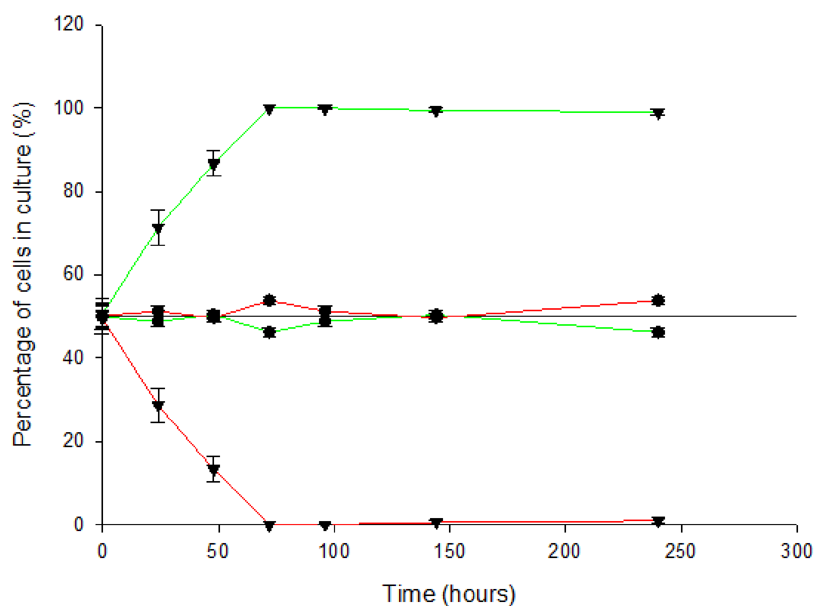


Figure S2. Population dynamics of a mixed culture strains between P5 (green lines) and P24 (red lines) growing in minimal medium (SD). The percentage of each strain was determined by flow cytometry during fermentation (0, 24, 48, 72, 96,144 and 240 h) at 15 ºC (▲) and 28 ºC (●).

Transcriptomic analysis revealed a key role of the sulfur assimilation pathway in adaptation at low temperature

To compare the transcriptome of P5 and P24 grown at 15 and 28 ºC with no interference by the different maximum specific growth rate at these two temperatures, these strains were grown in chemostat cultures at a fixed dilution rate of 0.028 h^{-1} at both temperatures. This dilution rate

corresponded to the μ_{max} of the strain P24 growing at 15 ºC. This experimental design allowed us to compare the transcriptional differences due to temperature in each strain (temperature effect) and the differences between strains at the same temperature (strain effect) (Figure 4A). A list of genes differentially expressed when comparing temperatures and strains and the MIPS functional categories analysis of these genes is provided as supplementary files (Tables S2-S6).

The comparison made by temperatures revealed that strains P5 and P24 had 211 and 128 differentially regulated genes at low temperature, respectively (Figure 4B). This temperature response was mainly strain-dependent because only 32 genes were commonly regulated in both strains (Figure 4B). Although these common genes showed a consistent up- or down-regulation in both strains, differences in the expression level were also observed in a simple view of the heat-map (Figure 4C). As these genes should play a crucial role in adaptation at low temperature, clear differences in their activity can explain the different phenotypic behavior of both strains at low temperature. For instance, genes *QDR2, SNZ1*and *SNO1* were much more markedly up-regulated by low temperature in P5 than in P24. *QDR2* is a plasma membrane transporter involved in the K^+ homeostasis induced by nitrogen limitation (Sá-Correia et al., 2009), whereas *SNZ1* and *SNO1* are involved in pyridoxine metabolism, which is essential for sphingolipids

biosynthesis, and are also up-regulated in response to nutrient starvation (Padilla et al., 1998). Conversely, genes *GPI12*, *CYB5, BTN2* and *DSE1* were more strongly down-regulated in P24 than in P5. *GPI12* is involved in the synthesis of glycosylphosphatidylinositol (GPI), the most important anchor of plasma membrane proteins (Watanabe et al., 2014). *CYB5* is involved in the sterol and lipid biosynthesis pathways, and acts as an electron donor to support sterol C5-6 desaturation (Lamb et al., 1999). *BTN2* modulates arginine uptake (Chattopadhyay and Pearce, 2002) and *DSE1* is involved in cell wall organization.

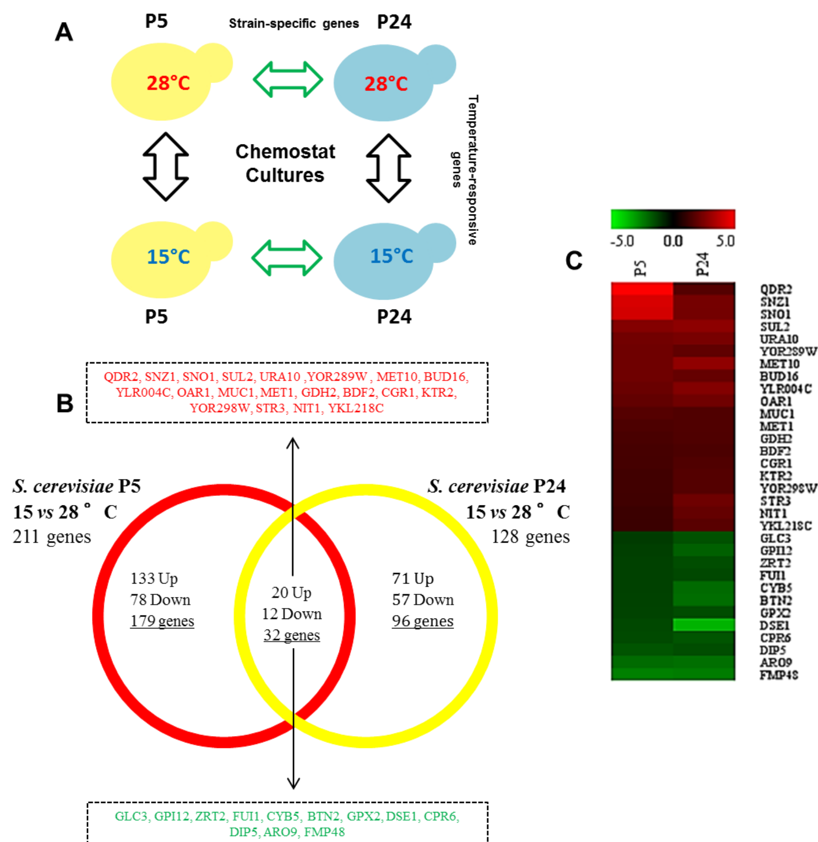


Figure 4. The transcriptional response at low temperature in the two selected strains. (A) Scheme of the experimental design: the transcriptomic changes in the same strain are due to growth temperature (temperature-responsive genes) or the transcriptomic changes at the same temperature depend on the wine strain (strain-specific genes). (B) Venn's diagram of the temperature-responsive genes in both strains. The common genes among strains are highlighted. Red indicates up-regulated genes, while green denotes down-regulation. (C) Heat map depicting the level of expression of the common genes in both strains at low temperature.

One interesting common trait in all the comparisons made of the transcriptional changes observed either by temperature or strains was the presence of functional categories "nitrogen, sulfur and selenium metabolism", "sulfur metabolism" and "metabolism of methionine" in the genes up-regulated at low temperature (Table 2). The genes included in these functional categories belong mainly to the sulfur assimilation pathway (Figure 5). This pathway incorporates extracellular sulfate into several key sulfur-containing compounds; e.g.; homocysteine, methionine, S-adenosylmethionine or glutathione (McIsaac et al., 2012; Thomas and Surdin-Kerjan, 1997). However, clear differences were observed in the transcriptomic activation of this route in both strains. Whereas most genes were overexpressed in strain P5, very few were also up-regulated in P24.

Table 2. Functional group analysis of common up-regulated MIPS categories of the transcriptomic data comparison.

Sample	No. of Genes	MIPS			
		Name	**No. of Genes**	***p*-value**	**Example of genes**
P5 15 ºC-28 ºC	32	Metabolism of methionine	10	$2 \cdot 10^{-6}$	ALT1:DAL7:DUR80 GDH2:MET1:MET3 MET5:MET10:MET14 MET16:MET17:MET28 MET32:NIT1:STR3:YHR112C
		Nitrogen, sulfur and selenium metabolism	16	$1 \cdot 10^{-6}$	
		Sulfur metabolism	6	$1 \cdot 10^{-5}$	
P24 15 ºC-28 ºC	11	Metabolism of methionine	3	$7 \cdot 10^{-3}$	ATO3:GDH2:MET1 MET10:NIT1:STR3
		Nitrogen, sulfur and selenium metabolism	6	$1 \cdot 10^{-3}$	
		Sulfur metabolism	2	$3 \cdot 10^{-3}$	
P5-P24 15 ºC	12	Metabolism of methionine	3	$1.99 \cdot 10^{-2}$	ASP3:DAL7:FMO1 MET3:MET14:MET32 OPT1
		Nitrogen, sulfur and selenium metabolism	7	$1 \cdot 10^{-4}$	
		Sulfur metabolism	2	$7.1 \cdot 10^{-3}$	
Common key genes 15 ºC	10	Metabolism of methionine	3	$1.81 \cdot 10^{-4}$	GDH2:MET1:MET10 NIT1:STR3
		Nitrogen, sulfur and selenium metabolism	5	$1.14 \cdot 10^{-5}$	
		Sulfur metabolism	2	$2.79 \cdot 10^{-4}$	

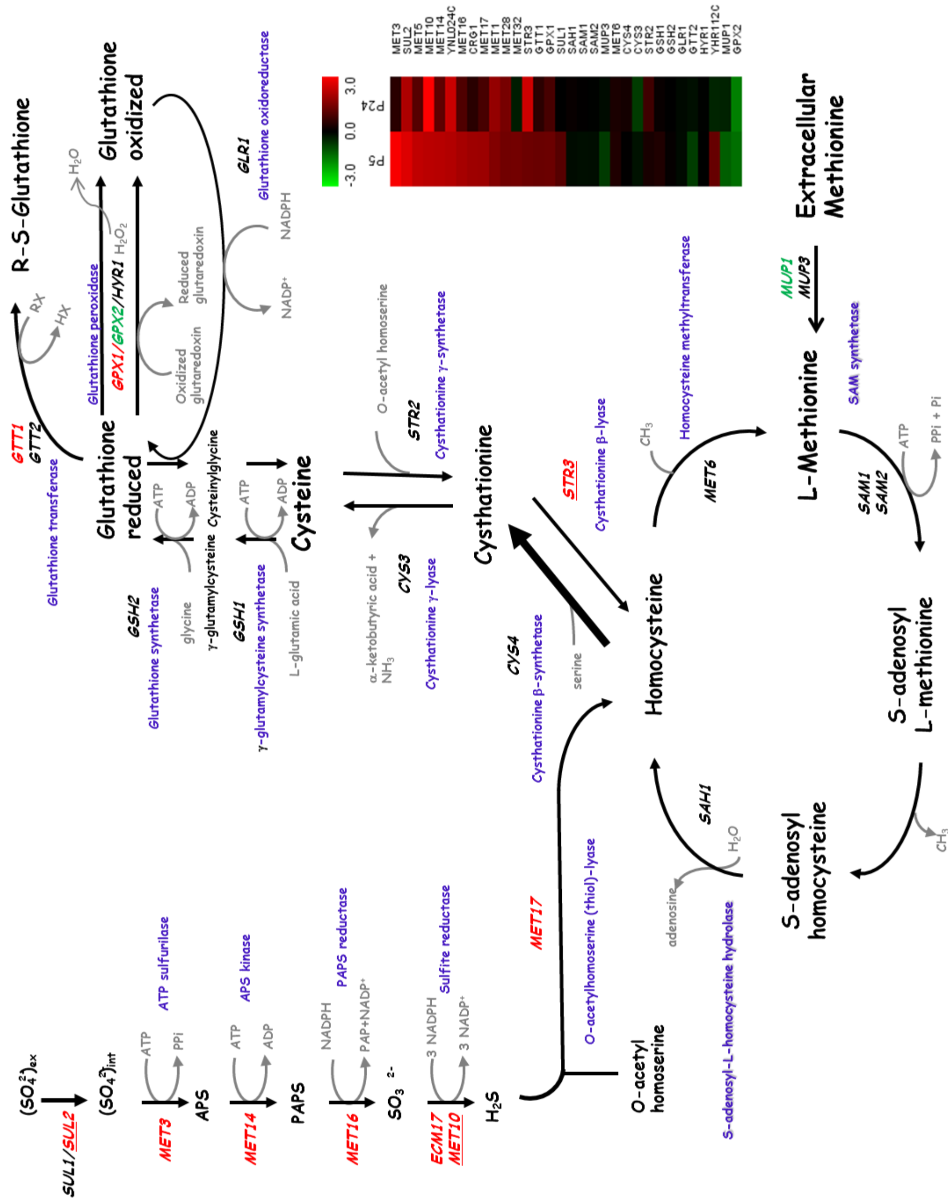


Figure 5. The sulfur assimilation pathway and the genes encoding the enzymes of the different biosynthetic steps. The genes in red and green represent up-regulation and down-regulation in strain P5 at low temperature, respectively. Underlined genes mean identical regulation in P24. Heat map shows the expression of the genes of this pathway that were differentially expressed in both strains.

The concentration of glycolytic and stress oxidative proteins increased at low temperature

A proteome analysis of the same samples used for the transcriptomic analysis was done to evaluate changes in proteins as a result of low temperature in each strain. As done previously, comparisons of 2D-PAGE gels were made to detect temperature-dependent and strain-dependent protein changes. Around 200 spots were detected on 2-D gels in both strains growing at 28 ºC (Table S7). This number of detected spots increased to 236 and 251 for strains P5 and P24, respectively, when cells were grown at low temperature. More interestingly, most of these detected proteins were matched on both 2-D gels but, between matched spots, very few showed statistically significant increases or decreases (Table S7 and Table 3).

The concentration of seven proteins increased at low temperature in P5 (Table 3); five were involved in glycolysis and glucose fermentation (Fba1p, Tpi1p, Eno2p, Cdc19p and the key fermentative enzyme Pcd1p), mainly belonging to the lower part of the glycolysis, the other two proteins were involved in oxidative stress (Ahp1p, Tsa1p). Tsa1p is a physiologically important antioxidant protein that is useful as enzymic defense against sulfur-containing radicals (Wong et al., 2002), thus providing protection against an oxidation system without thiol. Ahp1p is a similar peroxiredoxin to Tsa1p that forms a disulfide-linked homodimer

upon oxidation, and *in vivo* requires the presence of a thioredoxin system to perform its antioxidant protective function. Unlike Tsa1p, which is specific for H_2O_2, Ahp1p is also specific for organic peroxides (Lee et al., 1999). This latter protein shows one of the largest increases (20-fold or more) at low temperature. Regarding strain P24, three of the proteins with increased levels were implicated in the lower part of glycolysis and glucose fermentation (Tdh3p, Tdh1p and Gpm1p). Other proteins with increased levels at low temperature were Tef1p, a translational elongation factor and Rpl31Ap, a ribosomal 60S subunit protein, and both are implicated in translation. Only Eno2p showed significant changes in both strains, but in opposite directions. Low temperature increased the concentrations in P5, but lowered it in P24.

When examining the protein changes in strain P5 if compared to P24 (Table 4), ten proteins showed different concentrations at 15 °C, seven of which were more abundant in strain P5. Among these proteins, enzymes of the lower part of glycolysis (Eno2p, Fba1p and Adh1p), proteins implicated in oxidative stress and protein folding (Ahp1p and Ssa2p) and proteins implicated in methionine/cysteine biosynthesis (Met10p and Met17p), were detected. The remaining proteins with lower concentrations in P5 when compared with P24 were Tdh1p, which is induced during heat shock (Boucherié et al., 1995), Tdh3p (a key protein to pull the flux through the

ATP production stage in the lower part of glycolysis) and Met6p, involved in methionine biosynthesis. At the optimum temperature, only four proteins lowered statistically in P5 (or increased in P24): Ylr179Cp (of unknown function), Gre2p (a reductase implicated in the ergosterol metabolic pathway), Tpi1p (glycolysis) and Sah1p (S-Adenosyl-l-Homocysteine hydrolase involved in methionine biosynthesis).

Table 3. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) by at least 2-fold at 15 °C

Strain	Gene Name	Protein Name	Metabolic Function	Fold Change
P5	*TPI1*	Triosephosphateisomerase	Glycolisis	27.28
	AHP1	Peroxiredoxin type-2	Oxidative stress	20.07
	PDC1	Pyruvate DeCarboxylase	Glycolisis, Glucose fermentation	13.95
	TSA1	Thioredoxin peroxidase	Oxidative stress	5.28
	CDC19	Pyruvatekinase	Glycolisis, Glucose fermentation	5.16
	ENO2	Enolase II, phosphopyruvatehydratase	Glycolisis	3.28
	FBA1	Fructose 1,6-bisphosphate aldolase	Glycolisis	2.15
P24	*RPL31A*	Ribosomal Protein of the Large subunit	Structural constituent of ribosome	17.65
	TDH1	Glyceraldehyde-3-phosphate dehydrogenase	Glycolisis	7.11
	TEF1	Translational elongation factor EF-1 alpha	Translation	5.12
	TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolisis	4.76
	GPM1	Glycerate phosphomutase	Glycolisis	3.53
	ENO2	Enolase II, phosphopyruvatehydratase	Glycolisis	-4.09

Table 4. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) by at least 2-fold in P5 in comparison to P24.

Condition	Gene Name	Protein Name	Metabolic Function	Fold Change
15 ºC	*MET17*	O-acetylhomoserinesulfhydrilase/	Amino acid biosynthesis (methionine/cysteine)	12.24
	FBA1	Fructose 1,6-bisphosphate aldolase	Glycolisis	8.89
	MET10	Sulfite Reductase	Sulfate assimilation	6.55
	ENO2	Enolase II, phosphopyruvatehydratase	Glycolisis	5.51
	AHP1	Peroxiredoxin type-2	Oxidative stress	3.03
	ADH1	Alcohol dehydrogenase	Glucose fermentation	2.86
	SSA2	Heat shock protein 70	Protein Folding	2.56
	TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolisis	-3.11
	MET6	Cobalamine-independent Methioninesynthase	Methionine biosynthesis	-3.96
	TDH1	Glyceraldehyde-3-phosphate dehydrogenase	Glycolisis	-88.84
28 ºC	*YLR179C*	Unknown function	Unknown	-2.08
	SAH1	S-Adenosyl-l-Homocysteinehydrolase	Methionine metabolic process	-3.27
	GRE2	3-methylbutanal reductase/NADPH-dependent methylglyoxal reductase	Ergosterol metabolic process	-3.50
	TPI1	Triosephosphateisomerase	Glycolisis	-9.20

Addition of SAM and glutathione to SM suppressed growth differences at low temperature

As both transcriptomic and proteomic analyses indicated the importance of sulfur metabolism, sulfur amino acid and glutathione biosynthesis, we tested the impact that the addition of the key metabolites of these pathways (SAM, GSH and GSSG) to SM had on wine strain growth (Figure 6). As a control, we also incorporated the lab strain BY4743. Addition of GSH and GSSG significantly shortened the generation time (GT) of P5 at low temperature, while addition of SAM prolonged the GT by 2 h. Quite surprisingly, the presence of the three sulfur-containing compounds dramatically shortened the GT of P24 at low temperature and obtained similar values to these of P5. The behavior of the lab strain BY4743 was similar to P24 as it reduced GT from 17 h to around 8 h. Regarding growth at 28 °C, no statistically significant variations were noted in the GT of the three strains when grown in supplemented SM, which indicates a low temperature-dependent effect.

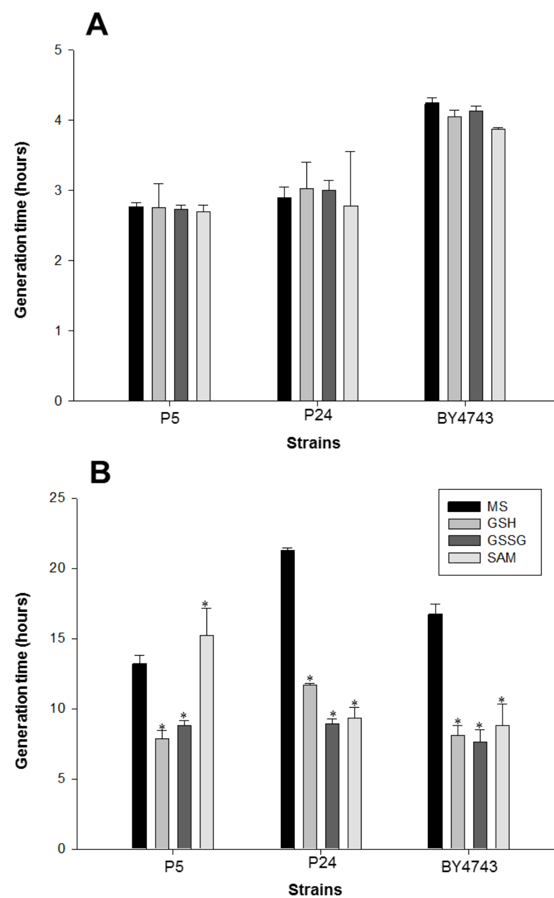


Figure 6. Generation time (hours) of P5, P24 and lab strain BY4743 at 28 ºC (A) and 15 ºC (B). The assay was carried out in synthetic must (SM) supplemented with different key sulfur-containing compounds (GSH, GSSG, SAM). *Significant differences compared with the strain growing in SM at the same temperature.

Strain P5 showed better oxidative stress recovery

The importance of glutathione metabolism and a higher concentration of some peroxiredoxins and thioredoxins at low temperature also suggest the

influence of oxidative stress defense on the better fitness of strain P5. To check this implication, both wine strains were incubated in PBS with different H_2O_2 concentrations for 1 h at 28 ºC. After this incubation, the oxidant agent was removed and cells were inoculated in SD and SM. Figure S3 shows the growth curves at 28 ºC for each strain after incubation with different H_2O_2 concentrations in both media. Increasing H_2O_2 concentrations affected mainly the lag phase, which was longer (from 3 to 13 h) the higher the oxidative agent concentration became. The longest lag phases were detected in the P24 growth curves, which revealed worse management or recovery after oxidative shock. Regarding the viability of the strains after incubation at different H_2O_2 concentrations, and before inoculation in SD or SM, no statistical differences were found between both strains and H_2O_2 concentrations (data not shown).

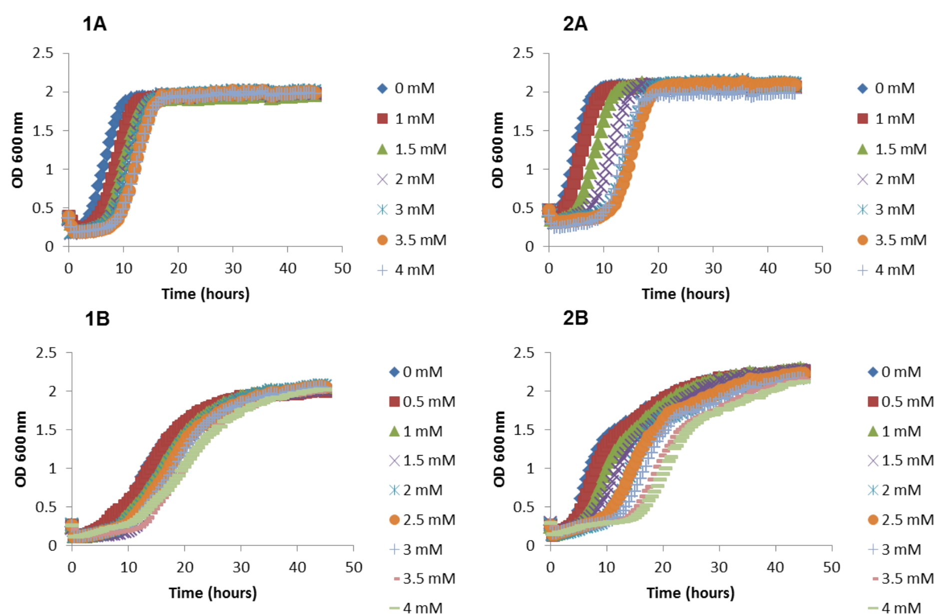


Figure S3. Recovery after oxidative stress. Cells were subjected to oxidative stress with different concentrations (0-4 mM) of hydrogen peroxide for 1 h. The oxidative agent was removed and the growth curves of P5 (1) and P24 (2) were analyzed immediately in SD (A) and SM (B) at 28 ºC.

Whole-genome comparison of the two wine strains

The genomes of the two wine strains were sequenced and compared with that of reference strain S288c (Figure S4). Based on the raw sequence data, we identified 44532 and 44030 mutations in strain P5 and strain P24, respectively, in comparison to the reference strain. This number of SNPs represents approximately 0.4% of the *S. cerevisiae* genome. When comparing the sequences between both wine strains, the number of SNPs

lowered to 6446 mutations, of which 90% gave homozygous changes. According to Liti et al., (2009), wine yeasts belong to the same cluster, the Wine/European lineage, while most lab strains, such as S288c, are mosaic strains between the Wine/European cluster and the other four clean lineages. Only 27% of the SNPs between both wine strains represented nonsynonymous changes in the coding region, which resulted in an amino acid change (Figure S4A). As these nonsynonymous changes could potentially explain the phenotypic differences observed between both strains, we identified mutations in the genes of the sulfur assimilation pathway (Table 5). Many genes of this route presented SNPs which involved amino acid changes in the biosynthetic enzymes and, most importantly, in regulators of the pathway, such as *MET4* and *MET28*. However, conversely to the transcriptomic analysis, the determination of the functional categories with significant overrepresentation among the nonsynonymous SNPs did not include the GO term “sulfur metabolism”, although the related “sulfate/sulfite transport” was included (Table S8).

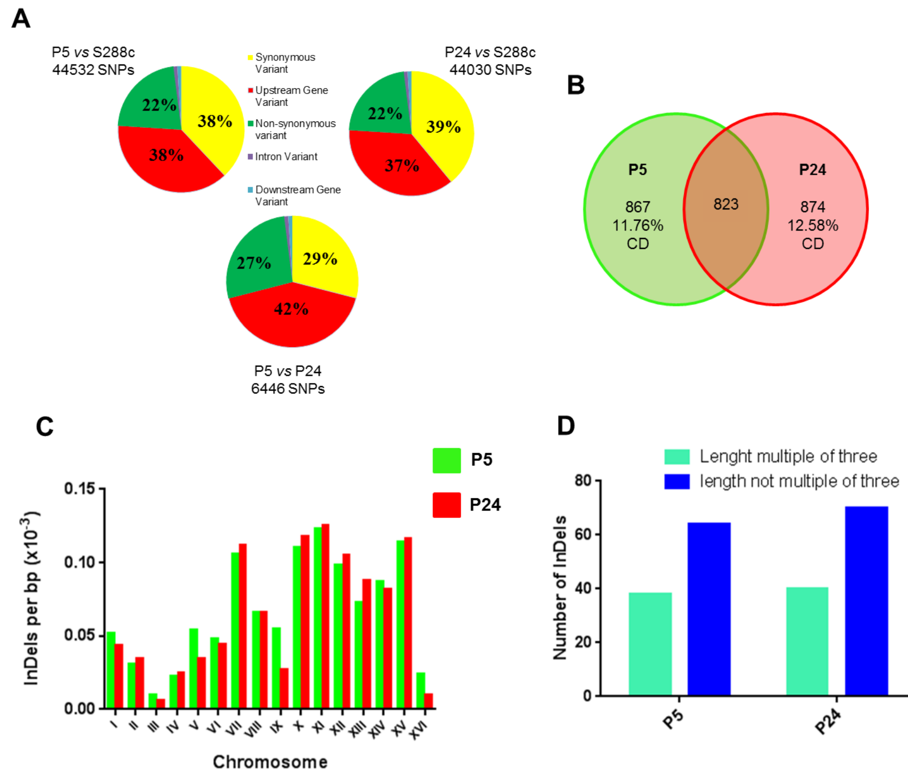


Figure S4. Genomic analysis of strains. (A) Single nucleotide polymorphism (SNPs) population distribution. SNPs were classified according to genome localization and change in protein sequence (nonsynonymous variant). (B) Venn's diagram of the indels in both strains compared with the reference strain. The common indels among strains are highlighted. (C) Distribution along the chromosomes of the unique indels. (D) Distribution of the unique indels present in the coding sequence according to their length.

Certain classes of variants, such as InDels, are expected to have dramatic consequences on gene products and, therefore, constitute particularly

interesting candidates for contributing to phenotypic variation (Table S8). In all, 1690 and 1697 InDels were found in P5 and P24, respectively, compared with the reference strain (Figure S4B). Of the total number of InDels, 823 were found in common between strains. When examining the distribution of the unique InDels for each strain along the chromosomes, some (chr XVI, X, XI, XV) showed an enrichment of variants (Figure S4C). Only 12% of the unique InDels for each strain were within the coding sequence. Within ORFs, the InDels with lengths that were multiples of three were highly enriched when compared with the noncoding sequence, which is consistent with the strong purifying selection against frameshifts (Figure S4D). Those InDels in the coding sequence with lengths that were not multiples of three were classified mainly as functionally uncharacterized. This demonstrates that this group of genes are, on average, under lower purifying selection pressure (Bergström et al., 2014). We also detected the larger copy number of genomic regions between both strains (CNV). Seventy-two CNV were detected across the genome comparison made between both strains (Table S8). Most of these CNV were classified mainly as transposable elements and subtelomeric regions. These results are in line with previous observations (Bergström et al., 2014), which found very limited CNV in nonsubtelomeric regions and extensive variation in subtelomeric regions.

Table 5. Genomic changes between strains P5 and P24 in the genes of the sulfur assimilation pathway and glutathione biosynthesis.

Genes	Mutations	Functions
MET4	Gln^{629}-His	Regulation of sulfur metabolism
MET28	Lys^{92}-Glu	Regulation of sulfur metabolism
MET1	Gly^{578}-Asp	Sulfate assimilation and methionine biosynthesis
GTT1	Gly^{231}-Asp	Glutathione metabolic process
SUL1	Ala^{99}-Val	Sulfur assimilation
HYR1	Lys^{172}-Glu	Cellular response to oxidative stress
GTO2	Trp^{172}-Leu	Glutathione metabolic process
SUL2	Ala^{809}-Ser	Sulfur assimilation
ECM5	Asn^{504}-Lys	Cellular response to oxidative stress
SAM3	Glu^{570}-Lys	S-adenosyl-L-methionine transport
SSE1	Asp^{691}-Gly	ATPase component of the heat shock protein Hsp90 chaperone complex
SSU1	Met^{19}-Val	Sulfite transport
CYS4	Ser^{504}-Asn	Cysteine biosynthesis
GSH1	Ser^{496}-Arg	Glutathione biosynthetic process
STR2	Gln^{403}-Lys	Cystathionine biosynthesis

4. Discussion

Several works have shown the marked importance of temperature on the growth of wine yeasts (Beltran et al., 2006; Fleet, 2003; López-Malo et al., 2013a; Redón et al., 2011) and the influence of this environmental factor on determining the natural distribution of wild species (Salvadó et al., 2011a). A direct effect of lowering temperature is to slow down the metabolic activity of yeast cells, which accounts for reduced growth and longer fermentation processes (Charoenchai et al., 1998). Thus, unraveling the molecular and physiological mechanisms that allow better adaptation and growth at low temperature is interesting. In this study we followed a global approach by comparing transcriptomic, proteomic and genomic changes in two commercial wine strains, which were selected as having clear differences in their growth and fermentation capacity at low temperature. The selection of these strains was based on the maximum growth rate in a synthetic grape must (SM) during miniaturized batch cultures at different temperatures. The fitness differences of the selected strains were corroborated by directly competing during fermentations at optimum and low temperature. These competition experiments highlighted the better competitiveness of P5 *vs.* P24 only at low temperature.

Although wine fermentations are operated in the batch mode, the proteomic and transcriptomic changes between both strains were determined in the steady-state of continuous cultures at the same dilution rate. In batch cultures, the specific growth rate (μ) is strongly affected by temperature. This means that it is impossible to dissect temperature effects on transcription and translation from specific growth rate effects (Tai et al., 2007). Recently, Vázquez-Lima et al., (2014) used chemostat cultures to mimic the different phases of a typical batch wine fermentation, and showed the potential of this experimental approach to systematically study the effect of environmental relevant factors such as temperature.

A global transcriptomic analysis has revealed key changes in the sulfur assimilation pathway at low temperature, with the up-regulation of key genes in both strains. This pathway incorporates extracellular sulfate into several key sulfur-containing compounds, including methionine, cysteine, homocysteine and S-adenosylmethionine (SAM) (Hickman et al., 2011). The biosynthetic genes of this pathway are controlled by a complex regulatory system, whose main transcriptional activator is Met4p. Met4p is recruited to specific promoters by site-specific DNA-binding transcription cofactors Met31p, Met32p and Cbf1p (Lee et al., 2010). The activity of this pathway has a widespread influence on other cellular pathways, some of which have a huge potential impact on adaptation at low temperature, such

as in the phospholipid (PL) biosynthesis pathway. Changes in the phospholipid composition of cellular membranes as a response to low temperature have been widely reported (Beltran et al., 2006; Henderson et al., 2013b; Redón et al., 2011; Tronchoni et al., 2012b). Phosphatidylcholine (PC), the major phospholipid (at least 30% of total PLs), is synthesized *de novo* from another PL, phosphatidylethanolamine (PE), in three SAM-consuming methyltransferase reactions catalyzed by Opi3p and Cho2p (Chin and Bloch, 1988). The enzymatic genes of this pathway are repressed by Opi1p, a protein that directly senses the levels of phosphatidic acid (PA), a precursor of PL biosynthesis. Hickman et al., (2011) reported coordinated regulation of the sulfur and phospholipid pathways with Met4p activating the genes involved in producing SAM and Opi1p repressing some of these genes. This coordinated regulation between both transcription factors ensures that cells maintain the requirement of methylation during the biosynthesis of cell membrane phospholipids. Thus we hypothesize that the higher demand of PC at low temperature (Chin and Bloch, 1988) increases the requirements of SAM for this biosynthesis, requiring the activation of the entire sulfur metabolism pathway. The better fitness of strain P5 at low temperature is consistent with the global transcriptional up-regulation of enzymatic genes and some transcription factors in comparison to strain P24. The greater transcriptional activation of this route in P5 also correlated with

the presence of higher concentrations of some Met proteins, such as Met10p and Met17p. However, P24's inability to synthesize SAM requirements at low temperature can be counterbalanced by the presence of this compound in the growth medium, which showed a similar growth rate to strain P5. These data also support the higher internal demand of SAM for growing at low temperature.

Greater activation of the sulfur assimilation pathway may also have a huge impact on other metabolic processes, such as the synthesis of the molecules involved in oxidative stress response. Thioredoxin and glutathione /glutaredoxin pathways are universal systems to maintain the redox homeostasis of the cell. The oxidized disulphide form of thioredoxin is reduced directly by NADPH and thioredoxin reductase, whereas glutaredoxin is reduced by glutathione (GSH) using electrons donated by NADPH (Figure 5). Thus according to our data, the coordinated up-regulation of the genes involved in the sulfur and glutathione pathways may lead to higher intracellular concentrations of glutathione, whose protective effect may contribute to improve the fermentation process. In a recent transcriptomic comparison of four wine strains showing different fermentation performances, Treu et al., (2014) correlated the induction of the genes involved in the biosynthesis of sulfur amino acids with the strains showing better fermentation performance. Specifically, the higher

expression of these genes, determined by the cooperation of TFs Met32p and Hap4p, contributed to more efficiently face stress induced by a high ethanol concentration and to improve strain fitness to starvation (Petti et al., 2011), which resulted in better fermentation performance. Not many reports have correlated low temperature and oxidative stress. Zhang et al., (2003) reported increased transcript levels of antioxidant genes *SOD1*, *CTT1* and *GSH1* in a rapid downshift in the growth temperature of *S. cerevisiae* from 30 to 10 ºC. Likewise, a previous proteomic study of our group (Salvadó et al., 2008) also detected an increase in Cys3p during wine yeast adaptation to low-temperature fermentation. Once again, the importance of glutathione biosynthesis in this cold adaptation is reinforced by growth data in the presence of both reduced (GSH) and oxidized glutathione (GSSG) in the culture medium. The GT of both strains significantly lowered, especially in strain P24, which showed similar values to P5. However, incubation in the presence of H_2O_2 clearly proved that strain P5 is more prone to cope with this stress oxidative. Once again, this is consistent with the up-regulation of practically all the enzymatic genes of both the sulfur and glutathione pathways, but also by the higher up-regulation of the key TFs of the route, such as Met32p and Met28p when compared with P24.

Once more, the proteomic comparison showed that strain P5 is better poised to deal with oxidative stress, as revealed by the higher concentration

of peroxiredoxin Ahp1p and thioredoxin Tsa1p at low temperature. Likewise, the higher concentration of the Met proteins in strain P5 when compared to P24 also agreed with the transcriptomic data and reinforced the greater metabolic activity of the sulfur pathway in this strain. However, the transcriptomic and proteomic data did not always correlate directly. No MIPS category that was related to glycolysis and glucose fermentation was significant in the transcriptomic analysis, but the concentration of several proteins in this category changed at low temperature in both strains. Most of these proteins represented the enzymes of the lower part of the pathway (the trioses phosphate branch) and ethanol production. With a similar experimental set-up to this study, Quirós et al., (2013) determined the distribution of metabolic fluxes during wine fermentations according to sugar concentration and temperature. In the upper part of glycolysis (the glucose 6-phosphate branch point), the C flux directed to glycolysis lowered at low temperature, which resulted in a higher C flux to the pentose phosphate pathway (PPP) and carbohydrate biosynthesis. These authors related this diversion of the C flux to these two minor branches with the higher biomass synthesis also observed at low temperature. Conversely in the lower part of glycolysis (the trioses phosphate node), the glycolytic flux was higher at low temperature, which resulted in a lower flux toward glycerol production. Biomass production is very high ATP-demanding.

Thus, this higher biomass synthesis at low temperature may result in a shortage of intracellular ATP. The increase of glycolytic and fermentative enzymes leading to ATP generation may balance this drain. The simultaneous overexpression of these enzymes enhanced the glycolytic flux and fermentative capacity of *S. cerevisiae* (Smits et al., 2000). In the comparison made of the strains, the extremely high concentration of Tdh1p (Glyceraldehyde-3-phosphate dehydrogenase) in strain P24 at low temperature was striking. Tdh1p catalyzed the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate, such as Tdh2p and Tdh3p, in the first step of the trioses phosphate branch. However Tdh2p and Tdh3p were detected in exponentially growing cells, whereas Tdh1p was detected primarily in the stationary phase (Delgado et al., 2001). It has therefore been suggested, but not confirmed, that Tdh1p may be involved in a process other than glycolysis because it is synthesized by cells in the stationary phase (Boucherié et al., 1995). Likewise, the higher concentration of the proteins involved in translation machinery (Tef1p, Rpl31Ap) can also be correlated with a greater impairment of translation in P24 at low temperature. A recent study done by our group (Tronchoni et al., 2014) showed that the better fitness of the cryophilic species *S. kudriavzevii* is given mainly by the enhanced translation efficiency of this species if compared to *S. cerevisiae*.

This suggests that translational efficiency might be an important target of adaptation evolution when cells face changing environments.

Human intervention has subjected wine yeasts to multiple rounds of independent domestication and thousands of generations of artificial selection, which has driven to a phylogenetic lineage denominated by Liti et al., (2009) as the Wine/European genetic clade. These authors also stated that despite a lineage formed by domesticated bred strains being expected to have lower phenotypic diversity, the Wine/European lineage showed similar or higher levels of diversity if compared to other clean lineages (Liti et al., 2009a). Our genomic data of the two wine strains confirm both concepts, phylogenetic proximity, but higher phenotypic diversity. They show a much smaller number of SNPs between them in comparison to reference lab strain S288c. However, despite a number of different SNPs representing less than 0.05% of the total genome, these genotype changes resulted in a clear divergent phenotype. Nonsynonymous changes in structural or regulatory genes can impact transcriptional regulation, mRNA stability or protein activity. A large number of mutations were detected in the genes of the sulfur and glutathione metabolic pathways. The changes in TFs such as *MET28* and *MET4,* which regulate the sulfur assimilation pathway, were particularly relevant. Hong et al., (2011) analyzed the mutations produced in strains evolved with improved galactose utilization and concluded that the

phenotypic changes observed in these evolved strains were the result of mutations in regulatory systems, which produced the overexpression and activation of some metabolic routes. Further work should be done in the future to evaluate whether these mutations cause the increase activity of this route.

5. Conclusions

The combination of a detailed phenotypic analysis, e.g., involving transcriptome and proteome analysis, with genome sequencing is a powerful strategy to provide a clear link between genomic and phenotypic differences. We firstly selected two strains with different fitnesses at a low, but not at an optimum, temperature. Our data highlight the importance of the sulfur assimilation and glutathione pathways to explain the phenotypic differences between both strains. In order to distinguish which of the genetic differences seen in this study is responsible for difference in growth at cold temperatures, we are currently undertaking a new comparative genetic study based on the QTLs analysis of a hybrid population generated by crossing these two strains, following the approach described by Parts et al., (2011).

AVAILABILITY OF SUPPORTING DATA

The data set supporting the results of this article is available in the Gene Expression Omnibus (GEO) Database repository GSE60140 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE60140) and in the Sequence Read Archive (SRA) database repository SRP048919 (http://www.ncbi.nlm.nih.gov/sra/?term=SRP048919). The data set supporting the results of this article is included in the article (and its additional files).

SUPPLEMENTARY MATERIAL

The additional files can be downloaded from:

http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-15-1059

CHAPTER 2

Correlation between low temperature adaptation and oxidative stress in *Saccharomyces cerevisiae*

Estéfani García-Ríos, Lucía Ramos-Alonso and José M. Guillamón

Frontiers in Microbiology (2016) 7:1199

ABSTRACT

Many factors, such as must composition, juice clarification, fermentation temperature or inoculated yeast strain, strongly affect the alcoholic fermentation and aromatic profile of wine. As fermentation temperature is effectively controlled by the wine industry, low-temperature fermentation (10-15 ºC) is becoming more prevalent in order to produce white and "rosé" wines with more pronounced aromatic profiles. Elucidating the response to cold in *Saccharomyces cerevisiae* is of paramount importance for the selection or genetic improvement of wine strains. Previous research has shown the strong implication of oxidative stress response in adaptation to low temperature during the fermentation process. Here we aimed first to quantify the correlation between recovery after shock with different oxidants and cold, and then to detect the key genes involved in cold adaptation that belong to sulfur assimilation, peroxiredoxins, glutathione-glutaredoxins and thioredoxins pathways. To do so, we analyzed the growth of knockouts from the EUROSCARF collection *S. cerevisiae* BY4743 strain at low and optimal temperatures. The growth rate of these knockouts, compared with the control, enabled us to identify the genes involved, which were also deleted and validated as key genes in the background of two commercial wine strains with a divergent phenotype

in their low-temperature growth. We identified three genes, *AHP1*, *MUP1* and *URM1*, whose deletion strongly impaired low-temperature growth. This affected phenotype may be due to the deregulation of the antioxidant defense system.

Keywords: Thioredoxins, glutathione, correlation analysis, ROS accumulation, *MUP1*, *URM1*

1. Introduction

Microorganisms constantly face environmental stimuli and stresses. The simplest response strategy to a stimulus is to monitor the environment and to respond directly to it using designated mechanisms. The environmental stress response in yeast is a complicated strategy in which responses to many stresses partially overlap (Causton et al., 2001; Gasch et al., 2000; Mitchell et al., 2009). Drops in ambient temperature are common in almost every ecological niche. In the yeast *Saccharomyces cerevisiae*, reductions in ambient temperature have widespread effects on growth and survival, which depend on the severity of stress. This is relevant for industrial yeast exploitations as several fermentations, like brewing and some wine fermentations, take place at around 12-15 ºC. In winemaking, fermentation at lower temperatures correlates with a fresh character and fruity notes in wines, and reduces the risk of bacterial contamination and the production of volatile acids (Beltran et al., 2002; Molina et al., 2007; Torija et al., 2003). The use of low temperature during the fermentation process improves product quality, but also prolongs the time needed to complete fermentation and, therefore, increases the economic cost and energy requirements. Industry is clearly interested in developing yeast strains with an enhanced capability to ferment at low temperatures. It is well-known that

cold induces biochemical, biophysical and physiological changes to cells. Cold strengthens the interactions between the two strands of DNA and the secondary structure of mRNA, so transcription and translation are impaired (Jones and Inouye, 1996). The fluidity of the lipid bilayer of membranes also diminishes and their rigidity increases, which decreases transport through cell membranes and increases passive permeability (Redón et al., 2011). Protein folding speed slows down as conformational instability increases. Cold can also induce protein denaturation and enzymatic activity generally decreases (Aguilera et al., 2007; Murata et al., 2006; Schade et al., 2004).

The release of reactive oxygen species (ROS) and the generation of oxidative stress can negatively impact yeast cell survival. ROS can be produced in the course of normal aerobic metabolism or when an organism is exposed to a variety of stress conditions. These include hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), and the superoxide anion ($O2^{\bullet-}$), which can damage proteins, lipids, carbohydrates and DNA (Herrero et al., 2008), which can lead to cell death. However, cells possess a variety of defenses, including cell-cycle delay (Flattery-O'Brien et al., 1993), the induction of enzymes, such as catalases, peroxidases, and superoxide dismutases, and the synthesis of antioxidants like glutathione, vitamins C

and E, and ubiquinol (Jamieson, 1998; Morano et al., 2012). In the last few years, some studies have connected low temperature with oxidative stress. Zhang et al. (2003) showed that a rapid downshift in the growth temperature of *Saccharomyces cerevisiae* from 30 to 10 ºC increased intracellular hydrogen peroxide (H_2O_2) levels and raised the transcript levels of antioxidant genes *SOD1*, *CTT1* and *GSH1*. Schade et al., (2004) also reported that a drop in the temperature induced a set of genes involved in oxidative stress response and implicated in detoxification processes, including *GTT2* (glutathione transferase), *HYR1* and *GPX1* (glutathione peroxidase isoforms), *TTR1* (glutaredoxin), and *PRX1* (thioredoxin peroxidase). Likewise, by means of an integrative analysis of transcriptome with metabolome and proteome data, recent studies by our group have revealed the up-regulation of the sulfur assimilation pathway and glutathione biosynthesis during adaptation to cold (García-Ríos et al., 2014). The sulfur pathway incorporates extracellular sulfate into several key sulfur-containing compounds, including methionine, cysteine, homocysteine and S-adenosylmethionine (SAM) (Hickman et al., 2011). Sulfur assimilation induction is easily understood in the oxidative stress context as cysteine is a component of molecules like glutathione, glutaredoxin and thioredoxin, which were all induced in response to oxidative stress (Sha et al., 2013), while methionine acts as a ROS scavenger (Campbell et al., 2016).

After considering all these data, this work aimed to identify the correlation between low-temperature growth and recovery after oxidative stress shock, and also the detection of the key genes related to the oxidative stress response, which play an important role in the adaptation of *S. cerevisiae* to low temperature. To achieve this objective, we analyzed the growth of several knockouts of sulfur assimilation, peroxiredoxins, glutathione-glutaredoxins and thioredoxins pathways in the lab strain BY4743 at 15 and 28 °C. The first screening of this laboratory strain enabled us to select genes in order to delete them from the genetic background of two commercial wine strains with divergent phenotypes in both low temperature and oxidative stress responses.

2. Materials and Methods

Yeast Strains and Media

In this study, 40 *Saccharomyces cerevisiae* strains were used, which were mainly industrial. These strains were typed by their interdelta sequences (Legras and Karst, 2003), and thus named according to their delta pattern (from P1 to P40). All the strains used in the correlation study herein are detailed in Table 1. Inocula were prepared by introducing one single colony from the pure cultures of each strain into 5 mL of YPD medium (1% yeast

extract, 2% peptone and 2% glucose). After 24 h of incubation at 30 °C, the volume required to obtain a concentration of about 2×10^6 cells mL^{-1} was inoculated in the different media. Correct inoculation size was always confirmed by surface spread on YPD agar plates. These yeast suspensions were used to inoculate the different experiments as described below. Forty mutants (Table S1) that belonged to the sulfur assimilation pathway and oxidative stress response of laboratory strain BY4743 (MATa/α, his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ), from the EUROSCARF collection (Frankfurt, Germany), were also used.

The growth media selected for the experiments were YPD (glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}) and synthetic grape must (SM). The latter was derived from that described by Quirós et al., (2013). The SM composition included 200 g L^{-1} of sugars (100 g L^{-1} glucose +100 g L^{-1} fructose), 6 g L^{-1} of malic acid, 6 g L^{-1} of citric acid, 1.7 g L^{-1} of yeast nitrogen base (YNB) without ammonium and amino acids, anaerobic factors (0.015 g L^{-1} ergosterol, 0.005 g L^{-1} sodium oleate and 0.5 mL L^{-1} tween 80) and 0.060 g L^{-1} of potassium disulfite. The assimilable nitrogen source used was 0.3 g N L^{-1} (0.12 g N L^{-1} as ammonium chloride and 0.18 g N L^{-1} in an amino acid form; the proportion of each amino acid was administered as previously proposed by Riou et al., (1997). The sporulation medium was KAc (potassium acetate 1%, agar 2%).

To test differential stress oxidative resistance, yeast cells were incubated in PBS with 4 mM of peroxide of hydrogen, 2mM of menadione, 0.5 mM of cumene hydroperoxide or 0.5 mM of tert-butyl hydroperoxide for 1 h at 28 ºC. These concentrations were selected after testing different amount of oxidants. The selected concentration produced a clear growth impairment but did not jeopardize the viability of the culture, such as was described in García-Ríos et al., (2014) for the peroxide of hydrogen. After this oxidative shock, cells were centrifuged at 10000 rpm for 3 min at room temperature and inoculated in SM at 28 ºC.

Table 1. The *Saccharomyces cerevisiae* strains used in this study. * These strains have no commercial name as they are already in the development stage.

Code Name	Strain designation	Origin/Source
P1	Lalvin®ICVD254	Lallemand Inc. (France)
P2	Uvaferm®WAM	Lallemand Inc. (France)
P3	Lalvin®ICVD80	Lallemand Inc. (France)
P4	Lalvin® Rhone2056	Lallemand Inc. (France)
P5	Lalvin®ICVGRE	Lallemand Inc. (France)
P6	Lalvin®EC1118	Lallemand Inc. (France)
P7	Lalvin®ICVD47	Lallemand Inc. (France)
P8	Uvaferm®CEG	Lallemand Inc. (France)
P9	Lalvin®Rhone2323	Lallemand Inc. (France)
P10	Uvaferm®BC	Lallemand Inc. (France)
P11	Uvaferm®VRB	Lallemand Inc. (France)
P12	Uvaferm®43	Lallemand Inc. (France)
P13	CrossEvolution®	Lallemand Inc. (France)
P14	Lalvin®71B	Lallemand Inc. (France)
P15	Lalvin®BM45	Lallemand Inc. (France)

P16	Enoferm®M1	Lallemand Inc. (France)
P17	Enoferm®M2	Lallemand Inc. (France)
P18	Uvaferm®BDX	Lallemand Inc. (France)
P19	Uvaferm®CM	Lallemand Inc. (France)
P20	Lalvin®ICVD21	Lallemand Inc. (France)
P21	Lalvin®Rhone2226	Lallemand Inc. (France)
P22	Lalvin®CY3079	Lallemand Inc. (France)
P23	*	Lallemand Inc. (France)
P24	*	Lallemand Inc. (France)
P25	*	Lallemand Inc. (France)
P26	*	Lallemand Inc. (France)
P27	*	Lallemand Inc. (France)
P28	QA23	Lallemand Inc. (France)
P29	S288c	Lab Strain
P30	AJ4	Lallemand Inc. (France)
P31	RVA	Agrovin Company (Ciudad Real, Spain)
P32	T73	Wine (Alicante, Spain)
P33	BMV60	Wine (Murviedro wineries,Valencia, Spain)

P34	PE35M	Masato (Greater San Marcos University, Lima, Peru)
P35	Temohaya-26	Agave juice (Technological Institute of Durango, Mexico)
P36	CPE7	Cachaça (Federal University of Minas Gerais, Brazil)
P37	Kyokai no.7	Sake (Japan)
P38	GB-FlorC	Jerez wine (González-Byass wineries, Jerez, Spain)
P39	CECT10131	*Centaurea alba* (CECT, Spain)
P40	Fermol Grand Rouge Nature	AEB group (Italy)

Growth Conditions

Growth was monitored by determining optical density at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 4 days after 20-second pre-shaking for all the experiments. At low temperatures (12-15 ºC), however, microplates had to be incubated outside the spectrophotometer to then be placed inside before being measured (every 3 h for 14 days). Microplate wells were filled with the required volume of inoculum and 0.25 mL of YPD or SM medium to always ensure an initial OD of approximately 0.1 (inoculum level of about 10^6 cells mL^{-1}). For each experimental series, non-inoculated wells were also included in the microplate to determine, and to

therefore subtract, the noise signal. All the experiments were carried out in triplicate. Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparametrized Gompertz equation proposed by Zwietering et al., (1990):

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

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h) (Aguilera et al., 2007). Generation time (GT) was calculated using the equation GT $=\ln 2/\mu_{max}$. The overall yeast growth was estimated as the area under the OD *vs*. time curve (70 and 250 h at 28 and 15 ºC, respectively). This parameter was calculated by integration using the OriginPro 8.0 software (OriginLab Corp., Northampton, MA).

Construction of Mutants in the Background of a Wine Strain

We constructed P5 and P24 homozygous and homothallic strains by autodiploidization of one ascospore and tested the fitness of the monosporic culture by comparing with the parental strain. Heterozygous mutants were constructed using the short flanking homology (SFH) method (Güldener et al., 1996) by transforming both wine strains according to the lithium acetate

procedure (Daniel Gietz and Woods, 2002) with a PCR fragment obtained by amplifying the KanMX4 cassette and flanking regions (about 500-pb upstream and downstream) from the corresponding mutant strain in the BY4743 background. After transformation, strains were selected using Geneticin (G418), added to the YPD solid media at a concentration of 200 mg L^{-1}. The total DNA from the transformants resistant to G418 was analyzed 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 in potassium acetate medium (KAc) 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, and were homozygous for the corresponding gene deletion, as verified by PCR.

Synthetic Wine Must Fermentation

Fermentations were performed at 28 and 15 °C with continuous orbital shaking at 100 rpm. Fermentations were carried out in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM. Fermentations were monitored by media density (g L^{-1}) in a densitometer (Densito 30PX, Mettler Toledo, Switzerland). Fermentations were considered complete when density reached 995 g L^{-1}. Yeast cell growth was determined by

absorbance at 600 nm and by plating on YPD. The kinetics of these fermentations was estimated by calculating the time needed to ferment 100% (T100) of sugars in SM.

Statistical Analysis

All the experiments were carried out at least in triplicate. Physiological data and correlation tests were analyzed by the Sigma Plot 12.5 software and the results are expressed as mean and standard deviation. To evaluate statistical significance, tailed t-student tests were applied with a *p*-value of 0.05. Phenotypic data were fitted to the reparametrized 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.

3. Results

Correlation analysis between low temperature and oxidative stress

In order to assess the correlation between low temperature and oxidative stress, hierarchical clustering analyses were performed using Euclidean distances with the area under the OD *versus* time curve (AUC) of each growth experiment (using the mean values of biological replicates). Figure 1 shows the clustering of the 40 strains under the seven assayed conditions. Low-temperature (12 and 15 ºC) growths clustered together with recovery

after H_2O_2 shock. A second cluster was observed between growth after both shocks with oxidants menadione and tert butyl hydroperoxide and growth in SM at 28 ºC, which highlights the weak correlation between the recovery of these two oxidant shocks and the low temperature during fermentations. Finally, growth after cumene hydroperoxide shock clustered alone. By considering sample clustering, we saw four major groups. Group 1 consisted in four strains isolated from two wineries, sugar cane and sake fermentation, and presented the worst recoveries after oxidative stress. Groups 2 and 3 were integrated mainly by wine yeasts and one strain isolated from the masato fermentation (P34). This set was characterized by good recoveries after oxidative shock, especially group 2. The characteristic by which these groups mainly differed was the behavior noted against cumene hydroperoxide since Group 2 presented better recoveries than Group 3. Group 4 was a mixture of strains from different environments (industrial, lab, agave, blueberries and *Centaurea alba* flowers) that displayed mosaic behavior to the different assayed conditions.

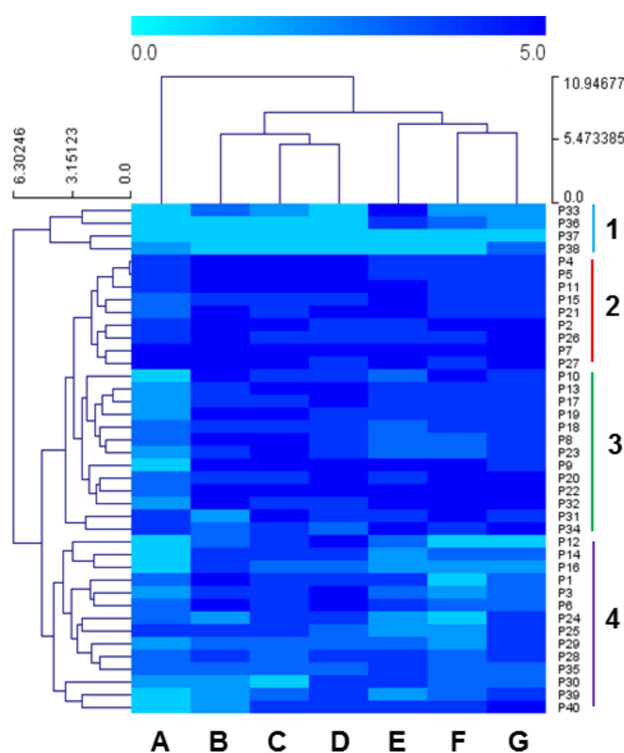


Figure 1. Heatmap illustrating the area under the curve (AUC) of 40 strains under seven different conditions. Growth experiments were performed in synthetic must (SM) at 12 ºC and 15 ºC for the low temperature analysis and SM at 28 ºC after oxidative shock to test the recovery capability. In order to normalize the different conditions, strains were divided into five groups depending on the minimum and maximum AUC value of the population per experiment. (A) Cumene hydroperoxide 0.5 mM at 28 ºC, (B) SM at 15 ºC, (C) SM at 12 ºC, (D) Hydrogen peroxide 4 mM at 28 ºC, (E) SM at 28 ºC, (F) Menadione 2 mM at 28 ºC, (G) Tert butyl hydroperoxide at 28 ºC. Sensitivity is indicated in light blue, and resistance in dark blue. The four groups separated by the Hierarchical clustering analysis (HCL) are marked by colors.

To quantitatively measure the correlations between growth temperatures (12, 15 and 28 ºC) and the capacity to deal with oxidative stress, we performed several Pearson correlation tests (Figure 2). It is noteworthy that all the correlations were positive, irrespectively of the oxidant agent used. However, the strongest correlations were found between H_2O_2/12 ºC (r=0.85) and H_2O_2/15 ºC (r=0.72), and this r value lowered (r=0.43) when the correlation was done with the control temperature (28 ºC/H_2O_2). Although significant in many cases, the correlations between low temperature and the rest of the oxidant agents were moderate and the differences between temperatures were not as clear as in H_2O_2.

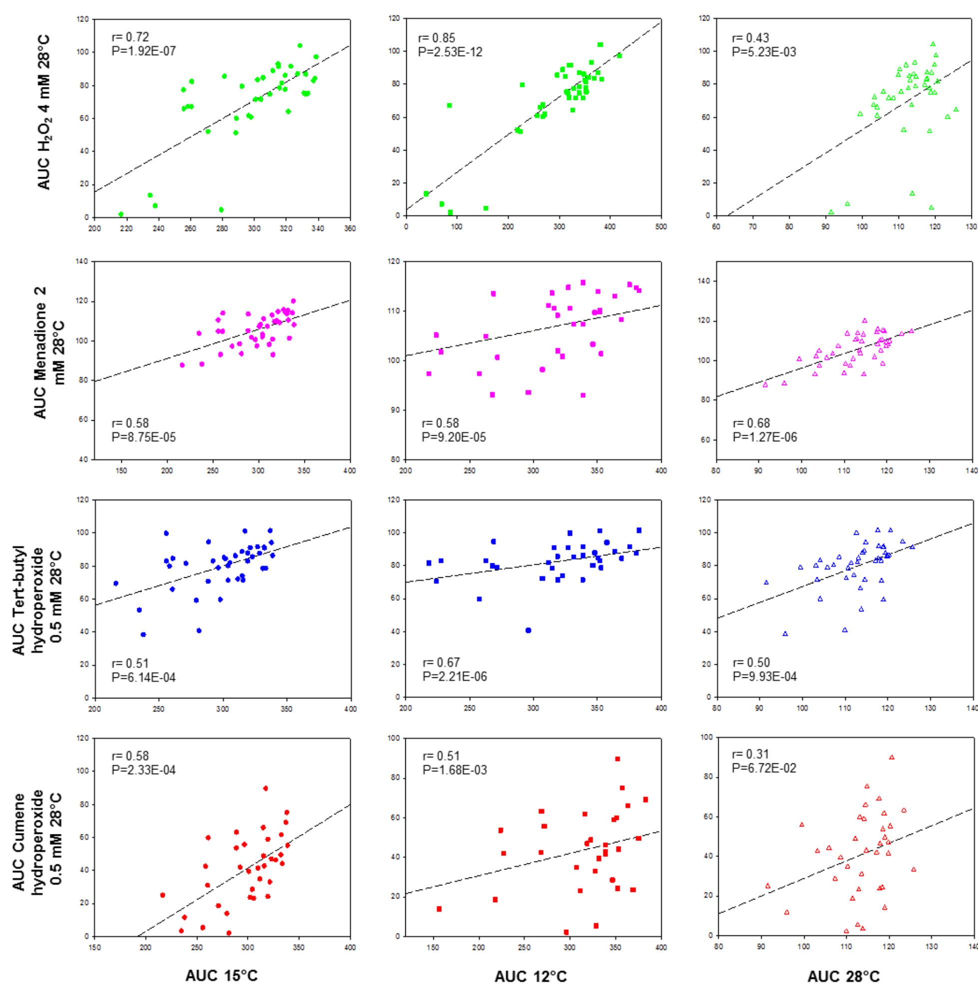


Figure 2. Correlation analysis between temperature and oxidant agents. The mean AUC value in SM at 15, 12 and 28 ºC was plotted against the mean AUC value under each oxidative condition. Linear regression (black line) is displayed. The squared Pearson correlation coefficient (r) and the p-value are provided in this figure.

Screening of genes related with the oxidative stress response and the sulfur assimilation pathway in the BY4743 mutant collection

Forty mutants of the BY4743 collection were chosen to phenotype them regarding their growth capacity at 15 and 28 ºC in YPD and SM (Figure 3). These genes belonged to four biochemical pathways: sulfur assimilation, peroxiredoxins, glutathione-glutaredoxins and thioredoxins. Figure 3 shows the relative AUC and the maximum specific growth rates (μ_{max}) compared with the BY4743 strain for each condition. Values below 1 meant that the mutant strain had impaired growth compared with the control strain. To select a gene as being determinant for low-temperature growth, its mutant strain had to show significantly impaired growth in at least two of the four growth conditions at 15 ºC (rate or AUC in YPD or SM) and for any of the growth conditions at 28 ºC. According to this criterion, ten genes (Table 2) were selected: *TRX3, AHP1, TSA1, SRX1, GLR1, GRX2, TRX2, GPX1, URM1* and *MUP1*. Fortunately, the four representative pathways related with oxidative stress, which we aimed to study, had several candidates among the ten chosen genes.

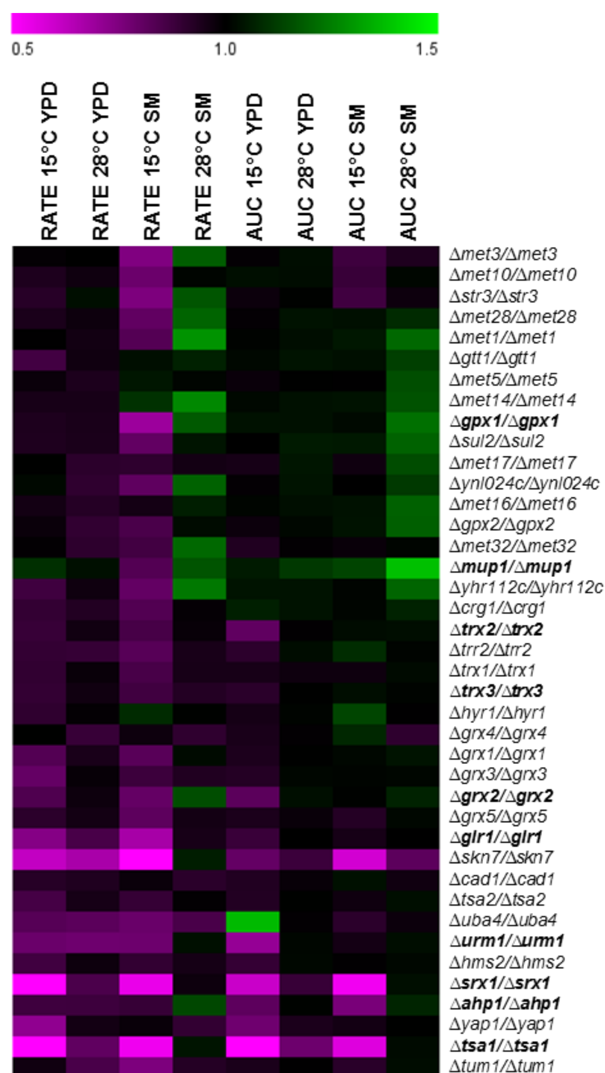


Figure 3. Growth parameters (rate and AUC) of 40 mutants strains of the BY4743 collection related to oxidative stress response. The values are relative compared with control strain BY4743. Values below 1 meant that the parameter was affected compared with the control strain. Sensitivity is indicated in purple, and resistance in green. The mutants in bold were selected for their construction in the wine strains.

Table 2. Selected genes with an affected phenotype at low temperature in the BY4743 strain.

Gene name	Description	Function
*MUP1/*YGR055W	High-affinity methionine permease	Methionine and cysteine uptake
*GPX1/*YKL026C	Glutathione peroxidase	Protects cells from phospholipid and nonphospholipid hydroperoxides during oxidative stress
*TRX2/*YGR209C	Cytoplasmic thioredoxin isoenzyme	Protects cells against oxidative and reductive stress
*TRX3/*YCR083W	Mitochondrial thioredoxin	Mantains the redox homeostasis of the cell
*GRX1/*YCL035C	Glutathione-dependent disulfide oxidoreductase	Hydroperoxide and superoxide-radical responsive, protects cells from oxidative damage
*GLR1/*YPL091W	Glutathione Reductase	Converts oxidized glutathione to reduced glutathione
*URM1/*YIL008W	Ubiquitin-Related Modifier	Receives sulfur from E1-like enzyme Uba4p and transfers it to tRNA
*AHP1/*YLR109W	Alkyl hydroperoxide reductase	Reduces hydroperoxides to protect against oxidative damage
*TSA1/*YML028W	Thioredoxin peroxidase	Acts as both ribosome-associated and free cytoplasmic antioxidant
*SRX1/*YKL086W	Sulfiredoxin	Contributes to oxidative stress resistance by reducing the cysteine-sulfinic acid groups in peroxiredoxin Tsa1p

Construction of oxidative stress response mutants in two Industrial *S. cerevisiae* strains with a divergent phenotype at low temperature

In a previous work (García-Ríos et al., 2014), a collection of industrial wine strains was phenotyped according to their capacity to grow and ferment at low temperature, and two strains (P5 and P24) were selected that displayed

divergent behavior. The P5 strain showed a much better fitness than P24 when grown at a low temperature, while both strains exhibited average growth at 28 ºC. These strains also exhibited the different capacity to deal with oxidative stress, which once again correlated well with its growth capacity at low temperature (P5 also obtained better growth recovery after oxidative stress). These previous data were confirmed in this study with the results shown in Figure 1: P5 clustered with Group 2, the more resistant strains for all the assayed conditions, while P24 belonged to Cluster 4, which is integrated by strains with worse recoveries against oxidative stress.

The ten genes that provoked a growth defect at low temperature in the BY4743 background were selected to construct heterozyogous and homozygous mutant strains in both wine strains. Heterozygous mutants were constructed by deleting one of the two copies in the parental strains (diploid). After the sporulation of heterozygous strains, homozygous mutants were obtained by autodiploidization of one spore, which harbored the deleted allele (G418 resistant). We analyzed the growth capacity of the 20 homo- and heterozygous mutants of each wine strain at 15 and 28 ºC in YPD and SM media. We also tested the recovery fitness of the mutants after the oxidative stress caused by H_2O_2. Figure 4 shows a heat-map of the relative growth rate (μ_{max}) of the mutants compared with each parental

strain. Values below 1 meant that the mutant strain had impaired growth compared with the parental strain. It is noteworthy, but not surprising, that all the homozygous and heterozygous mutants constructed in both strains showed significant growth impairment after oxidative shock. Moreover, practically all these mutants constructed in the background of P24 also displayed impaired growth in YPD at 15 ºC, whereas the growth of very few mutants of P5 was significantly affected under this condition. Curiously enough, the most affected mutants in P5 (*TRX3* and *AHP1*) were the less affected mutants in P24. This result reinforces the correlation between low temperature and oxidative stress in the P24 strain because the deletion of genes involved in response to this latter stress also affected its growth capacity at low temperature. However, this correlation was not confirmed in the P5 strain, in which most mutants were not affected when grown at a low temperature. The only gene whose deletion in both heterozygosity and homozygosity provoked growth impairment in the P5 strain was *AHP1*, which encodes a thiol-specific peroxiredoxin that reduces hydroperoxides to protect against oxidative damage (Trivelli et al., 2003).

It was noteworthy that mutants' growth capacity was not so widely affected in SM as in YPD at low temperature. This is striking because SM is a much more stressful medium because of high sugar and low nitrogen

concentrations, low pH, etc. Together with growth capacity, we were also interested in these mutants' fermentation capacity because they were constructed in two strains used industrially for wine fermentations.

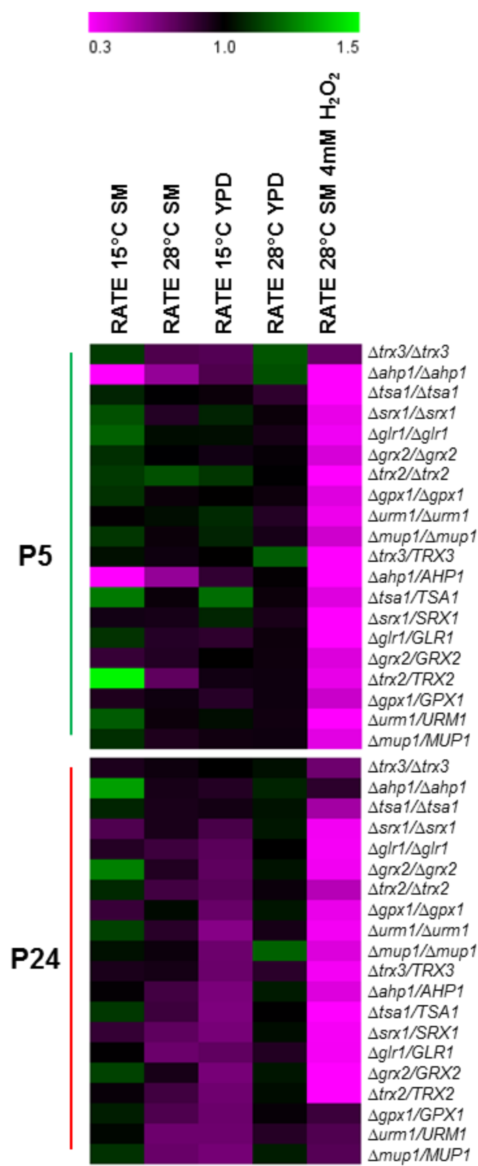
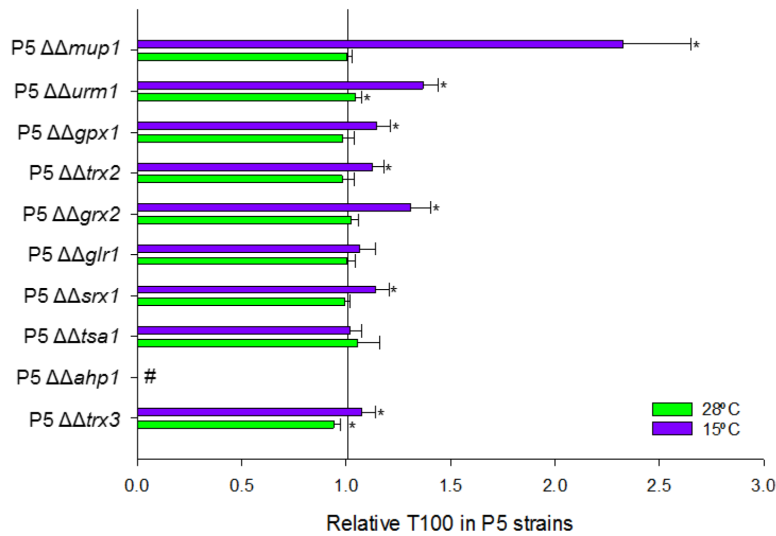


Figure 4. Relative growth rate of the mutants constructed in the background of a wine strain. The selected genes of the BY4743 collection were deleted in strains P5 and P24 and their fitness was tested under different conditions. Values below 1 meant that the parameter was affected compared with the control strain. Sensitivity is indicated in purple, and resistance in green.

Fermentation kinetics of oxidative stress response mutants

Fermentation activity was estimated by calculating the time required to ferment 100% (T100) of sugars in the SM at 15 and 28 ºC. T100 values below 1 indicated faster sugar consumption, while T100 values over 1 implied a delayed fermentation end compared with control strains P5 and P24. In this case, only the homozygous mutants were analyzed because we expected a higher impact on fermentation activity as a result of the deletion of both copies of a gene. Figure 5A shows the relative T100 of the P5 deletant strains. Conversely to growth data, a delay in the fermentation process at low temperature took place with most strains, except for genes *TSA1* and *GLR1*. The mutants with the most affected phenotypes were *MUP1,* and specially *AHP1*, whose deletion rendered this mutant incapable of ending fermentation at both temperatures. All the mutants constructed in the P24 background (Figure 5B), except *GRX2*, presented an affected phenotype at low temperature, but were not affected or barely impaired at

28 ºC. Generally speaking, end of fermentation was delayed longer for the mutants constructed in P24 than for the P5 mutants. The gene with the strongest impact was *URM1,* followed by *MUP1*. Conversely to that observed in the P5 strain, deletion of *AHP1* in P24 impaired fermentation activity at low temperature, but was unaffected at 28 ºC.



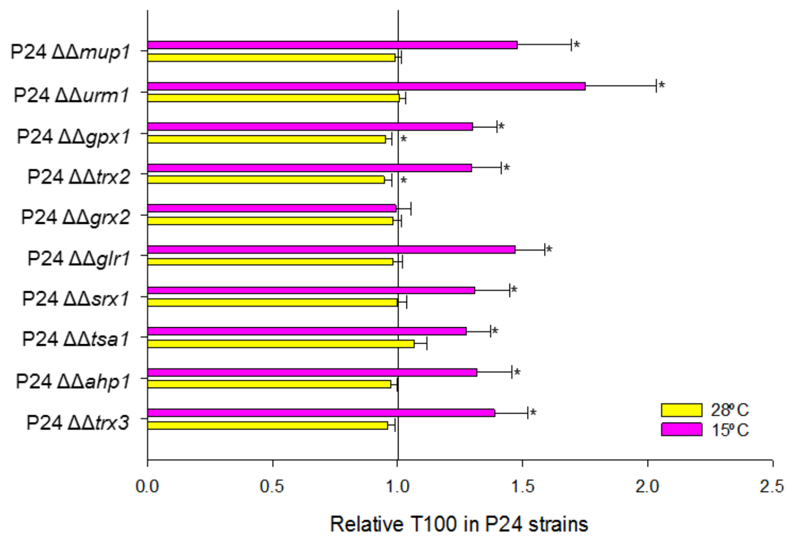


Figure 5. Fermentation kinetics in the mutant strains constructed in P5 (A) and P24 (B) at 15 and 28 ºC. T100 is the time needed to consume the total amount of sugars present in the must. The T100 value was compared with the control normalized as value 1. *Significant differences (*p*-value ≤0.05) compared with the control at the same temperature. # indicates a stuck fermentation before T100.

4. Discussion

In a previous study we selected two wine strains (P5 and P24) based on their divergent phenotype at a low, but not at optimum, temperature. We followed a global approach by using comparative transcriptomics, proteomics and genome sequencing between both strains for the purpose of clearing up the molecular basis under this divergent phenotype (García-Ríos et al., 2014). The transcriptomics analysis revealed key changes in the sulfur assimilation pathway and in other genes involved in oxidative stress defense at low temperature. So we hypothesized that low temperature adaptation and oxidative stress can share common protective mechanisms.

Hence the present work aimed to investigate the relationship between low-temperature adaptation and recovery after oxidative stress shock. For this purpose, we analyzed the growth of 40 *S. cerevisiae* strains under several temperature and oxidative stress conditions to establish a

mathematical correlation between them. Our results revealed that low-temperature growth correlated highly with the behavior of cells against most of the assayed oxidants, but particularly with hydrogen peroxide. Menadione obtained the worst correlation as the Pearson value was higher compared with 28 ºC than in the cold. Flattery-O'Brien et al., (1993) already reported a different response in *S. cerevisiae* cells treated with menadione in comparison to the same cells treated with hydrogen peroxide because these agents are thought to generate different ROS.

Thus our results clearly correlated low temperature and the oxidative stress produced by some strong oxidant molecules. However, one question remains: why does low temperature exert a stronger oxidative situation in cells than an optimum temperature? Previous studies have demonstrated that a downward shift in the growth temperature of *S. cerevisiae* from 30 to 10 ºC increases intracellular H_2O_2 levels (Zhang et al., 2003) and induces an antioxidant response (Schade et al., 2004). Recently, two independent studies (Ballester-Tomás et al., 2015; Paget et al., 2014) proved that low temperature produced a redox imbalance that needs to be corrected by the dynamic regulation of the NAD(P)/NAD(P)H ratio and the intracellular levels of these co-enzymes. Paget et al., (2014) corrected this redox imbalance by increasing glycerol accumulation or cytosolic acetaldehyde production by deleting *GUT2* and *ADH3*, respectively. Ballester-Tomás et

al., (2015) compensated this redox imbalance by overexpressing the *GDH2*-encoded glutamate dehydrogenase gene, which increased NADH oxidation. In both strategies, the *S. cerevisiae* strains displayed better fitness at low temperature, and both studies identified redox co-enzymes as key factors that governed yeast cold growth.

In order to assess a direct implication of the cellular mechanisms involved in the oxidative stress response during adaptation at low temperature, different homozygous mutants of the BY4743 strain were tested for their fitness to grow at low temperature. Although most of these genes showed impaired growth for any of the conditions tested at low temperature, we only selected ten genes, those that showed a severe growth defect, to construct heterozygous and homozygous mutants in both wine strains with different competitiveness at low temperature. One striking result was that the growth of practically all the heterozygous mutants (deletion of one copy) was strongly affected after oxidative shock, and exhibited a haploinsufficient growth defect. Haploinsufficiency is defined as a dominant phenotype in diploid organisms that are heterozygous for a loss-of-function allele. Deutschbauer et al., (2005) used the complete set of *S. cerevisiae* heterozygous deletion strains to survey the genome for haploinsufficiency by fitness profiling in rich (YPD) and minimal media. This assay revealed that approximately only 3% of all the 5900 tested genes

were haploinsufficient for growth in YPD, and concluded that haploinsufficiency is remarkably rare in *S. cerevisiae*. However in this set of genes related with oxidative stress, the retention of a single copy, which implies reduced gene dosage, was not enough to fight against and recover after an oxidative stress response. This denotes the importance of proper protein production on these oxidative response pathways. Likewise, practically all the heterozygous mutants of P24 also showed haploinsufficiency when grown at 15 ºC in YPD, which revealed the dependence of this strain on a suitable oxidative stress protection system for growth at low temperature. Conversely, few heterozygous or homozygous mutants of P5 displayed impaired growth at low temperature, which correlates with its better fitness for this condition and denotes a lower dependency of the oxidative stress protection. It is also interesting the different response obtained in both media, wherein growth capacity was less affected in SM. Even some homozygous and heterozygous mutants showed significant growth improvements (higher μ_{max}) when were grown in SM at 15 ºC. *S. cerevisiae* is an organism highly regulated with many genes and pathways to sense and respond appropriately to changing environmental conditions. The deletion of some genes that relaxed these strict controls might be beneficial in a stressful and complex medium as SM.

As P5 and P24 have a wine origin, we also tested the fermentation capacity of the homozygous mutants constructed in these strains. Similarly to growth rate, fermentation ended with a significant delay for most P24 mutants at 15 ºC, but not at 28 ºC. Regarding the P5 mutants, most of the genes also impacted fermentation activity at low temperature, but deletion of *MUP1* was quite remarkable as it increased the fermentation time by more than 2.5-fold. *MUP1*is a high-affinity methionine permease that is also involved in cysteine uptake (Kosugi et al., 2001). As explained above, this strain showed a very active up-regulated sulfur assimilation pathway at low temperature, and the phenotype observed for *mup1* also evidenced the need for sulfur amino acid uptake during wine fermentation at low temperature. Another gene to consider was *AHP1*, a thiol-specific peroxiredoxin that reduces hydroperoxides (Trivelli et al., 2003). The deletion of this gene in P5 strongly impaired the growth rate and produced a stuck fermentation in the strain P5 at both temperatures. However, this phenotype was temperature-independent and strain-dependent because the deletion of *AHP1* in P24 did not lead to the greatest reduction in growth and fermentation fitness. Strangely enough, the deletion with the strongest impact on the fermentation activity of P24 was *URM1*, a gene which encodes an ubiquitin-related protein that serves as a post-translational modification of other proteins. The Urm1p conjugation has been implicated

in the budding process and in nutrient sensing. Goehring et al., (2003) suggested that the conjugation of Urm1p to Ahp1p could regulate the Ahp1p function in the antioxidant stress response in *S. cerevisiae*. Nevertheless, if the Ahp1p-Urm1p conjugate was necessary for a proper antioxidant response, why did the disruption of one of these genes strongly affect the fitness in P5, but not in P24, and vice versa? Further future studies will be necessary to answer this question.

In conclusion, we clearly established herein a strong correlation between low temperature fitness and oxidative stress resistance in *S. cerevisiae*. Our hypothesis is that growing this yeast at a suboptimal temperature raises the intracellular levels of ROS and induces an antioxidant response. The fitter strains to fight against this oxidative stress are also the strains that display better growth and fermentation performance at low temperature. This correlation is also very interesting from an applied point of view because it could be a trait for future selections of industrial cryotolerant strains or for the genetic improvement of them. We have recently obtained an improved wine strain to ferment at low temperature by growing during many generations under low-temperature selective pressure (López-Malo et al., 2015). An alternative strategy could be the long-term culture of these strains in the presence of oxidants to obtain fitter genetic variants to cope with the oxidative stress and to better adapt to low

temperature. In order to assess this correlation at the molecular level, we constructed mutants of the genes involved on the main antioxidant response pathways in two wine strains with a divergent phenotype at low temperature. The growth and fermentation fitness of P24 was seen to strongly depend on an optimal oxidative stress response. With P5, this being the strain that displayed better competitiveness at low temperature, the deletion of key oxidative stress defense genes did not lead to a general reduction in its fitness. As low temperature adaptation is a trait that is regulated by many complex mechanisms at different levels in the cell, P5 must cope with this stress by other alternative mechanisms of stress response. However, the phenotype analysis of the mutants in this strain revealed the importance of the permease of sulfur amino acids *MUP1* to grow and ferment at low temperature and the paramount role of peroxiredoxin *AHP1* in the fermentation process.

SUPPLEMENTARY MATERIAL

The additional files can be downloaded from:

http://journal.frontiersin.org/article/10.3389/fmicb.2016.01199/full

CHAPTER 3

The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*

Estéfani García-Ríos, Miguel Morard, Leopold Parts, Gianni Liti and José M. Guillamón

BMC Genomics (Submitted)

ABSTRACT

Background

Low-temperature growth and fermentation of wine yeast can enhance wine aroma and make them highly desirable traits for the industry. Elucidating response to cold in *Saccharomyces cerevisiae* is, therefore, of paramount importance to select or genetically improve new wine strains. As most enological traits of industrial importance in yeasts, adaptation to low temperature is a polygenic trait regulated by many interacting loci.

Results

In order to unravel the genetic determinants of low-temperature fermentation, we mapped quantitative trait loci (QTLs) by bulk segregant analyses in the F13 offspring of two *Saccharomyces cerevisiae* industrial strains with divergent performance at low temperature. We detected four genomic regions involved in the adaptation at low temperature, three of them located in the subtelomeric regions (chromosomes XIII, XV and XVI) and one in the chromosome XIV. The QTL analysis revealed that subtelomeric regions play a key role in defining individual variation, which emphasizes the importance of these regions' adaptive nature.

Conclusions

The reciprocal hemizygosity analysis (RHA), run to validate the genes involved in low-temperature fermentation, showed that genetic variation in mitochondrial proteins, maintenance of correct asymmetry and distribution of phospholipid in the plasma membrane are key determinants of low-temperature adaptation.

Keywords: Quantitative trait loci, cold adaptation, industrial yeast, subtelomeres, lipid asymmetry, reciprocal hemizygosity analysis.

1. Introduction

Low temperature is one of the most important environmental stresses that influences the life and distribution of living organisms. In the yeast *Saccharomyces cerevisiae*, reductions in environmental temperature have widespread effects on growth and survival. At low, but permissive, temperatures (10–18 ºC), metabolic activity and growth rates lower. This is relevant for the industrial exploitation of yeast since brewing and some wine fermentations take place at around 12–15 ºC. Low temperatures are used in wine fermentations to enhance production and to retain flavor volatiles, which enable the production of white and "rosé" wines with greater aromatic complexity (Beltran et al., 2002; Torija et al., 2003).

Yeast undergoes considerable stress during wine fermentation from high concentrations of sugars in grape must, which leads to high osmotic pressure at the beginning of the process. As fermentation proceeds, ethanol accumulation, limiting nitrogen concentration, or even the presence of SO_2, impose further pressure. In addition to these inherent difficulties to the process, temperatures below the optimum range of growth (around 32 °C) (Salvadó et al., 2011b) affect yeast growth and fermentation rates, and give rise to both a prolonged lag phase and the production of stuck and sluggish fermentations (Bisson, 1999).

Changes in gene expression levels, ploidy and copy number variation (CNV) serve as the main genetic adaptive signatures of wine yeasts to fermentative processes (Infante et al., 2003). Other enological traits, such as ethanol production, residual sugar after fermentation, nitrogen uptake and volatile acidity, are complex traits determined by multiple quantitative trait loci (QTL) (Marullo et al., 2004). The genetic mechanisms that underlie their variation can be identified by a linkage analysis. This approach uses crosses between two phenotypically different strains, and searches for a statistical link between the phenotype and genetic markers of segregant strains (Mackay et al., 2009; Parts, 2014). QTL mapping has been successfully applied to high-temperature growth (Shapira and David, 2016; Sinha et al., 2008; Yang et al., 2013), sporulation (Ben-Ari et al., 2006; Deutschbauer and Davis, 2005; Ehrenreich et al., 2009; Katou et al., 2009), cell morphology (Nogami et al., 2007), drug sensitivity (Kim and Fay, 2007), ethanol tolerance and growth (Greetham et al., 2014; Katou et al., 2009; Voordeckers et al., 2015), protein abundance (Albert et al., 2014; Parts et al., 2014), and flocculation (Brauer et al., 2006; Li et al., 2013). QTL approaches have also been used to dissect the molecular basis of several wine yeast metabolic traits, such as acetic acid production, hydrogen sulfide production, and for the release of volatile phenol (Marullo et al., 2006), nitrogen utilization and metabolite production (Ambroset et al.,

2011), and the production of acetic acid, glycerol, and residual sugar concentrations (Salinas et al., 2012).

To identify the genetic variants that affect low-temperature adaptation in wine yeast, we performed QTL mapping using a set of segregants derived from a cross between two industrial wine yeast strains with a divergent phenotype at low temperature (García-Ríos et al., 2014), but with a very similar genotype. We identified four genomic regions located in the different chromosomes implicated in the fermentation process at low temperature in industrial wine yeast, and ran a reciprocal hemizygosity (RH) analysis for validation purposes. We identified subtelomeres to be an important source of genetic variation in industrial yeast (Liti et al., 2009b), and found both the mitochondria and proteins involved in maintaining correct asymmetry and distribution of phospholipid in the plasma membrane played a crucial role in low-temperature growth.

2. Materials and Methods

Strain selection and advanced intercross lines

We selected two industrial wine strains, P5 and P24, as parent strains for their marked phenotypic growth differences at low temperature (García-Ríos et al., 2014). Both strains were kindly provided by Lallemand Inc. (France). We generated derivative strains that were stable haploid and auxotrophic for uracil (ho::KanMX4 *ura3*Δ and ho::NatMX4 *ura3*Δ). We replaced the *LYS2* with *URA3* gene in the P5 strain. Such gene replacement restores the ability of growth in the absence of uracil and makes it unable to grow without lysine. The two parental strains with opposite mating types (P5 *mat α URA3+* and P24 *mat a LYS2+*) were crossed in complete media (YPD) and grown overnight. Patches were replica-plated in synthetic minimal media (SD) to select diploid F1 hybrids. Two F1 hybrid replicas were grown overnight (spread over a whole Petri dish) and replica-plated on KAc (potassium acetate 1%, agar 2%) at 30 ºC to be sporulated for 10 days. Sporulation efficiency (% of sporulating cells) was monitored until it reached >90%. The cells from the whole plate were carefully collected and resuspended in 0.5 ml of sterile water, treated with an equal amount of ether and vortexed for 10 minutes to selectively kill unsporulated cells (Dawes and Hardie, 1974). Cells were washed 4 times in sterile water, resuspended

in 900 µl of sterile water and treated with 100 µl of zymolase (10 mg mL^{-1}) to remove the ascus. Cell mixtures were vortexed for 5 minutes to increase spore dispersion and inter-ascus mating.

Cells were plated at high density to begin a second round of mating and meiosis (F2). The presence of two distinct markers (*LYS2* and *URA3*) at the same genomic position prevents them from co-segregating in haploid cells, and thus allows selection for diploid cells segregating at that locus. In order to confirm this system, we dissected 10 tetrads from the F6 pool, all of which had the correct 2:2 segregation of the *LYS*/*URA* markers. The sporulated pool was treated with zymolase and plated at high density to start the next intercross generation, as described in the paragraph above (Figure S1). This process was repeated until the F13 population was achieved to generate a large pool of segregants for sensitive and high resolution QTL mapping (Parts et al., 2011).

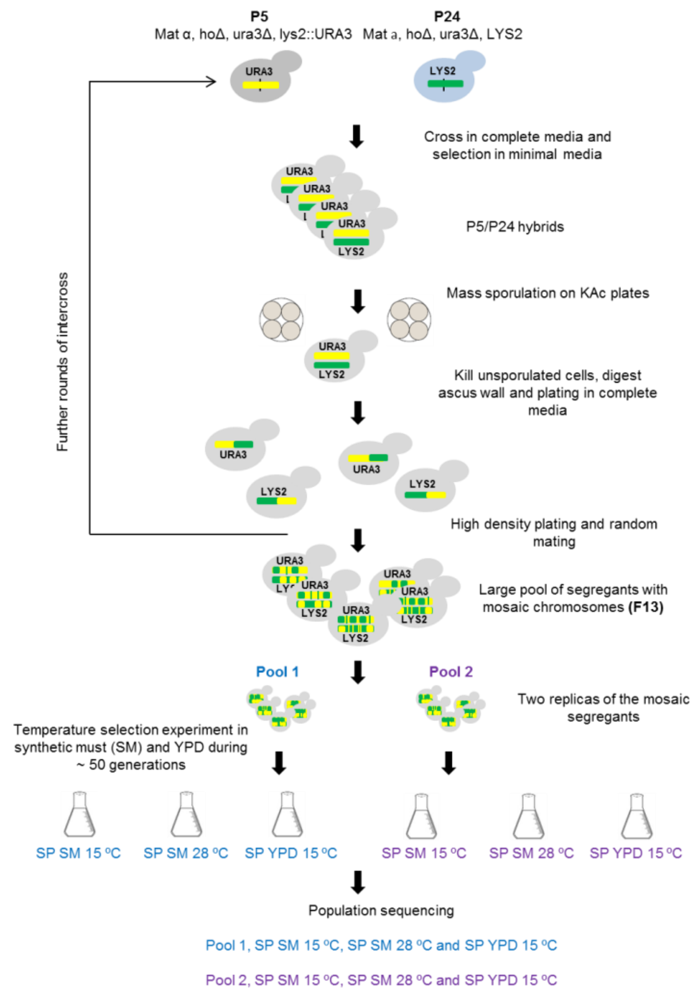


Figure S1: Outline of the construction of advanced intercross lines. We carried out a strategy that forces yeast cells through multiple rounds of random mating and sporulation to create advanced intercross lines (AILs). This step can improve genetic mapping in two ways: increasing resolution by reducing linkage and unlinking nearby QTLs.

Media and growth conditions

The growth media selected for the experiments were SD (Yeast Nitrogen Base (YNB, Difco) supplemented with 20 g L^{-1} of glucose as the carbon source), YPD (glucose 20 g L^{-1}, peptone 20 g L^{-1}, yeast extract 10 g L^{-1}) and synthetic grape must (SM), which was derived from that described by Riou et al., (1997). 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 (0.015 g 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 chloride and 0.18 g N L^{-1} in an amino acid form; the proportion of each amino acid was administered as previously proposed by Riou et al., (1997). Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 4 days after 20-second pre-shaking for all the experiments. At low temperatures (15 °C) however, microplates had to be incubated outside the spectrophotometer and then placed inside before being measured (every 3 h for 10 days). Microplate wells were filled with the required volume of inoculum and 0.25 mL of medium to always ensure an initial OD of approximately 0.1 (inoculum

level of about 10^6 cells mL^{-1}). Uninoculated wells for each experimental series were also included in the microplate to determine, and to therefore subtract, the noise signal. All the experiments were carried out at least in triplicate. Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al., (1990):

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

where $y = \ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D= \ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h).

For the spot assays, the cells were grown on YPD at 28 ºC 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, and followed by serial dilution. From each dilution, 3.5 µL were spotted onto YPD agar plates. Plates were incubated at 15 and 28 ºC for 9 and 2 days, respectively.

Selection experiment

The pools of the population size of 10-100 million cells (estimated by plating serial dilutions and CFU counting) were collected from the

sporulation media and treated with ether and zymolase, as described above. Spores were grown in complete media (YPD) and synthetic must (SM), and were incubated at either optimum temperature (28 ºC) or low temperature (15 ºC) until the stationary phase was reached. All the cells were carefully collected and resuspended in distilled water and a small volume (the volume required to inoculate at an OD of 0.2) of the expanded culture was transferred to 60 mL of fresh medium. Culture growth was monitored by measuring absorbance at 600 nm every 24 h at 28 ºC, and every 48 h at 15 ºC. The number of generations was calculated by the equation: $n = (\log N_t - \log N_0)/\log 2$, where n is the number of generations, N_0 is the initial OD and N_t is the OD at time t. The experiment was carried out 8 times, after which the selected populations were analyzed.

Sequencing

The genome sequencing of the selected populations was performed by 5500xl SOLiD sequencing. Genomic libraries were prepared following the manufacturer's standard instructions. Emulsion PCRs were performed using the SOLiD™ EZ Bead™ Systems. Sequencing was carried out by 75 nt single-end read exact call chemistry (ECC) following the manufacturer's standard protocols. The whole-genome sequences are deposited in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra/)

and are available with access number SRP048919. Breseq (v0.27.1) (Deatherage and Barrick, 2014) pipeline was used to first align the reads of the reference S288c genome (using Bowtie2 (Langmead and Salzberg, 2012), and to identify SNPs and indels with a frequency cutoff of 0.2. CNV detection was performed using CNV-seq (Xie and Tammi, 2009) with a window size of 121 nt (with a minimum log2 fold of 0.6 and a minimum p-value of 0.001). P5 was used as the "reference" genome and P24 as the "test genome". Only CNV larger than 900 bp were considered for the analysis. The sequences of seven informative loci (Ramazzotti et al., 2012) from 15 strains that represented pure groups were downloaded from the SGD (http://www.yeastgenome.org/) and the NCBI (https://www.ncbi.nlm.nih.gov/) to perform the phylogenetic analysis. Each gene sequence was aligned using mafft (v7.221) (Katoh and Standley, 2013) individually and then concatenated. The phylogenetic tree was constructed using a Maximum Likelihood Method (ML) with RAxML (Stamatakis, 2014) with 100 bootstrap replicates. The distribution of the SNPs among the strains was visualized using Circos 0.69.2 (http://circos.ca).

Linkage analysis

We retained variants that were covered with at least 30 sequencing reads in both control and selection experiments, and were present at 30-70% allele

frequency in the initial segregant population. This ensures that only confident segregating alleles are analyzed. We then calculated the allele frequency changes in both biological replicates for each retained site, and called QTLs the alleles with a frequency change of at least 0.1 in the same direction in both replicates.

Validation of QTLs

QTLs were validated by reciprocal hemizygosity using the *URA3* gene as a selectable marker (Steinmetz et al., 2002). Briefly, the haploid versions of the parental strains (P5 Mat α, ho::HygMX, ura3::KanMX or P24 Mat a, ho::NatMX, ura3::PhleoMX) were used to delete each target subtelomeric region or gene, and to construct all the possible combinations. After the deletions of the candidate regions, strains were crossed to generate the hybrid strains and selected in double drugs plates. Diploid hybrid strains were detected by the benomyl assay (Stearns et al., 1990; Upshall et al., 1977) and confirmed by Mat locus PCR (Huxley et al., 1990), and the deletions of the target genes were confirmed by PCR using specific primers. The uncharacterized single copy ORF *YMR317W* was used as a target to truncate the chromosome XIII right subtelomeric region, gene *YOL159C* was used to truncate the chromosome XV left subtelomeric region and gene *SGE1* was employed in the case of the chromosome XVI right subtelomeric

region. To identify the contribution of the alleles present in these regions, 60 hemizygote hybrids were constructed, each resulting from a cross between a derivative haploid P5 or a P24 strain and BY4741 that lacked one of these genes. A heterozygote hybrid strain with the wild-type BY4741 was also constructed and used as a control.

Fermentation conditions

Fermentations were performed at 28 ºC and 15 ºC, with continuous orbital shaking at 100 rpm. Fermentations were done in laboratory-scale fermenters using 100-mL bottles filled with 60 mL of SM. Fermentations were monitored by the density of media (g L^{-1}) using a densitometer (Densito 30PX, Mettler Toledo, Switzerland). Fermentations were considered complete when density reached 995 g L^{-1}. Yeast cell growth was determined by absorbance at 600 nm and by plating on YPD.

Statistical analysis

All the experiments were carried out at least in triplicate. Physiological data were analyzed with the Sigma Plot 12.5 software, and the results were expressed as mean and standard deviation. To evaluate statistical significance, two tailed t-student tests were applied with a p-value of 0.05. P-values were corrected for multiple testing by the Bonferroni test.

Phenotypic data were fitted to the reparameterized Gompertz model by nonlinear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0. Phenotype heritability H^2 was calculated as previously described (Brem and Kruglyak, 2005), i.e. $H^2 = ((Var_{seg} - Var_{env}) / Var_{seg})$ x100, where Var_{env} is the pooled variance among parental measurements and Var_{seg} is the variance among phenotype values for segregants. Transgressive segregation was defined as in (Brem and Kruglyak, 2005; Marullo et al., 2006) by the number of segregants whose phenotype level lay at least 2σ higher than the mean phenotype level of the higher parent, or was 2σ lower than the mean phenotype level of the lower parent. σ was the pooled standard deviation of parents.

3. Results

Genetic characterization of parental strains

We investigated the genetic basis of low-temperature adaptation in wine yeast in two *S. cerevisiae* enological strains, P5 and P24, characterized in a previous study (García-Ríos et al., 2014), as displaying extreme differences in fermentation ability at 15 ºC. P5 corresponds to commercial strain Lalvin®ICVGRE, which is marketed for temperature fermentations ranging from 15 to 30 ºC. P24 has no commercial name since it is undergoing its development stage. The genomes of these two wine strains were sequenced and compared with that of reference strain S288c. We used seven informative genes to classify the parental strain (Ramazzotti et al., 2012) to construct a maximum likelihood phylogenetic tree of 15 strains of different origins. Figure 1A shows the five clean lineage described by Liti et al., (2009a), which are well clustered. As expected, both parental strains belonged to the Wine/European lineage with a bootstrap value of 1.

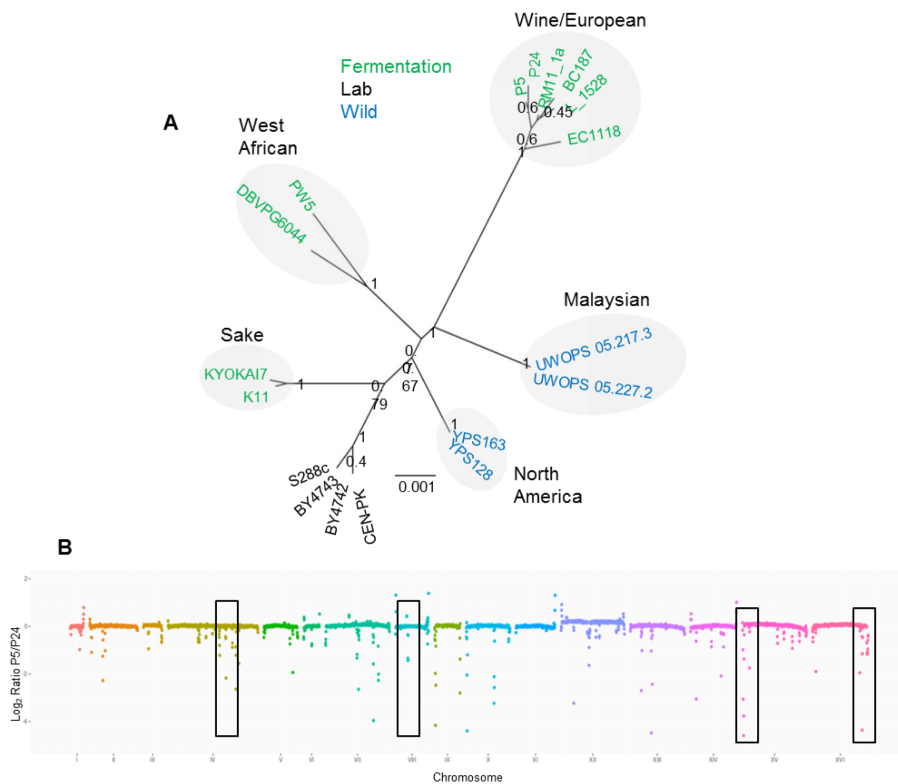


Figure 1. Genomic and phylogenetic analyses of parental strains. (A) The evolutionary history was inferred using the ML method with 1000 replicates of the bootstrap test based on concatenated nucleotide sequences of seven phylogenetic informative loci. Different colors denote the strain's origin. (B) Copy number variation across the genome (chromosomes along x-axis and log2 ratio in y-axis). The four regions with a CNV bigger than 4 kb are marked in the figure.

Based on the raw sequence data, 43666 and 42983 mutations in strain P5 and strain P24, respectively, were identified compared to the reference strain. This number of SNPs represents approximately 0.4% of the *S. cerevisiae* genome. Most of these changes were common to both strains (Table S1), and only 6836 and 6153 were strain specific mutations in P5 and P24 respectively, of which 86 and 82% were homozygous (Figure S2). Approximately 30% of the strain specific SNPs of both wine strains were nonsynonymous changes in the coding region, which resulted in an amino acid change.

Certain classes of variants, such as insertions and deletions (indels), are expected to have dramatic consequences for gene products, and therefore constitute particularly interesting candidates for contributing to phenotypic variation. In all, 1690 and 1697 indels were found in P5 and P24, respectively, compared with the reference strain. Of the total number of indels, 823 were common to both strains. When examining the distribution of private indels to each strain along chromosomes, some (chromosomes X, XI, XV and XVI) showed an enrichment of variants. Only 12% of the unique indels for each strain were within the coding sequence. Within ORFs, the indels with lengths that were multiples of three were highly enriched when compared with the noncoding sequence. This is consistent

with strong purifying selection against frameshifts. Those indels in the coding sequence with lengths that were not multiples of three were located mainly in functionally uncharacterized genes. This confirmed that this group of genes was, on average, under lower purifying selection pressure (Bergström et al., 2014).

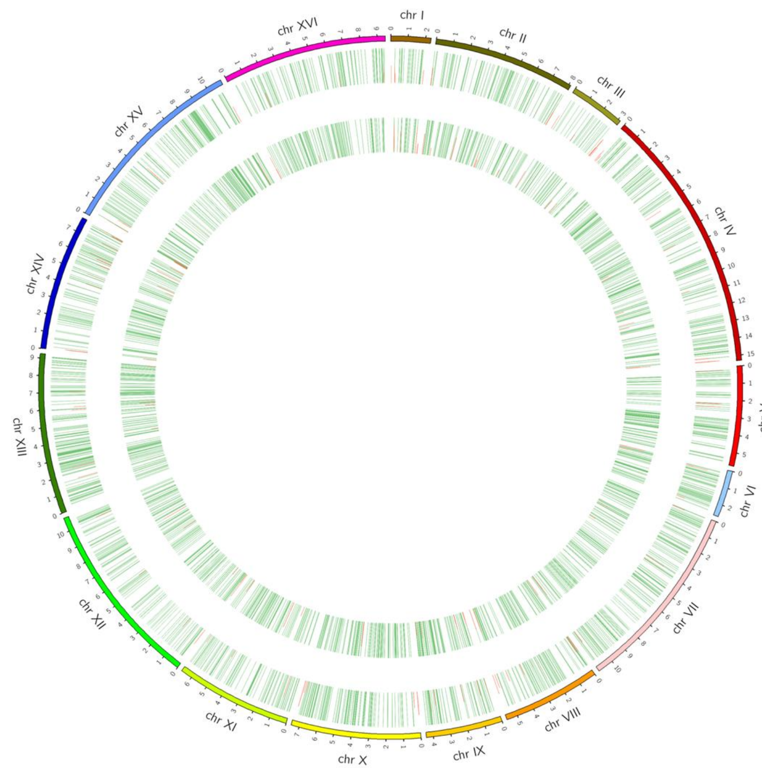


Figure S2: Distribution of private nonsynonymous SNPs in P5 and P24 compared to S288c. An external circle indicates P24 and an internal circle indicates P5. Homozygous changes are colored in green, while heterozygous changes are marked in red.

We also detected the copy number variation of the genomic regions between both strains (CNV). Ninety-three CNV longer than 900 bp were detected across the genome comparison made between both strains. Figure 1B shows the copy number variation (CNV) across chromosomes. Although there were some regions with variation, only four were larger than 4 kb. These four regions were located in chromosome IV and in the subtelomeric regions of chromosomes VIII, XV and XVI. Most of these CNV were classified as transposable elements and subtelomeric regions. These results are in line with previous observations (Bergström et al., 2014), which found very limited CNV in nonsubtelomeric regions and extensive variation in subtelomeric regions.

Genetic and phenotypic characteristics of segregants

To study the genetic diversity of segregant populations, five differential SNPs of chromosome III in 30 segregants of F6 were genotyped. A recombination frequency of 0.39 was assessed, and there was an average of 10 haplotypes per SNP (data not shown). After 13 rounds of segregant intercrosses (see Material and Methods), the F13 segregants were screened for their low temperature adaptation by calculating the maximum growth rate of each segregant and the two parental strains (Figure 2A).

Transgression levels, that is, the percentage of segregants that exceed the phenotypic range of their parents by at least 2 standard deviations (Marullo et al., 2006), can provide insight into the genetics that underlies complex traits. 16% of transgressive segregants better performed if compared with the superior parental, while just 0.66% of segregants presented a lower transgressive value than the inferior strain. Trait heritability was over 65%, which indicates the importance of genetic determinism under our conditions. An example of the growth curves of two transgressive segregants is offered in Figure 2B.

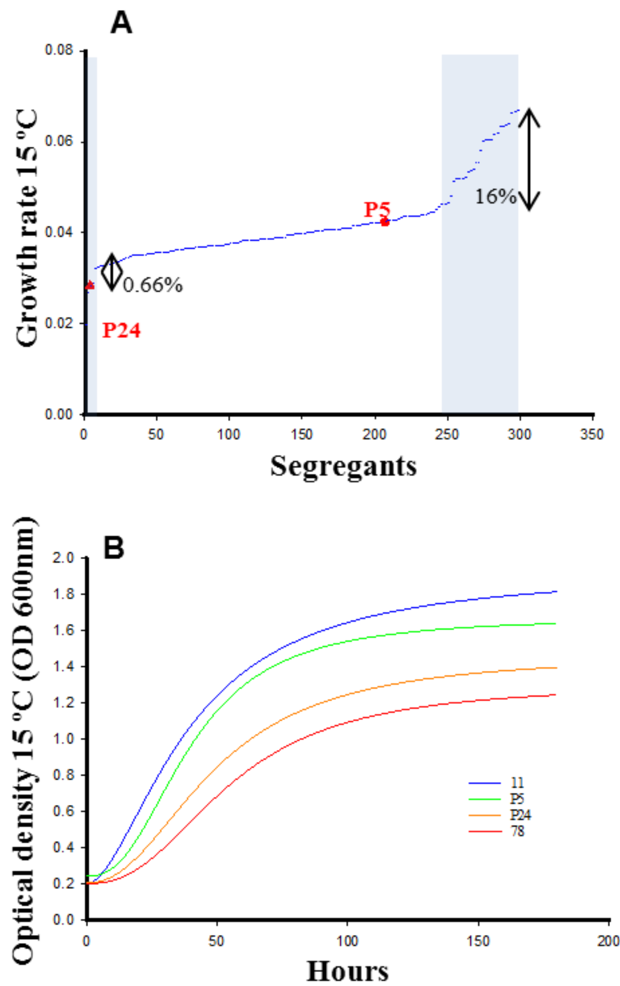


Figure 2. Growth rate variation at low temperature. (A) Growth rate values at 15 ºC are shown on the y-axis for 300 ranked segregants. Blue indicates transgressive phenotypic space, and segregants with transgressive phenotypes (exceeding two parental standard deviations). Red dots indicate parental strains. (B) Detailed growth curves of parental and transgressive segregants (11 and 77) with extreme traits in the P5xP24 cross.

Low-temperature selection of segregant populations

The F13 diploid population was subjected to a selection experiment (Figure S3) at 15 and 28 ºC for approximately 50 generations (8 rounds of batch serial dilution). Figure 3 shows the improvement in the growth rate (h^{-1}) of the selected population (SP) compared with the F13 population. At low temperature, the growth rate of the selected population improved in both YPD (97%, Figure 3A) and SM (66%, Figure 3C). The more restrictive growth conditions in SM might prevent better improvement in temperature selection terms. The growth rate improvement in synthetic must was also important as the mean generation time of F13 population diminished in approximately 7 h. The control population, which was cultured at 28 ºC, was also phenotyped in both media. The YPD selected population at 28 ºC showed an improved growth rate (57%, Figure 3B). No significant differences were obtained for the SM selected population at 28 ºC (Figure 3D).

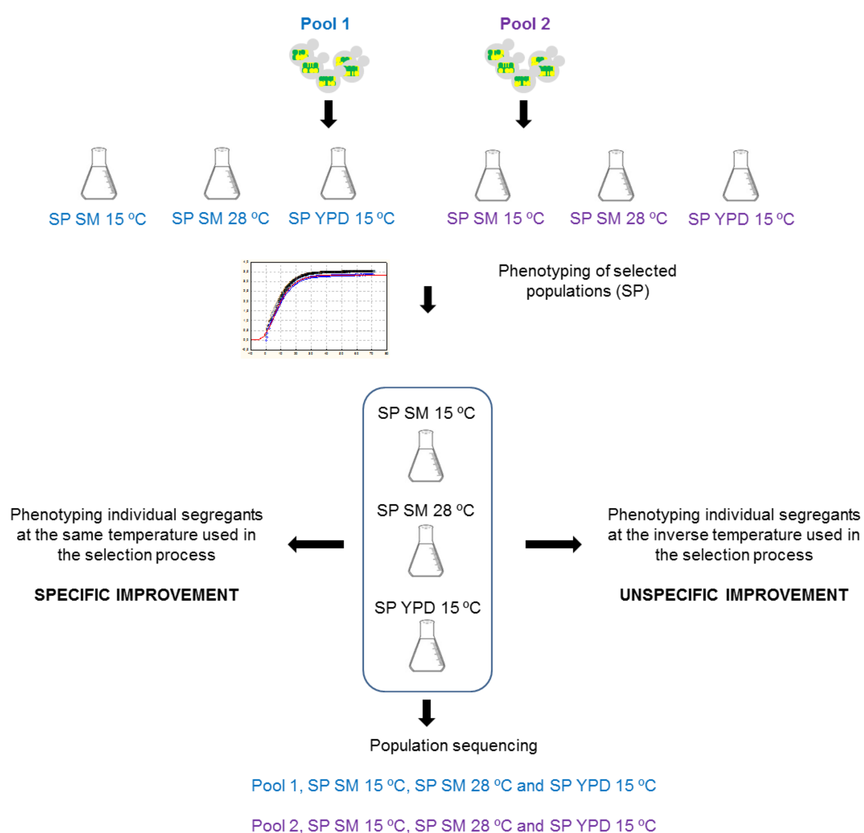


Figure S3: Workflow of populations' selection and sequencing. Cells were grown in complete media (YPD) and synthetic must (SM), and were incubated at either optimum temperature (28 ºC) or low temperature (15 ºC) until the stationary phase was reached. At this time, the volume required to inoculate at an OD of 0.2 was re-inoculated into 60 mL of fresh medium. The experiment was carried out 8 times after which the selected populations were analyzed and sequenced.

To study if the improved growth rate in populations was due to either the temperature selective pressure or an unselective stress, each set was exposed to the opposite temperature to which they were selected. This meant that the SP at 15 ºC was grown at 28 ºC and that selected at 28 ºC was grown at 15 ºC, and their growth rates were also compared with the F13 population (Figure S4). No significant differences were found between the populations in either YPD (Figure S4A-B) or SM (Figure S4C-D).

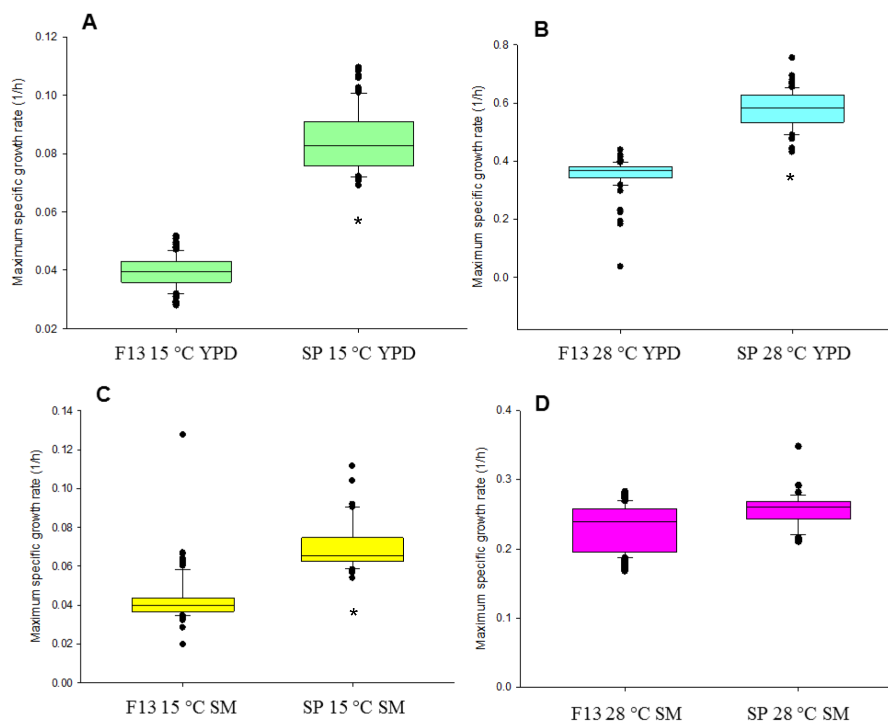


Figure 3. Phenotyping (growth rate) of the hybrid population after the selection experiment compared with the unselected F13 population. Selected

population (SP) in the YPD medium (A) and synthetic must (SM) (C) at 15 ºC. Selected population (SP) in YPD (B) and SM (D) at 28 °C. *Significant differences compared with the control population.

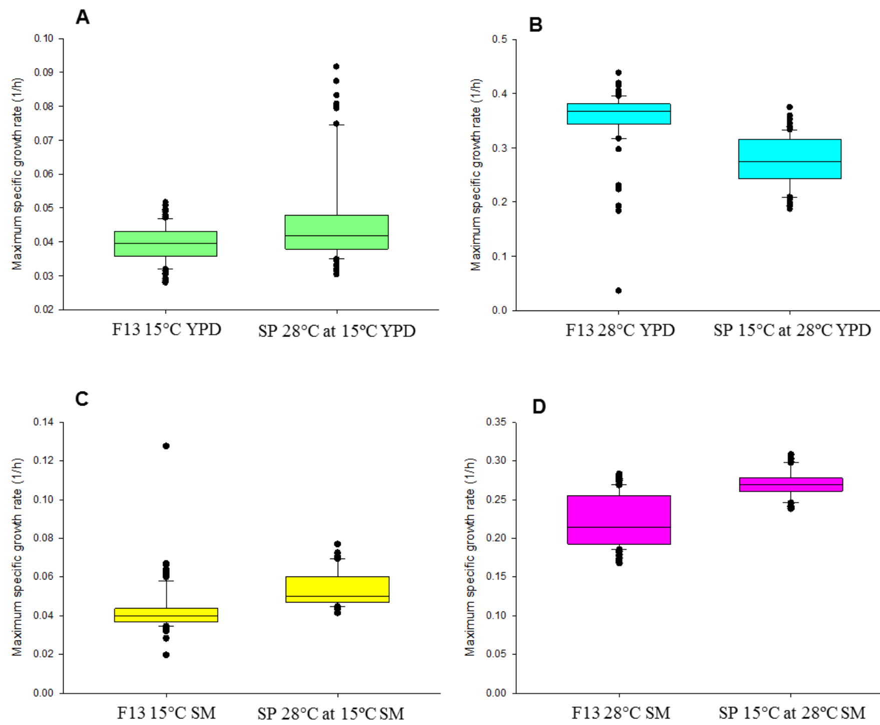


Figure S4: Hybrid population phenotyping after the selection experiment compared with the unselected F13 population using the opposite temperature to that used during the selection process (nonspecific improvement). The selected population (SP) in the YPD medium (A) and synthetic must (SM) (C) at 15 ºC. The selected population (SP) in YPD (B) and SM (D) at 28 °C.

Allele frequency analysis reveals that four QTLs are related to low-temperature adaptation during alcoholic fermentation

A pool of the selected populations at 15 ºC in YPD and SM and at 28 ºC in SM were whole-genome sequenced and compared with the whole-genome sequence of a pool of the F13 population before selective growth at both temperatures. The allele frequency analysis allowed us to map the QTL intervals responsible for phenotypic variation. Four distinct QTLs for the three assayed conditions (15 ºC in YPD, 15 ºC in SM and 28 ºC in SM) were found (Figure 4). QTLs were detected for 28 ºC (orange triangles) on chr I, for 15 ºC in YPD (red triangles) on chr XVI and for 15 ºC in SM (green triangles) on chr XIII, XIV, XV and XVI. The QTL found at low temperature in YPD overlapped with that located in chromosome XVI at low temperature in SM. This finding suggested that this region could be important for low-temperature adaptation, and independently of media. Except for the QTL located in chromosome XIV, all the others were located in the subtelomeric regions. These results reinforce the hypothesis that subtelomeric regions are a major source of the divergence of genome sequences and gene content in *S. cerevisiae,* and directly contribute to strain-specific adaptation processes.

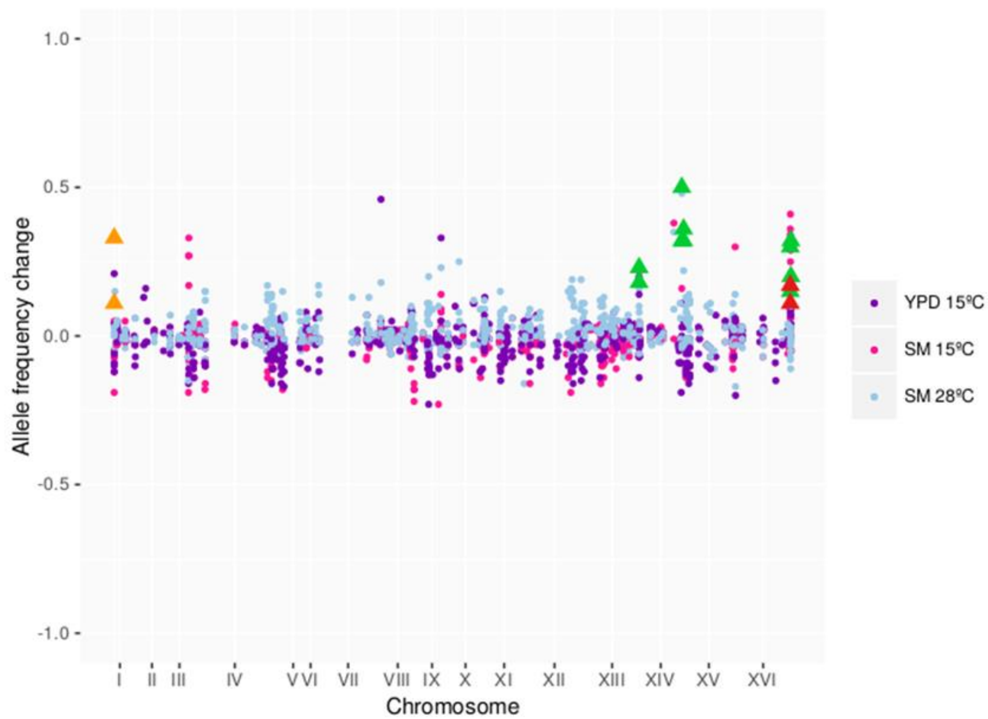


Figure 4. QTL analysis for low-temperature adaptation. Genomic DNA samples were extracted from an unselected pool (F13) and three pools of segregants were selected at 15 ºC (YPD and SM) and 28 ºC (SM). The figure shows the allele frequency change of the three selected pools compared with the unselected population. QTLs are indicated at the corresponding positions with orange triangles (28 ºC SM), red triangles (15 ºC YPD) and green triangles (15 ºC SM).

Validation of the QTLs detected in subtelomeric regions by reciprocal hemizygosity (RH) analyses

Subtelomeric regions are difficult to sequence and assemble due to their wide variation, duplication levels and shared homologies between different chromosome ends. Thus subtelomeres are generally incomplete in most genome projects, which precludes the identification of causative genes of a trait in these regions (Liti and Louis, 2012). To validate the involvement of subtelomeric regions as QTLs of low-temperature adaptation, two hemizygous diploid P5/P24 hybrid strains were constructed, which retained a single copy of the subtelomere from either the superior (P5) or inferior parent (P24), while the other copy was deleted. Subsequently, these strains were tested during low-temperature fermentations to estimate the phenotypic differences between the two reciprocal hemizygotes of each subtelomere (Figure 5). Fermentation activity was estimated by calculating the time required to ferment 100% (T100) of the sugars in the SM at 15 and 28 ºC. The fermentation activity of the hemizygous strains is presented in Figure 5 as the relative T100 compared with hybrid P5/P24. The parental origin of the subtelomere produced an opposite impact on fermentation activity in most cases. Thus the absence of the P5 right XVI-subtelomere caused a long delay of the end of fermentation at 28 ºC and the inability to

finish it at a low temperature. Conversely, lack of the same region that belonged to P24 significantly (p-value ≤0.05) improved fermentation at low temperature compared with the control hybrid strain P5/P24. Thus the P5 XVI-subtelomere must contain genes of paramount importance for fermentation activity in this strain, regardless of the temperature applied in the process. However, this same region cannot be very important in the P24 strain because its absence resulted in a haploproficient phenotype at low temperature.

Similarly, absence of the P24 right XIII-subtelomere caused a significant delay in the fermentation kinetics at both 15 ºC and 28 ºC, whereas absence of the same region that belonged to P5 significantly improved fermentation at low temperature. The hemizygosity of the left XV-subtelomere was the only one to have an impact on fermentation activity at low temperature. Absence of this region in P5 significantly delayed the end of the fermentation at 15 ºC, whereas lack of the P24 region improved this fermentation activity at low temperature.

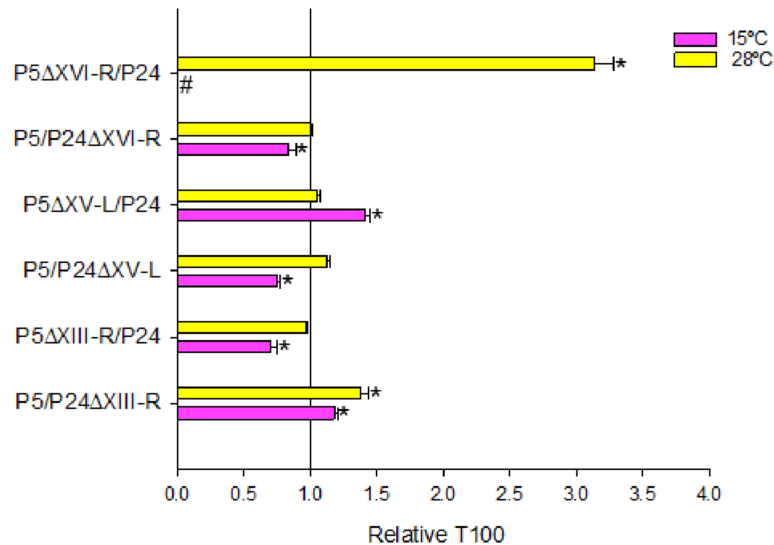


Figure 5. RH analysis of the QTLs detected in subtelomeric regions.

Fermentation kinetics of the hemizygous P5/P24 diploid hybrids retained a single copy of the subtelomere (XIII-R, XV-L and XVI-R) either from the superior (P5) or inferior parent (P24), while the other copy was deleted. The fermentation activity of the hemizygous strains is presented as the relative T100 compared with hybrid P5/P24. The T100 value was compared with the control normalized as value 1. *Significant differences compared with the control at the same temperature. # Indicates stuck fermentation before T100.

Identification of the causative genes of chromosome XIV QTL

The QTL located in chromosome XIV was the only one not to be located in a subtelomeric region. The four genes (*AGA1*, *COQ2*, *FPK1* and *PET494*) closest to the mapped QTL were selected for carrying out reciprocal hemizygosity analyses (Figure 6A). *MVD1* and *TRM112*, also in this region, were not selected because they are essential genes in the background of BY4741 strains. *AGA1* is an agglutinin involved in sexual reproduction whose deletion has no effect on fermentation activity (data not shown). However, the hemizygous hybrid strains of the other three genes clearly differed in fermentation activity at low temperature (Figure 6B).

PET494 and *COQ2* are mitochondrial proteins whose presence in hemizygosity produces impaired fermentation activity in both strains and at both temperatures, but mainly at a low temperature. *PET494* is a translational activator of one of the subunits (*COX3*) of cytochrome c oxidase (Naithani et al., 2003) and *COQ2* encodes a transferase that catalyzes the second step in ubiquinone (coenzyme Q) biosynthesis (Ashbysb et al., 1992). The deletion of both genes provokes the absence of respiratory growth.

Finally, *FPK1* is a Ser/Thr protein kinase that phosphorylates several aminophospholipid translocase family members (flippases) by regulating

phospholipid translocation and membrane asymmetry. The reciprocal hemizygote that carries the P5 allele has no effect on the fermentation kinetics at any temperature. However, the hemizygous strain that carries the P24 allele caused a substantial delay (~340 hours) in low-temperature fermentation. The impaired fermentation activity of P5 Δ*fpk1*/P24 was also confirmed by a spot assay (Figure 6C), which also showed an important growth defect of this hemizygous strain at low temperature. P24 has a substitution R520K in this gene that could be the cause of the inferior phenotype of this strain at low temperature.

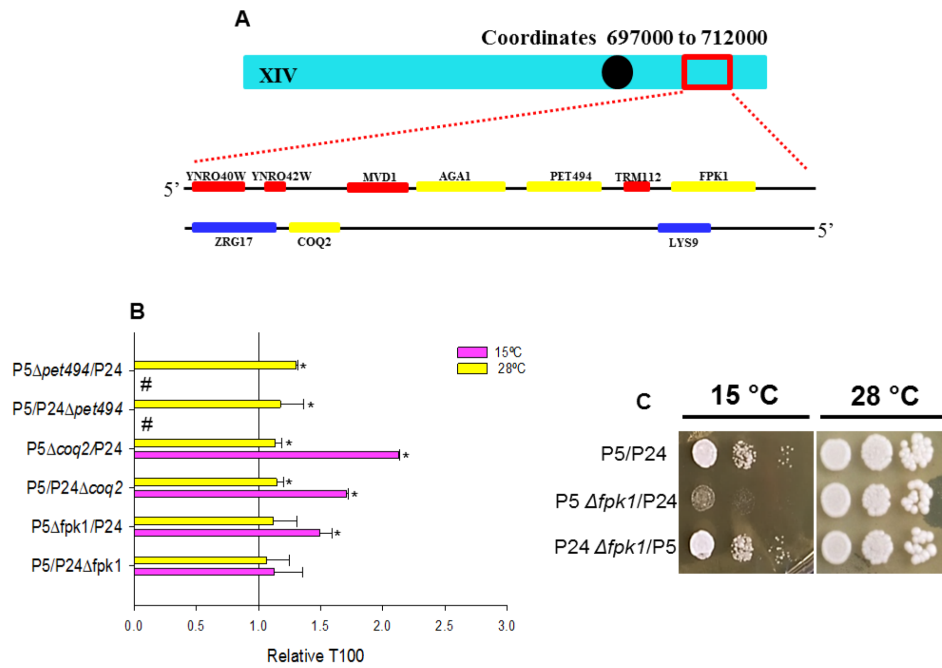


Figure 6. Identification of the causative genes in the QTL of chromosome XIV. (A) Distribution of the genes present in the QTL of chromosome XIV. The genes selected for RHA are colored in yellow. (B) Fermentation kinetics (T100) in hemizygous hybrids P5/P24 of genes *FPK1*, *COQ2* and *PET494*. The T100 value was compared with the control normalized as value 1. *Significant differences compared with the control at the same temperature. # Indicates stuck fermentation before T100. (C) Spot test between the control hybrid and the hemizygous deletion of the *FPK1* gene.

RH analysis of wine-lab strain hybrids for the presumptive individual genes contained in the QTL subtelomeric regions

The truncation of subtelomeric regions involved the deletion of some genes not connected with low temperature. As we have information only on the genes present in the first sequenced strain (S288c), in which special efforts were made to clone and sequence each telomere region, we used the sequence available in the *Saccharomyces* Genome Database (SGD: http://www.yeastgenome.org) to perform a large-scale RH analysis with the genes known to be present in subtelomeric regions (~20-30 Kb), and those that are not essential (Table S2). The haploid single gene deletion strains in the background of the BY4741 lab strain were crossed with the stable haploids of each parental wine strain by constructing hemizygous hybrid lab/wine strains (BY4741/P5 and BY4741/P24). The fermentation capacity of the resulting hemizygotes, which contained only the wine strain allele of each individual candidate gene, were tested on SM at 15 and 28 ºC and compared to the corresponding capacity of the lab/wine hybrid with both parental alleles intact (Figure 7). This strategy allowed us to compare the fitness of the different wine alleles and to attempt to discriminate the contribution made by each gene to the phenotype.

The RH analysis of individual genes showed both haploinsufficient and haploproficient strains in low-temperature fermentation activity. Haploproficiency meant that only the retention of the wine allele sufficed to improve the fermentation fitness of this hemizygous strain. Most haploproficient strains retained the P5 allele, which reinforces the superior fitness of this strain to grow and ferment at low temperature. Conversely, haploinsufficiency denoted a major function in low-temperature adaptation because a drop in the gene-dose impaired the fitness of the hemizygous strain. Most of these hemizygous strains had either a minimal or null impact on fermentation activity at 28 ºC.

In the chromosome XIII (Figure 7A), the genes whose hemizygosity produced haploinsuffiency in the hybrid of both wine strains were *YMR316C-A*, *DIA1* and *ADH6*. *YMR316C-A* and *DIA1* are proteins of unknown function whose ORFs overlap. *ADH6* is an alcohol dehydrogenase involved in alcohol, aldehyde, and furaldehyde metabolism. In the XV-subtelomere (Figure 7C), only two genes could be analyzed because the other genes contained in this region were essential. The hemizygosity of the protein of unknown function, *YOL159C,* severely affected the T100 in both hemizygous hybrid strains. Eleven genes were analyzed in the XVI-subtelomere region (Figure 7D). The hemizygosity of *ARR3*, a plasma

membrane permease that transports arsenite and antimonite, affected fermentation activity in both hybrid strains. Genes *QCR2*, *AQY1*, *YPR197c* and *ARR1* impaired only the low-temperature fermentation activity of the hemizygous P5/BY4741 strains, while genes *OPT2*, *YPR195c* and *YPR196c* affected this fermentation fitness at 15 ºC in the hemizygous P24/BY4741 strains. *QCR2* is also a component of the mitochondrial inner membrane electron transport chain and *AQY1* is a spore-specific aquaporin. Finally, *OPT2* is an oligopeptide transporter with a described role in the maintenance of lipid asymmetry between the inner and outer leaflets of the plasma membrane.

The same RHA analysis was also performed with the genes contained in the nonsubtelomeric QTL detected in chromosome XIV (Figure 7B). This analysis confirmed the relevance of the *FPK1* allele in the superior fitness of the P5 strain because hemizygous P5/BY4741 showed better fermentation activity, whereas hemizygous P24/BY4741 significantly delayed the end of fermentation. Furthermore, the importance of *PET494* and *COQ2* in low-temperature adaptation was also supported because both hemizygotes showed impaired fermentation activity at 15 ºC.

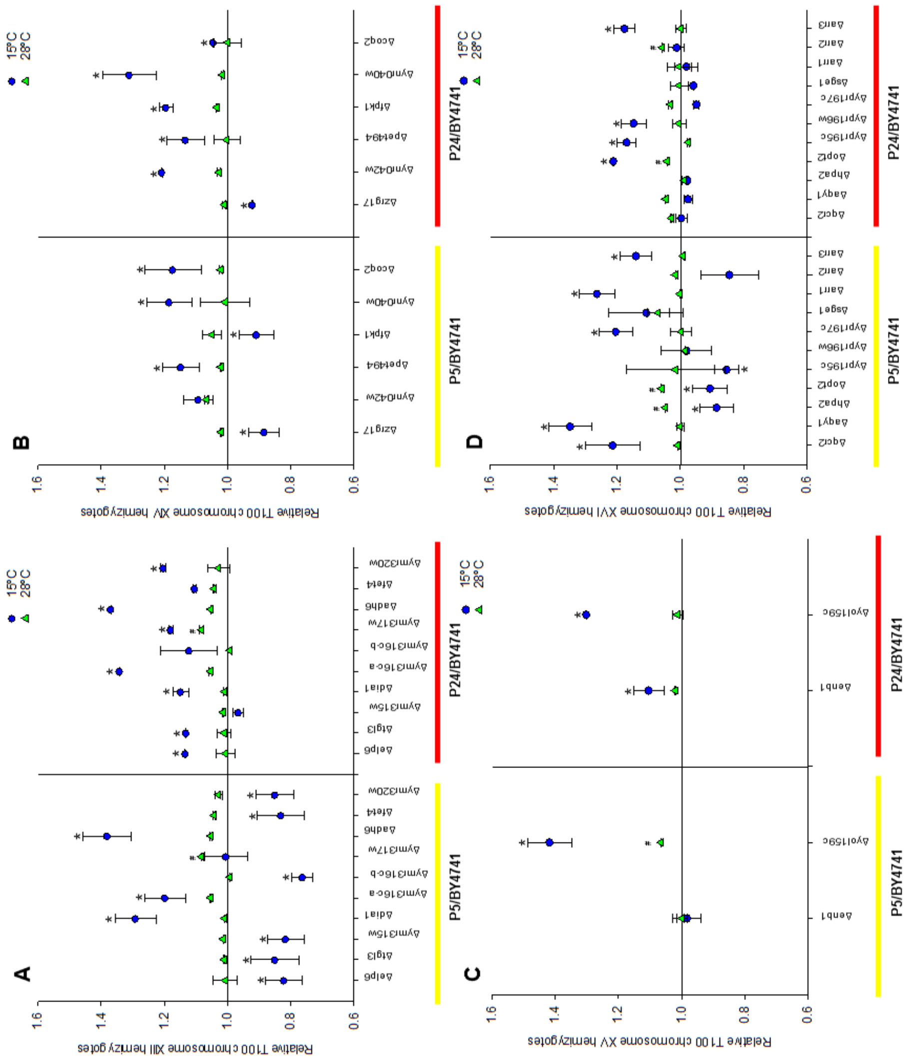


Figure 7. RH analysis of the genes present in the QTL regions of chromosomes XIII (A), XIV (B), XV (C) and XVI (D). Hybrids were constructed using the mutants of the BY4741 collection and haploid parental strains. T100 is the time needed to consume the total amount of sugars present in must. The T100 value was

compared with the control normalized as value 1. *Significant differences compared with the control at the same temperature.

4. Discussion

Temperature had a strong effect on many life history traits, including growth, development and reproduction (Lendenmann et al., 2016). To elucidate the genetic basis that underlies low-temperature adaptation, we followed a method that began with a hybrid generated by crossing two strains with a divergent phenotype. This hybrid was used to generate segregating populations of a very large size, which were selected at environmental pressure (low temperature). Finally, QTL mapping was performed by identifying the regions of allelic enrichment by sequencing the segregant populations. This strategy has been previously applied to identify the QTLs responsible for heat tolerance in *S. cerevisiae* (Ehrenreich et al., 2010; Parts et al., 2011; Yang et al., 2013), but not to detect the genetic structure of low-temperature adaptation. Our study has also some particularities in that it makes it different to other similar approaches.

Most QTL analyses use strains that are highly divergent with a high density of segregating sites. Liti et al., (2009) reported the presence of five genetically diverged clean lineages in *S. cerevisiae* (populations that do not

interbreed) and Parts et al., (2011) stated that the strains which belonged to clean lineages are ideal for linkage analyses as they have an even distribution of segregating sites across the genome, and these polymorphisms have coevolved in a specific genomic context. However, two strains that belonged to the same lineage were crossed (Wine/European) and were very close phylogenetically, as evidenced by the whole-sequence analysis of both strains. No more than 15% of the SNPs detected in both strains compared with the reference S228c were private mutations. While the low density of segregant sites between both strains proved to be an added difficulty for the QTL analysis since information about neighboring sites could not be used for more accurate allele frequency inferences, the strategy of intercrosses of segregants during the multiple generations that broke up linkage groups allowed individual QTLs to be determined and reduced to small numbers of variants (Liti and Louis, 2012; Parts et al., 2011).

Another different feature of our study compared with the commonly used bulk segregant analyses was the individual characterization of the 300 F13 segregants at their growth rate at low temperature. Segregants exhibited a wide range of cold tolerance phenotypes, but also a high percentage of these segregants displayed heterosis (hybrid vigor). This result revealed that

the arrangement of alleles between both strains produced some combinations that improved fitness for it to become superior to parentals. This suggests that both strains contained alleles which contributed to the cold tolerance phenotype of these transgressive segregants. However, this result was also very interesting for the industrial exploitation of these strains. The intercross during many generations of two industrial strains and the further selection of fitter genomic combinations by growth during several generations under selective pressure is a good genetic improvement method. These improved strains may be rapidly transferred and easily accepted by industry, which rejects the use of GMOs.

Our QTL analysis revealed the importance of the subtelomeric regions that contained a genetic variation responsible for traits of interest. Subtelomeric regions are generally incomplete in most genome projects. However, these regions cannot be ignored because, in yeast, 25% of all QTLs for many assessed traits map to beyond the last markers available. Yet the region contains only 8% of the genome (Cubillos et al., 2011). Subtelomeric gene families evolve faster than their internal counterparts, and subtelomeric regions are more frequently sites of gene duplication (Ames et al., 2010), which suggests a unique role of subtelomeres as hotbeds for genomic evolution and innovation (Brown et al., 2010). The

whole-sequence analysis of parental strains also supports the idea that some of this variation might be due to the copy number of the genes present in different subtelomeres (Liti and Louis, 2012).

The hemizygotic truncation of one copy of the subtelomere in isogenic hybrid strain P5/P24 had antagonistic effects depending on the parental origin of this region. Thus the P5 right subtelomere of chromosome XVI must contain genes of much importance for wine fermentation, regardless of the temperature of this process. Conversely, the same region in P24 must not comprise these fermentative genes as some gene has a detrimental effect on the fermentation activity at low temperature. However, the most paradigmatic example of genomic regions that confer superior fitness to the P5 strain at low temperature is the left subtelomere of chromosome XV. The fermentation performance of the hemizygous strain that lacked the P5 copy was clearly affected at low temperature, but not at 28 ºC, and the hybrid strain that carried the P5 copy in hemizygotes significantly improved. Application of new long read sequencing technologies would reveal genes residing in these subtelomeric regions, and allow identifying the causal genes involved in adaptation at low temperature.

We detected only a nonsubtelomeric QTL in chromosome XIV. In this case, the RH analysis showed two mitochondrial proteins, which are essential for respiratory growth and extremely important in fermentation activity at low temperature. Although we may think that the absence of respiratory metabolism could not be important during a process with a dominant fermentative metabolism, it is well-known that the yeast strains which lack the mitochondrial function are sensitive to oxidative stress caused by reactive oxygen species (ROS) (Grant et al., 1997). We recently proved that cells grown at low temperature are also subjected to stronger oxidative stress (García-Ríos et al., 2014, 2016). The RH analysis also showed that gene *FPK1* was very important for endowing better cold adaptation to P5 strain. This gene regulates flippase activity, which establishes plasma membrane asymmetry by flipping specific phospholipids from the extracellular to the cytosolic leaflet. The continuous remodeling of the phospholipid composition of cellular membranes as a response to low-temperature fermentations has been widely reported (Beltran et al., 2006; Henderson et al., 2013a; Redón et al., 2011; Tronchoni et al., 2012b). The implication of *FPK1* in low-temperature adaptation could be due to an improvement in the ability to maintain plasma membrane asymmetry. Finally, the RH analysis of the BY4741/wine hybrid strains revealed the key role during low-temperature fermentations of a gene (*QCR2*) that encodes a

protein of the mitochondrial inner membrane and a gene (*OPT2*) involved in lipid asymmetry maintenance between the inner and outer leaflets of the plasma membrane, among others. This demonstrates the importance of both a fit mitochondria and the capacity of lipid remodeling in the plasma membrane for coping with low temperatures.

5. Conclusions

We followed a thorough strategy, previously described by Parts et al., (2011), to elucidate the molecular basis that determines fitness or better adaptation during wine low-temperature fermentations. The QTL analysis and further RH analyses of the detected QTLs proved the importance of subtelomeres as a source of variation in industrial yeast and the need to invest efforts in sequencing these regions with new sequencing technologies with long reads from single molecules. We detected the individual genes involved in adaptation at low temperature fermentation by highlighting the importance of a fit mitochondria, perhaps for coping with greater oxidative stress at low temperature, and the maintenance of correct asymmetry and phospholipid distribution in the plasma membrane. Altogether this information is very useful for industrial yeast exploitation because the mechanisms involved in cold adaptation can be used as important traits for

future selections of industrial cryotolerant strains or for their genetic improvement.

AVAILABILITY OF SUPPORTING DATA

The data set supporting the results of this article is available in the Sequence Read Archive (SRA) database repository SRP048919 (http://www.ncbi.nlm.nih.gov/sra/?term=SRP048919). The data set supporting the results of this article is included in the article (and its additional files).

CHAPTER 4

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

Estéfani García-Ríos, Amparo Querol and José M. Guillamón

Journal of Proteomics (2016) 146: 70–79

ABSTRACT

Temperature is one of the most important parameters to affect the duration and rate of alcoholic fermentation and final wine quality. Wine produced at low temperature is often considered to have improved sensory qualities. However, there are certain drawbacks to low-temperature fermentations, such as slow growth rate, long lag phase, and sluggish or stuck fermentations. 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 species grown at 12 and 28 ºC, 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 and 28 ºC 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 *Sc* strain might explain such greater fermentation activity.

Keywords: *Saccharomyces*, Low temperature, Quantitative proteomic, Translation

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 (Beltran et al., 2008; Torija et al., 2003) . However, lowering fermentation temperatures has its disadvantages, including prolonged process duration and a higher risk of halted or sluggish fermentation (Bisson, 1999). 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 (Aguilera et al., 2007; Sahara et al., 2002; Schade et al., 2004; Tai et al., 2007). 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* (Gamero et al., 2014; Masneuf-Pomarède et al., 2010) or the hybrid strains of *S. cerevisiae* x *S. kudriavzevii* (González et al., 2006, 2007), have been shown to better adapt to low-temperature fermentations during winemaking. *Saccharomyces kudriavzevii* has been isolated only from natural environments (Lopes et al., 2010; Sampaio and Gonçalves, 2008). However, previous physiological and enological works have indicated the huge advantage of fermenting *S. kudriavzevii* at low temperature (Tronchoni et al., 2009, 2012b). The cryotolerant character of these two species, *S. uvarum and S. kudriavzevii*, in comparison to *S. cerevisiae,* has been well-established (Salvadó et al., 2011b). 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 (López-Malo et al., 2013b; Tronchoni et al., 2014). The metabolome comparison of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* grown at 12 ºC 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 (López-Malo et al., 2013b). Complementarily, the transcriptomic comparison of *S. cerevisiae* and *S. kudriavzevii* at low (12 ºC) and optimum (28 ºC) 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 (Tronchoni et al., 2014). 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 ºC and 28 ºC. 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 (Castrillo et al., 2007; Regenberg et al., 2006) 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 (Tai et al., 2007). Differences in protein composition were assessed by the iTRAQ technique, which is a powerful proteomic method used to quantify relative protein levels (Ross et al., 2004). 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 (Casado-Vela et al., 2010). 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 (Chiva et al., 2012; Salvadó et al.,

2008) and metabolic adaptations (López-Malo et al., 2013b; Tronchoni et al., 2012b) 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. Materials and methods

Yeast strains and 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) (Lopes et al., 2010) were used in this work. The strains were grown at a dilution rate (D) of 0.04 h^{-1} at 12 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.

Cultures were grown in the synthetic grape must (SM) that derived from that described by Riou et al., (1997). 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). 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. Biomass and extracellular metabolites were constant for at least five volume changes before sampling. When the steady state was reached, a volume of approximately 30 units of OD_{600} was centrifuged at 10000 g for 3 min at 4 ºC. 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 pellet was flash-frozen with liquid nitrogen and stored at -80 ºC.

HPLC analysis

Glucose, fructose, glycerol and ethanol were analyzed in all the supernatant samples. 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-mm 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 H_2SO_4 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.

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 (Dukes and Butzke, 1998). The results were expressed as mg N mL^{-1}.

Protein Extraction

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). To the supernatant, 150 µL of

extraction buffer were added. Proteins were allowed to precipitate at -20 °C for 1 h; the precipitate was recovered after centrifugation at 10000 g for 15 min. The pellet was washed with the2-D Clean-Up kit (GE Healthcare). The final pellet was air-dried and solubilized in 25 µL of 7 M urea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 2 M Tiourea, 20 mM Tris and milliQ water. Insoluble material was removed by centrifugation (13000 rpm, 5 min). The protein concentration was determined by Bradford, with BSA as a standard.

Sample Preparation

Proteins were precipitated with TCA (50 µL; Cf=10%) over night at 5 ºC. After centrifugation, the final pellets were washed with cold (-18 ºC) acetone with vigorous stirring, followed by centrifugation. Pellets were air-dried. Pellets were dissolved with 75 µL of 8 M Urea in 500 mM TEAB, pH 8 (Sigma), with sonication (5 min at 0 ºC). 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 Laemlhi buffer and loaded in 1D PAGE. The gel was stopped without

resolving samples and each slide was cut and digested (Shevchenko et al., 1996).

The sample was subjected to trypsin digestion with 10 µg of Promega sequencing grade modified trypsin in 0.5 mM TEAB. The volume was adjusted to 70 µL, and the final urea concentration was <2 M. Digestion was left overnight at 37 ºC. 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. 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 then be incubated at ambient temperature for 3 h. Then 300 µg of the peptide mixture were dissolved with 200 µL of 7 M urea/2 M thiourea/1.6% ampholytes. One IPG strip (GE; 13 cm, 3-11 NL) was hydrated with the peptide solution o.n. at room temperature. Peptides were isoelectrofocused with 5000 V to 30000 V h. After focusing, the strip was washed with milliQ grade water and cut into 15 equal pieces. Peptides were extracted with 100 µL of the ACN solutions: A. 5% ACN 0.1% TFA, B. 50% ACN 0.1% TFA, C. ACN 0.1% TFA. All the peptide fractions were combined, dried by vacuum centrifugation and re-dissolved with 2% ACN 0.1% TFA. The sample was cleaned and

concentrated by POROS R2. The clean peptide mixtures were dried by speed vacuum and resuspended to a concentration of ca. 0.3 µg $µL^{-1}$ in 2% ACN 0.1% TFA.

Mass spectrometry analysis

Liquid chromatography and tandem mass spectrometry (LC–MS/MS): 5 µL of each sample were loaded into a trap column (Nano LC Column, 3µ C18-CL, 75 µm x 15 cm; Eksigen) and desalted with 0.1% TFA at 2µL min^{-1} for 10 min. Peptides were then loaded into an analytical column (LC Column, 3 µ C18-CL, 75 µm x 25 cm, Eksigen) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5a35% B in A for 90 min (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nl min^{-1}. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (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. 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 Expasy protein database (515203 sequences; 181334896 residues) with parameters iTRAQ Quantitation.

Trypsin specificity, cys-alkylation (IAM), no taxonomy restriction, and the search effort set to throughout. 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.

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 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.

Statistical Analysis

The metabolic data are the result of five replicates per fermentation (temperature and strains) while 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.helmholtzmuenchen.de/funcatDB/). 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) (Franceschini et al., 2013). 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

Metabolic profiles comparison among the three species

To investigate how the yeast proteome changed under a suboptimal low growth temperature, we characterized the metabolic profile of the three *Saccharomyces* strains at both temperatures. Our experimental design was based on continuous-culture fermentations at low and optimum growth temperatures. This system offers a stable controlled environment for cells by maintaining constant biomass and the concentrations of nutrients and products (Clement et al., 2011), which make the comparison between fermentation conditions and strains more feasible. All the cultures were grown at the same dilution rate (D = 0.04 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 and the concentration of the main compounds in the supernatant in the steady state.

According to Vázquez-Lima et al., (2014) this dilution rate corresponded to the late exponential phase of a standard wine fermentation in the batch mode at 28 ºC. 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 ºC, 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 ºC is also far away from the optimum growth temperature for this species (Salvadó et al., 2011b). Sugar consumption correlated quite well with biomass production, which was higher at 28 ºC. In spite of the cryotolerance of *Sk*, this species yielded the lowest biomass production at 12 ºC. 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.

Table 1. Physiological characteristics of *Saccharomyces* strains and extracellular metabolites in the steady state of continuous cultures. [a]Significant differences (p value ≤ 0.05) in each strain compared with their control conditions (28 °C), [b]Significant differences (p value ≤ 0.05) in each strain at 12 °C compared with the control condition (*Sc* 12 °C), [c]Significant differences (p value ≤ 0.05) in each strain at 28°C compared with the control condition (*Sc* 28 °C)

	Sc 12 ºC	*Sc* 28 ºC	*Sk* 12 ºC	*Sk* 28 ºC	*Su* 12 ºC	*Su* 28 ºC
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±0.14b	0.63±0.00[c]	1.30±0.05[a]	1.12±0.04

Differences in metabolite concentration, and in the consumption and production specific rates, were used to perform hierarchical clustering (Figure 1), which grouped *Sc* 28 °C and *Su* 28 °C in a subcluster, and *Sc*, *Sk*, *Su* 12 ºC with *Sk* 28 ºC in another one. Intriguingly, the closest metabolic profiles were *Sc* 12 ºC and *Sk* 28 ºC, which once again reveals the problems of this latter species to grow at 28 ºC. Likewise, *Sc* and Su grown at 28 ºC presented a very similar metabolic profile.

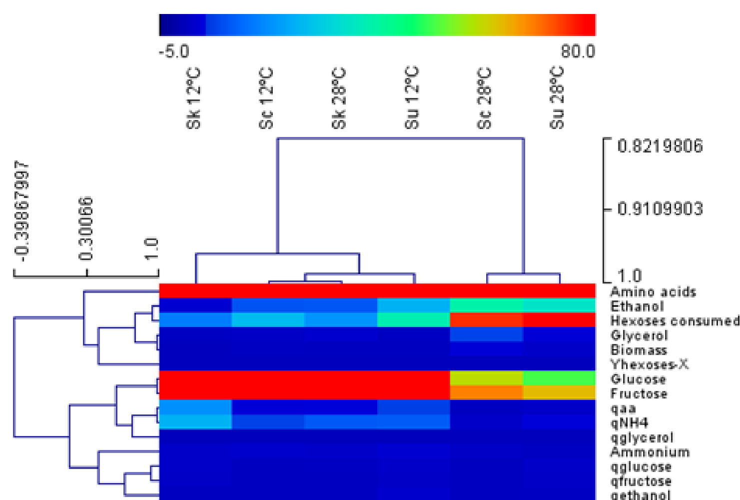


Figure 1. Global metabolic profile comparison of the three species. Hierarchical clustering of all the species at 12 and 28 °C.

Comparison of the proteomic profile at 12 and 28 ºC

By comparing the proteomes of the same strain at 12 and 28 ºC, 32, 129 and 226 significant proteins were identified for *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, respectively (Table S1). Figure 2 shows the relative abundance of the proteins that were over-represented at 12 and 28 ºC 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 *S.cerevisiae* (Figure 2A), the most strongly over-represented functional categories at 12 ºC were related to translation and proteins synthesis, together with glycolysis/gluconeogenesis, while at 28 ºC, the categories related more with purin nucleotide/nucleoside/nucleobase metabolism and amino acid metabolism. Likewise, *S.kudriavzevii* (Figure 2B) at 12 ºC showed a huge number of categories that related with translation and protein synthesis, and also with glycolysis and alcoholic fermentation. Conversely at 28 ºC, an over-representation of categories that

belonged to amino acid metabolism and G-protein-mediated signal transduction was observed. If we look at the *S. uvarum* (Figure 2C) analysis, an increase in the proteins that belonged to the translation category and also to oxidative stress response occurred at 12 ºC, whereas at 28 ºC the amino acid metabolism category (especially aspartate and the aromatic family) was the most important one.

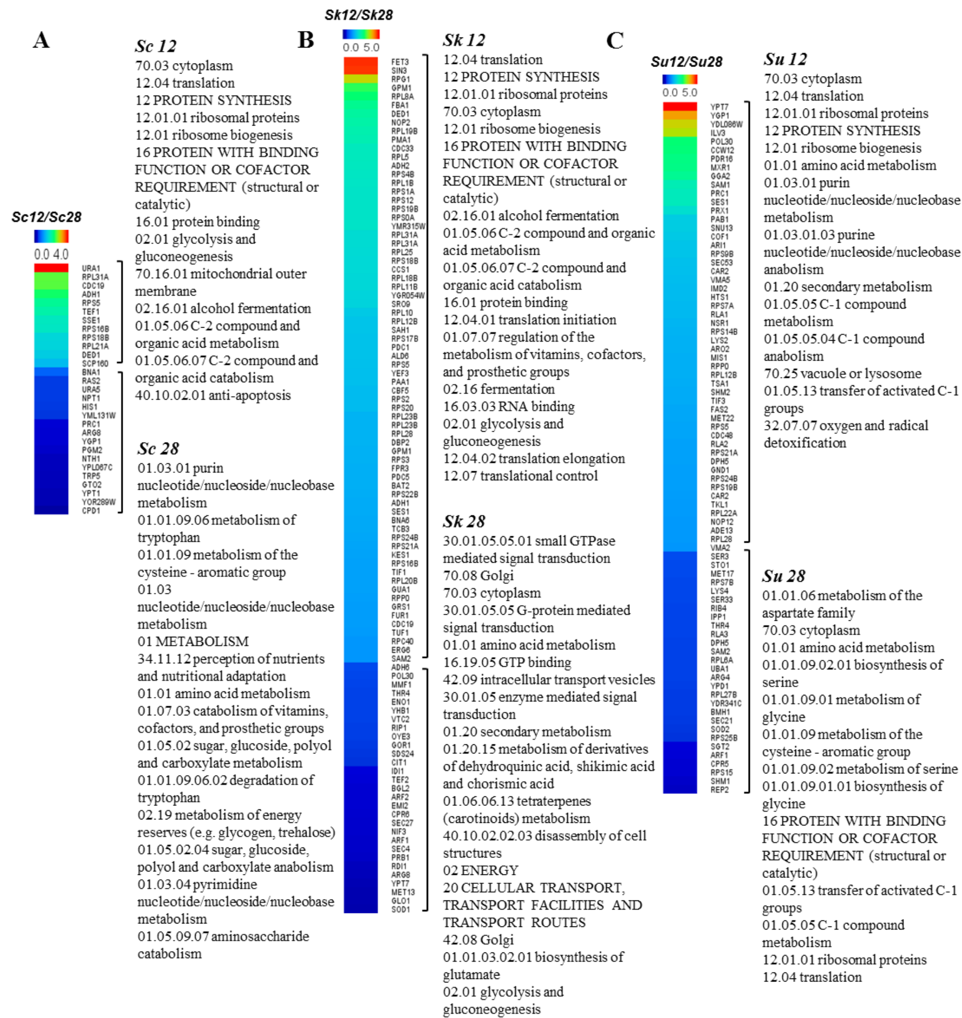


Figure 2. Heat maps depicting the significant concentration differences of proteins in the three strains when comparing 12 and 28 ºC. The enriched functional categories are sorted by level of significance.

The list of significant proteins in each *Saccharomyces* species was submitted individually to STRING 10 to elucidate the associations of these proteins (Figure 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 *S. cerevisiae* 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 *S. kudriavzevii* and *S. uvarum*, the common significant networks in both strains were ribosome, glycolysis/gluconeogenesis and biosynthesis of amino acids at both temperatures (Figures 3C to 3F). It is remarkable to note that the number of proteins that interconnected as a response to environmental temperature changes was much larger in *S. kudriavzevii* and *S. uvarum* than in *S. cerevisiae*, which may reveal the cryotolerant character of these species.

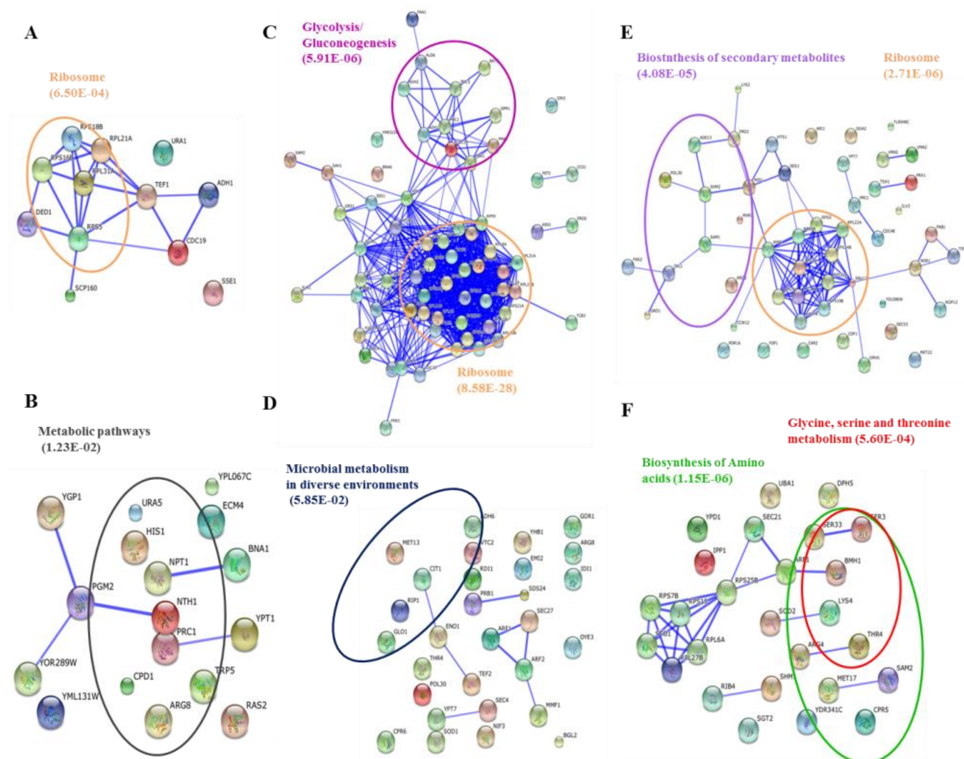


Figure 3. Network interactions of differentially expressed proteins at low temperature by a STRING analysis at the confidence level 0.7. *Sc*, *Sk* and *Su* are represented respectively by A-B, C-D and E-F. A-C-E indicate the over-represented proteins at 12 ºC and B-D-F the ones at 28 ºC.

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

Regarding the number of common proteins (Figure 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.

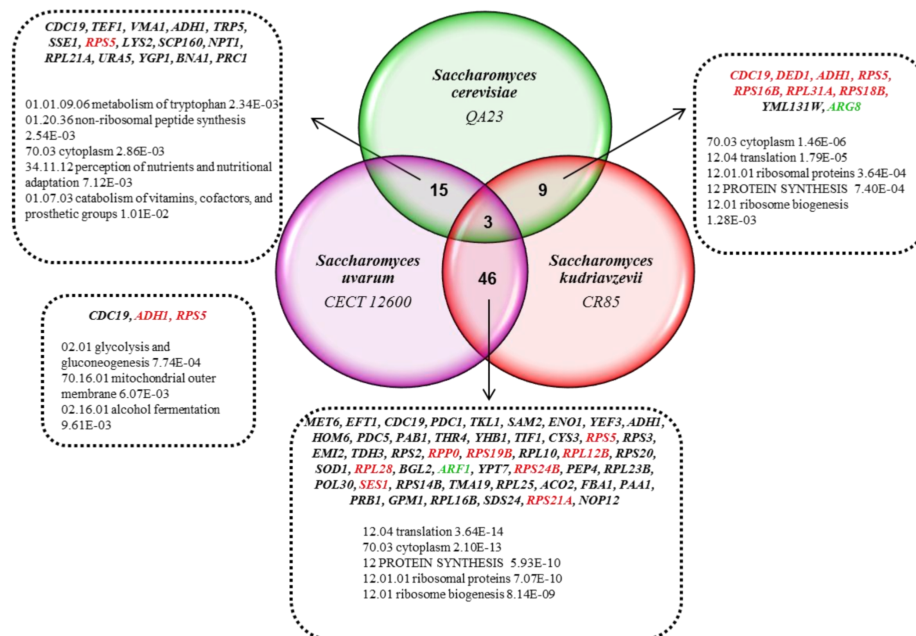
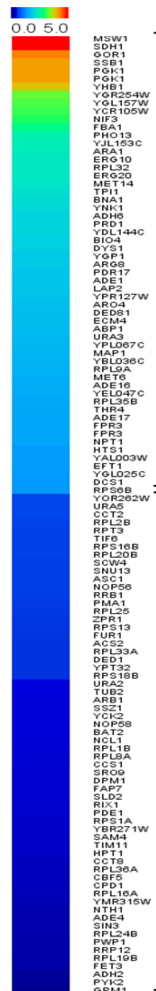


Figure 4. Overlap in the proteins regulated by temperature in the three strains. The proteins that are over-represented (red) and under-represented (green) are marked in the boxes.

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 ºC, respectively, (Figure 5). For *Sc/Sk* (Figure 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* (Figure 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 (García-Ríos et al., 2014). 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 (García-Ríos et al., 2014).



A

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
HOM6
INO1
CAR2
TKL1
PGI1
LYS2
GRE3
GLR1
YNL134C
BGL2
RPP2A
OYE2
EFT1
GSP1
IMD2
SOD2
LEU2
DCS1
HXK2
ERG13
EFB1
GPD1
RPS4B
PDC5
RPL4B
TRR1
TDH2
RPL9A
RPL5
RPS18B
MET17
GND2
RPS12
RPS5
SDS23
RPL14A
SRP1
ADH1
UBA1
RPL25
PGK1
SNU13

B

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/vitamine binding 1.03E-03
01.01 amino acid metabolism 1.36E-03
01.01.06.06.01 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
01.01.06 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

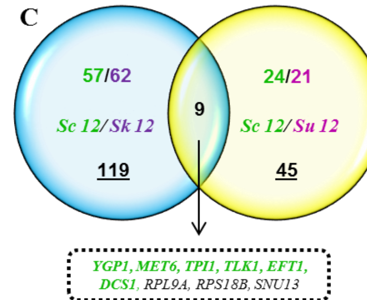


Figure 5. Proteomic landscape of the cryotolerant strains in the comparison made with *S. cerevisiae* at 12 ºC. (A) Heat map of the relative abundance of the protein in *Sc* and *Sk* at 12 ºC. (B) Heat map of the relative abundance of the protein in *Sc* and *Su* at 12 ºC. (C) Overlap in the proteins regulated by low temperature in both experiments.

Translation efficiency at low temperature

To demonstrate that *S. kudriavzevii* and *S.uvarum* adaptation to low temperature is related to enhanced translation efficiency; we tested its sensitivity to paromomycin, a potent translation inhibitor (Kurata et al., 2010). We studied the paromomycin (10, 20 and 40 µg) growth inhibition of yeast cells at either 12 or 28 °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.

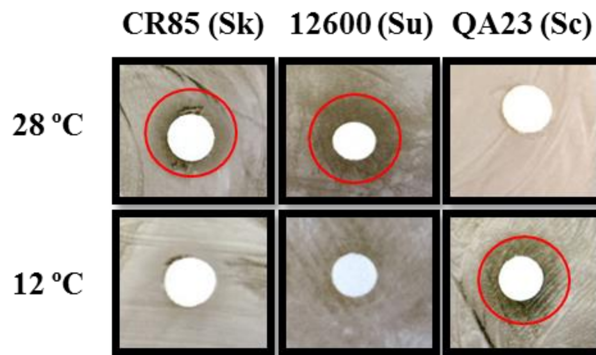


Figure 6. *S. kudriavzevii* and *S.uvarum* present increased translation efficiency at low temperature. The inhibitory effect of translation inhibitor paromomycin was evaluated by measuring the halo diameter generated in the three species lawns grown in YPD plates at 12 or 28 °C. It is only shown the result for the maximum paromomycin concentration (40 μg).

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., (2011) considered *S. cerevisiae* to be the most heat-tolerant strain within the genus *Saccharomyces*, with the highest optimum (32.3 ºC) growth temperature. *S. uvarum* and *S. kudriavzevii* were found to be the

most cryotolerant species of this genus, with the lowest optimum (26.2 and 23.6 ºC) growth temperatures. Our data of specific growth, consumption and production specific rates (Table 1) reinforce that *Sk* is the most psycrotolerant, 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, *S. cerevisiae* had the highest biomass yield. In spite of the fitness decrease in *S. cerevisiae* 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 with 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 (Tronchoni et al., 2014). Low

temperature causes hyperstabilization of RNA structures, which prevents the maturation of ribosomes and, consequently, the kinetics of translation initiation (Fortner et al., 1994; Hilliker et al., 2007; Li and Brow, 1996; Perriman and Ares, 2007; Staley and Guthrie, 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. 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 *S. cerevisiae*. Pizarro et al., (2008) 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.

This decrease in membrane fluidity might impair the activity of some permeases by modifying the profile of the up-taken amino acids (Abe and Horikoshi, 2000). 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 *TAT2* (Abe and Horikoshi, 2000). 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 bottle-neck produced by a non-optimal growth temperature. As Tronchoni et al., (2012) reported, a better adapted lipid membrane composition in *S. kudriavzevii* 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 (López-Malo et al., 2013b) of the three species at low temperature revealed that the main differences between the two cryophilic species and *S. cerevisiae* 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., (2014) used a genome-scale metabolic reconstruction of yeast metabolism combined with a thermodynamic analysis to identify the metabolic genes associated with cold adaptation in *S. cerevisiae* and *S. kudriavzevii*. 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 Δ*adh3* strain showed less fitness at cold temperatures, with *S. kudriavzevii* 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 (Paget et al., 2014). 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 specie was use and it is difficult to extend conclusions to the species level, 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. 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, *S. cerevisiae* 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 (Piskur et al., 2006), thus enabling better ethanol yields, which allow niche construction via ethanol production (Arroyo-López et al., 2010). A higher concentration of glycolytic and alcoholic fermentation enzymes in the *Sc* strain might explain such greater fermentation activity.

SUPPLEMENTARY MATERIAL

The additional files can be downloaded from:

http://www.sciencedirect.com/science/article/pii/S1874391916302627

GENERAL DISCUSSION

More than 26 billion liters of wine are annually produced worldwide, and winemaking plays a major role in the economies of many nations. Spain is one of the world's great wine producers: first in the ranking by surface, first by production of wine and grape juice in the 2014/2015 season, beating Italy and France and leader, in volume terms, in the export during 2015. Due to its importance in economic, social and environmental terms, as well as the importance of wine as image of the country abroad, the sector is of extraordinary importance in Spain.

Over the last century, yeasts with optimal characteristics were selected to inoculate fermentations with pure yeast cultures. Consequently, wine production quality vastly improved (Pretorius, 2000b). As the wine industry effectively controls fermentation temperatures, low temperature fermentation (10-15 ºC) is becoming more frequent in order to produce white and "rosé" wines with more pronounced aromatic profiles (Beltran et al., 2006; Llauradó et al., 2005; Molina et al., 2007; Torija et al., 2003). However, the optimal growth and fermentation temperature for *S. cerevisiae* is 25-28 ºC. Thus, there are certain drawbacks to low temperature fermentations, such as reduced growth rate, a long lag phase, and sluggish or stuck fermentations. To avoid this problem, it is important to study the

low temperature response, and to select or to develop well-adapted yeast strains.

The first and main objective of this thesis was to elucidate the physiological and molecular mechanisms involved in low temperature adaptation in the wine yeast *S. cerevisiae*. Low temperature adaptation in yeast, like most of the variable phenotype, has a complex genetic basis (Fay, 2013; Liti and Louis, 2012). It is a non-mendelian character controlled by more than a hundred of genes which makes its analysis more difficult. The use of the so-called “omics” techniques has allowed us to obtain a multidisciplinary snapshot of the cell while the adaptation process to this stress is happening. The employment of high-throughput methodologies, like transcriptomic, proteomic and whole genome sequencing, has enabled us to identify genes and genomic regions with changes that are contributing in different ways to the phenotype. Similar studies have previously attempted to elucidate the cold response in *S. cerevisiae* by a variety of high-throughput methodologies. Most of them, however, focused on cold shock on laboratory yeast strains (Homma et al., 2003; Murata et al., 2006; Sahara et al., 2002; Schade et al., 2004). A differential and novel feature of our study is the use of industrial or commercial strains of *S. cerevisiae* and in conditions mimicking industrial wine fermentations.

Phenotypic analysis and competition experiments

We have handled during the thesis work an important array of yeast strains that have been individually phenotyped, mainly in two parameters: maximum growth rate (μ_{max}) and fermentation activity. These strains showed a wide range of cold tolerance phenotypes, as it is expected of quantitative trait loci. This phenotypic analysis allowed us to select two strains with a divergent phenotype at low, but not at optimum, temperature, which were adopted as the model strains of this study. The fitness differences of the selected strains were corroborated by directly competing during fermentations at optimum and low temperature, proving the utility of the μ_{max} values, obtained during microtiter cultures, for calculating the fitness of different strains under specific conditions (García-Ríos et al., 2014).

A total of 300 segregants derived from a cross between the two selected strains were also phenotyped. These segregants also exhibited a wide range of growth rate at low temperature. However, the most remarkable result was the high percentage of trangressive segregants (16%), which showed better growth than the superior parental (P5 strain). This suggests that both strains contained alleles which contributed to the cold tolerance phenotype of these transgressive segregants. However, this result was also very interesting for

the industrial exploitation of these strains. The intercross during many generations of two industrial strains, and the further selection of fitter genomic combinations by growth during several generations under selective pressure, is a good genetic improvement method.

Physiological and metabolic mechanisms involved in adaptation at low temperature

One of the most interesting results of this thesis is the importance of the sulfur assimilation pathway at low temperature. So far, this metabolic pathway has not been related with cold adaptation. However, our transcriptomic and proteomic data clearly evidenced that differential activity in this route can explain differences in yeast adaptation at low temperature. The activity of this pathway can determine the cellular availability of two metabolites, SAM and glutathione, which can impact adaptation at low temperature by influencing two additional metabolic mechanisms: phospholipid metabolism and oxidative stress response. The importance of these compounds was corroborated by growth data improvement observed when these molecules were present in the culture medium.

S. cerevisiae cell membrane is considered as the first assaulting target of low temperature. Cold can influence the cell membrane integrity and function, thereby impacting on the exchange between environment and cell

and decreasing the cell viability. Moreover*, S. cerevisiae* cells might change their membrane compositions or structure to try to adapt to fluctuating temperatures. Previous studies have shown that temperature modifies the cellular lipid composition of yeast by increasing the degree of unsaturation at the beginning of fermentation, shortening chain length as fermentation progresses and a lowered ratio of phosphatidilcholine (PC)/phosphatidylethanolamine (PE) to increase the membrane fluidity (Beltran et al., 2008; López-Malo et al., 2013a, 2013b; Redón et al., 2011; Torija et al., 2003; Tronchoni et al., 2012). Our data corroborated the importance of a fit phospholipid (PL) biosynthesis and a proper distribution of these PL between the exoplasmic and the cytoplasmic leaflet of cell membranes. Previous works of our group (López-Malo et al., 2013a) showed the strong impairment of the mutants Δ*opi3* and Δ*cho2* at low temperature. These genes encode the enzymes involved in synthesis of the most important plasma membrane phospholipids, PE and PC, by the *de novo* pathway (Daum et al., 1998). In this thesis work, we have not directly detected genes of PL metabolism connected with a better performance at low temperature. However, the overexpression of the sulfur pathway may connect both metabolic routes. Phosphatidylcholine (PC), the major phospholipid (at least 30% of total PLs), is synthesized *de novo* from another PL, phosphatidylethanolamine (PE), in three SAM-consuming

methyltransferase reactions catalyzed by Opi3p and Cho2p (Chin and Bloch, 1988). In addition, the use of QTL mapping allowed us to identify and validate the relevance in cold adaptation of the genes *FPK1* and *OPT2*, both of them involved in lipid asymmetry maintenance between the inner and outer leaflets of the plasma membrane (PM). *FPK1* is a Ser/Thr protein kinase that regulates phospholipid translocation and membrane asymmetry and *OPT2* is an oligopeptide transporter that has a role in maintenance of lipid asymmetry between the inner and outer leaflets of the PM. Curiously the deletion of this gene also leads to major defects in maintaining peroxisomal, mitochondrial, and cytosolic glutathione redox homeostasis because gluthatione is synthesized *de novo* solely in the cytosol and must subsequently be transported to other cellular compartments through this permease (Elbaz-Alon et al., 2014). Thus, we cannot rule out the involvement of this gene in another important metabolic process in cold adaptation as it is stress oxidative defense.

One of the primary metabolic effects of the PM rigidification is an important impairment of the nutrient uptake, mainly the nitrogen compounds. Pizarro et al., (2008) reported that the physiological and transcriptional response of laboratory and wine yeast strains to stress at low temperature was similar to grow under nitrogen-limiting conditions. 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 (Abe and Horikoshi, 2000). In our data, we also detected a key role of a permease of amino acids in the fitness of a *S. cerevisiae* strain growing at low temperature. This is the case of *MUP1*, a high affinity methionine permease that is also involved in cysteine uptake (Kosugi et al., 2001). According to the important role of the sulfur assimilation pathway, the uptake of sulfur amino acids at low temperature can be also a limiting-step at low temperature, similar to the many evidences reported for tryptophan. Moreover, in the context of the proteomic study, one of the groups with differential concentration in the strains of the three species of *Saccharomyces* was related with the biosynthesis of amino acids, maybe as a cellular response to surpass a metabolic bottle-neck produced in this metabolic pathway. Finally, in a recent work of our group (Salvadó et al., 2016), 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. In this study, 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 the two proteomic studies carried out in this thesis (the comparison between P5/P24 and the comparison between the three species of *Saccharomyces*) showed the increase in the concentration of proteins of the glycolysis at low temperature, mainly enzymes of the lower part of this pathway (the trioses phosphate branch). According to Quirós et al., (2013), in the upper part of glycolysis (the glucose 6-phosphate branch point), the C flux directed to glycolysis lowered at low temperature, which resulted in a higher C flux to the pentose phosphate pathway (PPP) and carbohydrate biosynthesis. These authors related this diversion of the C flux to these two minor branches with the higher biomass synthesis also observed at low temperature. Biomass production is very high ATP-demanding. Thus, this higher biomass synthesis at low temperature may result in a shortage of intracellular ATP. The increase of glycolytic and fermentative enzymes leading to ATP generation may balance this drain. Another plausible explanation is the redox imbalance produced at low temperature. Recently, two independent studies (Ballester-Tomás et al., 2015; Paget et al., 2014) proved that low temperature produced a redox imbalance that needs to be corrected by the dynamic regulation of the NAD(P)/NAD(P)H ratio and the intracellular levels of these co-enzymes. Paget et al., (2014) corrected this redox imbalance by increasing glycerol accumulation or cytosolic acetaldehyde production by deleting *GUT2* and *ADH3*, respectively.

Ballester-Tomás et al., (2015) compensated this redox imbalance by overexpressing the *GDH2*-encoded glutamate dehydrogenase gene, which increased NADH oxidation. In both strategies, the *S. cerevisiae* strains displayed better fitness at low temperature, and both studies identified redox co-enzymes as key factors that governed yeast cold growth.

This last statement links directly with another of the most important physiological mechanisms observed in this work to cope with low temperature adaptation in wine yeasts. We refer to the “oxidative stress defense”. In all the studies of this thesis we have observed the clear trigger of this mechanism with the upregulation of genes and proteins involved in this defense. Despite not many reports have correlated low temperature and oxidative stress, in chapter 2, we stablished a strong correlation between the behavior of the cells at low temperature and in the ability to cope with a strong oxidative stress situation in presence of peroxide of hydrogen. This response against both stresses seems to have overlapping protective mechanisms. We have connected the greater activation of the sulfur assimilation pathway with the oxidative stress response. Cysteine is a component of molecules like glutathione, glutaredoxin and thioredoxin, which were all induced in response to oxidative stress (Sha et al., 2013), while methionine acts as a ROS scavenger (Campbell et al., 2016).

Although this data has not been shown in any of the chapters of this thesis, we quantified intracellular glutathione and, in both strains P5 and P24, the amount of this oligopeptide was greater at low temperature. The proteomic comparison between P5/P24 showed higher concentration of peroxiredoxin Ahp1p and thioredoxin Tsa1p in the strain better poised to deal with oxidative stress. According to previous studies, growth at low temperature produces redox imbalance, and probably raises the intracellular levels of ROS. However, as far as we know, there is not a clear hypothesis to explain this redox imbalance and ROS accumulation. By using the QTL mapping, we were able to identify genes related with the oxidative stress and mitochondrial functions. *COQ2* encodes a transferase that catalyzes the second step in ubiquinone (coenzyme Q) biosynthesis (Ashbysb et al., 1992) and *PET494* is a translational activator of one of the subunits (*COX3*) of cytochrome c oxidase (Naithani et al., 2003). Both genes belong to mitochondria and are essential for respiratory growth. However, the lack of these genes provokes a strong impairment in the fermentation activity at low temperature. We may think that the absence of two mitochondrial proteins would not be so important during alcoholic fermentation where the respiration is not the main metabolic process, however, our results show that are contributing in some way during the low temperature adaptation. Some authors have reported that defects in the mitochondrial functions give as a

result more sensitive strains to oxidative stress (Collinson and Dawes, 1992; Grant et al., 1997). It could be possible that the generation of ROS at low temperature together with the increased sensitivity in these mutants could produce an accumulative situation that the cell is not able to counterbalance. Another possibility could be that cold leads to a more ordered membrane structure and hence a reduction in fluidity of the mitochondrial inner membrane, affecting to the electron transport chains. In any case, our data evidenced the importance of a fit mitochondria to cope with the cold response.

Molecular and genetic basis underlying low-temperature adaptation

The molecular mechanisms involved in adapting *S. 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 (Sahara et al., 2002, Homma et al., 2003, Schade et al., 2004, Murata et al., 2006), but Tai et al., (2007) also analysed transcriptional changes during long-term exposure of yeast to low temperature.

At the gene expression level, transcription data have provided evidence for most of the adaptation strategies that have been previously described. As

mentioned above, we have seen upregulation or differential activity in genes involved in oxidative stress, lipid and amino acid metabolism, glycolysis, etc., when the cells were grown at low temperature. Moreover, the transcriptomic data also revealed the upregulation of many genes involved in translation. 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. In chapter one, we also found an up-regulation at low temperature of some genes belonging to functional categories related with the ribosomes and also higher concentrations of two proteins: *TEF1* and *RPL31A*. *TEF1* is a

translational activator while *RPL31A* is a part of the large subunit of the ribosome. In chapter 4, the proteomic comparison between the species of *Saccharomyces* showed that the improvement of the translation efficiency was the common mechanism of the three species, but differences in the number of proteins involved and in the effectiveness of this response were encountered. *S. kudriavzevii* and *S. uvarum* presented a strong proteomic response due to low temperature and this fact could be one of the reasons why these yeasts are better-adapted to cold environments. In our recent genome-wide study to detect genes that contribute to low temperature adaptation (Salvadó et al., 2016), two genes related to ribosome biosynthesis (*SNU66* and *PAP2*) were essentials for growing and fermenting 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).

Many of these genes that determine a better low temperature adaptation can be located in subtelomeric regions. Our QTL analysis revealed the importance of the subtelomeric regions, containing genetic variation responsible for differences in cold adaptation. Unfortunately, so far, the sequencing technology did not allow mapping the genes located in these regions, being generally incomplete in most genome projects. We are

currently involved in a project for sequencing again the P5 and P24 strains by new long read sequencing technologies. Hopefully, the sequencing data with this new technology would reveal genes residing in these subtelomeric regions, and allow identifying the causal genes involved in adaptation at low temperature.

Future work

In this thesis work, we have followed a holistic approach with the use of different high-throughput technologies to unravel the physiological mechanisms and molecular basis underlying low temperature adaptation in wine yeasts. This is a powerful strategy to provide a clear link between genomic and phenotypic differences among wine strains. We have been able to identify key pathways, enzymes and genes related to a particular phenotype adapted to grow at low temperature. In future work, we should go into depth of some of these mechanisms to understand much better their involvement in cold adaptation. Moreover, in our opinion, this information is very useful for obtaining strains more adapted to these high demanding industrial processes. Adaptation and tolerance of yeast strains to temperatures beyond the optimum range is crucial for economic and eco-efficient production processes for new and traditional fermentations. From an industrial perspective, such knowledge is important to come up with

better metabolic engineering strategies that take into account the impact of novel genes and pathways on cellular economics and to develop cost-effective bio-based processes. Accordingly, we will design rational genetic improvement strategies to obtain more robust and adapted yeast strains to grow and ferment at low temperature. For this strain improvement, non-recombinant strategies, based on evolutionary engineering and intra- and inter-specific hybridization, are appealing because they may generate improved strains that are not considered GMOs, and will most likely be better accepted by the general public.

CONCLUSIONS

The main conclusions drawn from the results obtained in this thesis are:

1. The metabolic activity of the sulfur assimilation pathway explain phenotypic differences in yeast adaptation at low temperature. This work is the first report relating this metabolic pathway with cold adaptation.
2. The different transcriptomic data obtained in this thesis also revealed the importance of the phospholipid metabolism and the maintenance of lipid asymmetry between the inner and outer leaflets of the plasma membrane during low temperature adaptation. Our QTL analysis showed two genes involved in phospholipid flippase activity, *FPK1* and *OPT2*, as causative genes of better fitness at low temperature.
3. The rigidification of the plasma membrane at low temperature provokes difficulties in the uptake of nutrients, mainly nitrogen compounds. This metabolic bottle-neck is counteracted by the upregulation of genes and proteins involved in amino acid metabolism, such as the permease of cysteine and methionine *MUP1*.
4. Low temperature increases oxidative stress and induces an antioxidant response. In all the studies of this thesis we have

observed the clear trigger of this mechanism with the upregulation of genes and proteins involved in this defense. The greater activation of the sulfur assimilation pathway at low temperature can be connected with the oxidative stress response by synthesizing cysteine and methionine, substrate of many cellular antioxidant compounds.

5. There is a strong correlation between the behavior of the cells at low temperature and in the ability to cope with a strong oxidative stress situation in presence of peroxide of hydrogen. This correlation is also very interesting from an applied point of view because it could be a trait for future selections of industrial cryotolerant strains or for the genetic improvement of them.
6. The QTL analysis revealed the importance of a fit mitochondria for low temperature adaptation. The deletion of two genes involved in the electron transport chain (*COQ2* and *PET494*) provoked a strong impairment in the fermentation activity at low temperature.
7. The main and common molecular mechanism of cold adaptation observed for the three species of *Saccharomyces* studied (*S. cerevisiae*, *S. kudriavzevii* and *S. uvarum*) was the induction of genes and proteins involved in translation. *S. kudriavzevii* and *S. uvarum* presented a strong proteomic response due to low

temperature and this fact could be one of the reasons why these yeasts are better-adapted to cold environments.

8. The proteome analysis of the three *Saccharomyces* species also revealed 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.
9. The QTL analysis and further RH analyses of the detected QTLs proved the importance of subtelomeres as a source of variation in industrial yeast and the need to invest efforts in sequencing these regions with new sequencing technologies with long reads from single molecules.
10. The information obtained in this thesis is very useful from an industrial perspective for obtaining strains more adapted to low temperature fermentation processes. For instance, the high percentage of heterosis (hybrid vigor) obtained in the phenotyping of the P5/P24 segregants can be exploited for obtained genetically improved strains.

SPANISH SUMMARY

1. Introducción

Se cree que las uvas fueron domesticadas entre el Mar Negro e Irán durante el periodo del 7000-4000 aC. Las primeras evidencias de elaboración de vino provienen de la presencia de ácido tartárico en un tarro antiguo que data de 5400 - 5000 aC en el yacimiento neolítico de Tepe en Mesopotamia y de los restos de la extracción del zumo de uva en el yacimiento neolítico de Dikili Tash en Grecia (5000 aC). La colonización de los romanos extendió la elaboración del vino por todo el Mediterráneo; en el 500 aC el vino ya se producía en Italia, Francia, España, Portugal y el norte de África. Posteriormente también fue extendido a los Balcanes, Alemania y otras partes del norte de Europa. En 1530, los conquistadores españoles introdujeron la vid en México, Argentina, Perú y Chile. De la misma manera, en 1655 los holandeses plantaron vides en Sudáfrica (Pretorius, 2000a; Sicard and Legras, 2011).

Sin embargo, no fue hasta 1860, cuando Louis Pasteur descubrió que la levadura era la responsable de la conversión del azúcar en etanol y dióxido de carbono, cuando el proceso de elaboración del vino recibió el nombre de fermentación. Con el conocimiento de que la levadura era responsable de la fermentación, los productores podrían controlar el proceso

desde la viña hasta la planta embotelladora. Más tarde, en 1890, Müller - Thurgau introdujo el concepto de la inoculación de las fermentaciones con el cultivo de levaduras (Pretorius, 2000). En la actualidad la mayor parte de la producción se basa en el uso de cultivo iniciadores de levaduras seleccionadas como práctica enológica para generar vinos con características deseables y para garantizar la homogeneidad de las cosechas sucesivas.

Una de las principales demandas del sector vitivinícola está asociada a resolver los problemas generados por el cambio climático. El disponer de levaduras que produzcan un menor rendimiento en etanol, o que incrementen el contenido en glicerol en los vinos pueden ser buenas estrategias para resolver este tipo de problemas. Además de las características fisiológicas mencionadas, las levaduras también deben adaptarse a las actuales prácticas enológicas.

Dentro de estas tendencias se encuentra la realización de fermentaciones a baja temperatura para vinos blancos y rosados, ya que se obtienen vinos con una mayor complejidad organoléptica (Beltran et al., 2006; Molina et al., 2007; Torija et al., 2003). Esta práctica se limita a fermentaciones de vinos blancos y rosados ya que en vinos tintos es necesario el uso de temperaturas más altas (22-28 ºC) para la extracción de

los compuestos fenólicos de la piel de la uva. Las bajas temperaturas aumentan no sólo la retención sino también la producción de algunos compuestos volátiles (Killian and Ough, 1979). En estas condiciones se produce una mayor cantidad de compuestos aromáticos, especialmente de ésteres que imparten aromas dulces y afrutados, a la vez que se disminuye la producción de algunos compuestos desagradables, tales como ciertos alcoholes superiores y ácido acético (Beltrán et al., 2006). Otro aspecto interesante es que las bajas temperaturas reducen notablemente el crecimiento de bacterias lácticas y acéticas, lo que facilita el control del proceso (Ribéreau-Gayon et al., 2000).

A pesar de que las fermentaciones a baja temperatura presentan ventajas interesantes; esta práctica también tiene algunas desventajas principalmente relacionadas con la disminución de la tasa de crecimiento de las levaduras. La temperatura óptima de crecimiento de *S. cerevisiae* es alrededor de 32 ºC (Salvadó et al., 2011), por lo que la baja temperatura aumenta la fase de latencia, ralentizando el proceso fermentativo e incluso dando lugar a paradas del mismo (Bisson, 1999).

Por otra parte, los factores moleculares y fisiológicos que determinan una mejor adaptación de las diferentes especies a las bajas temperaturas durante los procesos de fermentación no son del todo bien conocidos.

Varios estudios han puesto de manifiesto la importancia de la composición lipídica en la respuesta adaptativa de las levaduras a las temperaturas ambientales (Beltran et al., 2008; López-Malo et al., 2013a; Redón et al., 2011; Torija et al., 2003; Tronchoni et al., 2012b). El efecto más comúnmente descrito debido a la bajada de temperatura, es un aumento en el grado de insaturación de los ácidos grasos, que se traduce en un aumento en la fluidez de membrana. Sin embargo, esta respuesta podría no ser universal para todas las levaduras. Existen otras formas de aumentar la fluidez de la membrana, como puede ser disminuir la longitud de cadena de los ácidos grasos. Este efecto ha sido descrito en fermentaciones a escala industrial (Torija *et al*., 2003; Beltrán *et al*., 2008). De la misma forma que es importante conocer los factores fisiológicos que intervienen en la adaptación a la baja temperatura, también lo es conocer cuáles son las diferencias en la expresión génica que pueden justificar una mejor o peor adaptación en ambientes fríos. Beltrán et al., (2006) realizó un análisis del transcriptoma utilizando la cepa comercial vínica de *S. cerevisiae* QA23 en condiciones de fermentación industriales a baja temperatura. Observaron que los genes relacionados con el ciclo celular, el crecimiento celular, el destino celular se expresaban menos en la fase de crecimiento exponencial a 13 ºC comparado con 25 ºC. Mientras que los genes cuya expresión se activaba durante la fase exponencial a 13 ºC eran esencialmente genes del

metabolismo lipídico y nitrogenado y genes de transporte de nutrientes, junto con genes de respuesta a estrés ambiental previamente descrita por Gasch et al., (2000).

Teniendo en cuenta estos antecendentes, el objetivo global de la tesis es el estudio de las bases moleculares y de los mecanismos fisiológicos que determinan una mayor tolerancia a la baja temperatura en levaduras vínicas. Este objetivo global se abordó en distintos objetivos parciales que corresponden con los cuatro capítulos de la presente tesis.

2. Resultados y discusión

CAPÍTULO 1

En este objetivo realizamos un análisis fenotípico de una colección de 27 levaduras vínicas industriales pertenecientes al género *Saccharomyces* en base a su temperatura de crecimiento. Con el fin de seleccionar dos levaduras que presentaran un comportamiento divergente frente a la baja temperatura.

Se llevó a cabo un fenotipado de la colección de levaduras en el rango de temperatura comprendido entre 4-45 ºC tanto en medio mínimo (SD) como en mosto sintético (SM). Se seleccionaron dos cepas, P5 y P24, que presentaban un comportamiento divergente a baja temperatura (15 ºC),

pero comportamientos similares a su temperatura óptima de crecimiento (28 ºC). P5 fue seleccionada como la levadura que presentaba un buen comportamiento a baja temperatura mientras que P24 fue seleccionada por presentar un crecimiento más afectado en frío. Para tener un control de que la selección se había realizado correctamente marcamos la cepa P5 con la proteína verde fluorescente (GFP) y realizamos cultivos mixtos (P5/P24) a ambas temperaturas, de manera que pudimos comprobar que a 15 ºC, la P5 se imponía de manera clara a la P24 desplazándola del cultivo, mientras que a 28 ºC los porcentajes de ambas cepas de mantenían cercanos al 50% durante todo el proceso.

A continuación, con las cepas seleccionadas, llevamos a cabo fermentaciones en continúo usando la misma velocidad de crecimiento, tanto a baja temperatura como a temperatura óptima. Mediante el uso de este sistema podemos separar los efectos causados por la propia velocidad de crecimiento de cada cepa de los efectos provocados por la baja temperatura. Las células obtenidas se usaron para realizar estudios transcriptómicos y proteómicos, así como la secuenciación de los genomas de ambas cepas.

En cuanto a la comparación de los transcriptomas de cada cepa por separado a baja temperatura, obtuvimos 211 y 128 genes diferencialmente

expresados en frío en P5 y P24, respectivamente. Cabe destacar que la cepa con un mejor comportamiento a baja temperatura presentaba una respuesta transcripcional más fuerte, como se observa en el mayor número de genes regulados por la temperatura. Sólo 32 de estos genes eran comunes a ambas comparaciones, poniendo de manifiesto el gran carácter cepa-dependiente de la respuesta frente a la baja temperatura.

Tanto en las comparaciones dentro de la misma cepa a distintas temperaturas como entre cepas a la misma temperatura, siempre surgían categorías funcionales relacionadas con el metabolismo del azufre y del aminoácido metionina. Además, los genes incluidos en estas categorías se situaban en su gran mayoría dentro de la ruta de captación de azufre, con un mayor número de genes regulados positivamente en la cepa P5.

Siguiendo el mismo procedimiento realizamos un análisis proteómico mediante 2D-PAGE y posterior espectrometría de masas. El número de proteínas con una concentración diferencial a baja temperatura fue de 7 y 6 en P5 y P24 respectivamente. Estas proteínas pertenecían principalmente a la glucolisis, el proceso de traducción y respuesta frente a estrés oxidativo. Lo más interesante surgió de la comparación entre ambas cepas a 15 ºC donde, de manera similar al análisis transcriptómico, algunas de las proteínas diferenciales pertenecían a la ruta de captación de azufre.

Teniendo en cuanta tanto los datos de expresión como de proteínas, decidimos suplementar a las células con tres de los compuestos finales (S-adenosil metionina, glutatión oxidado y glutatión reducido) de la ruta de captación de azufre para ver su impacto sobre las fermentaciones a baja temperatura. La adición de estos compuestos provocaba, excepto el SAM en P5, una disminución del tiempo de generación, especialmente en P24 que alcanzaba velocidades de crecimiento similares a P5. De la misma manera, testamos la capacidad de recuperación tras un choque con un agente oxidante, y observamos que la cepa P5 también presentaba una mejor recuperación, manteniéndose el comportamiento divergente frente a la baja temperatura también frente al estrés oxidativo.

La actividad de esta ruta tiene mucha influencia en otros procesos celulares, muchos de ellos con gran importancia en la adaptación a la baja temperatura como la síntesis de fosfolípidos. Cambios en la composición de la membrana plasmática como respuesta adaptativa al frío han sido ampliamente descritos (Beltran et al., 2006; Henderson et al., 2013b; Redón et al., 2011; Tronchoni et al., 2012b). La fosfatidilcolina (PC) es el fosfolípido más abundante de la membrana (~30%) y es sintetizada *de novo* a partir de la fosfatidiletanolamina (PE) mediante 3 metilaciones que utilizan como donador de grupos metilo a la S-Adenosilmetionina (Chin and

Bloch, 1988). De modo que la alta demanda de PC a baja temperatura podría dar como lugar un incremento en la demanda de SAM y por tanto una mayor activación transcripcional de la ruta de captación de azufre. Otra posible explicación sería que el mayor estrés oxidativo al que se ven sometidas las células a baja temperatura (Ballester-Tomás et al., 2015; Paget et al., 2014; Zhang et al., 2003) haga necesario producir una mayor cantidad de glutatión que ayudaría a mantener el equilibrio redox de la célula.

Complementariamente a los análisis transcriptómico y proteómico, se secuenciaron los genomas de ambas cepas y se compararon con la cepa de referencia S288c. Se encontraron 6446 SNPs entre ambas cepas vínicas, un número notablemente pequeño, pero lógico debido a que son cepas que se encuentran filogenéticamente muy próximas. Muchos de los polimorfismos entre ambas cepas se encontraban nuevamente en genes relacionados con la ruta de captación de azufre.

CAPÍTULO 2

En base a los resultados obtenidos en el primer capítulo, decidimos estudiar en más detalle la posible correlación entre la respuesta frente a estrés oxidativo y la respuesta frente a la baja temperatura.

Para ello realizamos un estudio del comportamiento a baja temperatura (12 y 15 ºC) y frente a distintos oxidantes (H_2O_2, menadiona, terbutilo y cumeno hidroperóxido) empleando 40 levaduras del género *Saccharomyces* pertenecientes a diversos procesos y orígenes geográficos. Analizamos el área bajo la curva (AUC) de crecimiento de las distintas levaduras en las diversas condiciones y realizamos un agrupamiento jerárquico de las mismas. El resultado más significativo fue el fuerte agrupamiento que se producía entre las condiciones de baja temperatura y el comportamiento de la población frente al peróxido de hidrógeno. Poniendo de manifiesto la posible existencia de un mecanismo de respuesta común.

A continuación, seleccionamos 40 mutantes homocigotos, de la colección de la levadura de laboratorio BY4743, en genes relacionados con la respuesta a estrés oxidativo para realizar un muestreo frente a baja temperatura. Se analizaron diversos parámetros de crecimiento tanto en medio YPD como en mosto sintético (SM) y aquellos mutantes que presentaban un fenotipo más afectado a baja temperatura, pero no a 28 ºC, fueron seleccionados para posteriores estudios. Se seleccionaron 10 genes (*TSA1*, *MUP1*, *GPX1*, *GLR1*, *GRX1*, *TRX2*, *TRX3*, *URM1*, *SRX1* y *AHP1*), pertenecientes a diversas rutas metabólicas relacionadas con la respuesta

frente a estrés oxidativo, para construir mutantes en el fondo genético de las levaduras vínicas seleccionadas en el capítulo anterior por su comportamiento divergente frente a la baja temperatura. La eliminación de prácticamente cualquiera de los 10 genes en la cepa P24 daba como resultado un fenotipo afectado a baja temperatura. Sin embargo, este efecto fue más acusado en los mutantes para los genes *MUP1* y *URM1*. En el caso de P5, los mutantes que presentaban un mayor retraso en la fermentación a baja temperatura fueron *MUP1* y *AHP1*, aunque este último se encontraba muy afectado también a temperatura óptima, con lo cual no se trataba de un efecto debido a la temperatura.

Nuestros resultados corroboran que existe una alta correlación entre la respuesta fisiológica a la baja temperatura y el estrés oxidativo, más concretamente frente al peróxido de hidrógeno. Una posible explicación a este hecho podría ser que el frío provoca una situación de mayor estrés oxidativo, debido a que se produce un desequilibrio redox, que debe ser corregido mediante la regulación dinámica del ratio NAD(P)+/NAD(P)H (Ballester-Tomás et al., 2015; Paget et al., 2014). Nuestro análisis de los mutantes en genes de estrés oxidativo a baja temperatura mostró la importancia de los genes *MUP1* y *URM1* durante las fermentaciones en mosto sintético. *MUP1* codifica para una permeasa de alta afinidad de

metionina, implicada también en la captación de cisteína (Kosugi et al., 2001). De nuevo, observamos la importancia de la ruta de captación de azufre a baja temperatura, de modo que el mutante para este transpotador en P5 presentaba un fenotipo muy afectado. *URM1* codifica para un modificador post-traduccional de otras proteínas y que también está implicado en la señalización por nutrientes (Goehring et al., 2003). Para esclarecer la posible función de este gen en la adaptación a baja temperatura es necesario realizar más análisis en un futuro.

CAPÍTULO 3

Dado que la adaptación a la baja temperatura en levaduras, como una gran parte de los caracteres de interés industrial, está bajo el control de múltiples genes (QTLs), decidimos realizar un mapeo para localizar los genes o regiones genéticas implicadas en esta adaptación.

Para ello construimos una población híbrida entre las cepas seleccionadas en el capítulo 1 (P5 y P24) y la sometimos a 13 rondas de esporulación e hibridación con el fin de generar una población final que presentara un genoma mosaico; disminuyendo así el ligamiento entre QTLs cercanos. Para estar seguros de que la recombinación se estaba produciendo de manera efectiva, secuenciamos 6 SNPs situados a lo largo del

cromosoma III y reconstruimos los haplotipos de 30 individuos de la población F6. Fuimos capaces de encontrar 27 haplotipos diferentes, comprobando así que el proceso de recombinación estaba ocurriendo de manera eficaz. Una vez conseguida esta población mosaico fue sometida a un proceso de selección en YPD y SM tanto a 15 como a 28 ºC, tras lo cual, todas poblaciones seleccionadas, así como la población sin seleccionar, fueron secuenciadas.

A continuación las frecuencias alélicas de las poblaciones fueron comparadas con las de la población sin seleccionar con el fin de encontrar genes o regiones en los cuales se produjera un aumento en la frecuencia alélica y por tanto una fijación de esa variante durante el proceso de selección. Localizamos 4 regiones del genoma en las cuales se producía un cambio en la frecuencia alélica cuando la población era seleccionada en mosto sintético a baja temperatura. Tres de ellas se situaban en las regiones subteloméricas de los cromosomas XIII, XV y XVI y una cuarta localizada en el brazo derecho del cromosoma XIV. En la selección en YPD, solo localizamos un pico que era coincidente con el localizado en SM en el cromosoma XVI.

Las regiones subteloméricas son muy difíciles de secuenciar y ensamblar debido a su gran variación, presencia de duplicaciones y zonas

con muchas repeticiones con homología entre los distintos cromosomas. Este hecho hace que las regiones cercanas a los telómeros estén incompletas en la mayoría de los proyectos de secuenciación y, por tanto, se dificulta la localización de los genes responsables de un fenotipo localizados en estas zonas. Para validar la importancia de estas regiones en ambas cepas seguimos la estrategia de hemicigosis recíproca (RH), en la cual se utilizan derivados haploides de las cepas parentales, a los cuales se les elimina una de las copias del gen o región en cuestión y se hibrida con el otro derivado haploide sin ninguna deleción. En este caso se eliminaron las tres regiones subteloméricas detectadas tanto en el derivado haploide de P5 como de P24, y se analizaron los fenotipos de los híbridos en una fermentación a baja temperatura. La falta de la zona subtelomérica del cromosoma XVI de P5 causaba un retraso muy importante en la fermentación a baja temperatura pero también a 28 ºC, aunque no de manera tan dramática. La falta de esta misma región perteneciente a la cepa P24 mejoraba el proceso, denotando la presencia en esta región de la cepa P5 de genes con una gran importancia para la fermentación alcohólica a ambas temperaturas. De la misma forma, la falta de la región subtelomérica del cromosoma XIII de P24 daba como resultado un fenotipo afectado a ambas temperaturas. Sin embargo, la falta de esta misma zona de P5 mejoraba el proceso. La única de las regiones subteloméricas detectadas que presentaba una dependencia de la baja

temperatura fue la perteneciente al cromosoma XV en la cepa P5, cuya ausencia retrasaba el proceso fermentativo, mientras que la falta de la misma perteneciente a P24 mejoraba el proceso.

La región del cromosoma XIV fue la única que no estaba situada en la zona subtelomérica, y para tratar de identificar los genes responsables del fenotipo, se realizaron análisis de RH con los cuatro genes más cercanos al QTL (*AGA1*, *PET494*, *COQ2* y *FPK1*). *AGA1* es una aglutinina implicada en el proceso de reproducción sexual y su deleción no tenía ningún efecto a baja temperatura. Sin embargo, el análisis de las cepas hemicigotas para cualquiera de los otros genes generaba claras diferencias durante la fermentación a 15 ºC. *PET494* y *COQ2* son proteínas mitocondriales cuya deleción tanto en P5 como en P24 afecta al proceso fermentativo a ambas temperaturas, pero especialmente a 15 ºC. *PET494* es un activador traduccional de una de las subunidades (*COX3*) de la citocromo c oxidasa (Naithani et al., 2003) mientras que *COQ2* es una transferasa que cataliza el segundo paso de la síntesis de la ubiquinona (Ashbysb et al., 1992). La falta de ambos genes hace que la mitocondria funcione de manera incorrecta y las células no puedan respirar. Varios autores han descrito que la falta de mitocondrias funcionales genera fenotipos más sensibles al estrés oxidativo (Grant et al., 1997), de modo que el estrés generado a baja temperatura junto

con esta deficiencia podría ser un efecto aditivo, que la levadura no es capaz de contrarrestar. En cuanto al gen *FPK1*, codifica para una “Ser/Thr protein kinasa” que fosforila varias translocasas de lípidos y, por tanto, interviene en el mantenimiento de la asimetría de la membrana plasmática. La eliminación del alelo del alelo de la cepa P24 no tenía efecto en la cepa hemicigota, sin embargo, la deleción del alelo proveniente de P5 provocaba un retraso de ~340 horas en la fermentación a baja temperatura. Al comparar las secuencias de ambos genes observamos que la cepa P24 presentaba una sustitución aminoacídica (R520K) en este gen, que podría ser el responsable del fenotipo inferior de este alelo.

También realizamos análisis de RH construyendo híbridos entre los derivados haploides de las cepas vínicas y los mutantes para los genes situados en las regiones subtelómericas de la cepa de laboratorio BY4741. La capacidad fermentativa de los híbridos generados, los cuales sólo mantenían la copia vínica de cada uno de los genes analizados, fue testada tanto a 15 como a 28 ºC. En el cromosoma XIII, los genes cuya hemicigosis producía haploinsuficiencia en el híbrido de ambas cepas vínicas fueron *YMR316C-A*, *DIA1* y *ADH6*. *YMR316C-A* y *DIA1* son proteínas de función desconocida, cuyas ORFs se superponen. *ADH6* es una alcohol deshidrogenasa. En la región subtelomérica del cromosoma XV, sólo dos

genes pudieron ser analizados debido a que los otros genes contenidos en esta región eran esenciales. La hemicigosis de la proteína de función desconocida, *YOL159C*, afectó severamente a la fermentación en ambas cepas híbridas. En cuanto al cromosoma XVI, la hemicigosis del gen *ARR3*, una permeasa de membrana plasmática que transporta arsenito y antimonio, también afectaba en ambas cepas. Los genes *QCR2*, *AQY1*, *YPR197c* y *ARR1* afectaban a la baja temperatura sólo en el caso de la cepa P5/BY4741, mientras que los genes *OPT2*, *YPR195c* e *YPR196c* afectaban en el caso de la cepa P24/BY4741. *QCR2* es un componente de la cadena de transporte de electrones de la membrana mitocondrial interna y *AQY1* es una acuaporina. Finalmente, *OPT2* es un transportador de oligopéptidos implicado también en el mantenimiento de la asimetría de la membrana plasmática.

Este trabajo pone de manifiesto la importancia de las regiones subteloméricas como fuente de variabilidad genética de muchos de los caracteres de importancia industrial (Cubillos et al., 2011). Los genes situados en estas regiones evolucionan más rápidamente que sus homólogos internos, debido a que en estas zonas se producen más fácilmente duplicaciones (Ames et al., 2010). De nuevo, el mantenimiento de una adecuada composición lipídica de la membrana plasmática, así como una

eficiente respuesta al estrés oxidativo, se sitúan como mecanismos clave en la adaptación a la baja temperatura.

CAPÍTULO 4

En un intento de detectar diferencias metabólicas inter-específicas, llevamos a cabo un estudio proteómico comparando dos levaduras criotolerantes del género *Saccharomyces* (*S. uvarum* y *S. kudriadzevii*) con *S. cerevisiae* tanto a baja temperatura como a temperatura óptima.

Con este fin realizamos fermentaciones en continuo de cada cepa a ambas temperaturas en mosto sintético, y con las células obtenidas utilizamos la técnica iTRAQ, que permite cuantificar el proteoma. Además también analizamos los metabolitos de cada una de las fermentaciones en continuo.

En cuanto a las diferencias metabólicas de las tres especies, observamos que *S. kudriavzevii* es la más eficaz a baja temperatura ya que consume más azúcares y nitrógeno con la menor biomasa. Al comparar los proteomas de la misma especie creciendo a ambas temperaturas, encontramos 32, 129 y 226 proteínas con cambios significativos en su concentración en *S. cerevisiae* (*Sc*), *S. kudriavzevii* (*Sk*) y *S. uvarum* (*Su*), respectivamente. En las tres especies a baja temperatura las categorías

funcionales más representativas fueron "Traducción y síntesis proteica" y "Glucolisis y gluconeogénesis", mientras que a 28 ºC era principalmente la categoría "Metabolismo de aminoácidos". Aunque la respuesta en las tres especies comprendía en general, los mismos mecanismos, la cantidad de proteínas en cada una de estas categorías era mucho mayor en el caso de las especies criotolerantes que en el caso de *Sc*.

Si comparamos los proteomas de las tres especies a cada una de las temperaturas, es decir, *Sc*, *Sk* y *Su* a baja temperatura y *Sc*, *Sk* y *Su* a 28 ºC, lo que obtenemos es que *Sc* a baja temperatura se caracteriza por tener mayores cantidades de proteínas relacionadas con el metabolismo de carbohidratos y de aminoácidos mientras que *Sk* y *Su*, de nuevo, presentan grandes cantidades de proteínas relacionadas con la traducción.

Para intentar demostrar que la mejor adaptación de *Sk* y *Su* a baja temperatura esta principalmente relacionada con una mejor capacidad para iniciar la traducción, decidimos hacer un ensayo en presencia del antibiótico paramomicina. Este antibiótico es un potente inhibidor de la traducción de modo que la levadura que tenga un proceso traduccional más eficiente se verá menos afectada por esta droga. En ambas especies criotolerantes vimos un halo de inhibición a 28 ºC, mientras que a 12 ºC no se encontraban afectadas. Sin embargo, en *Sc* sucedía lo contrario, y encontramos halo de

inhibición a baja temperatura. Estos resultados demuestran que uno de los mecanismos más potentes de adaptación en estas especies es la presencia de una traducción más eficiente que en *Sc* a baja temperatura y, por tanto, una capacidad de respuesta mucho mayor.

3. Conclusiones

Las conclusiones principales que se extraen de esta tesis doctoral son las siguientes:

1. La actividad metabólica de la ruta de asimilación de azufre explica las diferencias fenotípicas en la adaptación de las levaduras a la baja temperatura. Este trabajo es el primero que establece una relación entre esta ruta y la adaptación a la baja temperatura.
2. Los resultados de transcriptómica obtenidos en esta tesis ponen de manifiesto la importancia del metabolismo de los fosfolípidos y del mantenimiento de la asimetría entre la parte interna y la externa de la membrana plasmática en la adaptación a la baja temperatura. El análisis de QTLs reveló la importancia de dos genes implicados en el mantenimiento de la asimetría,

FPK1 y *OPT2*, como responsables del mejor comportamiento a baja temperatura.

3. La rigidificación de la membrana plasmática a baja temperatura dificulta la captación de nutrientes, principalmente compuestos nitrogenados. Este cuello de botella metabólico es contrarrestado por la regulación positiva de genes y proteínas implicados en el metabolismo de aminoácidos, tales como la permeasa de cisteína y metionina *MUP1*.
4. La baja temperatura aumenta el estrés oxidativo e induce una respuesta antioxidante. En todos los resultados de esta tesis hemos observado la inducción de este mecanismo mediante la regulación positiva de genes y proteínas implicados en esta defensa. La mayor activación de la vía de asimilación de azufre a baja temperatura se puede conectar con la respuesta al estrés oxidativo mediante la síntesis de cisteína y metionina, sustratos de muchos compuestos antioxidantes de las células.
5. Hay una fuerte correlación positiva entre el comportamiento de las células a baja temperatura y su capacidad de hacer frente a una situación de estrés oxidativo en presencia de peróxido de hidrógeno. Esta correlación también es muy interesante desde un punto de vista aplicado, ya que podría ser una característica para

futuras selecciones de cepas industriales criotolerantes o para la mejora genética de las mismas.

6. El análisis de QTL mostró la importancia de una mitocondria en buen estado en la adaptación a la baja temperatura. La deleción de dos genes implicados en la cadena de transporte de electrones (*COQ2* y *PET494*) provocó un fuerte deterioro en la actividad fermentativa a baja temperatura.
7. El principal mecanismo común de adaptación al frío observado para las tres especies de *Saccharomyces* estudiadas (*S. cerevisiae*, *S. kudriavzevii* y *S. uvarum*) fue la inducción de genes y proteínas implicados en la traducción. *S. kudriavzevii* y *S. uvarum* presentaron una intensa respuesta proteómica debido a la baja temperatura mediante el aumento de un gran número de proteínas relacionadas con la traducción y este hecho podría ser una de las razones por las que estas levaduras están mejor adaptadas a ambientes fríos.
8. El análisis del proteoma de las tres especies de *Saccharomyces* también reveló una inducción de enzimas fermentativas y glucolíticas a baja temperatura, siendo mayor este aumento en *S. cerevisiae*, lo que en parte podría explicar el mayor flujo glucolítico de esta especie. Otra explicación plausible es el

desequilibrio redox producido como resultado de la cinética más lenta de las alcohol deshidrogenasas a baja temperatura, que daría lugar a la acumulación de NADH y el bloqueo del flujo glucolítico.

9. El análisis de QTLs y su posterior validación mediante RH resaltó la importancia de las regiones subteloméricas como fuente de variación genética en cepas industriales y la necesidad de invertir esfuerzos en proyectos de secuenciación de estas zonas con nuevas tecnologías que permitan lecturas más largas.
10. La información obtenida en esta tesis es muy útil desde el punto de vista industrial para la obtención de cepas más adaptadas a los procesos de fermentación a baja temperatura. Por ejemplo, el alto porcentaje de heterosis (vigor híbrido) obtenido en el fenotipado de los segregantes P5/P24 puede ser explotado obtener cepas mejoradas a baja temperatura.

REFERENCES

Abe, F., and Horikoshi, K. (2000). Tryptophan permease gene TAT2 confers high-pressure growth in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 20, 8093–102. doi:10.1128/MCB.20.21.8093-8102.2000.

Abiola, O., Angel, J. M., Avner, P., Bachmanov, A. A., Belknap, J. K., Bennett, B., et al. (2003). The nature and identification of quantitative trait loci: a community's view. *Nat. Rev. Genet.* 4, 911–6. doi:10.1038/nrg1206.

Abramova, N., Sertil, O., Mehta, S., and Lowry, C. V (2001). Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in Saccharomyces cerevisiae. *J. Bacteriol.* 183, 2881–7. doi:10.1128/JB.183.9.2881-2887.2001.

Aguilera, J., Randez-Gil, F., and Prieto, J. A. (2007). Cold response in Saccharomyces cerevisiae: new functions for old mechanisms. *FEMS Microbiol. Rev.* 31, 327–41. doi:10.1111/j.1574-6976.2007.00066.x.

Albert, F. W., Treusch, S., Shockley, A. H., Bloom, J. S., and Kruglyak, L. (2014). Genetics of single-cell protein abundance variation in large yeast populations. *Nature* 506, 1–19. doi:10.1038/nature12904.

Alexandre, H., Ansanay-Galeote, V., Dequin, S., and Blondin, B. (2001). Global gene expression during short-term ethanol stress in Saccharomyces cerevisiae. *FEBS Lett.* doi:10.1016/S0014-5793(01)02503-0.

Alexandre, H., and Charpentier, C. (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol.* 20, 20–27. doi:10.1038/sj.jim.2900442.

Al-Fageeh, M. B., and Smales, C. M. (2006). Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem. J.* 397, 247–59. doi:10.1042/BJ20060166.

Ambroset, C., Petit, M., Brion, C., Sanchez, I., Delobel, P., Guérin, C., et al. (2011). Deciphering the molecular basis of wine yeast fermentation traits using a combined genetic and genomic approach. *G3 (Bethesda).* 1, 263–81. doi:10.1534/g3.111.000422.

Ames, R. M., Rash, B. M., Hentges, K. E., Robertson, D. L., Delneri, D., and Lovell, S. C.

(2010). Gene duplication and environmental adaptation within yeast populations. *Genome Biol. Evol.* 2, 591–601. doi:10.1093/gbe/evq043.

Arroyo-López, F. N., Pérez-Torrado, R., Querol, A., and Barrio, E. (2010). Modulation of the glycerol and ethanol syntheses in the yeast Saccharomyces kudriavzevii differs from that exhibited by Saccharomyces cerevisiae and their hybrid. *Food Microbiol.* 27, 628–637. doi:10.1016/j.fm.2010.02.001.

Ashbysb, M. N., Kutsunais, S. Y., Ackermany, S., Tzagoloffll, A., and Edwards, P. A. (1992). COQ2 Is a Candidate for the Structural Gene Encoding puru-Hydroxybenzoate: Polyprenyltransferase. *J. Biol. Chem.* 267, 4128–4136.

Attfield, P. V. (1997). Stress tolerance: The key to effective strains of industrial baker’s yeast. *Nat. Biotechnol.* 15, 1351–1357. doi:10.1038/nbt1297-1351.

Ballester-Tomás, L., Randez-Gil, F., Pérez-Torrado, R., and Prieto, J. A. (2015). Redox engineering by ectopic expression of glutamate dehydrogenase genes links NADPH availability and NADH oxidation with cold growth in Saccharomyces cerevisiae. *Microb. Cell Fact.* 14, 100. doi:10.1186/s12934-015-0289-2.

Barnett, J. A. (2000). A history of research on yeasts: Louis Pasteur and his contemporaries, 1850-1880. *Yeast* 16, 755–71.

Barta, I., and Iggo, R. (1995). Autoregulation of expression of the yeast Dbp2p “DEAD-box” protein is mediated by sequences in the conserved DBP2 intron. *EMBO J.* 14, 3800–8.

Bauer, F. F., and Pretorius, I. S. (2000). Yeast Stress Response and Fermentation Efficiency : How to Survive the Making of Wine - A Review. *South African J. Enol. Vitic.* 21, 27–51.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Hayes, N. S., Green, B. G., and Cascieri, M. A. (1988). Expression, purification and characterization of recombinant human insulin-like growth factor I in yeast. *Gene* 66, 235–44.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., and Rozés, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *Int. J. Food Microbiol.* 121, 169–177. doi:10.1016/j.ijfoodmicro.2007.11.030.

Beltran, G., Novo, M., Leberre, V., Sokol, S., Labourdette, D., Guillamón, J. M., et al. (2006). Integration of transcriptomic and metabolic analyses for understanding the global responses of low-temperature winemaking fermentations. *FEMS Yeast Res.* 6, 1167–1183. doi:10.1111/j.1567-1364.2006.00106.x.

Beltran, G., Rozès, N., Mas, A., and Guillamón, J. M. (2007). Effect of low-temperature fermentation on yeast nitrogen metabolism. *World J. Microbiol. Biotechnol.* 23, 809–815. doi:10.1007/s11274-006-9302-6.

Beltran, G., Torija, M. J., Novo, M., Ferrer, N. N., Poblet, M., Guillamón, J. M., et al. (2002). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. *Syst Appl Microbiol* 25, 287–93. doi:10.1078/0723-2020-00097.

Ben-Ari, G., Zenvirth, D., Sherman, A., David, L., Klutstein, M., Lavi, U., et al. (2006). Four linked genes participate in controlling sporulation efficiency in budding yeast. *PLoS Genet.* 2, 1815–1823. doi:10.1371/journal.pgen.0020195.

Benjamini, Y., and Hochberg, Y. (1995). Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* 57, 289–300. doi:10.2307/2346101.

Bergström, A., Simpson, J. T., Salinas, F., Barré, B., Parts, L., Zia, A., et al. (2014). A high-definition view of functional genetic variation from natural yeast genomes. *Mol. Biol. Evol.* 31, 872–88. doi:10.1093/molbev/msu037.

Bertolini, L., Zambonelli, C., Giudici, P., and Castellari, L. (1996). Higher alcohol production by cryotolerant Saccharomyces strains: technical brief. *Am. J. Enol. Vitic.* 47, 343–345.

Bhatia, A., Yadav, A., Zhu, C. C., Gagneur, J., Radhakrishnan, A., Steinmetz, L. M., et al. (2014). Yeast Growth Plasticity Is Regulated by Environment-Specific Multi-QTL Interactions. *G3-Genes Genomes Genet.* 4, 769–777. doi:10.1534/g3.113.009142.

Bidenne, C., Blondin, B., Dequin, S., and Vezinhet, F. (1992). Analysis of the chromosomal DNA polymorphism of wine strains of Saccharomyces cerevisiae. *Curr. Genet.* 22, 1–7.

Bisson, L. F. (1999). Stuck and Sluggish Fermentations. *Am. J. Enol. Vitic.* 50, 107–119.

Blomberg, A. (1997). Osmoresponsive proteins and functional assessment strategies in Saccharomyces cerevisiae. *Electrophoresis* 18, 1429–40. doi:10.1002/elps.1150180818.

Boer, V. M., de Winde, J. H., Pronk, J. T., and Piper, M. D. W. (2003). The genome-wide transcriptional responses of Saccharomyces cerevisiae grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J. Biol. Chem.* 278, 3265–74. doi:10.1074/jbc.M209759200.

Borevitz, J. O., and Chory, J. (2004). Genomics tools for QTL analysis and gene discovery. *Curr. Opin. Plant Biol.* doi:10.1016/j.pbi.2004.01.011.

Borneman, A. R., Desany, B. A., Riches, D., Affourtit, J. P., Forgan, A. H., Pretorius, I. S., et al. (2011). Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of Saccharomyces cerevisiae. *PLoS Genet.* 7, e1001287. doi:10.1371/journal.pgen.1001287.

Borneman, A. R., Forgan, A. H., Pretorius, I. S., and Chambers, P. J. (2008). Comparative genome analysis of a Saccharomyces cerevisiae wine strain. *FEMS Yeast Res.* 8, 1185–95. doi:10.1111/j.1567-1364.2008.00434.x.

Borneman, A. R., and Pretorius, I. S. (2015). Genomic insights into the Saccharomyces sensu stricto complex. *Genetics* 199, 281–91. doi:10.1534/genetics.114.173633.

Borneman, A. R., Pretorius, I. S., and Chambers, P. J. (2013). Comparative genomics: a revolutionary tool for wine yeast strain development. *Curr. Opin. Biotechnol.* 24, 192–9. doi:10.1016/j.copbio.2012.08.006.

Boucherié, H., Bataille, N., Fitch, I. T., Perrot, M., and Tuite, M. F. (1995). Differential synthesis of glyceraldehyde-3-phosphate dehydrogenase polypeptides in stressed yeast cells. *FEMS Microbiol. Lett.* 125, 127–133. doi:10.1016/0378-1097(94)00484-9.

Boynton, P. J., and Greig, D. (2014). The ecology and evolution of non-domesticated Saccharomyces species. *Yeast* 31, 449–62. doi:10.1002/yea.3040.

Brauer, M. J., Christianson, C. M., Pai, D. A., and Dunham, M. J. (2006). Mapping novel traits by array-assisted bulk segregant analysis in Saccharomyces cerevisiae. *Genetics* 173, 1813–1816. doi:10.1534/genetics.106.057927.

Brem, R. B., and Kruglyak, L. (2005). The landscape of genetic complexity across 5,700 gene expression traits in yeast. *Proc. Natl. Acad. Sci. U. S. A.* 102, 1572–7. doi:10.1073/pnas.0408709102.

Brown, C. A., Murray, A. W., and Verstrepen, K. J. (2010). Rapid Expansion and Functional Divergence of Subtelomeric Gene Families in Yeasts. *Curr. Biol.* 20, 895–903. doi:10.1016/j.cub.2010.04.027.

Brown, C. J., Todd, K. M., and Rosenzweig, R. F. (1998). Multiple duplications of yeast hexose transport genes in response to selection in a glucose-limited environment. *Mol. Biol. Evol.* 15, 931–42.

Burke, M. K., Liti, G., and Long, A. D. (2014). Standing genetic variation drives repeatable experimental evolution in outcrossing populations of saccharomyces cerevisiae. *Mol. Biol. Evol.* 31, 3228–3239. doi:10.1093/molbev/msu256.

Campbell, K., Vowinckel, J., Keller, M. A., and Ralser, M. (2016). Methionine metabolism alters oxidative stress resistance via the pentose phosphate pathway. *Antioxid. Redox Signal.* 14, 1–14. doi:10.1089/ars.2015.6516.

Cardona, F., Carrasco, P., Pérez-Ortín, J. E., del Olmo, M. lí, and Aranda, A. (2007). A novel approach for the improvement of stress resistance in wine yeasts. *Int. J. Food Microbiol.* 114, 83–91. doi:10.1016/j.ijfoodmicro.2006.10.043.

Carlson, M. (1987). Regulation of sugar utilization in Saccharomyces species. *J. Bacteriol.* 169, 4873–7.

Carlson, M., Celenza, J. L., and Eng, F. J. (1985). Evolution of the dispersed SUC gene family of Saccharomyces by rearrangements of chromosome telomeres. *Mol. Cell. Biol.* 5, 2894–2902. doi:10.1128/MCB.5.11.2894.

Casado-Vela, J., Martínez-Esteso, M. J., Rodriguez, E., Borrás, E., Elortza, F., and Bru-Martínez, R. (2010). iTRAQ-based quantitative analysis of protein mixtures with large fold change and dynamic range. *Proteomics* 10, 343–347. doi:10.1002/pmic.200900509.

Castrillo, J. I., Zeef, L. A., Hoyle, D. C., Zhang, N., Hayes, A., Gardner, D. C. J., et al. (2007). Growth control of the eukaryote cell: a systems biology study in yeast. *J. Biol.* 6, 4. doi:10.1186/jbiol54.

Causton, H., Ren, B., Koh, S., Harbison, C., Kanin, E., Jennings, E., et al. (2001). Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* 12, 323–337. doi:10.1091/mbc.12.2.323.

Charoenchai, C., Fleet, G. H., and Henschke, P. A. (1998). Effects of Temperature, pH, and Sugar Concentration on the Growth Rates and Cell Biomass of Wine Yeasts. *Am. J. Enol. Vitic.* 49, 283–288.

Chattopadhyay, S., and Pearce, D. A. (2002). Interaction with Btn2p is required for localization of Rsg1p: Btn2p-mediated changes in arginine uptake in Saccharomyces cerevisiae. *Eukaryot. Cell*. doi:10.1128/EC.1.4.606-612.2002.

Chin, J., and Bloch, K. (1988). Phosphatidylcholine synthesis in yeast. *J. Lipid Res.* 29, 9–14.

Chiva, R., López-Malo, M., Salvadó, Z., Mas, A., and Guillamón, J. M. (2012). Analysis of low temperature-induced genes (LTIG) in wine yeast during alcoholic fermentation. *FEMS Yeast Res.* 12, 831–843. doi:10.1111/j.1567-1364.2012.00834.x.

Ciani, M., Mannazzu, I., Marinangeli, P., Clementi, F., and Martini, A. (2004). Contribution of winery-resident Saccharomyces cerevisiae strains to spontaneous grape must fermentation. *Antonie Van Leeuwenhoek* 85, 159–164. doi:10.1023/B:ANTO.0000020284.05802.d7.

Clement, T., Perez, M., Mouret, J. R., Sablayrolles, J. M., and Camarasa, C. (2011). Use of a continuous multistage bioreactor to mimic winemaking fermentation. *Int. J. Food Microbiol.* 150, 42–49. doi:10.1016/j.ijfoodmicro.2011.07.016.

Coleman, M. C., Fish, R., and Block, D. E. (2007). Temperature-dependent kinetic model for nitrogen-limited wine fermentations. *Appl. Environ. Microbiol.* 73, 5875–84. doi:10.1128/AEM.00670-07.

Collinson, L. P., and Dawes, I. W. (1992). Inducibility of the Response of Yeast-Cells To Peroxide Stress. *J. Gen. Microbiol.* 138, 329–335.

Costa, V., and Moradas-Ferreira, P. (2001). Oxidative stress and signal transduction in Saccharomyces cerevisiae: insights into ageing, apoptosis and diseases. *Mol. Aspects Med.* 22, 217–246. doi:10.1016/S0098-2997(01)00012-7.

Cubillos, F. A. (2016). Exploiting budding yeast natural variation for industrial processes. *Curr. Genet.* 62, 745–751. doi:10.1007/s00294-016-0602-6.

Cubillos, F. A., Billi, E., Zörgö, E., Parts, L., Fargier, P., Omholt, S., et al. (2011). Assessing the complex architecture of polygenic traits in diverged yeast populations. *Mol. Ecol.* 20, 1401–1413. doi:10.1111/j.1365-294X.2011.05005.x.

Culotta, V. C. (2000). Superoxide dismutase, oxidative stress, and cell metabolism. *Curr. Top. Cell. Regul.* 36, 117–32.

Culotta, V. C., Yang, M., and O'Halloran, T. V. (2006). Activation of superoxide dismutases: Putting the metal to the pedal. *Biochim. Biophys. Acta - Mol. Cell Res.* 1763, 747–758. doi:10.1016/j.bbamcr.2006.05.003.

D'Amato, D., Corbo, M. R., Nobile, M. A. Del, and Sinigaglia, M. (2006). Effects of temperature, ammonium and glucose concentrations on yeast growth in a model wine system. *Int. J. Food Sci. Technol.* 41, 1152–1157. doi:10.1111/j.1365-2621.2005.01128.x.

Daniel Gietz, R., and Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol.* 350, 87–96. doi:10.1016/S0076-6879(02)50957-5.

Darvasi, A., and Pisanté-Shalom, A. (2002). Complexities in the genetic dissection of quantitative trait loci. *Trends Genet.* 18, 489–91.

Daum, G., Lees, N. D., Bard, M., and Dickson, R. (1998). Biochemistry, cell biology and molecular biology of lipids ofSaccharomyces cerevisiae. *Yeast* 14, 1471–1510. doi:10.1002/(SICI)1097-0061(199812)14:16<1471::AID-YEA353>3.0.CO;2-Y.

Davies, K. J. (1995). Oxidative stress: the paradox of aerobic life. *Biochem. Soc. Symp.* 61, 1–31. doi:10.1042/bss0610001.

Dawes, I. W., and Hardie, I. D. (1974). Selective killing of vegetative cells in sporulated yeast cultures by exposure to diethyl ether. *Mol. Gen. Genet.* 131, 281–289. doi:10.1007/BF00264859.

Dean, R. T., Fu, S., Stocker, R., and Davies, M. J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J.* 324, 1–18. doi:10.1042/bj3240001.

Deatherage, D. E., and Barrick, J. E. (2014). Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. *Methods Mol. Biol.* 1151, 165–88. doi:10.1007/978-1-4939-0554-6_12.

Delgado, M. L., O'Connor, J. E., Azorín, I., Renau-Piqueras, J., Gil, M. L., and Gozalbo, D. (2001). The glyceraldehyde-3-phosphate dehydrogenase polypeptides encoded by the Saccharomyces cerevisiae TDH1, TDH2 and TDH3 genes are also cell wall proteins. *Microbiology* 147, 411–417.

Delneri, D., Brancia, F. L., and Oliver, S. G. (2001). Towards a truly integrative biology through the functional genomics of yeast. *Curr. Opin. Biotechnol.* 12, 87–91.

Demuyter, C., Lollier, M., Legras, J.-L., and Le Jeune, C. (2004). Predominance of Saccharomyces uvarum during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. *J. Appl. Microbiol.* 97, 1140–8. doi:10.1111/j.1365-2672.2004.02394.x.

DeRisi, J. L., Iyer, V. R., Brown, P. O., Schena, M., Shalon, D., Davis, R. W., et al. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278, 680–6. doi:10.1126/science.278.5338.680.

Deutschbauer, A. M., and Davis, R. W. (2005). Quantitative trait loci mapped to single-nucleotide resolution in yeast. *Nat. Genet.* 37, 1333–1340. doi:10.1038/ng1674.

Deutschbauer, A. M., Jaramillo, D. F., Proctor, M., Kumm, J., Hillenmeyer, M. E., Davis, R. W., et al. (2005). Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. *Genetics* 169, 1915–25. doi:10.1534/genetics.104.036871.

van Dijken, J. P., Weusthuis, R. A., and Pronk, J. T. (1993). Kinetics of growth and sugar consumption in yeasts. *Antonie Van Leeuwenhoek* 63, 343–352. doi:10.1007/BF00871229.

Duina, A. A., Miller, M. E., and Keeney, J. B. (2014). Budding yeast for budding geneticists: A primer on the Saccharomyces cerevisiae model system. *Genetics* 197, 33–48. doi:10.1534/genetics.114.163188.

Dukes, B. C., and Butzke, C. E. (1998). Rapid Determination of Primary Amino Acids in Grape Juice Using an o-Phthaldialdehyde/N-Acetyl-L-Cysteine Spectrophotometric Assay. *Am. J. Enol. Vitic.* 49, 125–134.

Dunham, M. J. (2010). Experimental evolution in yeast: A practical guide. *Methods Enzymol.* 470, 487–507. doi:10.1016/S0076-6879(10)70019-7.

Ehrenreich, I. M., Gerke, J. P., and Kruglyak, L. (2009). Genetic dissection of complex traits in yeast: Insights from studies of gene expression and other phenotypes in the BYxRM cross. in *Cold Spring Harbor Symposia on Quantitative Biology*, 145–153. doi:10.1101/sqb.2009.74.013.

Ehrenreich, I. M., Torabi, N., Jia, Y., Kent, J., Martis, S., Shapiro, J. A., et al. (2010). Dissection of genetically complex traits with extremely large pools of yeast segregants. *Nature* 15, 1030–1042. doi:10.1038/nature08923.

Elbaz-Alon, Y., Rosenfeld-Gur, E., Shinder, V., Futerman, A. H., Geiger, T., and Schuldiner, M. (2014). A dynamic interface between vacuoles and mitochondria in yeast. *Dev. Cell* 30, 95–102. doi:10.1016/j.devcel.2014.06.007.

Engel, S. R., Dietrich, F. S., Fisk, D. G., Binkley, G., Balakrishnan, R., Costanzo, M. C., et al. (2014). The reference genome sequence of Saccharomyces cerevisiae: then and now. *G3 (Bethesda).* 4, 389–98. doi:10.1534/g3.113.008995.

Erten, H. (2002). Relations between elevated temperatures and fermentation behaviour of Kloeckera apiculata and Saccharomyces cerevisiae associated with winemaking in mixed cultures. *World J. Microbiol. Biotechnol.* 18, 377–382. doi:10.1023/A:1015221406411.

Farrugia, G., Balzan, R., Madeo, F., and Breitenbach, M. (2012). Oxidative stress and programmed cell death in yeast. *Front. Oncol.* 2, 1–21. doi:10.3389/fonc.2012.00064.

Fay, J. C. (2013). The molecular basis of phenotypic variation in yeast. *Curr. Opin. Genet. Dev.* 23, 672–677. doi:10.1016/j.gde.2013.10.005.

Fay, J. C., and Benavides, J. A. (2005). Evidence for Domesticated and Wild Populations of Saccharomyces cerevisiae. *PLoS Genet.* 1, e5. doi:10.1371/journal.pgen.0010005.

Flattery-O'Brien, J., Collinson, L. P., and Dawes, I. W. (1993). Saccharomyces cerevisiae has an inducible response to menadione which differs from that to hydrogen peroxide. *J. Gen. Microbiol.* 139, 501–507. doi:10.1099/00221287-139-3-501.

Fleet, G. H. (1998). "The microbiology of alcoholic beverages," in *Microbiology of*

Fermented Foods (Boston, MA: Springer US), 217–262. doi:10.1007/978-1-4613-0309-1_9.

Fleet, G. H. (2003). Yeast interactions and wine flavour. *Int. J. Food Microbiol.* 86, 11–22. doi:10.1016/S0168-1605(03)00245-9.

Fleet, G. H. (2008). Wine yeasts for the future. *FEMS Yeast Res.* 8, 979–998. doi:10.1111/j.1567-1364.2008.00427.x.

Förstemann, K., and Lingner, J. (2001). Molecular Basis for Telomere Repeat Divergence in Budding Yeast Molecular Basis for Telomere Repeat Divergence in Budding Yeast. *Mol. Cell. Biol.* 21, 7277–7286. doi:10.1128/MCB.21.21.7277.

Fortner, D. M., Troy, R. G., and Brow, D. A. (1994). A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. *Genes Dev.* 8, 221–33. doi:10.1101/GAD.8.2.221.

Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., et al. (2013). STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* 41, D808-15. doi:10.1093/nar/gks1094.

Galeote, V., Novo, M., Salema-Oom, M., Brion, C., Valério, E., Gonçalves, P., et al. (2010). FSY1, a horizontally transferred gene in the Saccharomyces cerevisiae EC1118 wine yeast strain, encodes a high-affinity fructose/H+ symporter. *Microbiology* 156, 3754–61. doi:10.1099/mic.0.041673-0.

Gamero, A., Belloch, C., Ibañez, C., and Querol, A. (2014). Molecular analysis of the genes involved in aroma synthesis in the species S. cerevisiae, S. kudriavzevii and S. bayanus var. uvarum in winemaking conditions. *PLoS One* 9, e97626. doi:10.1371/journal.pone.0097626.

Gamero, A., Belloch, C., and Querol, A. (2015). Genomic and transcriptomic analysis of aroma synthesis in two hybrids between Saccharomyces cerevisiae and S. kudriavzevii in winemaking conditions. *Microb. Cell Fact.* 14, 128. doi:10.1186/s12934-015-0314-5.

Gamero, A., Tronchoni, J., Querol, A., and Belloch, C. (2013). Production of aroma compounds by cryotolerant Saccharomyces species and hybrids at low and moderate fermentation temperatures. *J. Appl. Microbiol.* 114, 1405–14. doi:10.1111/jam.12126.

Gan, Z. R. (1991). Yeast thioredoxin genes. *J. Biol. Chem.* 266, 1692–6.

Gancedo, J. M. (1998). Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* 62, 334–61.

Gao, C., and Fleet, G. H. (1988). The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae, Candida stellata* and *Kloeckera apiculata*. *J. Appl. Bacteriol.* 65, 405–409. doi:10.1111/j.1365-2672.1988.tb01909.x.

García-Ríos, E., López-Malo, M., and Guillamón, J. M. (2014). Global phenotypic and genomic comparison of two Saccharomyces cerevisiae wine strains reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature fermentations. *BMC Genomics* 15, 1059. doi:10.1186/1471-2164-15-1059.

García-Ríos, E., Ramos-Alonso, L., and Guillamón, J. M. (2016). Correlation between Low Temperature Adaptation and Oxidative Stress in Saccharomyces cerevisiae. *Front. Microbiol.* 7, 1–11. doi:10.3389/fmicb.2016.01199.

Gasch, A. P. (2003). “The environmental stress response: a common yeast response to diverse environmental stresses,” in *Yeast Stress Responses* (Springer Berlin Heidelberg), 11–70. doi:10.1007/3-540-45611-2_2.

Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., et al. (2000). Genomic Expression Programs in the Response of Yeast Cells to Environmental Changes. *Mol. Biol. Cell* 11, 4241–4257. doi:10.1091/mbc.11.12.4241.

Gasch, A. P., and Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Funct. Integr. Genomics* 2, 181–192. doi:10.1007/s10142-002-0058-2.

Gasmi, N., Jacques, P. E., Klimova, N., Guo, X., Ricciardi, A., Robert, F., et al. (2014). The switch from fermentation to respiration in Saccharomyces cerevisiae is regulated by the Ert1 transcriptional activator/repressor. *Genetics* 198, 547–560. doi:10.1534/genetics.114.168609.

Gerngross, T. U. (2004). Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat. Biotechnol.* 22, 1409–14. doi:10.1038/nbt1028.

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. *Nature* 418, 387–91. doi:10.1038/nature00935.

Giudici, P., Zambonelli, C., Passarelli, P., and Castellari, L. (1995). Improvement of Wine Composition with Cryotolerant Saccharomyces Strains. *Am. J. Enol. Vitic.* 46, 143–147.

Glazier, A. M. (2002). Finding Genes That Underlie Complex Traits. *Science (80-.).* 298, 2345–2349. doi:10.1126/science.1076641.

Goddard, M. R. (2008). Quantifying the complexities of Saccharomyces cerevisiae's ecosystem engineering via fermentation. *Ecology* 89, 2077–82. doi:10.1890/07-2060.1.

Goehring, A. S., Rivers, D. M., and Sprague, G. F. (2003). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryot. Cell* 2, 930–936. doi:10.1128/EC.2.5.930-936.2003.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., et al. (1996). Life with 6000 genes. *Science* 274, 546, 563–7. doi:10.1126/science.274.5287.546.

González, S. S., Barrio, E., Gafner, J., and Querol, A. (2006). Natural hybrids from Saccharomyces cerevisiae, Saccharomyces bayanus and Saccharomyces kudriavzevii in wine fermentations. *FEMS Yeast Res.* 6, 1221–34. doi:10.1111/j.1567-1364.2006.00126.x.

González, S. S., Gallo, L., Climent, D., Barrio, E., Querol, A., Climent, M. A. D., et al. (2007). Enological characterization of natural hybrids from Saccharomyces cerevisiae and S. kudriavzevii. *Int. J. Food Microbiol.* 116, 11–18. doi:10.1016/j.ijfoodmicro.2006.10.047.

Görner, W., Durchschlag, E., Martinez-Pastor, M. T., Estruch, F., Ammerer, G., Hamilton, B., et al. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* 12, 586–97.

Grant, C. M., MacIver, F. H., and Dawes, I. W. (1997). Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces

cerevisiae. *FEBS Lett.* 29, 511–515. doi:10.1016/S0014-5793(97)00592-9.

Greetham, D., Wimalasena, T. T., Leung, K., Marvin, M. E., Chandelia, Y., Hart, A. J., et al. (2014). The genetic basis of variation in clean lineages of Saccharomyces cerevisiae in response to stresses encountered during bioethanol fermentations. *PLoS One* 9, e103233. doi:10.1371/journal.pone.0103233.

Greig, D., Borts, R. H., Louis, E. J., and Travisano, M. (2002a). Epistasis and hybrid sterility in Saccharomyces. *Proc. Biol. Sci.* 269, 1167–71. doi:10.1098/rspb.2002.1989.

Greig, D., Louis, E. J., Borts, R. H., Travisano, M., Coyne, J. A., Johnson, P. A., et al. (2002b). Hybrid speciation in experimental populations of yeast. *Science* 298, 1773–5. doi:10.1126/science.1076374.

Güldener, U., Heck, S., Fielder, T., Beinhauer, J., and Hegemann, J. H. (1996). A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24, 2519–24.

Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F. N., and Guillamon, J. M. (2012). Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. *Food Microbiol.* 31, 25–32. doi:10.1016/j.fm.2012.02.012.

Halliwell, B., and Gutteridge, J. M. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* 246, 501–14.

Hauser, N. C., Fellenberg, K., Gil, R., Bastuck, S., Rg, J., Hoheisel, D., et al. (2001). Whole genome analysis of a wine yeast strain. *Comp. Funct. Genomics* 2, 69–79. doi:10.1002/cfg.73.

Henderson, C. M., Lozada-Contreras, M., Jiranek, V., Longo, M. L., and Block, D. E. (2013a). Ethanol production and maximum cell growth are highly correlated with membrane lipid composition during fermentation as determined by lipidomic analysis of 22 Saccharomyces cerevisiae strains. *Appl. Environ. Microbiol.* 79, 91–104. doi:10.1128/AEM.02670-12.

Henderson, C. M., Zeno, W. F., Lerno, L. A., Longo, M. L., and Block, D. E. (2013b).

Fermentation Temperature Modulates Phosphatidylethanolamine and Phosphatidylinositol Levels in the Cell Membrane of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 79, 5345–5356. doi:10.1128/AEM.01144-13.

Herrero, E., Ros, J., Bellí, G., and Cabiscol, E. (2008). Redox control and oxidative stress in yeast cells. *Biochim. Biophys. Acta* 1780, 1280–1235. doi:10.1016/j.bbagen.2007.12.004.

Hickman, M. J., Petti, A. A., Ho-Shing, O., Silverman, S. J., McIsaac, R. S., Lee, T. A., et al. (2011). Coordinated regulation of sulfur and phospholipid metabolism reflects the importance of methylation in the growth of yeast. *Mol. Biol. Cell* 22, 4192–204. doi:10.1091/mbc.E11-05-0467.

Hilliker, A. K., Mefford, M. A., and Staley, J. P. (2007). U2 toggles iteratively between the stem IIa and stem IIc conformations to promote pre-mRNA splicing. *Genes Dev.* 21, 821–34. doi:10.1101/gad.1536107.

Hiltunen, J. K., Mursula, A. M., Rottensteiner, H., Wierenga, R. K., Kastaniotis, A. J., and Gurvitz, A. (2003). The biochemistry of peroxisomal beta-oxidation in the yeast Saccharomyces cerevisiae. *FEMS Microbiol. Rev.* 27, 35–64.

Hittinger, C. T. (2013). Saccharomyces diversity and evolution: a budding model genus. *Trends Genet.* 29, 309–17. doi:10.1016/j.tig.2013.01.002.

Hittinger, C. T., and Carroll, S. B. (2007). Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* 449, 677–81. doi:10.1038/nature06151.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J. Biol. Chem.* 264, 13963–6.

Homma, T., Iwahashi, H., and Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. *Cryobiology* 46, 230–237. doi:10.1016/S0011-2240(03)00028-2.

Hong, K.-K., Vongsangnak, W., Vemuri, G. N., and Nielsen, J. (2011). Unravelling evolutionary strategies of yeast for improving galactose utilization through integrated systems level analysis. *Proc. Natl. Acad. Sci.* 108, 12179–12184. doi:10.1073/pnas.1103219108.

Hoskisson, P. A., and Hobbs, G. (2005). Continuous culture--making a comeback? *Microbiology* 151, 3153–9. doi:10.1099/mic.0.27924-0.

Hughes, T. R., Roberts, C. J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., et al. (2000). Widespread aneuploidy revealed by DNA microarray expression profiling. *Nat. Genet.* 25, 333–337. doi:10.1038/77116.

Huxley, C., Green, E. D., and Dunham, I. (1990). Rapid assessment of S. cerevisiae mating type by PCR. *Trends Genet.* 6, 236.

Infante, J. J., Dombek, K. M., Rebordinos, L., Cantoral, J. M., and Young, E. T. (2003). Genome-Wide Amplifications Caused by Chromosomal Rearrangements Play a Major Role in the Adaptive Evolution of Natural Yeast. *Genetics* 165, 1745–1759.

Inouye, M., and Phadtare, S. (2004). Cold shock response and adaptation at near-freezing temperature in microorganisms. *Sci STKE* 2004, pe26. doi:10.1126/stke.2372004pe26.

Izawa, S., Maeda, K., Sugiyama, K.-I., Mano, J. 'ichi, Inoue, Y., and Kimura, A. (1999). Thioredoxin Deficiency Causes the Constitutive Activation of Yap1, an AP-1-like Transcription Factor in Saccharomyces cerevisiae. *J. Biol. Chem.* 40, 28459–28465.

Jackson, C. L., and Hartwell, L. H. (1990). Courtship in Saccharomyces cerevisiae: an early cell-cell interaction during mating. *Mol. Cell. Biol.* 10, 2202–13.

Jamieson, D. J. (1998). Oxidative Stress Responses of the Yeast Saccharomyces cerevisiae. *Yeast* 14, 1511–1527. doi:10.1002/(SICI)1097-0061(199812)14:16<1511::AID-YEA356>3.0.CO;2-S.

Jones, P. G., and Inouye, M. (1996). RbfA, a 30S ribosomal binding factor, is a cold-shock protein whose absence triggers the cold-shock response. *Mol. Microbiol.* 21, 1207–1218. doi:10.1111/j.1365-2958.1996.tb02582.x.

Kaeberlein, M., Powers, R. W., Steffen, K. K., Westman, E. A., Hu, D., Dang, N., et al. (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* 310, 1193–6. doi:10.1126/science.1115535.

Kandror, O., Bretschneider, N., Kreydin, E., Cavalieri, D., and Goldberg, A. L. (2004). Yeast adapt to near-freezing temperatures by STRE/Msn2,4-dependent induction of trehalose synthesis and certain molecular chaperones. *Mol. Cell* 13, 771–81.

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software

version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–80. doi:10.1093/molbev/mst010.

Katou, T., Namise, M., Kitagaki, H., Akao, T., and Shimoi, H. (2009). QTL mapping of sake brewing characteristics of yeast. *J. Biosci. Bioeng.* 107, 383–393. doi:10.1016/j.jbiosc.2008.12.014.

Kazemi Seresht, A., Cruz, A. L., de Hulster, E., Hebly, M., Palmqvist, E. A., van Gulik, W., et al. (2013). Long-term adaptation of Saccharomyces cerevisiae to the burden of recombinant insulin production. *Biotechnol. Bioeng.* 110, 2749–63. doi:10.1002/bit.24927.

Kessi-Pérez, E. I., Araos, S., García, V., Salinas, F., Abarca, V., Larrondo, L. F., et al. (2016). RIM15 antagonistic pleiotropy is responsible for differences in fermentation and stress response kinetics in budding yeast. *FEMS Yeast Res.* 16, fow021. doi:10.1093/femsyr/fow021.

Khrameeva, E. E., Fudenberg, G., Gelfand, M. S., and Mirny, L. A. (2016). History of chromosome rearrangements reflects the spatial organization of yeast chromosomes. *J. Bioinform. Comput. Biol.* 14, 1641002. doi:10.1142/S021972001641002X.

Killian, E., and Ough, C. S. (1979). Fermentation Esters-Formation and Retention as Affected by Fermentation Temperature. *Am. J. Enol. Vitic.* 30, 301–305.

Kim, H. S., and Fay, J. C. (2007). Genetic variation in the cysteine biosynthesis pathway causes sensitivity to pharmacological compounds. *Proc. Natl. Acad. Sci. U. S. A.* 104, 19387–19391. doi:10.1073/pnas.0708194104.

Kitano, H. (2002). Systems biology: a brief overview. *Science* 295, 1662–4. doi:10.1126/science.1069492.

Kondo, K., and Inouye, M. (1991). TIP1, a cold shock-inducible gene of Saccharomyces cerevisiae. *J. Biol. Chem.* 266, 17537–44.

Kosugi, A., Koizumi, Y., Yanagida, F., and Udaka, S. (2001). MUP1, high affinity methionine permease, is involved in cysteine uptake by Saccharomyces cerevisiae. *Biosci. Biotechnol. Biochem.* 65, 728–31. doi:http://doi.org/10.1271/bbb.65.728.

Kuge, S., and Jones, N. (1994). YAP1 dependent activation of TRX2 is essential for the

response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. *EMBO J.* 13, 655–64.

Kurata, S., Nielsen, K. H., Mitchell, S. F., Lorsch, J. R., Kaji, A., and Kaji, H. (2010). Ribosome recycling step in yeast cytoplasmic protein synthesis is catalyzed by eEF3 and ATP. *Proc. Natl. Acad. Sci. U. S. A.* 107, 10854–9. doi:10.1073/pnas.1006247107.

Kwast, K. E., Lai, L.-C., Menda, N., James, D. T., Aref, S., and Burke, P. V (2002). Genomic analyses of anaerobically induced genes in Saccharomyces cerevisiae: functional roles of Rox1 and other factors in mediating the anoxic response. *J. Bacteriol.* 184, 250–65.

Lai, L.-C., Kosorukoff, A. L., Burke, P. V, and Kwast, K. E. (2005). Dynamical remodeling of the transcriptome during short-term anaerobiosis in Saccharomyces cerevisiae: differential response and role of Msn2 and/or Msn4 and other factors in galactose and glucose media. *Mol. Cell. Biol.* 25, 4075–91. doi:10.1128/MCB.25.10.4075-4091.2005.

Lamb, D. C., Kelly, D. E., Manning, N. J., Kaderbhai, M. A., and Kelly, S. L. (1999). Biodiversity of the P450 catalytic cycle: Yeast cytochrome b5/NADH cytochrome b5 reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction. *FEBS Lett.* 462, 283–288. doi:10.1016/S0014-5793(99)01548-3.

Landry, C. R., Townsend, J. P., Hartl, D. L., and Cavalieri, D. (2006). Ecological and evolutionary genomics of Saccharomyces cerevisiae. *Mol. Ecol.* 15, 575–91. doi:10.1111/j.1365-294X.2006.02778.x.

Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–9. doi:10.1038/nmeth.1923.

Lee, J., Spector, D., Godon, C., Labarre, J., and Toledano, M. B. (1999). A new antioxidant with alkyl hydroperoxide defense properties in yeast. *J. Biol. Chem.* 274, 4537–44.

Lee, T. A., Jorgensen, P., Bognar, A. L., Peyraud, C., Thomas, D., and Tyers, M. (2010). Dissection of combinatorial control by the Met4 transcriptional complex. *Mol. Biol. Cell* 21, 456–69. doi:10.1091/mbc.E09-05-0420.

Legras, J.-L., and Karst, F. (2003). Optimisation of interdelta analysis for *Saccharomyces*

cerevisiae strain characterisation. *FEMS Microbiol. Lett.* 221, 249–255. doi:10.1016/S0378-1097(03)00205-2.

Lendenmann, M. H., Croll, D., Palma-Guerrero, J., Stewart, E. L., and McDonald, B. A. (2016). QTL mapping of temperature sensitivity reveals candidate genes for thermal adaptation and growth morphology in the plant pathogenic fungus Zymoseptoria tritici. *Heredity (Edinb).* 116, 384–94. doi:10.1038/hdy.2015.111.

Li, J., Wang, L., Wu, X., Fang, O., Wang, L., Lu, C., et al. (2013). Polygenic molecular architecture underlying non-sexual cell aggregation in budding yeast. *DNA Res.* 20, 55–66. doi:10.1093/dnares/dss033.

Li, Y., Zhang, W., Zheng, D., Zhou, Z., Yu, W., Zhang, L., et al. (2014). Genomic evolution of Saccharomyces cerevisiae under Chinese rice wine fermentation. *Genome Biol. Evol.* 6, 2516–2526. doi:10.1093/gbe/evu201.

Li, Z., and Brow, D. A. (1996). A spontaneous duplication in U6 spliceosomal RNA uncouples the early and late functions of the ACAGA element in vivo. *RNA* 2, 879–94.

Libkind, D., Hittinger, C. T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., et al. (2011). Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. *Proc. Natl. Acad. Sci. U. S. A.* 108, 14539–44. doi:10.1073/pnas.1105430108.

Liti, G., Barton, D. B. H., and Louis, E. J. (2006). Sequence Diversity, Reproductive Isolation and Species Concepts in Saccharomyces. *Genetics* 174, 839–850.

Liti, G., Carter, D. M., Moses, A. M., Warringer, J., Parts, L., James, S. A., et al. (2009a). Population genomics of domestic and wild yeasts. *Nature* 458, 337–341. doi:10.1038/nature07743.

Liti, G., Haricharan, S., Cubillos, F. A., Tierney, A. L., Sharp, S., Bertuch, A. A., et al. (2009b). Segregating YKU80 and TLC1 alleles underlying natural variation in telomere properties in wild yeast. *PLoS Genet.* 5, e1000659. doi:10.1371/journal.pgen.1000659.

Liti, G., and Louis, E. J. (2012). Advances in Quantitative Trait Analysis in Yeast. *PLoS Genet.* 8, e1002912. doi:10.1371/journal.pgen.1002912.

Llauradó, J. M., Rozès, N., Constantí, M., Mas, A., Josep M. Llauradó, Nicolas Rozès, et al. (2005). Study of some Saccharomyces cerevisiae strains for winemaking after preadaptation at low temperature. *J. Agric. Food Chem.* 53, 1003–1011. doi:10.1021/jf049324n.

Llauradó, J., Rozès, N., Bobet, R., Mas, A., and Constantí, A. M. (2002). Low Temperature Alcoholic Fermentations in High Sugar Concentration Grape Musts. *J. Food Sci.* 67, 268–273.

Llopis, S., Querol, A., Heyken, A., Hube, B., Jespersen, L., Fernández-Espinar, M., et al. (2012). Transcriptomics in human blood incubation reveals the importance of oxidative stress response in Saccharomyces cerevisiae clinical strains. *BMC Genomics* 13, 419. doi:10.1186/1471-2164-13-419.

Long, A., Liti, G., Luptak, A., and Tenaillon, O. (2015). Elucidating the molecular architecture of adaptation via evolve and resequence experiments. *Nat. Rev. Genet.* 16, 567–582. doi:10.1038/nrg3937.

Lopandic, K., Pfliegler, W. P., Tiefenbrunner, W., Gangl, H., Sipiczki, M., and Sterflinger, K. (2016). Genotypic and phenotypic evolution of yeast interspecies hybrids during high-sugar fermentation. *Appl. Microbiol. Biotechnol.* 100, 6331–43. doi:10.1007/s00253-016-7481-0.

Lopes, C. A., Barrio, E., and Querol, A. (2010). Natural hybrids of S. cerevisiae x S. kudriavzevii share alleles with European wild populations of Saccharomyces kudriavzevii. *FEMS Yeast Res.* 10, 412–21. doi:10.1111/j.1567-1364.2010.00614.x.

López, S., Prieto, M., Dijkstra, J., Dhanoa, M. S., and France, J. (2004). Statistical evaluation of mathematical models for microbial growth. *Int. J. Food Microbiol.* 96, 289–300. doi:10.1016/j.ijfoodmicro.2004.03.026.

López-Malo, M., Chiva, R., Rozes, N., Guillamón, J. M., and Guillamon, J. M. (2013a). Phenotypic analysis of mutant and overexpressing strains of lipid metabolism genes in Saccharomyces cerevisiae: Implication in growth at low temperatures. *Int. J. Food Microbiol.* 162, 26–36. doi:10.1016/j.ijfoodmicro.2012.12.020.

López-Malo, M., García-Ríos, E., Chiva, R., and Guillamon, J. M. (2014a). Functional analysis of lipid metabolism genes in wine yeasts during alcoholic fermentation at

low temperature. *Microb. cell* 1, 1–11. doi:10.15698/mic2014.11.174.

López-Malo, M., García-Ríos, E., Chiva, R., Guillamon, J. M., and Martí-Raga, M. (2014b). Effect of deletion and overexpression of tryptophan metabolism genes on growth and fermentation capacity at low temperature in wine yeast. *Biotechnol. Prog.* 30, 776–783. doi:10.1002/btpr.1915.

López-Malo, M., García-Rios, E., Melgar, B., Sanchez, M. R., Dunham, M. J., and Guillamón, J. M. (2015). Evolutionary engineering of a wine yeast strain revealed a key role of inositol and mannoprotein metabolism during low-temperature fermentation. *BMC Genomics* 16, 537. doi:10.1186/s12864-015-1755-2.

López-Malo, M., Querol, A., and Guillamón, J. M. (2013b). Metabolomic Comparison of Saccharomyces cerevisiae and the Cryotolerant Species S. bayanus var. uvarum and S. kudriavzevii during Wine Fermentation at Low Temperature. *PLoS One* 8, e60135. doi:10.1371/journal.pone.0060135.

Louis, E. J., and Borts, R. H. (1995). A complete set of marked telomeres in Saccharomyces cerevisiae for physical mapping and cloning. *Genetics* 139, 125–36.

Lydall, D. (2003). Hiding at the ends of yeast chromosomes: telomeres, nucleases and checkpoint pathways. *J. Cell Sci.* 116, 4057–65. doi:10.1242/jcs.00765.

Lynch, M., Force, A., Allendorf, F. W., Utter, F. M., May, B. P., Amores, A., et al. (2000). The probability of duplicate gene preservation by subfunctionalization. *Genetics* 154, 459–73. doi:10.1126/science.282.5394.1711.

Mackay, T. F. C., Stone, E. a, and Ayroles, J. F. (2009). The genetics of quantitative traits: challenges and prospects. *Nat. Rev. Genet.* 10, 565–577. doi:10.1038/nrg2612.

Magwene, P. M. (2014). Revisiting mortimer’s genome renewal hypothesis: Heterozygosity, homothallism, and the potential for adaptation in yeast. *Adv. Exp. Med. Biol.* 781, 37–48. doi:10.1007/978-94-007-7347-9-3.

Marcet-Houben, M., Gabaldón, T., Jaillon, O., Aury, J.-M., Wincker, P., Coghlan, A., et al. (2015). Beyond the Whole-Genome Duplication: Phylogenetic Evidence for an Ancient Interspecies Hybridization in the Baker’s Yeast Lineage. *PLOS Biol.* 13, e1002220. doi:10.1371/journal.pbio.1002220.

Marks, V. D., Ho Sui, S. J., Erasmus, D., Van Der Merwe, G. K., Brumm, J., Wasserman, W. W., et al. (2008). Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. *FEMS Yeast Res.* 8, 35–52. doi:10.1111/j.1567-1364.2007.00338.x.

Martínez-Moreno, R., Morales, P., Gonzalez, R., Mas, A., and Beltran, G. (2012). Biomass production and alcoholic fermentation performance of Saccharomyces cerevisiae as a function of nitrogen source. *FEMS Yeast Res.* 12, 477–85. doi:10.1111/j.1567-1364.2012.00802.x.

Martínez-Pastor, M. T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H., and Estruch, F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J.* 15, 2227–35.

Martini, A., Federici, F., and Rosini, G. (1980). A new approach to the study of yeast ecology of natural substrates. *Can. J. Microbiol.* 26, 856–859. doi:10.1139/m80-149.

Marullo, P., Bely, M., Masneuf-Pomarede, I., Aigle, M., and Dubourdieu, D. (2004). Inheritable nature of enological quantitative traits is demonstrated by meiotic segregation of industrial wine yeast strains. *FEMS Yeast Res.* 4, 711–719. doi:http://dx.doi.org/10.1016/j.femsyr.2004.01.006.

Marullo, P., Bely, M., Masneuf-Pomarède, I., Pons, M., Aigle, M., and Dubourdieu, D. (2006). Breeding strategies for combining fermentative qualities and reducing off-flavor production in a wine yeast model. *FEMS Yeast Res.* 6, 268–279. doi:10.1111/j.1567-1364.2006.00034.x.

Marullo, P., Yvert, G., Bely, M., Aigle, M., and Dubourdieu, D. (2007). Efficient use of DNA molecular markers to construct industrial yeast strains. *FEMS Yeast Res.* 7, 1295–1306. doi:10.1111/j.1567-1364.2007.00281.x.

Masneuf-Pomarede, I., Bely, M., Marullo, P., and Albertin, W. (2016). The genetics of non-conventional wine yeasts: Current knowledge and future challenges. *Front. Microbiol.* 6, 1563. doi:10.3389/fmicb.2015.01563.

Masneuf-Pomarède, I., Bely, M., Marullo, P., Lonvaud-Funel, A., and Dubourdieu, D. (2010). Reassessment of phenotypic traits for Saccharomyces bayanus var. uvarum

wine yeast strains. *Int. J. Food Microbiol.* 139, 79–86. doi:10.1016/j.ijfoodmicro.2010.01.038.

McIsaac, R. S., Petti, A. A., Bussemaker, H. J., and Botstein, D. (2012). Perturbation-based analysis and modeling of combinatorial regulation in the yeast sulfur assimilation pathway. *Mol. Biol. Cell* 23, 2993–3007. doi:10.1091/mbc.E12-03-0232.

Merhej, J., Delaveau, T., Guitard, J., Palancade, B., Hennequin, C., Garcia, M., et al. (2015). Yap7 is a transcriptional repressor of nitric oxide oxidase in yeasts, which arose from neofunctionalization after whole genome duplication. *Mol. Microbiol.* 96, 951–72. doi:10.1111/mmi.12983.

Mertens, S., Steensels, J., Saels, V., De Rouck, G., Aerts, G., and Verstrepen, K. J. (2015). A large set of newly created interspecific Saccharomyces hybrids increases aromatic diversity in lager beers. *Appl. Environ. Microbiol.* 81, 8202–8214. doi:10.1128/AEM.02464-15.

Michiels, C., Raes, M., Toussaint, O., and Remacle, J. (1994). Importance of SE-glutathione peroxidase, catalase, and CU/ZN-SOD for cell survival against oxidative stress. *Free Radic. Biol. Med.* 17, 235–248. doi:10.1016/0891-5849(94)90079-5.

Mitchell, A., Romano, G. H., Groisman, B., Yona, A., Dekel, E., Kupiec, M., et al. (2009). Adaptive prediction of environmental changes by microorganisms. *Nature* 460, 220–4. doi:10.1038/nature08112.

Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S., and Agosin, E. (2007). Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Appl. Microbiol. Biotechnol.* 77, 675–687. doi:10.1007/s00253-007-1194-3.

Morales, L., and Dujon, B. (2012). Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. *Microbiol. Mol. Biol. Rev.* 76, 721–39. doi:10.1128/MMBR.00022-12.

Morano, K. A., Grant, C. M., and Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in saccharomyces cerevisiae. *Genetics* 190, 1157–1195. doi:10.1534/genetics.111.128033.

Mortimer, R. K. (2000). Evolution and variation of the yeast (Saccharomyces) genome.

Genome Res. 10, 403–409. doi:10.1101/gr.10.4.403.

Mortimer, R. K., and Johnston, J. R. (1986). Genealogy of principal strains of the yeast genetic stock center. *Genetics* 113, 35–43.

Mortimer, R. K., Romano, P., Suzzi, G., and Polsinelli, M. (1994). Genome renewal: A new phenomenon revealed from a genetic study of 43 strains ofSaccharomyces cerevisiae derived from natural fermentation of grape musts. *Yeast* 10, 1543–1552. doi:10.1002/yea.320101203.

Munna, M. S., Humayun, S., and Noor, R. (2015). Influence of heat shock and osmotic stresses on the growth and viability of Saccharomyces cerevisiae SUBSC01. *BMC Res. Notes* 8, 369. doi:10.1186/s13104-015-1355-x.

Murata, Y., Takayuki, A., Ae, H., Kitagawa, E., Momose, Y., Sato, M. S., et al. (2006). Genome-wide expression analysis of yeast response during exposure to 4°C. *Extremophiles* 10, 117–128. doi:10.1007/s00792-005-0480-1.

Nadal, D., Carro, D., Fernández-Larrea, J., and Piña, B. (1999). Analysis and dynamics of the chromosomal complements of wild sparkling-wine yeast strains. *Appl. Environ. Microbiol.* 65, 1688–95.

Naithani, S., Saracco, S. A., Butler, C. A., and Fox, T. D. (2003). Interactions among COX1, COX2, and COX3 mRNA-specific translational activator proteins on the inner surface of the mitochondrial inner membrane of Saccharomyces cerevisiae. *Mol. Biol. Cell* 14, 324–33. doi:10.1091/mbc.E02-08-0490.

Nakagawa, Y., Sakumoto, N., Kaneko, Y., and Harashima, S. (2002). Mga2p is a putative sensor for low temperature and oxygen to induce OLE1 transcription in Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* 291, 707–13. doi:10.1006/bbrc.2002.6507.

Naseeb, S., Carter, Z., Minnis, D., Donaldson, I., Zeef, L., and Delneri, D. (2016). Widespread Impact of Chromosomal Inversions on Gene Expression Uncovers Robustness via Phenotypic Buffering. *Mol. Biol. Evol.* 33, 1679–96. doi:10.1093/molbev/msw045.

Naumov, G. ., Nguyen, H.-V., Naumova, E. ., Michel, A., Aigle, M., and Gaillardin, C. (2001). Genetic identification of Saccharomyces bayanus var. uvarum, a cider-

fermenting yeast. *Int. J. Food Microbiol.* 65, 163–171. doi:10.1016/S0168-1605(00)00515-8.

Naumov, G. I., Gazdiev, D. O., and Naumova, E. S. (2003). The Finding of the Yeast Species Saccharomyces bayanus in Far East Asia. *Microbiology* 72, 738–743. doi:10.1023/B:MICI.0000008378.41367.19.

Naumov, G. I., James, S. A., Naumova, E. S., Louis, E. J., and Roberts, I. N. (2000a). Three new species in the Saccharomyces sensu stricto complex: Saccharomyces cariocanus, Saccharomyces kudriavzevii and Saccharomyces mikatae. *Int. J. Syst. Evol. Microbiol.* 50, 1931–42. doi:10.1099/00207713-50-5-1931.

Naumov, G. I., Masneuf, I., Naumova, E. S., Aigle, M., and Dubourdieu, D. (2000b). Association of Saccharomyces bayanus var. uvarum with some French wines: genetic analysis of yeast populations. *Res. Microbiol.* 151, 683–91.

Naumov, G. I., Naumova, E. S., Masneuf, I., Aigle, M., Kondratieva, V. I., and Dubourdieu, D. (2000c). Natural Polyploidization of Some Cultured Yeast Saccharomyces Sensu Stricto: Auto- and Allotetraploidy. *Syst. Appl. Microbiol.* 23, 442–449. doi:10.1016/S0723-2020(00)80076-4.

Navarro-Tapia, E., Nana, R. K., Querol, A., and P??rez-Torrado, R. (2016). Ethanol cellular defense induce unfolded protein response in yeast. *Front. Microbiol.* 7, 1–12. doi:10.3389/fmicb.2016.00189.

Nguyen, H. V, Lepingle, A., and Gaillardin, C. A. (2000). Molecular typing demonstrates homogeneity of Saccharomyces uvarum strains and reveals the existence of hybrids between S. uvarum and S. cerevisiae, including the S. bayanus type strain CBS 380. *Syst. Appl. Microbiol.* 23, 71–85.

Nogami, S., Ohya, Y., and Yvert, G. (2007). Genetic complexity and quantitative trait loci mapping of yeast morphological traits. *PLoS Genet.* 3, 0305–0318. doi:10.1371/journal.pgen.0030031.

Novo, M., Dé, F., Bigey, R., Beyne, E., Galeote, V., Gavory, R., et al. (2009). Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast Saccharomyces cerevisiae EC1118. *Proc. Natl. Acad. Sci.* 106, 16333–16338.

Oliveira, B. M., Barrio, E., Querol, A., and Pérez-Torrado, R. (2014). Enhanced enzymatic

activity of glycerol-3-phosphate dehydrogenase from the cryophilic Saccharomyces kudriavzevii. *PLoS One* 9, e87290. doi:10.1371/journal.pone.0087290.

Oliver, D. J., Nikolau, B., and Wurtele, E. S. (2002). Functional genomics: high-throughput mRNA, protein, and metabolite analyses. *Metab. Eng.* 4, 98–106. doi:10.1006/mben.2001.0212.

Oliver, S. G. (2002). Functional genomics: lessons from yeast. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 357, 17–23. doi:10.1098/rstb.2001.1049.

Padilla, P. A., Fuge, E. K., Crawford, M. E., Errett, A., and Werner-Washburne, M. (1998). The highly conserved, coregulated SNO and SNZ gene families in Saccharomyces cerevisiae respond to nutrient limitation. *J. Bacteriol.* 180, 5718–5726.

Paget, C. M., Schwartz, J. M., and Delneri, D. (2014). Environmental systems biology of cold-tolerant phenotype in Saccharomyces species adapted to grow at different temperatures. *Mol. Ecol.* 23, 5241–5257. doi:10.1111/mec.12930.

Panadero, J., Pallotti, C., Rodríguez-Vargas, S., Randez-Gil, F., and Prieto, J. A. (2006). A downshift in temperature activates the high osmolarity glycerol (HOG) pathway, which determines freeze tolerance in Saccharomyces cerevisiae. *J. Biol. Chem.* 281, 4638–45. doi:10.1074/jbc.M512736200.

Parts, L. (2014). Genome-wide mapping of cellular traits using yeast. *Yeast* 31, 197–205. doi:10.1002/yea.3010.

Parts, L., Cubillos, F. A., Warringer, J., Jain, K., Salinas, F., Bumpstead, S. J., et al. (2011). Revealing the genetic structure of a trait by sequencing a population under selection. *Genome Res.* 21, 1131–1138. doi:10.1101/gr.116731.110.

Parts, L., Liu, Y.-C., Tekkedil, M. M., Steinmetz, L. M., Caudy, A. A., Fraser, A. G., et al. (2014). Heritability and genetic basis of protein level variation in an outbred population. *Genome Res.* 24, 1363–70. doi:10.1101/gr.170506.113.

Pedrajas, J. R., McDonagh, B., Hernández-Torres, F., Miranda-Vizuete, A., González-Ojeda, R., Martínez-Galisteo, E., et al. (2015). Glutathione is the resolving thiol for thioredoxin peroxidase activity of 1-Cys Peroxiredoxin without being consumed during the catalytic cycle. *Antioxid. Redox Signal.* 24, 115–128. doi:10.1089/ars.2015.6366.

Pérez-Ortín, J. E., Querol, A., Puig, S., and Barrio, E. (2002). Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. *Genome Res.* 12, 1533–9. doi:10.1101/gr.436602.

Pérez-Torrado, R., Llopis, S., Jespersen, L., Fernández-Espinar, T., and Querol, A. (2012). Clinical Saccharomyces cerevisiae isolates cannot cross the epithelial barrier in vitro. *Int. J. Food Microbiol.* 157, 59–64. doi:10.1016/j.ijfoodmicro.2012.04.012.

Pérez-Torrado, R., and Querol, A. (2016). Opportunistic Strains of Saccharomyces cerevisiae: A Potential Risk Sold in Food Products. *Front. Microbiol.* 6, 1–5. doi:10.3389/fmicb.2015.01522.

Peris, D., Langdon, Q. K., Moriarty, R. V., Sylvester, K., Bontrager, M., Charron, G., et al. (2016a). Complex Ancestries of Lager-Brewing Hybrids Were Shaped by Standing Variation in the Wild Yeast Saccharomyces eubayanus. *PLOS Genet.* 12, e1006155. doi:10.1371/journal.pgen.1006155.

Peris, D., Lopes, C. A., Belloch, C., Querol, A., and Barrio, E. (2012). Comparative genomics among Saccharomyces cerevisiae × Saccharomyces kudriavzevii natural hybrid strains isolated from wine and beer reveals different origins. *BMC Genomics* 13, 1. doi:10.1186/1471-2164-13-407.

Peris, D., Pérez-Través, L., Belloch, C., and Querol, A. (2016b). Enological characterization of Spanish Saccharomyces kudriavzevii strains, one of the closest relatives to parental strains of winemaking and brewing Saccharomyces cerevisiae X S. kudriavzevii hybrids. *Food Microbiol.* 53, 31–40. doi:10.1016/j.fm.2015.07.010.

Perriman, R. J., and Ares, M. (2007). Rearrangement of competing U2 RNA helices within the spliceosome promotes multiple steps in splicing. *Genes Dev.* 21, 811–20. doi:10.1101/gad.1524307.

Petranovic, D., and Nielsen, J. (2008). Can yeast systems biology contribute to the understanding of human disease? *Trends Biotechnol.* 26, 584–90. doi:10.1016/j.tibtech.2008.07.008.

Petti, A. A., Crutchfield, C. A., Rabinowitz, J. D., and Botstein, D. (2011). Survival of starving yeast is correlated with oxidative stress response and nonrespiratory mitochondrial function. *Proc. Natl. Acad. Sci. U. S. A.* 108, 1089–98.

doi:10.1073/pnas.1101494108.

Piskur, J., Rozpedowska, E., Polakova, S., Merico, A., and Compagno, C. (2006). How did Saccharomyces evolve to become a good brewer? *Trends Genet.* 22, 183–6. doi:10.1016/j.tig.2006.02.002.

Pizarro, F. J., Jewett, M. C., Nielsen, J., and Agosin, E. (2008). Growth temperature exerts differential physiological and transcriptional responses in laboratory and wine strains of Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 74, 6358–68. doi:10.1128/AEM.00602-08.

Pizarro, F., Varela, C., Martabit, C., Bruno, C., Pérez-Correa, J. R., and Agosin, E. (2007). Coupling kinetic expressions and metabolic networks for predicting wine fermentations. *Biotechnol. Bioeng.* 98, 986–98. doi:10.1002/bit.21494.

Pretorius, I. S. (2000a). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of wine making. *Yeast* 16, 675–729. doi:10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B.

Pretorius, I. S. (2000b). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16, 675–729. doi:10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B.

Pryde, F. E., and Louis, E. J. (1997). Saccharomyces cerevisiae telomeres. A review. *Biochem. Biokhimii͡a* 62, 1232–41.

Puig, S., Querol, A., Barrio, E., and Pérez-Ortín, J. E. (2000). Mitotic recombination and genetic changes in Saccharomyces cerevisiae during wine fermentation. *Appl. Environ. Microbiol.* 66, 2057–61.

Querol, A., Barrio, E., and Ramón, D. (1992). A Comparative Study of Different Methods of Yeast Strain Characterization. *Syst. Appl. Microbiol.* 15, 439–446. doi:10.1016/S0723-2020(11)80219-5.

Querol, A., Belloch, C., Fernández-Espinar, M. T., and Barrio, E. (2003a). Molecular evolution in yeast of biotechnological interest. *Int. Microbiol.* 6, 201–5. doi:10.1007/s10123-003-0134-z.

Querol, A., Fernández-Espinar, M. T., del Olmo, M. l., and Barrio, E. (2003b). Adaptive

evolution of wine yeast. *Int. J. Food Microbiol.* 86, 3–10. doi:10.1016/S0168-1605(03)00244-7.

Quirós, M., Martínez-Moreno, R., Albiol, J., Morales, P., Vázquez-Lima, F., Barreiro-Vázquez, A., et al. (2013). Metabolic Flux Analysis during the Exponential Growth Phase of Saccharomyces cerevisiae in Wine Fermentations. *PLoS One* 8, 1–14. doi:10.1371/journal.pone.0071909.

Quirós, M., Rojas, V., Gonzalez, R., and Morales, P. (2014). Selection of non-Saccharomyces yeast strains for reducing alcohol levels in wine by sugar respiration. *Int. J. Food Microbiol.* 2, 85–91. doi:10.1016/j.ijfoodmicro.2014.04.024.

Radford, A. (1991). Methods in Yeast Genetics — A Laboratory Course Manual. *Biochem. Educ.* 19, 101–102. doi:10.1016/0307-4412(91)90039-B.

Rainieri, S., Kodama, Y., Kaneko, Y., Mikata, K., Nakao, Y., and Ashikari, T. (2006). Pure and mixed genetic lines of Saccharomyces bayanus and Saccharomyces pastorianus and their contribution to the lager brewing strain genome. *Appl. Environ. Microbiol.* 72, 3968–74. doi:10.1128/AEM.02769-05.

Rainieri, S., and Pretorius, I. (2000). Selection and improvement of wine yeasts. *Ann. Microbiol.* 50, 15–31.

Ramazzotti, M., Berná, L., Stefanini, I., and Cavalieri, D. (2012). A computational pipeline to discover highly phylogenetically informative genes in sequenced genomes: Application to Saccharomyces cerevisiae natural strains. *Nucleic Acids Res.* 40, 3834–3848. doi:10.1093/nar/gks005.

Redón, M., Guillamón, J. M., Mas, A., and Rozés, N. (2011). Effect of growth temperature on yeast lipid composition and alcoholic fermentation at low temperature. *Eur. Food Res. Technol.* 232, 517–527. doi:10.1007/s00217-010-1415-3.

Regenberg, B., Grotkjaer, T., Winther, O., Fausbøll, A., Akesson, M., Bro, C., et al. (2006). Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in Saccharomyces cerevisiae. *Genome Biol.* 7, R107. doi:10.1186/gb-2006-7-11-r107.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., and Lonvaud, A. (2005). *Handbook of Enology*. Chichester, UK: John Wiley & Sons, Ltd doi:10.1002/0470010363.

Riou, C., Nicaud, J. M., Barre, P., and Gaillardin, C. (1997). Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast* 13, 903–15. doi:10.1002/(SICI)1097-0061(199708)13:10<903::AID-YEA145>3.0.CO;2-1.

Roberts, G. G., and Hudson, A. P. (2006). Transcriptome profiling of Saccharomyces cerevisiae during a transition from fermentative to glycerol-based respiratory growth reveals extensive metabolic and structural remodeling. *Mol. Genet. Genomics* 276, 170–86. doi:10.1007/s00438-006-0133-9.

Robzyk, K., and Kassir, Y. (1992). A simple and highly efficient procedure for rescuing autonomous plasmids from yeast. *Nucleic Acids Res.* 20, 3790.

Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B., Parker, K., Hattan, S., et al. (2004). Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154–69. doi:10.1074/mcp.M400129-MCP200.

Rossignol, T., Dulau, L., Julien, A., and Blondin, B. (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* 20, 1369–1385. doi:10.1002/yea.1046.

Rossignol, T., Kobi, D., Jacquet-Gutfreund, L., and Blondin, B. (2009). The proteome of a wine yeast strain during fermentation, correlation with the transcriptome. *J. Appl. Microbiol.* 107, 47–55. doi:10.1111/j.1365-2672.2009.04156.x.

Roustan, J. L., and Sablayrolles, J.-M. (2002). Modification of the acetaldehyde concentration during alcoholic fermentation and effects on fermentation kinetics. *J. Biosci. Bioeng.* 93, 367–75.

Sá-Correia, I., dos Santos, S. C., Teixeira, M. C., Cabrito, T. R., and Mira, N. P. (2009). Drug:H+ antiporters in chemical stress response in yeast. *Trends Microbiol.* 17, 22–31. doi:10.1016/j.tim.2008.09.007.

Sahara, T., Goda, T., and Ohgiya, S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J. Biol. Chem.* 277, 50015–50021. doi:10.1074/jbc.M209258200.

Salinas, F., Cubillos, F. A., Soto, D., Garcia, V., Bergström, A., Warringer, J., et al. (2012). The Genetic Basis of Natural Variation in Oenological Traits in Saccharomyces

cerevisiae. *PLoS One* 7, e49640. doi:10.1371/journal.pone.0049640.

Salvadó, Z., Arroyo-López, F. N., Barrio, E., Querol, A., and Guillamón, J. M. (2011a). Quantifying the individual effects of ethanol and temperature on the fitness advantage of Saccharomyces cerevisiae. *Food Microbiol.* 28, 1155–1161. doi:10.1016/j.fm.2011.03.008.

Salvadó, Z., Arroyo-López, F. N., Guillamón, J. M., Salazar, G., Querol, A., Barrio, E., et al. (2011b). Temperature adaptation Markedly Determines evolution within the genus Saccharomyces. *Appl. Environ. Microbiol.* 77, 2292–2302. doi:10.1128/AEM.01861-10.

Salvadó, Z., Chiva, R., Rodríguez-Vargas, S., Rández-Gil, F., Mas, A., and Guillamón, J. M. (2008). Proteomic evolution of a wine yeast during the first hours of fermentation. *FEMS Yeast Res.* 8, 1137–46. doi:10.1111/j.1567-1364.2008.00389.x.

Salvadó, Z., Chiva, R., Rozès, N., Cordero-Otero, R., and Guillamón, J. M. (2012). Functional analysis to identify genes in wine yeast adaptation to low-temperature fermentation. *J. Appl. Microbiol.* 113, 76–88. doi:10.1111/j.1365-2672.2012.05308.x.

Salvadó, Z., Ramos-Alonso, L., Tronchoni, J., Penacho, V., García-Ríos, E., Morales, P., et al. (2016). Genome-wide identification of genes involved in growth and fermentation activity at low temperature in Saccharomyces cerevisiae. *Int. J. Food Microbiol.* 236, 38–46. doi:10.1016/j.ijfoodmicro.2016.07.010.

Sampaio, J. P., and Gonçalves, P. (2008). Natural populations of Saccharomyces kudriavzevii in Portugal are associated with oak bark and are sympatric with S. cerevisiae and S. paradoxus. *Appl. Environ. Microbiol.* 74, 2144–52. doi:10.1128/AEM.02396-07.

Santos, J., Sousa, M. J., Cardoso, H., Inácio, J., Silva, S., Spencer-Martins, I., et al. (2008). Ethanol tolerance of sugar transport, and the rectification of stuck wine fermentations. *Microbiology* 154, 422–30. doi:10.1099/mic.0.2007/011445-0.

Schade, B., Jansen, G., Whiteway, M., Entian, K. D., and Thomas, D. Y. (2004). Cold Adaptation in Budding Yeast. *Mol. Biol. Cell* 15, 5492–5502. doi:10.1091/mbc.E04.

Schafer, F. Q., and Buettner, G. R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic.*

Biol. Med. 30, 1191–1212. doi:10.1016/S0891-5849(01)00480-4.

Schmitt, A. P., and McEntee, K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 93, 5777–82.

Schuller, D., Alves, H., Dequin, S., and Casal, M. (2005). Ecological survey of Saccharomyces cerevisiae strains from vineyards in the Vinho Verde Region of Portugal. *FEMS Microbiol. Ecol.* 51, 167–77. doi:10.1016/j.femsec.2004.08.003.

Selmecki, A. M., Maruvka, Y. E., Richmond, P. A., Guillet, M., Shoresh, N., Sorenson, A. L., et al. (2015). Polyploidy can drive rapid adaptation in yeast. *Nature* 519, 349–52. doi:10.1038/nature14187.

Sha, W., Martins, A. M., Laubenbacher, R., Mendes, P., and Shulaev, V. (2013). The Genome-Wide Early Temporal Response of Saccharomyces cerevisiae to Oxidative Stress Induced by Cumene Hydroperoxide. *PLoS One* 8, 1–15. doi:10.1371/journal.pone.0074939.

Shapira, R., and David, L. (2016). Genes with a Combination of Over-Dominant and Epistatic Effects Underlie Heterosis in Growth of Saccharomyces cerevisiae at High Temperature. *Front. Genet.* 7, 72. doi:10.3389/fgene.2016.00072.

Sharf, R., and Margalith, P. (1983). The effect of temperature on spontaneous wine fermentation. *Eur. J. Appl. Microbiol. Biotechnol.* 17, 311–313. doi:10.1007/BF00508027.

Sheehan, D., Meade, G., Foley, V. M., and Dowd, C. A. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem. J.* 360, 1–16.

Sheff, M. A., and Thorn, K. S. (2004). Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. *Yeast* 21, 661–70. doi:10.1002/yea.1130.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V, Sagliocco, F., Wilm, M., Vorm, O., et al. (1996). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. *Proc. Natl. Acad. Sci. U. S. A.* 93, 14440–5. doi:10.1073/pnas.93.25.14440.

Sicard, D., and Legras, J.-L. (2011). Bread, beer and wine: yeast domestication in the Saccharomyces sensu stricto complex. *C. R. Biol.* 334, 229–36. doi:10.1016/j.crvi.2010.12.016.

Sierkstra, L. N., Verbakel, J. M., and Verrips, C. T. (1992). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of Saccharomyces cerevisiae. *J. Gen. Microbiol.* 138, 2559–66. doi:10.1099/00221287-138-12-2559.

Sinha, H., David, L., Pascon, R. C., Clauder-M??nster, S., Krishnakumar, S., Nguyen, M., et al. (2008). Sequential elimination of major-effect contributors identifies additional quantitative trait loci conditioning high-temperature growth in yeast. *Genetics* 180, 1661–1670. doi:10.1534/genetics.108.092932.

Smits, H. P., Hauf, J., Müller, S., Hobley, T. J., Zimmermann, F. K., Hahn-Hägerdal, B., et al. (2000). Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of Saccharomyces cerevisiae. *Yeast* 16, 1325–1134. doi:10.1002/1097-0061(200010)16:14<1325::AID-YEA627>3.0.CO;2-E.

Speers, R. A., Rogers, P., and Smith, B. (2003). Non-Linear Modelling of Industrial Brewing Fermentations. *J. Inst. Brew.* 109, 229–235. doi:10.1002/j.2050-0416.2003.tb00163.x.

Stadtman, E. R., Moskovitz, J., and Levine, R. L. (2003). Oxidation of methionine residues of proteins: biological consequences. *Antioxid. Redox Signal.* 5, 577–82. doi:10.1089/152308603770310239.

Stadtman, E. R., Van Remmen, H., Richardson, A., Wehr, N. B., and Levine, R. L. (2005). Methionine oxidation and aging. *Biochim. Biophys. Acta - Proteins Proteomics* 1703, 135–140. doi:10.1016/j.bbapap.2004.08.010.

Staley, J. P., and Guthrie, C. (1999). An RNA Switch at the 5′ Splice Site Requires ATP and the DEAD Box Protein Prp28p. *Mol. Cell* 3, 55–64. doi:10.1016/S1097-2765(00)80174-4.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–3. doi:10.1093/bioinformatics/btu033.

Stearns, T., Hoyt, M. A., and Botstein, D. (1990). Yeast mutants sensitive to antimicrotubule drugs define three genes that affect microtubule function. *Genetics* 124, 251–262.

Steinmetz, L. M., Sinha, H., Richards, D. R., Spiegelman, J. I., Oefner, P. J., McCusker, J. H., et al. (2002). Dissecting the architecture of a quantitative trait locus in yeast. *Nature* 416, 326–30. doi:10.1038/416326a.

Tai, S. L., Daran-Lapujade, P., Walsh, M. C., Pronk, J. T., and Daran, J.-M. (2007). Acclimation of Saccharomyces cerevisiae to Low Temperature: A Chemostat-based Transcriptome Analysis. *Mol. Biol. Cell* 18, 5100–5112. doi:10.1091/mbc.E07.

Taxis, C., Keller, P., Kavagiou, Z., Jensen, L. J., Colombelli, J., Bork, P., et al. (2005). Spore number control and breeding in Saccharomyces cerevisiae: a key role for a self-organizing system. *J. Cell Biol.* 171, 627–40. doi:10.1083/jcb.200507168.

Thomas, D., and Surdin-Kerjan, Y. (1997). Metabolism of sulfur amino acids in Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* 61, 503–32.

Torija, M., Jesús, Rozés, N., Poblet, M., Guillamón, J. M., and Mas, A. (2003). Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. *Int. J. Food Microbiol.* 80, 47–53. doi:10.1016/S0168-1605(02)00144-7.

Treu, L., Toniolo, C., Nadai, C., Sardu, A., Giacomini, A., Corich, V., et al. (2014). The impact of genomic variability on gene expression in environmental Saccharomyces cerevisiae strains. *Environ. Microbiol.* 16, 1378–97. doi:10.1111/1462-2920.12327.

Trevisol, E. T. V, Panek, A. D., Mannarino, S. C., and Eleutherio, E. C. A. (2011). The effect of trehalose on the fermentation performance of aged cells of Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 90, 697–704. doi:10.1007/s00253-010-3053-x.

Trivelli, X., Krimm, I., Ebel, C., Verdoucq, L., Prouzet-Mauléon, V., Chartier, Y., et al. (2003). Characterization of the Yeast Peroxiredoxin Ahp1 in Its Reduced Active and Overoxidized Inactive Forms Using NMR. *Biochemistry* 42, 14139–14149. doi:10.1021/bi035551r.

Tronchoni, J., Gamero, A., Arroyo-López, F. N., Barrio, E., and Querol, A. (2009). Differences in the glucose and fructose consumption profiles in diverse

Saccharomyces wine species and their hybrids during grape juice fermentation. *Int. J. Food Microbiol.* 134, 237–243. doi:10.1016/j.ijfoodmicro.2009.07.004.

Tronchoni, J., Medina, V., Guillamón, J. M., Querol, A., and Pérez-Torrado, R. (2014). Transcriptomics of cryophilic Saccharomyces kudriavzevii reveals the key role of gene translation efficiency in cold stress adaptations. *BMC Genomics* 15, 432. doi:10.1186/1471-2164-15-432.

Tronchoni, J., Rozés, N., Querol, A., and Guillamón, J. M. (2012a). Lipid composition of wine strains of Saccharomyces kudriavzevii and Saccharomyces cerevisiae grown at low temperature. *Int. J. Food Microbiol.* doi:10.1016/j.ijfoodmicro.2012.02.004.

Tronchoni, J., Rozès, N., Querol, A., and Guillamón, J. M. (2012b). Lipid composition of wine strains of Saccharomyces kudriavzevii and Saccharomyces cerevisiae grown at low temperature. *Int. J. Food Microbiol.* 155, 191–198. doi:10.1016/j.ijfoodmicro.2012.02.004.

Upshall, a., Giddings, B., and Mortimore, I. D. (1977). The Use of Benlate for Distinguishing Between Haploid and Diploid Strains of Aspergillus nidulans and Aspergillus terreus. *J. Gen. Microbiol.* 100, 413–418. doi:10.1099/00221287-100-2-413.

Valero, E., Cambon, B., Schuller, D., Casal, M., and Dequin, S. (2007). Biodiversity of Saccharomyces yeast strains from grape berries of wine-producing areas using starter commercial yeasts. *FEMS Yeast Res.* 7, 317–29. doi:10.1111/j.1567-1364.2006.00161.x.

Vázquez-Lima, F., Silva, P., Barreiro, A., Martínez-Moreno, R., Morales, P., Quirós, M., et al. (2014). Use of chemostat cultures mimicking different phases of wine fermentations as a tool for quantitative physiological analysis. *Microb. Cell Fact.* 13, 85. doi:10.1186/1475-2859-13-85.

Verghese J, Abrams J, Wang Y, M. K. (2012). Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (Saccharomyces cerevisiae) as a Model System. *Microbiol. Mol. Biol. Rev.* 76, 115–158. doi:10.1128/MMBR.05018-11.

Viladevall, L., Serrano, R., Ruiz, A., Domenech, G., Giraldo, J., Barceló, A., et al. (2004). Characterization of the calcium-mediated response to alkaline stress in

Saccharomyces cerevisiae. *J. Biol. Chem.* 279, 43614–24. doi:10.1074/jbc.M403606200.

Voordeckers, K., Brown, C. A., Vanneste, K., van der Zande, E., Voet, A., Maere, S., et al. (2012). Reconstruction of Ancestral Metabolic Enzymes Reveals Molecular Mechanisms Underlying Evolutionary Innovation through Gene Duplication. *PLoS Biol.* 10, e1001446. doi:10.1371/journal.pbio.1001446.

Voordeckers, K., Kominek, J., Das, A., Espinosa-Cantú, A., De Maeyer, D., Arslan, A., et al. (2015). Adaptation to High Ethanol Reveals Complex Evolutionary Pathways. *PLoS Genet.* 11, e1005635. doi:10.1371/journal.pgen.1005635.

Wagner, A. (1998). The fate of duplicated genes: loss or new function? *Bioessays* 20, 785–8. doi:10.1002/(SICI)1521-1878(199810)20:10<785::AID-BIES2>3.0.CO;2-M.

Wagner, A. (2000). Robustness against mutations in genetic networks of yeast. *Nat. Genet.* 24, 355–61. doi:10.1038/74174.

Warringer, J., Zörgö, E., Cubillos, F. A., Zia, A., Gjuvsland, A., Simpson, J. T., et al. (2011). Trait variation in yeast is defined by population history. *PLoS Genet.* 7, e1002111. doi:10.1371/journal.pgen.1002111.

Watanabe, T., Irokawa, H., Ogasawara, A., Iwai, K., and Kuge, S. (2014). Requirement of peroxiredoxin on the stationary phase of yeast cell growth. *J. Toxicol. Sci.* 39, 51–8. doi:10.2131/jts.39.51.

Wei, W., McCusker, J. H., Hyman, R. W., Jones, T., Ning, Y., Cao, Z., et al. (2007). Genome sequencing and comparative analysis of Saccharomyces cerevisiae strain YJM789. *Proc. Natl. Acad. Sci. U. S. A.* 104, 12825–30. doi:10.1073/pnas.0701291104.

Wellinger, R. J., and Zakian, V. A. (2012). Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: Beginning to end. *Genetics* 191, 1073–1105. doi:10.1534/genetics.111.137851.

Wilkening, S., Lin, G., Fritsch, E. S., Tekkedil, M. M., Anders, S., Kuehn, R., et al. (2014). An evaluation of high-throughput approaches to QTL mapping in Saccharomyces cerevisiae. *Genetics* 196, 853–865. doi:10.1534/genetics.113.160291.

Winzeler, E. A., Castillo-Davis, C. I., Oshiro, G., Liang, D., Richards, D. R., Zhou, Y., et al. (2003). Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. *Genetics* 163, 79–89. doi:10.1111/j.1432-1033.1970.tb00817.x.

Wolfe, K. H. (2015). Origin of the Yeast Whole-Genome Duplication. *PLoS Biol.* 13, e1002221. doi:10.1371/journal.pbio.1002221.

Wolfe, K. H., and Shields, D. C. (1997). Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* 387, 708–713. doi:10.1038/42711.

Wong, C.-M., Zhou, Y., Ng, R. W. M., Kung Hf, H., and Jin, D.-Y. (2002). Cooperation of yeast peroxiredoxins Tsa1p and Tsa2p in the cellular defense against oxidative and nitrosative stress. *J. Biol. Chem.* 277, 5385–94. doi:10.1074/jbc.M106846200.

Wood, Z. A., Schröder, E., Robin Harris, J., and Poole, L. B. (2003). Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem. Sci.* 28, 32–40. doi:10.1016/S0968-0004(02)00003-8.

Xie, C., and Tammi, M. T. (2009). CNV-seq, a new method to detect copy number variation using high-throughput sequencing. *BMC Bioinformatics* 10, 80. doi:10.1186/1471-2105-10-80.

Yale, J., and Bohnert, H. J. (2001). Transcript Expression in Saccharomyces cerevisiae at High Salinity. *J. Biol. Chem.* 276, 15996–16007. doi:10.1074/jbc.M008209200.

Yamada, M., Hayatsu, N., Matsuura, A., and Ishikawa, F. (1998). Y'-Help1, a DNA helicase encoded by the yeast subtelomeric Y' element, is induced in survivors defective for telomerase. *J. Biol. Chem.* 273, 33360–6.

Yang, Y., Foulquié-Moreno, M. R., Clement, L., Erdei, É., Tanghe, A., Schaerlaekens, K., et al. (2013). QTL Analysis of High Thermotolerance with Superior and Downgraded Parental Yeast Strains Reveals New Minor QTLs and Converges on Novel Causative Alleles Involved in RNA Processing. *PLoS Genet.* 9, e1003693. doi:10.1371/journal.pgen.1003693.

Zagarese, H. E., Diaz, M., Pedrozo, F., Ferraro, M., Cravero, W., and Tartarotti, B. (2001). Photodegradation of natural organic matter exposed to fluctuating levels of solar radiation. *J. Photochem. Photobiol. B.* 61, 35–45.

Zara, S., Bakalinsky, A. T., Zara, G., Pirino, G., Demontis, M. A., and Budroni, M. (2005). FLO11-Based Model for Air-Liquid Interfacial Biofilm Formation by Saccharomyces cerevisiae. *Appl. Environ. Microbiol.* 71, 2934–2939. doi:10.1128/AEM.71.6.2934-2939.2005.

Zavanelli, M. I., Britton, J. S., Igel, A. H., and Ares, M. (1994). Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. *Mol. Cell. Biol.* 14, 1689–97. doi:10.1128/MCB.14.3.1689.

Zhang, L., Onda, K., Imai, R., Fukuda, R., Horiuchi, H., and Ohta, A. (2003). Growth temperature downshift induces antioxidant response in Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* 307, 308–314. doi:10.1016/S0006-291X(03)01168-9.

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., et al. (2001). Global analysis of protein activities using proteome chips. *Science* 293, 2101–5. doi:10.1126/science.1062191.

Zimmer, A., Durand, C., Loira, N., Durrens, P., Sherman, D. J., and Marullo, P. (2014). QTL dissection of lag phase in wine fermentation reveals a new translocation responsible for Saccharomyces cerevisiae adaptation to sulfite. *PLoS One* 9, 37–39. doi:10.1371/journal.pone.0086298.

Zuzuarregui, A., Carrasco, P., Palacios, A., Julien, A., and Olmo, M. (2005). Analysis of the expression of some stress induced genes in several commercial wine yeast strains at the beginning of vinification. *J. Appl. Microbiol.* 98, 299–307. doi:10.1111/j.1365-2672.2004.02463.x.

Zuzuarregui, A., Monteoliva, L., Gil, C., and del Olmo, M. lí (2006). Transcriptomic and proteomic approach for understanding the molecular basis of adaptation of Saccharomyces cerevisiae to wine fermentation. *Appl. Environ. Microbiol.* 72, 836–47. doi:10.1128/AEM.72.1.836-847.2006.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M., van ’t Riet, K., and Van, K. (1990). Modeling of the Bacterial Growth Curve. *Appl. Environ. Microbiol.* 56, 1875–81.

Annex 1: Material and Methods

YEAST STRAINS

The yeasts used in the present thesis are shown in Tables 1-4. All strains are belonging to genus *Saccharomyces*. *S. uvarum* 12600 and *S. kudriavzevii* CR85 were used as cryotolerant model yeast species.

Table 1. Commercial wine strains belonging to *S. cerevisiae* species used in this work.

Strain	Source
Lalvin®ICVD254 (P1)	Lallemand Inc. (France)
Uvaferm®WAM (P2)	Lallemand Inc. (France)
Lalvin®ICVD80 (P3)	Lallemand Inc. (France)
Lalvin® Rhone2056 (P4)	Lallemand Inc. (France)
Lalvin®ICVGRE (P5)	Lallemand Inc. (France)
Lalvin®EC1118 (P6)	Lallemand Inc. (France)
Lalvin®ICVD47 (P7)	Lallemand Inc. (France)
Uvaferm®CEG (P8)	Lallemand Inc. (France)
Lalvin®Rhone2323 (P9)	Lallemand Inc. (France)
Uvaferm®BC (P10)	Lallemand Inc. (France)
Uvaferm®VRB (P11)	Lallemand Inc. (France)
Uvaferm®43 (P12)	Lallemand Inc. (France)
CrossEvolution® (P13)	Lallemand Inc. (France)
Lalvin®71B (P14)	Lallemand Inc. (France)
Lalvin®BM45 (P15)	Lallemand Inc. (France)
Enoferm®M1 (P16)	Lallemand Inc. (France)
Enoferm®M2 (P17)	Lallemand Inc. (France)
Uvaferm®BDX (P18)	Lallemand Inc. (France)
Uvaferm®CM (P19)	Lallemand Inc. (France)
Lalvin®ICVD21 (P20)	Lallemand Inc. (France)

Strain	Source
Lalvin®Rhone2226 (P21)	Lallemand Inc. (France)
Lalvin®CY3079 (P22)	Lallemand Inc. (France)
P23	Lallemand Inc. (France)
P24	Lallemand Inc. (France)
P25	Lallemand Inc. (France)
P26	Lallemand Inc. (France)
P27	Lallemand Inc. (France)
QA23	Lallemand Inc. (France)
AJ4	Lallemand Inc. (France)
RVA	Agrovin Company (Ciudad Real, Spain)
T73	Lallemand Inc. (France)
BMV60	Lallemand Inc. (France)
Fermol Grand Rouge Nature	AEB group (Italy)

The enological features of the strains can be obtained from the company's website

(http://www.lallemandwine.com) (http://www.agrovin.com) (http://www.aebiberica.com)

Table 2. Environmental and non-*cerevisiae* strains used in this work.

Strain	Specie	Source
PE35M	*S. cerevisiae*	Masato (Greater San Marcos University, Lima, Peru)
Temohaya-26	*S. cerevisiae*	Agave juice (Technological Institute of Durango, Mexico)
CPE7	*S. cerevisiae*	Cachaça (Federal University of Minas Gerais, Brazil)
Kyokai no.7	*S. cerevisiae*	Sake (Japan)
GB-FlorC	*S. cerevisiae*	Jerez wine (González-Byass wineries, Jerez, Spain)
CECT10131	*S. cerevisiae*	*Centaurea alba* (CECT, Spain)
CECT12600	*S.uvarum*	CECT
CR85	*S. kudriavzevii*	Lopes *et al.,* (2010)

Table 3. Lab strains used in this work.

Strain	Genotype	Source
S288c	*MATα SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	(Mortimer and Johnston, 1986)
BY4743	MATa/α; *his3Δ* 1; *leu2Δ* 0; *lys2Δ* 0; *ura3Δ* 0	EUROSCARF
BY4741	MATa; *his3Δ* 1; *leu2Δ* 0; *lys2Δ* 0; *ura3Δ* 0	EUROSCARF
BY4742	MATα; *his3Δ* 1; *leu2Δ* 0; *lys2Δ* 0; *ura3Δ* 0	EUROSCARF
BY-*met3*	BY4743 met3 Δ::kanMX4	EUROSCARF
BY-*met10*	BY4743 met10 Δ::kanMX4	EUROSCARF

Strain	Genotype	Source
BY-*str3*	BY4743 str3 Δ::kanMX4	EUROSCARF
BY-*met28*	BY4743 met28 Δ::kanMX4	EUROSCARF
BY-met1	BY4743 met1 Δ::kanMX4	EUROSCARF
BY-gtt1	BY4743 gtt1 Δ::kanMX4	EUROSCARF
BY-met5	BY4743 met5 Δ::kanMX4	EUROSCARF
BY-met14	BY4743 met14 Δ::kanMX4	EUROSCARF
BY-gpx1	BY4743 gpx1 Δ::kanMX4	EUROSCARF
BY-sul2	BY4743 sul2 Δ::kanMX4	EUROSCARF
BY-met17	BY4743 met17 Δ::kanMX4	EUROSCARF
BY-efm6	BY4743 efm6 Δ::kanMX4	EUROSCARF
BY-met16	BY4743 met16 Δ::kanMX4	EUROSCARF
BY-gpx2	BY4743 gpx2 Δ::kanMX4	EUROSCARF
BY-met32	BY4743 met32 Δ::kanMX4	EUROSCARF
BY-mup1	BY4743 mup1 Δ::kanMX4	EUROSCARF
BY-yhr112c	BY4743 yhr112c Δ::kanMX4	EUROSCARF
BY-crg1	BY4743 crg1 Δ::kanMX4	EUROSCARF
BY-trx2	BY4743 trx2 Δ::kanMX4	EUROSCARF
BY-trr2	BY4743 trr2 Δ::kanMX4	EUROSCARF
BY-trx1	BY4743 trx1 Δ::kanMX4	EUROSCARF
BY-trx3	BY4743 trx3 Δ::kanMX4	EUROSCARF

Strain	Genotype	Source
BY-hyr1	BY4743 hyr1 Δ::kanMX4	EUROSCARF
BY-grx4	BY4743 grx4 Δ::kanMX4	EUROSCARF
BY-grx1	BY4743 grx1 Δ::kanMX4	EUROSCARF
BY-grx3	BY4743 grx3 Δ::kanMX4	EUROSCARF
BY-grx2	BY4743 grx2 Δ::kanMX4	EUROSCARF
BY-grx5	BY4743 grx5 Δ::kanMX4	EUROSCARF
BY-glr1	BY4743 glr1 Δ::kanMX4	EUROSCARF
BY-skn7	BY4743 skn7 Δ::kanMX4	EUROSCARF
BY-cad1	BY4743 cad1 Δ::kanMX4	EUROSCARF
BY-tsa2	BY4743 tsa2 Δ::kanMX4	EUROSCARF
BY-uba4	BY4743 uba4 Δ::kanMX4	EUROSCARF
BY-urm1	BY4743 urm1 Δ::kanMX4	EUROSCARF
BY-hms2	BY4743 hms2 Δ::kanMX4	EUROSCARF
BY-srx1	BY4743 srx1 Δ::kanMX4	EUROSCARF
BY-ahp1	BY4743 ahp1 Δ::kanMX4	EUROSCARF
BY-yap1	BY4743 yap1 Δ::kanMX4	EUROSCARF
BY-tsa1	BY4743 tsa1 Δ::kanMX4	EUROSCARF
BY-tum1	BY4743 tum1 Δ::kanMX4	EUROSCARF
BY-elp6	BY4741 elp6 Δ::kanMX4	EUROSCARF
BY-tgl3	BY4741 tgl3 Δ::kanMX4	EUROSCARF

Strain	Genotype	Source
BY-ymr315w	BY4741 YMR315w Δ::kanMX4	EUROSCARF
BY-dia1	BY4741 dia1 Δ::kanMX4	EUROSCARF
BY-ymr316c-a	BY4741 ymr316c-a Δ::kanMX4	EUROSCARF
BY-ymr316c-b	BY4741 ymr316c-b Δ::kanMX4	EUROSCARF
BY-ymr317w	BY4741 ymr317w Δ::kanMX4	EUROSCARF
BY-adh6	BY4741 adh6 Δ::kanMX4	EUROSCARF
BY-fet4	BY4741 fet4 Δ::kanMX4	EUROSCARF
BY-ymr320w	BY4741 ymr320w Δ::kanMX4	EUROSCARF
BY-zrg17	BY4741 zrg17 Δ::kanMX4	EUROSCARF
BY-ynr042w	BY4741 ynr042w Δ::kanMX4	EUROSCARF
BY-pet494	BY4741 pet494 Δ::kanMX4	EUROSCARF
BY-fpk1	BY4741 fpk1 Δ::kanMX4	EUROSCARF
BY-ynr040w	BY4741 ynr040w Δ::kanMX4	EUROSCARF
BY-coq2	BY4741 coq2 Δ::kanMX4	EUROSCARF
BY-enb1	BY4741 enb1 Δ::kanMX4	EUROSCARF
BY-css3	BY4741 css3 Δ::kanMX4	EUROSCARF
BY-qcr2	BY4741 qcr2 Δ::kanMX4	EUROSCARF
BY-aqy1	BY4741 aqy1 Δ::kanMX4	EUROSCARF
BY-hpa2	BY4741 hpa2 Δ::kanMX4	EUROSCARF
BY-opt2	BY4741 opt2 Δ::kanMX4	EUROSCARF

Strain	Genotype	Source
BY-ypr195c	BY4741 ypr195c Δ::kanMX4	EUROSCARF
BY-ypr196w	BY4741 ypr196w Δ::kanMX4	EUROSCARF
BY-ypr197c	BY4741 ypr197c Δ::kanMX4	EUROSCARF
BY-sge1	BY4741 sge1 Δ::kanMX4	EUROSCARF
BY-arr1	BY4741 arr1 Δ::kanMX4	EUROSCARF
BY-arr2	BY4741 arr2 Δ::kanMX4	EUROSCARF
BY-arr3	BY4741 arr3 Δ::kanMX4	EUROSCARF

Table 4. Strains constructed in this thesis work.

Strain	Genotype	Chapter
P5-*gal1*	P5 MATa/α Δgal1 Δ::kanMX4	1
P5 -gpx1	P5 MATa/α Δgpx1 Δ::kanMX4	2
P5-glr1	P5 MATa/α Δglr1 Δ::kanMX4	2
P5-tsa1	P5 MATa/α Δtsa1 Δ::kanMX4	2
P5-ahp1	P5 MATa/α Δahp1 Δ::kanMX4	2
P5-srx1	P5 MATa/α Δsrx1 Δ::kanMX4	2
P5-grx1	P5 MATa/α Δgrx1 Δ::kanMX4	2
P5-mup1	P5 MATa/α Δmup1 Δ::kanMX4	2
P5-trx2	P5 MATa/α Δtrx2 Δ::kanMX4	2
P5-trx3	P5 MATa/α Δtrx3 Δ::kanMX4	2
P5-urm1	P5 MATa/α Δurm1 Δ::kanMX4	2
P24-gpx1	P24 MATa/α Δgpx1 Δ::kanMX4	2
P24-glr1	P24 MATa/α Δglr1 Δ::kanMX4	2
P24-tsa1	P24 MATa/α Δtsa1 Δ::kanMX4	2
P24-ahp1	P24 MATa/α Δahp1 Δ::kanMX4	2
P24 -srx1	P24 MATa/α Δsrx1 Δ::kanMX4	2
P24 -grx1	P24 MATa/α Δgrx1 Δ::kanMX4	2

Strain	Genotype	Chapter
P24-mup1	P24 MATa/α Δmup1 Δ::kanMX4	2
P24-trx2	P24 MATa/α Δtrx2 Δ::kanMX4	2
P24-trx3	P24 MATa/α Δtrx3 Δ::kanMX4	2
P24-urm1	P24 MATa/α Δurm1 Δ::kanMX4	2
P5 ho a	P5 MATa, ho::HygMX, ura3::KanMX	3
P5 ho α	P5 MATα, ho::HygMX, ura3::KanMX	3
P5 ho α lys2	P5 MATα, ho::HygMX, ura3::KanMX lys2::URA3	3
P24 ho a	P24 MATa, ho::NatMX, ura3::PhleoMX	3
P24 ho α	P24 MATα, ho::NatMX, ura3::PhleoMX	3
P5/BY4741	P5 ho/BY4741elp6 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741tgl3 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741YMR315w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741dia1 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ymr316c-a Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ymr316c-b Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ymr317w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741adh6 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741fet4 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ymr320w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741zrg17 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ynr042w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741pet494 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741fpk1 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ynr040w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741coq2 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741enb1 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741css3 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741qcr2 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741aqy1 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741hpa2 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741opt2 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ypr195c Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ypr196w Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741ypr197c Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741sge1 Δ::kanMX4	3

Strain	Genotype	Chapter
P5/BY4741	P5 ho/BY4741arr1 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741arr2 Δ::kanMX4	3
P5/BY4741	P5 ho/BY4741arr3 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741elp6 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741tgl3 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741YMR315w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741dia1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ymr316c-a Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ymr316c-b Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ymr317w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741adh6 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741fet4 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ymr320w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741zrg17 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ynr042w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741pet494 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741fpk1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ynr040w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741coq2 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741enb1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741css3 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741qcr2 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741aqy1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741hpa2 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741opt2 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ypr195c Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ypr196w Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741ypr197c Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741sge1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741arr1 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741arr2 Δ::kanMX4	3
P24/BY4741	P24 ho/BY4741arr3 Δ::kanMX4	3

CULTURE MEDIA

Minimal medium. Synthetic complete drop out (SC) medium

Difco Yeast Nitrogen Base (w/o amino acids)	6.7 g
Glucose	20 g
*Drop-out mix	0.83 g
H_2O (distilled)	1 L
Agar (for solid media preparation)	20 g

* Synthetic complete drop-out mix (2 g Histidine, 4 g Leucine, 2 g Lysine and 1.2 g Uracil).

YEPD or YPD (Yeast Extract Peptone Dextrose) medium.

Glucose	20 g
Bacteriological peptone	20 g
Yeast extract	10 g
H_2O (distilled)	1 L
Agar (for solid media preparation)	15 g

Sporulation medium: Potassium acetate

Potassium acetate	10 g

Agar	20 g
H_2O (distilled)	1 L

Phosphate-buffered saline (PBS) medium

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.42 g
KH_2PO_4	0.24 g
H_2O (distilled)	1L

Synthetic grape must (SM)

Synthetic grape must adapted from Riou et al., (1997)

To mimic the wine fermentation conditions synthetic must medium was used that reproduces a standard natural must composition. This medium is very useful to make laboratory micro-fermentations in a reproducible manner.

Media composition for 1 L

<u>Sugars</u>

Glucose	100 g
Fructose	100 g

Organic acids

Malic acid	5 g
Citric acid	0.5 g
Tartaric acid	3 g

Mineral salts

KH_2PO_4	0.75 g
K2SO4	0.5 g
$MgSO_4$ $7H_2O$	0.250 g
$CaCl_2$ H_2O	0.155 g
NaCl	0.2 g
NH_4Cl	0.46 g

Add the previous prepared stocking solution of:

Amino acids	13.09 mL
Oligoelements	1 mL
Vitamins	10 mL

pH = 3.3 with pellets of NaOH

Add distilled water (up to 1 L).

Filter the whole volume using a 0.22 μm filter.

Aminoacids stocking solution (1 L)

Tyrosine (Tyr)	1.5 g
Tryptophan (Trp)	13.4 g
Isoleucine (Ile)	2.5 g
Aspartic Acid (Asp)	3.4 g
Glutamic Acid (Glu)	9.2 g
Arginine (Arg)	28.3 g
Leucine (Leu)	3.7 g
Threonine (Thr)	5.8 g
Glycine (Gly)	1.4 g
Glutamine (Gln)	38.4 g
Alanine (Ala)	11.2 g
Valine (Val)	3.4 g
Methionine (Met)	2.4 g
Phenylalanine (Phe)	2.9 g
Serine (Ser)	6 g

Histidine (His)	2.6 g
Lysine (Lys)	1.3 g
Cysteine (Cys)	1.5 g
Proline (Pro)	46.1 g

Keep at -20 ºC

Vitamins stocking solution (1 L)

Myo-inositol	2 g
Calcium pantothenate	15 g
Thiamine hydrochloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine	0.025 g
* Biotin	3 mL

*(stocking biotin solution 100 mg L^{-1})

Keep at -20 ºC

Oligoelements stocking solution (1 L)

$MnSO_4$. H_20	4 g

$ZnSO_4$. $7H_2O$	4 g
$CuSO_4$. $5H_2O$	1 g
KI	1 g
$CoCl_2$. $6H_2O$	0.4 g
H_3BO_3	1 g
$(NH_4)_6Mo_7O_{24}$	1 g

Keep at -20 ºC

***Synthetic grape must adapted from* Martínez-Moreno et al., (2012)**

This synthetic must is very similar to the previous one described by Riou et al., (1997) with some variations that simplify its preparation.

Media composition for 1 L:

The medium composition included 100 g glucose and 100 g fructose, but only had two organic acids: 6 g malic acid and 6 g citric acid. The vitamins, oligoelements and mineral salts were substituted by 1.7 g YNB without ammonium and amino acids. This synthetic must also included anaerobic factors (0.015 g ergosterol, 0.005 g sodium oleate and 0.5 mL tween 80) and 0.006 g potassium disulfite. The assimilable nitrogen used was the same

sources and amount (total amount of 300 mg N L^{-1}) than in the other synthetic must.

After weighting the different substances and adding distilled water (up to 1 L), the medium is autoclaved at 121 ºC for 20 min. Afterwards the anaerobic factors and amino acids were added. The pH was adjusted to 3.3 by pellets of NaOH and the whole volume was filtered by 0.22 µm filter.

CULTURE TECHNIQUES

Chemostat cultures (continuous cultures)

A chemostat is a growth vessel into which fresh medium is delivered at a constant rate and cells and spent medium overflow at the same rate. Thus, the culture is forced to divided to keep up with the dilution, and the system exists in a steady state where inputs match outputs (Dunham, 2010).

Continuous cultures were performance at 12, 15 and 28 ºC in 0.5 L reactor (MiniBio, Applikon Biotechnology) with a working volume of 0.35 L. A temperature probe connected to a cryostat controlled the temperature cultures. pH was measured online and kept constant at 3.6 by the automatic addition of 2 M NaOH and 1 M HCl. The stirrer was set at 100 rpm. The population inoculated in the chemostat was approximately OD = 0.2. The initial inocula came from an overnight culture in YPD at 30 ºC. Previously to start the continuous culture, cells were allowed to grow, at the same

temperature than the continuous culture, through a normal growth curve to achieve enough biomass (batch phase). Yeast cells were grown at dilutions rates (D) between 0.028 and 0.04 h^{-1}.

Microfermentations

In order to simulate the wine fermentation conditions, a synthetic must media was used that reproduces a standard natural must composition. Fermentations were performed at 28 and 15 ºC, with continuous orbital shaking at 100 rpm. Fermentations were done in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM. The population inoculated in the synthetic grape-must came from an overnight culture in YPD at 30 ºC. After counting under the microscope, the appropriate dilution of the overnight culture was transferred to synthetic must to achieve an initial cell concentration of 2 x 10^6 cells mL^{-1}. Fermentation was considered to be completed when density was below 998 g L^{-1}.

Spot assays

For the spot assays, the cells, after grown on YPD at 28 ºC up to the stationary phase (OD_{600} ~ 7) were harvested by centrifugation, washed with sterile water, resuspended in sterile water to an OD_{600} value of 0.5, and followed by serial dilution. From each dilution, 3.5 µL were spotted onto

YPD agar plates. Plates were incubated at 28 and 15 ºC for 2 and 9 days, respectively.

FERMENTATION MONITORING

Fermentations were sampling every day at 28 ºC and every two days at 15 ºC. In order to homogenize the fermentations, previously to take the sample, the fermentations were shaken.

Density measurements

Density measurement is a good fermentation monitoring approach, because the decrease density during the fermentation is directly proportional to sugar consumption. The grape must density usually is between 1070 and 1120 g L^{-1} and the wine density is between 990 and 998 g L^{-1}. The decrease of density is due to the transformation of sugars into ethanol.

To measure the density, cells were removed from the must by centrifugation. Afterwards samples were measured with Densito 30PX densitometer (Mettler Toledo, Switzerland).

The fermentation kinetics were estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the synthetic must. The fermentation kinetics were calculated by directly fitting density measurements versus time to the four-parameter logistic equation proposed

by Speers et al., (2003). The estimation was done using Sigmaplot software (Systa Software Inc. USA).

$$Pt = P_D / \{1 + e^{[-B * (t - M)]}\} + P\infty$$

where P_t is the Plato values (density values) at time t, $P\infty$ is the Plato values at equilibrium, P_D represents the changes in the Plato during the fermentation (P_0 - $P\infty$), B is the fermentation rate and M is the time where the exponential fermentation rate is maximal. When the data was fitted to four-parameter logistic equation we also obtained an estimation of time for every density value. These values were used to calculate the T5, T50 and T100 (Figure 1)

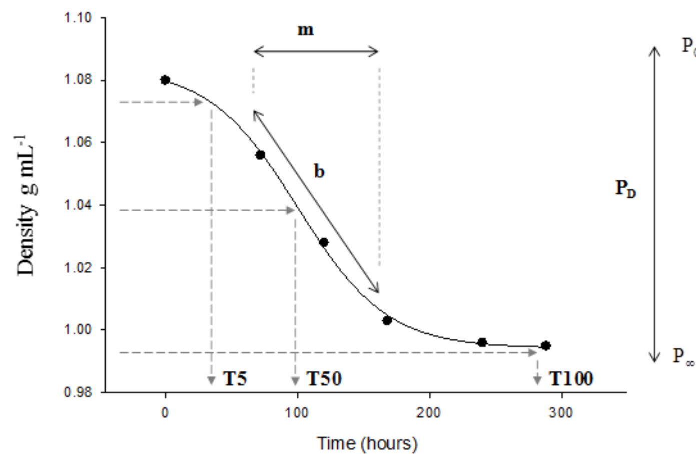


Figure 1. Estimation of fermentation parameters

Optical density measurements

During the fermentation the yeast growth was monitoring by optical density measurements. The absorbance measurement at 600 nm is directly proportional to the yeast cell biomass, between the ranges 0.1 and 0.8. The samples of the fermentation were properly diluted with distilled water, using as blank also distilled water.

Viable yeast counting

To calculate the viable yeast during the fermentation, yeast cells were plated on YPD agar at an adequate dilution and incubated for 2 days at 28 ºC.

Biomass dry weight determination

Biomass dry weight (g DW L^{-1}) was determined by centrifuging a known volume of culture broth (approximately 30 units of OD_{600}) 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.

HPLC analysis

Glucose, fructose, glycerol and ethanol were analyzed in supernatant samples. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a

refraction index detector, autosampler and a UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min, and samples were diluted 5 or 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 μm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL min^{-1} and a column temperature of 50 ºC. The concentration of each was calculated using external standards. Each sample was analyzed in duplicate.

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 (Dukes and Butzke, 1998). The results were expressed as mg N L^{-1}.

GROWTH RATE ANALYSIS

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12, 15 and 28 ºC. Measurements were made every half hour during 3 days after a pre-shaking of 20 sec at 28

ºC. However, at low temperature, the microplate had to be incubated outside the spectrophotometer and then transferred into it to take the measurements every 8 h during lag phase and every 3 h during exponential phase. The wells of the microplate were filled with 0.25 mL of media and inoculated with an initial OD of approximately 0.1 (inoculum level of 10^6 cells mL^{-1}). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out at least in triplicate. Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparametized Gompertz equation proposed by Zwietering *et al.*, (1990):

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

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h) (Salvadó et al., 2011b).

Area under the curve determination

The overall yeast growth was estimated as the area under the OD vs. time curve (70 and 250 h at 28 and 12-15 ºC, respectively). This parameter was

calculated by integration using the OriginPro 8.0 software (OriginLab Corp., Northampton,MA).

MOLECULAR TECNIQUES

DNA extraction

The extraction of yeast DNA was carried out from 3 mL overnight culture in YPD at 30 ºC, using the method described by Querol *et al*., (1992). 5 mL of the cell culture was centrifuged at 10000 rpm for 2 min and the YPD was removed. The cell pellet was washed with 1 mL of distilled water and centrifuged at 10000 rpm for 2 min to remove the water. 0.5 mL of Buffer 1 (sorbitol 0.9 M, EDTA 0.1 M pH 7.5) and 30 µL of Zymolyase (Seikagaku Corporation, Japan) were added. The samples were vortexed and the tubes were incubated at 37 ºC for 20 min. Samples were centrifuged at 10000 rpm for 2 min to remove the supernatant. Protoplasts were re-suspended in 0.5 mL of Buffer 2 (Tris 50 mM pH 7.4, EDTA 20 mM). Afterwards 13 µL of SDS 10% was added and the tubes were incubated at 65 ºC for 5 min. After the incubation, 0.2 mL of potassium acetate was added, the samples were mixed and incubated in ice for 5 min. Samples were centrifuged 15 min at 12000 rpm at 4 ºC to ensure the elimination of SDS. Supernatant was added to 0.7 mL of isopropanol (v/v) and incubated at room temperature for 5 min. Samples were centrifuged 10 min at 12000 rpm at 4 ºC. Supernatant was

removed and 0.5 mL of ethanol 70% was added. Samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA was re-suspended in 40 µL of TE (Tris 10 mM ph 7.4, EDTA 1 mM pH 8.0).

RNA extraction

Yeast RNA was extracted from frozen cell samples (10^8 cell mL^{-1}), harvested from fermentation, using the method described by (Sierkstra et al., 1992). Previous to freeze the samples, cells were centrifuged at 4 ºC and washed with distilled water. The supernatant was removed and then the pellet was frozen with liquid nitrogen and then stored at -80 ºC until the RNA extraction.

Cells were defrost in ice and washed with 1 mL of Extraction Buffer (Tris-HCl 100 mM pH 7.4, LiCl 100 mM, EDTA 0.1 mM). Samples were centrifuged and then 0.5 mL of Vortex Buffer (LiCl 100 mM, EDTA 10 mM, Lithium Dodecyl Sulfate 0.5 % , pH 7.4) was added to the pellet. The suspension was added to 1 g of glass beads. Cells were broken vortexing vigorously with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Afterwards phenol:chloroform:isoamyl alcohol (25:24:1) (v/v) was added, then samples were centrifuged 5 min at 10000 rpm at 4 ºC and the aqueous

phase was transferred to new tube (this step was repeated until non inter-phase was observed). The samples were centrifuged with chloroform-isoamyl alcohol (24:1, v/v), to extract the aqueous phase. The aqueous phase was transferred to new tube and 1/10 volume of 3 M NaAc (pH 5.6) and 2.5 volumes of ethanol 96% were added. Then it was precipitated for 15 min at -80 ºC. The samples were centrifuged at 4 ºC for 30 min. Pellets were washed with ethanol 70% and samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally RNA was re-suspended with 100 µL DEPC-treated water.

Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the quality of the RNA was verified electrophoretically on a 0.8% agarose gel.

Solutions and equipment were treated, so that they were RNase free, as outlined in (Radford, 1991). All the solutions, with the exception of Tris-HCl, were treated with DEPC (Diethyl pyrocarbonate) to eliminate the possible RNases. For each 50 mL of solution 50 µL of DEPC was added, leaving work overnight and then was autoclaved (121 ºC for 20 min) to inactivate traces of DEPC. Plastic and glass materials were treated with

RNAseZap (Ambion, Canada), which also inactivate the RNase, and then were autoclaved (121 ºC for 20 min).

Protein Extraction

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). To the supernatant, 150 µL of extraction buffer were added. Proteins were allowed to precipitate at -20 °C for 1 h; the precipitate was recovered after centrifugation at 10000 g for 15 min. The pellet was washed with the2-D Clean-Up kit (GE Healthcare). The final pellet was air-dried and solubilized in 25 µL of 7 M urea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 2 M Tiourea, 20 mM Tris and milliQ water. Insoluble material was removed by centrifugation (13000 rpm, 5 min). The protein concentration was determined by Bradford, with BSA as a standard.

Plasmid extraction

For the yeast plasmid extraction a modified protocol described by Robzyk and Kassir, (1992) was used. Cells were growing in 5 mL overnight culture

in YPD with G418 Geneticin at 0.2 g L^{-1} concentration in order to maintain the plasmid. 1.5 mL of the culture was transferred to new tube and centrifuged at 6000 rpm for 5 min. Supernatant was removed and then cells were resuspended in 100 µL of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl pH 8, 50 mM EDTA). 0.2 g glass beads (0.45 mm) were added, and then samples were mixed with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Another 100 µL of STET were added, samples were mixed briefly and were incubated in boiling water for 3 min. The samples were cooled in ice and then were centrifuged at 12000 rpm for 10 min at 4 ºC. 100 µL of the supernatant were transferred to new tube and 50 µL of ammonium acetate 7.5 M was added. Samples were centrifuged at 12000 rpm for 10 min at 4 ºC. 100 µL of supernatant was added to 200 µL of absolute ethanol, and then it was precipitated for 1 h at -20 ºC. Samples were centrifuged at 12000 rpm for 15 min at 4 ºC. Pellets were washed with ethanol 70% and samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA plasmid was re-suspended with 20 µL distilled water.

Oligonucleotides

Oligonucleotides used in this study are shown in Table 5. Primer pairs were designed using the Web Primer tool and genome sequences from the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/), with the exception of K2 and K3 described by Güldener *et al.*, (1996), and delta12 and delta21 described by Legras and Karst, (2003). The sequences were sent to Invitrogen to be synthesized. Primers were used to amplify DNA in PCR.

Table 5. Primers used in this work.

Primer	Sequence 5' to 3'	Purpose
Galgfp-F	AAAATTGTTAATATACCTCT ATACTTTAACGTCAAGGAGA AAAAACTATAATGTCTAAAGG TGAAGAAT	to amplify KanMX4 cassette to delete GAL1
Galgfp-R	ATTATCATACAATCATTTATTA AGTAGTTGAAGCATGTATGAA CTATAAACTAGTGGATCTGAT ATCATC	to amplify KanMX4 cassette to delete GAL1
Galcp-F	CGTTCCTGAAACGCAGATG	to check the deletion of GAL1
Galcp-R	CTCCTCGCGCTTGTCTACTAA	to check the deletion of GAL1
Delta12	TCAACAATGGAATCCCAAC	to amplify delta elements
Delta21	CATCTTAACACCGTATATGA	to amplify delta elements
Glr1-R	TGAATATCCAAGCGAGTCCAC	to amplify KanMX4 cassette to delete GLR1
Gpx1-F	TTCATGGTCCTGGAATCGTC	to amplify KanMX4 cassette to delete GPX1

Primer	Sequence 5' to 3'	Purpose
Gpx1-R	ATCAATCGTTGGCAGGTCA	to amplify KanMX4 cassette to delete GPX1
Grx2-F	TTCCTGGCAAGATTATCTCCA	to amplify KanMX4 cassette to delete GRX2
Grx2-R	GCTGAAGCGTTACCGTTAAG	to amplify KanMX4 cassette to delete GRX2
Mup1-F	ACTGTGGAATTGCCGTTCA	to amplify KanMX4 cassette to delete MUP1
Mup1-R	CACTGTTCCGCCAACTCAG	to amplify KanMX4 cassette to delete MUP1
Skn7-F	TGCTTATACCTCACCATTCCA	to amplify KanMX4 cassette to delete SKN7
Skn7-R	AACATGAGGAACTGAGGTTGC	to amplify KanMX4 cassette to delete SKN7
Srx1-F	GGATGCGATGCAATACATGTC	to amplify KanMX4 cassette to delete SRX1
Srx1-R	TGGCGAACAAGTCATCACG	to amplify KanMX4 cassette to delete SRX1
Str3-F	AAGTGTGGCATGTGCTTCAC	to amplify KanMX4 cassette to delete STR3
Trx2-F	CCACTGGACGACAAGATCCTT	to amplify KanMX4 cassette to delete TRX2
Trx2-R	GCTGACGAACGTCATTGACA	to amplify KanMX4 cassette to delete TRX2

Primer	Sequence 5' to 3'	Purpose
Trx3-F	GACCTGCATCAAGATCTCGA	to amplify KanMX4 cassette to delete TRX3
Trx3-R	CGTTGTATCCGGCTCATTAG	to amplify KanMX4 cassette to delete TRX3
Tsa1-F	GTGCGCAACCTCATCTCTACA	to amplify KanMX4 cassette to delete TSA1
Tsa1-R	CATCTGCAGTAATTGGCGACT	to amplify KanMX4 cassette to delete TSA1
Urm1-F	GCATCGCATCGACCTAATG	to amplify KanMX4 cassette to delete URM1
Urm1-R	ATGGACTCTGCGTTGGTGAC	to amplify KanMX4 cassette to delete URM1
Ahp1cp-F	TCCTGATTACTCAATTTCTGAA TGT	to check the deletion of AHP1
Glr1cp-F	CTGTCGCTCTTATGATCCGTT	to check the deletion of GLR1
Gpx1cp-F	AAGACCGAACACGAAAGCA	to check the deletion of GPX1
Grx2cp-F	ACCACCGATGAAGAGAACCA	to check the deletion of GRX2
Mupcp-F	GGCATGACTTCAGGGATCAA	to check the deletion of MUP1
Skn7cp-F	CGTTCTCAAAGATGTAGGACC AT	to check the deletion of SKN7
Str3cp-F	TCGTGAAGATGAACGCGA	to check the deletion of STR3

Primer	Sequence 5' to 3'	Purpose
Trx2cp-F	GCAAATGGTGGTTGGGAA	to check the deletion of TRX2
Trx3cp-F	TACCATCAACGGCACTAACAA	to check the deletion of TRX3
Tsa1cp-F	CAGTTGTCGATGAACGTCAAA	to check the deletion of TSA1
Urm1cp-F	GAATCTTCAAAAGCCGCA	to check the deletion of URM1
K3	CCTCGACATCATCTGCCC	to check the integration of *Kan*MX4 cassette
K2	GGGACAATTCAACGCGTCTG	to check the integration of *Kan*MX4 cassette
Lys2mUra3-F	TGTATTCGAATGAAAGAGTAACCATTGTTGCGGACCAATTTACTCAATATTTGACTGCTGCGCTAAGCGATCCATCCAATTGAGCTTTTCAATTCAATTC	to amplify URA3 cassette to delete LYS2
Lys2mUra3-R	CTTGAAACAATCTCCAATTCATCATTGATGTAATCTTCTACTAGTTGATCCAATTGTCCAGCAGCTCTAATAACAATCAATCCGATTTAGTGCTTTAC	to amplify URA3 cassette to delete LYS2
LYS2cp-F	AGAGTAACCGGTGACGATGAT	to check the integration of *URA3* cassette
LYS2cp-R	ATGGTGTAGTAGCTTGGGCA	to check the integration of URA3 cassette
Ho-F hyg	AGACATCGCAAACGTCACGGCTAACTCTTACGTTATGTGCGCAGATGGCTCGTACGCTGCAGGTCGACG	to amplify kygMX4 cassette to delete HO
Ho-R hyg	ACTCTTATGAGGCCCGCGGACAGCATGAAACTGTAAGATTCCGCCACATTATATCAGATCCACTAGTGGC	to amplify kygMX4 cassette to delete HO
Ho-R nat	ACTCTTATGAGGCCCGCGGACAGCATGAAACTGTAAGATTCCGCCACATTATATCAGATCCACTAG	to amplify natMX4 cassette to delete HO
Hocp-F	GAGGTTTGCAGAAGCTTGTTGA	to check the disruption of HO gene

Primer	Sequence 5' to 3'	Purpose
Hocp-R	TTGGCGTATTTCTACTCCAGCAT	to check the disruption of HO gene
mUrakan-F	TCTTAACCCA ACTGCACAGA ACAAAAACCT GCAGGAAACGAAGATAAATCATGGTACGCTGCAGGTCGACA	to amplify KanMX4 cassette to delete URA3
mUrakan-R	GCTCTAATTTGTGAGTTTAGTATACATGCATTTACTTATAATACAGTTTTACTAGTGGATCTGATATC	to amplify KanMX4 cassette to delete URA3
mUrapheo-F	TCTTAACCCAACTGCACAGAACAAAAACCTGCAGGAAACGAAGATAAATCATGGTACGCTGCAGGTCGACA	to amplify PhleoMX4 cassette to delete URA3
mUraphleo-R	GCTCTAATTTGTGAGTTTAGTATACATGCATTTACTTATAATACAGTTTTACTAGTGGATCTGATATC	to amplify PhleoMX4 cassette to delete URA3
Uracp-F	TTCGAGTGAAACACAGGAAGA	to check the disruption of URA3 gene
Uracp-R	GAGCACTTCATGATGCATGTT	to check the disruption of URA3 gene
MAT-F	AGTCACATCAAGATCGGTTATGG	Mating type verification
MATα-R	GCACGGAATATGGGACTACTTCG	Mating type verification
MATa-R	ACTCCACTTCAAGTAAGAGTTTG	Mating type verification
chrXV-F	ATGCTGGAAACTGATCACTCTAGGAATGACAATTTAGACGATAAAAGCACTGTCTGCTACAGCGAAAAGAAGCTTTTCAATTCATCA	to amplify URA3 cassette to delete chomosome XV left subtelomere

Primer	Sequence 5' to 3'	Purpose
chrXVI-F	ATGAAGAGTACTTTGAGTTTAACTTTATGTGTTATATCGCTTCTATTAACCCTTTTTCTGGCGGCCTTGGATATTGTTATAGCTTTTCAATTCATCA	to amplify URA3 cassette to delete chomosome XVI right subtelomere
chrtrunc-R	CTCACTATAGGGCGAATTC	to amplify URA3 cassette to delete chomosome subtelomeres
ChrXIIIcp-R	AGTGACAGCCGATCCTGAG	to check the deletion of subtelomere
ChrXVcp-R	ACGTCGATGACGAAGGACAT	to check the deletion of subtelomere
ChrXVIcp-R	ACGATATAGCGAGTGCCGT	to check the deletion of subtelomere
m-AGA1ura3-F	ATGACATTATCTTTCGCTCATTTTACCTACCTGTTCACAATATTGTTGGGATTAACTAATATTGCCTTGGCGGCATCAGAGCAGATTGTACTG	to amplify URA3 cassette to delete AGA1
m-AGA1ura3-R	TTAACTGAAAATTACATTGCAAGCAACTGCCATGATGGCGAAGACCAACTTTCCTAATGGCAAACGCGACACCGCAGGGTAATAACTG	to amplify URA3 cassette to delete AGA1
AGA1cp-F	CTGCACGTGAATTGGATCTTG	to check the integration of URA3 cassette
m-FPK1ura3-F	AAGGTTCAGGGGCCTTGAAGGTAGTATCATTTGGAAAACAGCAAGAGAATAAAACCTCTGACACATGCAGC	to amplify URA3 cassette to delete FPK1
FPK1cp-F	GGAGATACAGATTCTAAGAG	to check the integration of URA3 cassette

Primer	Sequence 5' to 3'	Purpose
m-COQ2ura3-F	ATGTTTATTTGGCAGAGAAAGAGTATTTTACTAGGGAGGTCCATTCTGGGAAAACCTCTGACACATGCAGC	to amplify URA3 cassette to delete COQ2
m-COQ2ura3-R	CTACAAGAATCCAAACAGTCTCAAGATGTAGTCGACTGCCAAGGCGTACGTTAATCGCCTTGCAGCACAT	to amplify URA3 cassette to delete COQ2
COQ2cp-F	CCATTACCAGCACGATGGAT	to check the integration of URA3 cassette
m-PET494ura3-F	ATGCATTTGAAAAAGGGGAAGAGGAGTATTAGCACAGTATGGCGACTTTTAAAACCTCTGACACATGCAG	to amplify URA3 cassette to delete PET494
m-PET494ura3-R	TTAAGAAGAGTCCGCCTGGATATCAGAAATGCGTTTCAGCAGCGCAACCATTAATCGCCTTGCAGCACAT	to amplify URA3 cassette to delete PET494
PET494cp-F	TCGCCATCATGGCAGTTG	to check the integration of URA3 cassette
S8	CCTCTAGGTTCCTTTGTTACTTCT	to check the integration of URA3 cassette
SNP1-F	GCAGAAACGACAAATGGTTC	to check the recombination of F6 population in chrIII
SNP1-R	CATGCTTTAGCTTCTGTCATG	to check the recombination of F6 population in chrIII
SNP2-F	GTGTTGCACACGACCAAC	to check the recombination of F6 population in chrIII
SNP2-R	GAACATCTGAGCTCACTAACC	to check the recombination of F6 population in chrIII

Primer	Sequence 5' to 3'	Purpose
SNP3-R	GCTCTTTCAGGATTCATTGAG	to check the recombination of F6 population in chrIII
SNP4-F	GTAGCACGATATCCGCAAG	to check the recombination of F6 population in chrIII
SNP4-R	ACAAGGATTGCGAATTCTTAG	to check the recombination of F6 population in chrIII
SNP5-F	GTCACGCCACGAGTAGGAT	to check the recombination of F6 population in chrIII
SNP5-R	CACTATCGCCCTCCTTCATC	to check the recombination of F6 population in chrIII
SNP6-F	GTTGGCCCTAGATAAGAATCC	to check the recombination of F6 population in chrIII
SNP6-R	TTGATTCAAAGGCTATCCTTG	to check the recombination of F6 population in chrIII

Underlined sequences correspond to sequences homologous to the plasmids.

Polymerase chain reaction (PCR)

Standard DNA amplification was performed via PCR in a GenAmp PCR System 2700 (Applied Biosystems, USA) to synthesize the deletion cassettes, to check the deletion and for typing by delta elements amplification. A typical 50 µL reaction was performed, contained 1-5 µL,

0.1-100 ng DNA, 2 μL 200 μM dNTPs, 5 μL 10 μM primers, 5 μL 10 x PCR buffer, 3-5 μL 50 mM $MgCl_2$, 1 μL of Taq polymerase, 0.03 U μL^{-1} and water to complete the 50 μL. PCR products were stored at -20 ºC until required.

Deletion cassette amplification

The deletion cassette was amplified from different plasmids (Table 6) or by amplifying the KanMX4 cassette and flanking regions (about 500-pb upstreamand downstream) from the corresponding mutant strain in the BY4743 background. Primers used have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The Taq polymerase EcoTaq (Ecogen, Spain) was used. Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at the correct temperature for each plasmid for 30 sec and elongation at 72 ºC for 2 min. The amplification terminated with an extended incubation at 72 ºC for 5 min and cooling to 4 ºC.

Table 6. Plasmids used in this work.

Plasmid	Characteristics/marker
pUG6	kanMX4
pAG25	natMX4
pAG32	hygMX4
pUG66	phleoMX4
pAG36	URA3
pGL32	URA3-CORE X-TG1-3
pKT127	kanMX4-GFP

Deletion verification

The genomic DNA of possible deletion mutants was analyzed by PCR using primers upstream and downstream of the deleted region, and a combination with primers of the *Kan*MX gene (K2 and K3, Table 5). The Taq polymerase EcoTaq (Ecogen, Spain) was used. Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 50-55 ºC (depending on the different primers) for 30 sec and elongation at 72 ºC for 2-5.5 min (depending on the different PCR product length) . The amplification terminated with an extended incubation at 72 ºC for 5 min and cooling to 4 ºC.

Yeast transformation

Yeast transformation was performed using a lithium acetate protocol describe by Daniel Gietz and Woods, (2002). To construct the deletion strains P5 and P24 strains were transformed with the deletion cassette, obtained by PCR.

Yeast cells were inoculated in 50 mL YPD, overnight at 30 ºC and 200 rpm. This culture was used to inoculate an OD of 0.2 in 5 mL YPD and was incubated at 30 ºC and 200 rpm until the cells were completed at least 3 divisions (OD of approximately 0.8). Then 5 mL culture was transferred to a new tube and centrifuged at 5000 rpm for 5 min. The media was poured off and cells were resuspended in 2.5 mL of sterile water and centrifuged again. The water was removed and cells were resuspended in 100 µL of 0.1 M LiAc. Cells were pellet a top speed for 15 sec and LiAc was removed with a micropipette. Cells were resuspended in 40 µL of 0.1 M LiAc, centrifuged and LiAc was removed again with a micropipette. Then transformation mix was added in this order: 240 µL of PEG (50% w/v), 36 µL of 1 M LiAc, 50 µL of ss-DNA 2 mg mL^{-1} (previously boiled for 5 min and quickly chilled in ice) and 34 µL of deletion cassette. Each tube was vigorously vortexed until the cell pellet had been completely mixed. Tubes were incubated at 30 ºC for 30 min, then 42 ºC for 30-60 min. Tubes were centrifuged at 7000 rpm for 15 sec and the transformation mix was removed with a

micropipette. Into each tube 1 mL of water was added and pellet was resuspended by pipetting it up and down gently. Then tubes were centrifuged at 7000 rpm for 15 sec and water was removed. Pellet was resuspended in 500 µL YPD and incubated at 30 ºC for 3 h with a gentle shaking at 125 rpm. 200 µL (twice for each transformation) were plated onto selection plates and incubated at 30 ºC until colonies appeared (2-3 days).

Spores and mating type

Potassium acetate medium was used to induce the sporulation of strains. The plates were incubated at 30 ºC during 5 days. After this time, we tested that the yeast cells have sporulated by examining them under optical microscope. Yeast strains differed in their efficiency and the required time in sporulation. When we can see the tetrad of spores, we continued with the microdissection. Took a small bit of cells of the sporulation plate with a toothpick and mixed with 5 µL glucuronidase in an eppendorf tube. Following the digestion of the ascus wall, the spores of the tetrad can be separated and grown. This solution was incubated for 5 minutes at 30 ºC. To stop the reaction was added 5 µL sorbitol 1M, and the tube was placed on

ice. The dissection of spores was done with micromanipulator equipment (Singer instruments, United Kingdom) in YPD plates. After, the spores were grown in YPD plates with antibiotic resistance. To test the mating type of

each spore, we carried out a PCR with MAT, MATa and MATα primer (Huxley et al., 1990). The specific primers produce a characteristic PCR product that differentiates if the strain is a/α haploid or diploid. The size of the band is 544 bp (haploid a) or 404 bp (haploid α). If it is a diploid we observed both bands. PCR was done under the following conditions: 94 ºC for 5 min, 30 cycles at 94 ºC for 1 min, 58 ºC for 2 min and 72 ºC for 2 min, and 72 ºC for 7 min.

Annex 2: Publications

Chapter 1

http://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-15-1059

García-Ríos *et al. BMC Genomics* 2014, **15**:1059
http://www.biomedcentral.com/1471-2164/15/1059

RESEARCH ARTICLE Open Access

Global phenotypic and genomic comparison of two *Saccharomyces cerevisiae* wine strains reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature fermentations

Estéfani García-Ríos[1], María López-Malo[1,2] and José Manuel Guillamón[1*]

Abstract

Background: The wine industry needs better-adapted yeasts to grow at low temperature because it is interested in fermenting at low temperature to improve wine aroma. Elucidating the response to cold in *Saccharomyces cerevisiae* is of paramount importance for the selection or genetic improvement of wine strains.

Results: We followed a global approach by comparing transcriptomic, proteomic and genomic changes in two commercial wine strains, which showed clear differences in their growth and fermentation capacity at low temperature. These strains were selected according to the maximum growth rate in a synthetic grape must during miniaturized batch cultures at different temperatures. The fitness differences of the selected strains were corroborated by directly competing during fermentations at optimum and low temperatures. The up-regulation of the genes of the sulfur assimilation pathway and glutathione biosynthesis suggested a crucial role in better performance at low temperature. The presence of some metabolites of these pathways, such as S-Adenosilmethionine (SAM) and glutathione, counteracted the differences in growth rate at low temperature in both strains. Generally, the proteomic and genomic changes observed in both strains also supported the importance of these metabolic pathways in adaptation at low temperature.

Conclusions: This work reveals a novel role of the sulfur assimilation pathway in adaptation at low temperature. We propose that a greater activation of this metabolic route enhances the synthesis of key metabolites, such as glutathione, whose protective effects can contribute to improve the fermentation process.

Keywords: Wine yeast, Cold adaptation, Transcriptomics, Proteomics, Genomics, Oxidative stress, Glutathione biosynthesis, Genotype-phenotype association

Chapter 2

http://journal.frontiersin.org/article/10.3389/fmicb.2016.01199/full

ORIGINAL RESEARCH
published: 03 August 2016
doi: 10.3389/fmicb.2016.01199

Correlation between Low Temperature Adaptation and Oxidative Stress in *Saccharomyces cerevisiae*

Estéfani García-Ríos, Lucía Ramos-Alonso and José M. Guillamón *

Food Biotechnology Department, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Valencia, Spain

Many factors, such as must composition, juice clarification, fermentation temperature, or inoculated yeast strain, strongly affect the alcoholic fermentation and aromatic profile of wine. As fermentation temperature is effectively controlled by the wine industry, low-temperature fermentation (10–15°C) is becoming more prevalent in order to produce white and "rosé" wines with more pronounced aromatic profiles. Elucidating the response to cold in *Saccharomyces cerevisiae* is of paramount importance for the selection or genetic improvement of wine strains. Previous research has shown the strong implication of oxidative stress response in adaptation to low temperature during the fermentation process. Here we aimed first to quantify the correlation between recovery after shock with different oxidants and cold, and then to detect the key genes involved in cold adaptation that belong to sulfur assimilation, peroxiredoxins, glutathione-glutaredoxins, and thioredoxins pathways. To do so, we analyzed the growth of knockouts from the EUROSCARF collection *S. cerevisiae* BY4743 strain at low and optimal temperatures. The growth rate of these knockouts, compared with the control, enabled us to identify the genes involved, which were also deleted and validated as key genes in the background of two commercial wine strains with a divergent phenotype in their low-temperature growth. We identified three genes, *AHP1*, *MUP1*, and *URM1*, whose deletion strongly impaired low-temperature growth.

Keywords: thioredoxins, glutathione, correlation analysis, ROS accumulation, *MUP1*, *URM1*

OPEN ACCESS

Edited by:
Joaquin Bautista-Gallego, Rovira i Virgili University, Spain

Reviewed by:
Carmen Portillo, Rovira i Virgili University, Spain
Jose Antonio Curiel, Instituto de Ciencias de la Vid y del Vino, Spain
Manuel Ramirez, University of Extremadura, Spain

***Correspondence:**
José M. Guillamón
guillamon@iata.csic.es

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *23 June 2016*
Accepted: *19 July 2016*
Published: *03 August 2016*

Citation:
García-Ríos E, Ramos-Alonso L and Guillamón JM (2016) Correlation between Low Temperature Adaptation and Oxidative Stress in Saccharomyces cerevisiae. Front. Microbiol. 7:1199. doi: 10.3389/fmicb.2016.01199

INTRODUCTION

Microorganisms constantly face environmental stimuli and stresses. The simplest response strategy to a stimulus is to monitor the environment and to respond directly to it using designated mechanisms. The environmental stress response in yeast is a complicated strategy in which responses to many stresses partially overlap (Gasch et al., 2000; Causton et al., 2001; Mitchell et al., 2009). Drops in ambient temperature are common in almost every ecological niche. In the yeast *Saccharomyces cerevisiae*, reductions in ambient temperature have widespread effects on growth and survival, which depend on the severity of stress. This is relevant for industrial yeast exploitations as several fermentations, like brewing and some wine fermentations, take place at around 12–15°C. In winemaking, fermentation at lower temperatures correlates with a fresh character and fruity notes in wines, and reduces the risk of bacterial contamination and the

Chapter 4

http://www.sciencedirect.com/science/article/pii/S1874391916302627

Journal of Proteomics 146 (2016) 70–79

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

Estéfani García-Ríos, Amparo Querol, José Manuel Guillamón *

Departamento de Biotecnología de los alimentos, Instituto de Agroquímica y Tecnología de los Alimentos (CSIC), Avda. Agustín Escardino, 7, E-46980 Paterna, Valencia, Spain

A R T I C L E I N F O

Article history:
Received 14 January 2016
Received in revised form 11 May 2016
Accepted 20 June 2016
Available online 22 June 2016

Keywords:
Saccharomyces
Low temperature
Quantitative proteomic
Translation

A B S T R A C T

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

© 2016 Elsevier B.V. All rights reserved.

CSIC

iata

Instituto de Agroquímica y Tecnología de Alimentos

Vniversitat de València