Transcriptional Profile of the Industrial Hybrid *Saccharomyces pastorianus* Reveals Temperature-Dependent Allele Expression Bias and Preferential Orthologous Protein Assemblies

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Abstract

***Saccharomyces pastorianus* is a natural yeast evolved from different hybridization events between the mesophilic *S. cerevisiae* and the cold-tolerant *S. eubayanus*. This complex aneuploid hybrid carries multiple copies of the parental alleles alongside specific hybrid genes and encodes for multiple protein isoforms which impart novel phenotypes, such as the strong ability to ferment at low temperature. These characteristics lead to agonistic competition for substrates and a plethora of biochemical activities, resulting in a unique cellular metabolism. Here, we investigated the transcriptional signature of the different orthologous alleles in *S. pastorianus* during temperature shifts. We identified temperature-dependent media-independent genes and showed that 35% has their regulation dependent on extracellular leucine uptake, suggesting an interplay between leucine metabolism and temperature response. The analysis of the expression of ortholog parental alleles unveiled that the majority of the genes expresses preferentially one parental allele over the other and that *S. eubayanus*-like alleles are significantly over-represented among the genes involved in the cold acclimatization. The presence of functionally redundant parental alleles may impact on the nature of protein complexes established in the hybrid, where both parental alleles are competing. Our expression data indicate that the majority of the protein complexes investigated in the hybrid are likely to be either exclusively chimeric or unispecific and that the redundancy is discouraged, a scenario that fits well with the gene balance hypothesis. This study offers the first overview of the transcriptional pattern of *S. pastorianus* and provides a rationalization for its unique industrial traits at the expression level.**

***Key words:* *S. pastorianus*, yeast hybrid, transcriptome, protein complexes, temperature acclimatization, orthologous alleles.**

Introduction

Natural or artificial hybridization between strains or species is a common phenomenon that occurs in almost all sexually reproducing group of organisms including bacteria, yeasts, plants, and animals (Li et al. 2012). *Saccharomyces pastorianus* is a natural evolved allopolyploid and sterile hybrid between the mesophilic *S. cerevisiae* and the cold-tolerant *S. eubayanus*. *Saccharomyces pastorianus* is used in 89% of brewed beer worldwide (Gorter de Vries et al. 2019). This species has been isolated, domesticated, and maintained by human selection for colder brewing temperatures of 8–15 °C (lager fermentation environment; Nakao et al. 2009). Before the discovery of *S. eubayanus* species, the non-*S. cerevisiae* portion of the genome of *S. pastorianus* was considered as being *S. uvarum* and/or *S. bayanus* genome that are closely related to *S. eubayanus* (Vaughan-Martini and Martini 1993). *Saccharomyces cerevisiae* was isolated in Europe, whereas *S. eubayanus* have been isolated from Nothofagus trees in Patagonia and in East Asia (Tibet; Bing et al. 2014). The connection of Asia and Europe via the silk road could explain how the hybridization occurred between those two species. The analysis of transposon sequence distribution within subtelomeric regions and recombination breakpoints in the genome of *S. pastorianus* strains suggested its separation into two genomically distinct groups, which may have arisen from multiple hybridization events (Monerawela and Bond 2018). One hypothesis states that an initial spontaneous hybridization event occurred between a haploid *S. cerevisiae* strain and

a diploid *S. eubayanus* strain (Bond et al. 2004). That event led to a progenitor of the Group I strains, which evolved through further reduction of the *S. cerevisiae* genome content, to produce an aneuploid and an approximate triploid genome. In parallel, the progenitor strain made a second hybridization event with a different *S. cerevisiae* strain and evolved to give strains with an approximate tetraploid genome that are classified in Group II. A more recent theory supports that a single hybridization event occurred between a diploid *S. cerevisiae* and a diploid *S. eubayanus*, resulting in a tetraploid progenitor that evolved to give the Group II strains (Okuno et al. 2016). In parallel, this progenitor underwent chromosomal deletions of the *S. cerevisiae* subgenome, leading to a progenitor approximately triploid, that evolved to give the Group I strains (Salazar et al. 2019; Alsammar and Delneri 2020). Therefore, Group II strains possess about two to three times more *S. cerevisiae* genomic content than the Group I strains. Moreover, *S. pastorianus* lager strains inherited the mitochondrial DNA (mtDNA) from the *S. eubayanus* parental strain which confers an advantage at cold temperatures (Okuno et al. 2016; Baker et al. 2019). Several factors may be behind the dynamic leading to the selection of a parental mtDNA, such as the fitness of the different mitotypes in the selected environment and/or growing temperature (Solieri et al. 2008; Albertin et al. 2013; Li et al. 2019; Hewitt et al. 2020). Interestingly, in *S. cerevisiae*/*S. uvarum* synthetic hybrids, it has been shown that specific mitotypes can be created according to the environmental conditions where they have been formed (Hewitt et al. 2020).

The difference in the genome composition of Group I and Group II strains results in different brewing properties. Group I strains appear to be more cold-tolerant (*i.e. S. eubayanus* trait) whereas Group II strains show a better maltotriose consumption (*i.e. S. cerevisiae* trait) (Brouwers et al., 2019). More specifically, *S. eubayanus* and most of the Group I strains are not able to utilize efficiently maltotriose, which might be due to the absence or nonfunctionality of the maltotriose transporters, encoded by *AGT1* or *MTT1* genes (Magalhães et al. 2016). The difference in maltotriose utilization could also be related to the copy number of the transporter encoding genes or gene sequence itself (Smit et al. 2007; Vidgren et al. 2009; Duval et al. 2010; Vidgren et al. 2011). Group I and II strains are also different in terms of ester production. Group I strains showed 2- to 6-fold less amount of ethyl caprylate (apple, aniseed flavor), ethyl acetate (fruity flavor), and 3-methylbutyl acetate (banana, pear flavor) flavor compounds compared with the Group II strains, whereas higher alcohol and ester production by *S. eubayanus* was similar to that of Group II strains (Saerens et al. 2010; Gibson et al. 2013). Group I include Saaz-type and Carlsberg-type strains from Czech Republic and Denmark breweries, respectively. Group II, also referred to Frohberg-type, includes strains brewed in Canada, the Netherlands (Heineken, Oranjeboom, and other breweries), and Denmark (non-Carlsberg breweries; Dunn and Sherlock 2008). The *S. cerevisiae* parental strain of the Group I yeasts is related to those used for Ale beer production in Europe, whereas the one of the Group II contains, in addition to the Ale-like gene content, Stout-like subgenome. *Saccharomyces eubayanus* parental strain possesses similar fermentation capacities to Saaz group at cold temperature (10 °C) and is more resistant to cold temperatures compared with the strains belonging to the Frohberg group and *S. cerevisiae* Ale yeasts (Gibson et al. 2013). The polyploid nature of *S. pastorianus* may impact on the transcriptome, the proteome, and the metabolome, including the production of higher alcohols and esters behind aromas. Additionally, *S. pastorianus* has also the ability to ferment under stressful conditions such as anaerobiosis, high hydrostatic pressure, and solutions with high sugar concentration, producing complex metabolites leading to further unique flavors and aromas (Monerawela and Bond 2017a). The parental chromosomes of *S. pastorianus* underwent homeologous recombination, which resulted in unique hybrid chromosomes (Monerawela and Bond 2017b).

Strong evidence suggests that the ancestry of *Saccharomyces sensu stricto* yeast underwent an event of whole-genome duplication (WGD) that may have occurred 100 Ma (Wolfe 2015). The doubling of an organism's genetic content leads immediately to a reproductive barrier with their relative species and ancestors (Wolfe 2015). However, in the yeasts, two ancestral species mated before the WGD which restored its fertility (Marcet-Houben and Gabaldón 2015; Wolfe, 2015). WGD is a rare evolutionary event with great consequences as new paralogs are the basis of speciation, as they may subfunctionalize or neofunctionalize (Wolfe 2015). Both WGD and the hybridization events shaped the genome of *S. pastorianus* and are responsible of the emergence of functionally redundant genes. Therefore, this hybrid species encounters issues raised by the global increase of gene dosage, in addition to the potential incompatibilities between genes and proteins encoded by the parental subgenomes (Sriswasdi et al. 2016). These incompatibilities between redundant paralogs may be tackled by the hybrid via gene conversion (Takuno et al. 2008; McGrath et al. 2014). Extensive loss of heterozygoty (Li et al. 2012; Louis et al. 2012; Stukenbrock et al. 2012) and gene expression reprogramming (Tirosh et al. 2009) have also been identified as mechanism of actions used by fungal hybrids to stabilize their genome. Additionally, comparative genomic analysis on the genus *Trichosporon* (that went through WGD and hybridization events) revealed that redundant genes display a deceleration of evolution rates, alongside a large-scale gene loss, which suggests a global compensatory mechanism against increased gene dosages (Sriswasdi et al. 2016).

Therefore, multiple copies of *S. cerevisiae*-like, *S. eubayanus*-like, and hybrid gene alleles coexist in the *S. pastorianus* genome. Consequently, the presence of multiple protein isoforms can lead to agonistic competition for substrates. Protein redundancy can also broaden the types of protein complexes established in the cell, since these can be either unispecific (composed uniquely by *S. cerevisiae*-like or *S. eubayanus*-like members) or chimeric (Piatkowska et al. 2013). This mixture of proteomes results in flexible biochemical activities leading to a unique cellular metabolism. The *S. cerevisiae*-like and *S. eubayanus*-like alleles of three *S. pastorianus* Group I strains (CBS 1513, CBS 1503, and CBS 1538) and one Group II strain (WS34/70) have been predicted using

HybridMine (Timouma et al. 2020), an open-source predictive tool our group developed (https://github.com/Sookie-S/HybridMine). To this date, there is no study published on transcriptomic differences between Group I and Group II strains in brewing conditions.

Here, we focused on the Group I *S. pastorianus* CBS 1513, also known as *S. pastorianus carlsbergensis*, which is the first established lager yeast strain (Hewitt et al. 2014; Walther et al. 2014). This bottom fermenting strain has been used in breweries for lager-style production since its isolation by Emil Chr. Hansen in 1883 (Hansen 1883). The brewing and distilling industries are constantly looking to improve their production strains for specific properties such as temperature tolerance, maltose utilization, and balanced flavor profiles (Gorter de Vries et al. 2020; Giannakou et al. 2021; Naseeb et al. 2021). In *Saccharomyces*, a large number of studies focused on transcriptional divergence in different species and presented temperature as a factor that influences several traits, including fermentation, cell division, allelic expression, and protein synthesis (Hartwell and McLaughlin 1968; Borneman et al. 2007; Ciani et al. 2016; Li and Fay 2017; Hewitt et al. 2020; Hovhannisyan et al. 2020). At low temperature (<15 °C), the beer fermentation process is slowed down, which causes a positive impact on volatile flavor production and retention, and increases the consumption of the wort sugars (García-Ríos et al. 2019). However, low temperatures can also be responsible for the termination of the fermentation process. Hybrids of cryo- and thermo-tolerant strains like *S. pastorianus* have a favorable genetic background to ferment at different temperatures.

Historically, temperature treatments on *Saccharomyces* yeast had been used for industrial purposes, with beer brewing being one of the main applications. In this study, we analyzed the transcriptome data of *S. pastorianus* CBS 1513 at different environmental conditions to determine the genes involved in temperature stress that are media-independent. We observed the presence of a skew toward the expression of one or the other parental subgenome (*S. cerevisiae* or *S. eubayanus*) in the different conditions. Using the genomic features and expression data, we predicted the proportion of protein complexes that are more likely to be chimeric, unispecific, fully or partially redundant in *S. pastorianus* CBS 1513. We identified that the majority of the protein complexes tend to be either chimeric or unispecific even if both parental alleles are present in the genome. This study highlights that functional redundancy is efficiently exploited by *S. pastorianus* by using preferentially one allele over the other to adapt its environment. As this strain is still evolving, most of the redundant alleles could be predicted to be eventually lost. Our study is the first to provide insights into the genetics of *S. pastorianus* CBS 1513 at gene expression level and a rationalization for its unique industrial traits.

Results and Discussion

Temperature Variations Induce Larger Transcriptional Changes Than Nutritional Media

To determine the genes affected by temperature stress in different culture media, the expression profiles of the *S. pastorianus* CBS 1513 (Group I strain) at 13 °C, 22 °C, and 30 °C in synthetic dextrose (SD) media, wort (maltose rich medium), SD media with 6% ethanol, and SD media without leucine were analyzed. These media were chosen given their relevance to *S. pastorianus* domesticated habitat, as wort is used during the beer fermentation, alcohol is produced during this process, and the leucine amino acid metabolism is responsible for important flavor compound production, such as the isoamyl-acetate production (fruity flavor; Stewart 2017). The cells were grown aerobically and the yeast growth on these media and their comparison is reported in supplementary figure S1 and tables S1 and S2, Supplementary Material online. The expression of six *S. cerevisiae*-like and six *S. eubayanus*-like alleles was validated by RT-qPCR (supplementary fig. S2, Supplementary Material online).

To find patterns of expression or groupings between the different conditions a multidimensional scaling (MDS) analysis of the RNAseq libraries was performed (fig. 1). When comparing the temperatures with the media, as expected, we observed that biological replicates clustered tightly together. The highest amount of variance is detected at different temperatures, where samples taken at the lowest temperature clusters at the opposite end to those at a highest temperature (*i.e.* larger spread of data on the first dimension). In a consistent trend, samples taken at 22 °C clustered in between. The media show less variance with the exception of SD +6% ethanol at 30 °C. The combined presence of ethanol and high temperature may increase the stress in the cell compared with the other conditions, which could explain the variance in the data. Overall, these results suggest that temperature stress has a higher effect on the transcriptome than the nutritional media and that temperature impacts on the growth of *S. pastorianus* in all media (supplementary table S1, Supplementary Material online).

To assess whether the type of culture media had an impact on the expression network at different temperatures, we compared the absolute number of differentially expressed (DE) genes (absolute fold change >2 and *P*-adjusted value <0.05) obtained in each temperature condition, for each media. The absolute number of DE genes up- and downregulated at different temperatures varied significantly according to the culture media (fig. 2). As expected, at extreme temperature conditions (13 °C vs. 30 °C), changes in the transcriptome are higher in each culture media compared with the other temperatures (13 °C vs. 22 °C and 22 °C vs. 30 °C), with the exception of wort where the total number of DE genes is similar for all temperatures (fig. 2). Within the SD media, there are less DE genes at 22 °C versus 30 °C compared with the other temperatures. Given that *S. pastorianus* CBS 1513 optimal growth temperature is around 25 °C (Fischer et al. 2016), it is somehow expected that a shift between two temperatures close to its optimal would not induce a drastic difference in the gene expression compared with a shift involving colder temperatures (13 °C). Surprisingly, in SD w/o leucine and SD + 6% ethanol, the absolute number of genes DE is gradually increasing when the shift goes toward the warmest temperature of 30 °C. Furthermore, the changes in *S. pastorianus* CBS 1513 transcriptome appeared to be

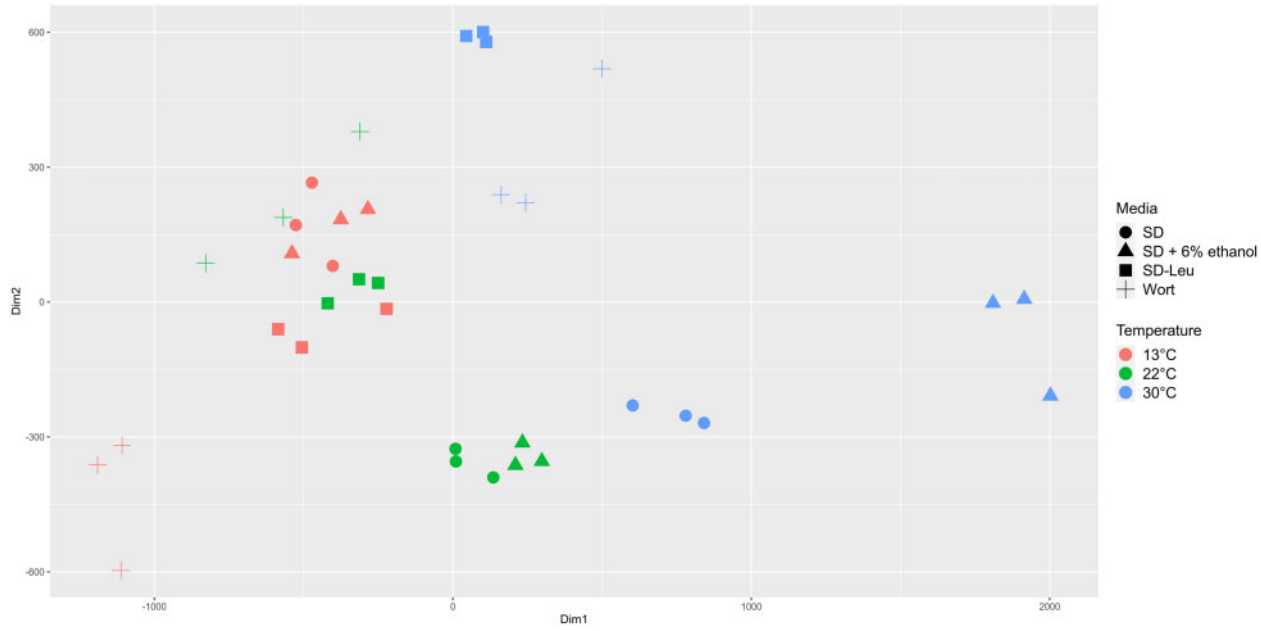

FIG. 1. MDS plot of RNA-seq expression profiles of *Saccharomyces pastorianus* CBS 1513, in two dimensions. For visual aid, samples cultured at 13 °C, 22 °C, and 30 °C are colored in red, green, and blue, respectively. Samples cultured in the different media are represented with circles, triangles, squares, and crosses shapes for SD + 6% ethanol, SD with all amino acids, SD w/o leucine, and wort media, respectively.

very low in SD media w/o leucine between 13 °C and 22 °C. It has been shown in a recent study in *Staphylococcus aureus* that amino acid uptake and release are correlated to environmental changes, such as temperature, pH, and osmolality, to help the bacteria to adapt (Alreshidi et al. 2020). In *S. cerevisiae*, the addition of ethanol induces stress responses and causes cell cycle delay, where heat shock proteins are induced and trehalose is accumulated (Stanley et al. 2010). So, when the temperature rises in presence of ethanol, the transcriptional changes may be exacerbated.

Temperature-Dependent Media-Independent Genes Are Primarily Located in the Cell Wall, Plasma Membrane, and Mitochondria

Given that media have an impact on temperature acclimatization, we isolated and analyzed the genes that are temperature-dependent and media-independent. When comparing the expression network at 13 °C versus 30 °C, 94 temperature-dependent genes were common for all media (fig. 3A). Using the pathway enrichment tool (yeastMine), we found a significant over-representation of the glycerol degradation pathways. The GO term analysis showed a significant enrichment of mono- and dicarboxylic acids catabolic processes, glutamine metabolic process, oxidoreductase activity, vitamin binding, protein folding, and coenzyme binding (fig. 3B). Several of these temperature-dependent proteins were located in the cell wall, plasma membrane, and mitochondrial membrane. Within the core genes, we identified *GUT2* (glycerol-3-phosphate dehydrogenase), a mitochondrial gene previously described as partially responsible for cryo-tolerance in *S. kudriazevii* (Paget et al. 2014). Similar analysis was carried out for the other temperature shifts. We observed that 13 and 29 genes are temperature-dependent and media-independent at 13 °C versus 22 °C (supplementary fig. S3A, Supplementary Material online) and 22 °C versus 30 °C (supplementary fig. S3C, Supplementary Material online), respectively. The GO enrichment analysis showed that these genes are mainly involved in transport, regulation of fatty acid metabolic process, short-chain fatty acid catabolism, phosphatidylcholine biosynthetic process, fermentation, and oxidoreductions. Seven out of the 13 genes at 13 °C versus 22 °C are associated with mitochondria subcellular localization (supplementary fig. S3B and D, Supplementary Material online). This reflects recent findings in laboratory hybrids *S. cerevisiae*/*S. uvarum* where the transcriptional changes at different temperatures were correlated to the parent donating the mtDNA (Hewitt et al. 2020). The functional annotation of the ature-dependent, media-independent genes is presented in supplementary table S3, Supplementary Material online.

We identified a small proportion of DE genes in the temperature-dependent group that are affecting growth; such as *DFG16*, *CIS3*, and *WHI3*, involved in invasion during filamentous growth, stability of cell wall, and positive regulation of transcription, respectively. In fact, temperature has an impact on the growth rate through cell division, which makes the biomass yield varying with temperature (Zakhartsev et al. 2015). The majority of temperature-dependent genes were nongrowth related, such as *CUR1* (involved in cellular response to heat and chaperone binding) and *SSA1* (heat shock protein involved in chaperone cofactor-dependent protein refolding). Indeed, the temperature can trigger the production of chaperones, cold shock or heat shock proteins to adapt to the environment (Verghese et al. 2012). Temperature can also affect the folding of the proteins, which results in a modified activity. This phenomenon could explain why temperature has a higher impact on the transcriptome

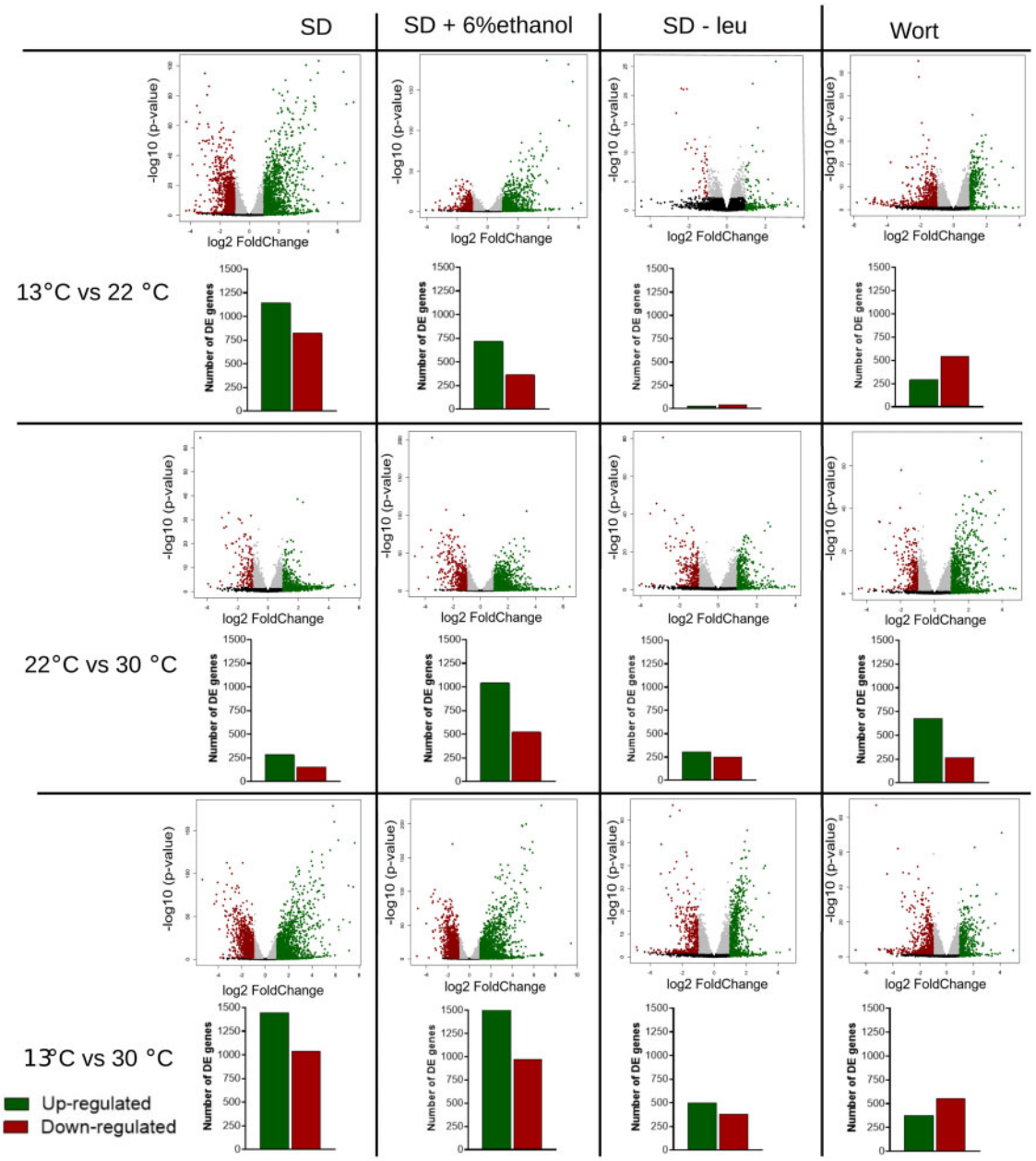

Fig. 2. Volcano plots and histograms representing the differential expression and absolute number of DE genes of *Saccharomyces pastorianus* CBS 1513 genes in SD, SD +6% ethanol, SD w/o leucine, and wort media, in each temperature shifts (13 °C vs. 22 °C, 22 °C vs. 30 °C, and 13 °C vs. 30 °C). Up- and downregulated genes are represented in green and red, respectively (absolute fold change >2 and adjusted *P* value <0.05). Genes with significant, but low expression change (absolute fold change <2 and adjusted *P* value <0.05) are colored in gray, and the ones not significant in black (adjusted *P* value >0.05).

than the different media. Vitamin biosynthesis, lipid/fatty acid processes, and oxido-reduction reactions have also been described in *S. cerevisiae* by Paget and coworkers (2014) to be highly affected by the cold condition. Our transcriptome data in *S. pastorianus* support this scenario.

In complete SD media, the extracellular leucine uptake is increased at cold temperature as *OPT1* (encoding for an oligopeptide transporter 1 of tetra- and pentapeptides including leucine) is upregulated. Strikingly, 35% (33 genes) of the 94 core temperature-dependent genes have a unique and opposite behavior in SD w/o leucine compared with all the other media (fig. 3*C*). Such result suggests an impact of leucine uptake on temperature acclimatization in *S. pastorianus*. Among these genes, *GUT2* is highly upregulated at 30 °C in all media (*i.e.* 11, 10, and 4 folds in SD, SD + 6% Ethanol, and Wort, respectively) with the exception of SD media w/o leucine. In this medium, *GUT2* shows an opposite trend as it is 2.6 times less expressed at 30 °C. Such differential expression has been validated via RT-qPCR for both parental alleles of *S. pastorianus* and also measured for the parental strains *S. cerevisiae* 96.2 and *S. eubayanus* PYCC 6148 type strains (fig. 3*D*). *GUT2* expression in medium w/o leucine seems to be hybrid specific as *S. cerevisiae* and *S. eubayanus* parental strains show a different trend: in *S. cerevisiae* 96.2, *GUT2* is

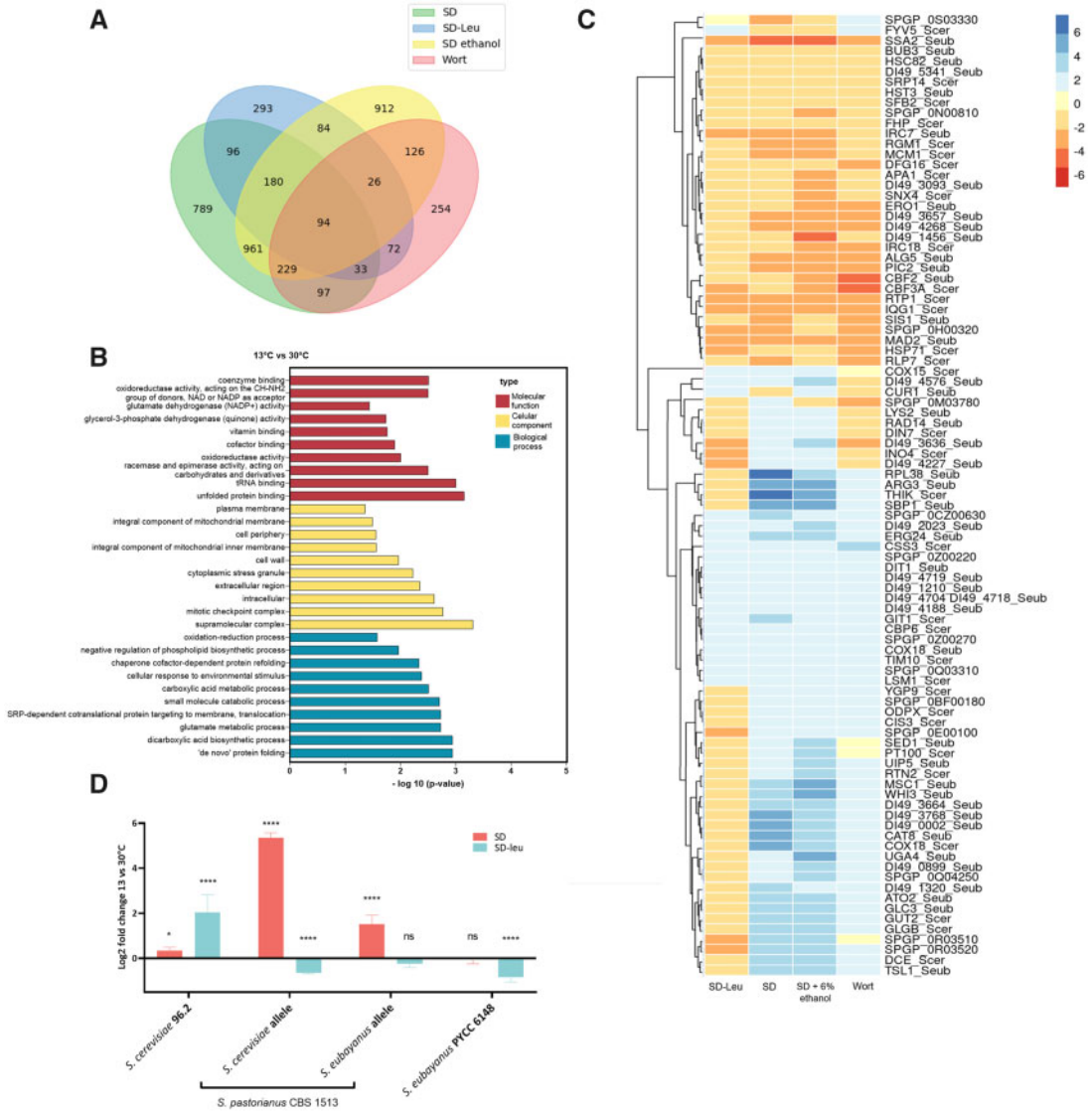

FIG. 3. DE genes temperature-dependent media-independent. (*A*) Venn diagram of the genes DE between growth at 13 °C and 30 °C in standard medium (SD; green), standard media without leucine (SD-Leu; blue), standard media with 6% ethanol (SD ethanol; yellow), and maltose rich medium (Wort; pink). The genes present in the intersection of all media conditions are considered temperature-dependent media-independent. (*B*) Histogram representing the significance (-log10[*P*-value]) of the GO terms enriched of the core DE genes at 13 °C versus 30 °C. Molecular function, cellular component, and biological process are colored in red, yellow, and blue, respectively. (*C*) Heatmap showing individual expression of the 94 temperature-dependent media-independent core genes at 13 °C versus 30 °C in SD-Leu, SD, SD+ 6% ethanol, and wort. The color key represents the log2 fold change (negative and positive fold change in shades of red and blue, respectively). (*D*) *GUT2* differential expression at 13 °C versus 30 °C, for both parental alleles of *Saccharomyces pastorianus* CBS 1513 (*Saccharomyces cerevisiae*-like and *Saccharomyces eubayanus*-like alleles), and for the type strains *S. cerevisiae* 96.2 and *S. eubayanus* PYCC 6148, in SD (red) and SD w/o leucine (blue) media. Error bars denote standard deviations and *P* values are indicated as: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$; ns = no significant change.

upregulated at 30 °C in both media and the *S. eubayanus* PYCC 6148 is not DE in SD and downregulated in SD w/o leucine (fig. 3*D*). Both leucine and *GUT2* are involved in fatty acids regulation, by acting as precursor of branched fatty acids and phospholipids, respectively (Kerkhoven et al. 2017; Ferreira et al. 2018). Temperature impacts on the physical state of membranes that needs to stay in a lamellar liquid crystalline phase to function properly. As adaptation mechanism, the yeasts avoid the formation of lamellar gel phase caused by cold temperature by changing the membrane lipids composition, which alters its fluidity (Gunde-Cimerman et al. 2014). Short-chain lengths and/or unsaturated fatty acids and triacyl-glycerides abundance increase at cold temperatures, whereas the ratio phosphatidylcholine/phosphatidylethanolamine and the phosphatidic acid content decreases (Martin et al. 2007; Redón et al. 2011). Here, it is possible that the lack of leucine is already sufficient to limit the amount of phosphatidic acid produced without the need of *GUT2* downregulation at cold, which would be shifting free glycerol toward glycerone phosphate. In addition, phosphatidylcholine fatty acid is known to be essential for efficient functioning of the mitochondrial Gut2p in *S. cerevisiae* (Rijken et al. 2007).

The absence of extracellular leucine could lead to a decreased production of phosphatidylcholine, which negatively impacts on Gut2p efficiency that is compensated by an upregulation at the transcriptome level at 13 °C in SD media w/o leucine compared with 30 °C.

GO analysis was also carried out on the genes with unique behavior in media lacking leucine. Out of the 33 genes showing a unique behavior in media lacking leucine, 6 and 1 genes are specific to *S. pastorianus* CBS 1513 and to *S. eubayanus* subgenome, respectively, and have an unknown function. Although no significant enrichment has been found on the remaining 26 genes after applying the Bonferroni correction at a confidence interval of 95% ($q = 0.434$), 4 out 26 genes (*GDH3*, *ARG3*, *PUT1*, and *GAD1*) participate in the glutamine family amino acid metabolic process with a significant *P* value ($P = 8.110805e{-}5$; supplementary table S4, Supplementary Material online). Glutamate–glutamine pathway is involved in fatty acid biosynthesis (He et al. 2018) and glutamine uptake has been linked to low temperature fermentation (Beltran et al. 2007).

We also carried out a similar analysis for the media-dependent, temperature-independent genes (supplementary file 1, Supplementary Material online). Genes involved in wort utilization are mainly acting in the super-pathway of glucose fermentation (*ADH1*, *ADH2*, *ADH5*, *GLK1*, *HXK1*, *TDH1*, *PDC5*, and *FBP1*). The core temperature-independent genes, triggered by the absence of leucine in the media, are also involved in the super-pathway of glucose fermentation. Interestingly, they are not enriched with leucine biosynthesis biological process, which is concordant with the hypothesis that leucine metabolism is temperature-dependent. Finally, the genes media-dependent temperature-independent involved in ethanol tolerance are enriched with the L-lysine biosynthesis IV pathway and the super-pathway of allantoin degradation that allows the yeast to use nitrogen as nutrient source by converting the allantoin to ammonia and carbon dioxide.

Analysis of Expression Profiles in Parental Alleles Shows That *S. eubayanus*-Like Alleles Are Primarily Involved in Temperature Response

We broke down the contribution of the specific parental alleles to the expression of the core temperature-dependent DE genes. We first analyzed at the genomic level the pool of the 94 temperature-dependent media-independent core genes of which 24 have maintained both parental copies in the genome.

Out of the 53 *S. eubayanus*-like alleles and 33 *S. cerevisiae*-like alleles, there are 40 and 22 alleles that have lost the *S. cerevisiae* and *S. eubayanus* copy, respectively (table 1). There were also 8 *S. pastorianus*-specific genes (table 1). Only three genes had both alleles DE in the same direction. One DE orthologous pair (*GLC3*) that have both alleles downregulated at 13 °C, encodes for a 1,4-alpha-glucan-branching enzyme a protein involved in the pathway glycogen biosynthesis, previously described to have been affected by temperature in the rainbow trout, human, and mouse (Seibert 1985; Naperalsky et al. 2010; Hanya and Katz 2018). The two other pairs, *CBF2* and *RTP1*, that encode the centromere DNA-binding part of the protein complex CBF3, and RNA polymerase II assembly factor, respectively, have all their alleles strongly upregulated at 13 °C. These are genes involved in cell division and transcription processes, so it is possible that their change in expression could be due to the different growth rate of the yeast at 13 °C and 30 °C, rather than the temperature itself. In the core genes involving cold temperature (13 °C), there is ∼37% more *S. eubayanus* alleles which are DE, whereas there is a similar amount of *S. eubayanus*-like and *S. cerevisiae*-like alleles for higher temperatures (22 °C vs. 30 °C; table 1). These results suggest that *S. eubayanus* alleles are those who are responding to large temperature shifts and are primarily involved in transcriptional changes at cold temperatures. The 40 and 22 *S. eubayanus*-like and *S. cerevisiae*-like alleles temperature-dependent media-independent may have lost their *S. cerevisiae*-like and *S. eubayanus*-like copy, respectively, in response to the evolutionary trajectory at cold temperature, as *S. pastorianus* hybrids have only been encountered in brewing-related environments (Gorter de Vries et al. 2019).

We expanded this analysis to all DE genes detected in our transcription study to see whether a parental subgenome is more likely to be DE under temperature stress. *Saccharomyces pastorianus* CBS 1513 has evolved from a precursor that appeared after a hybridization event between a haploid *S. cerevisiae* and a diploid *S. eubayanus*. There are 5,228 and 3,751 *S. eubayanus*-like and *S. cerevisiae*-like alleles detected in *S. pastorianus* CBS 1513 genome (Timouma et al. 2020). Therefore, if there is no allele bias, out of the total of the DE genes, we would expect to identify ∼58% *S. eubayanus*-like alleles and 42% *S. cerevisiae*-like alleles. We compared the expected and observed number of *S. cerevisiae*-like and *S. eubayanus*-like alleles that show a difference in expression (absolute FC > 1.5 and *P*-adjusted value <0.05) in all the temperature conditions for each growth media (table 2). We observed a statistical over-representation of the *S. eubayanus*-like alleles in all the temperature conditions, for all the media except wort for the temperature shifts to 30 °C (confidence interval of 95%). In the case of wort, there is not a statistically significant difference between the two types of alleles, but there are *ca.* 7% more DE *S. cerevisiae*-like alleles than expected (rather than less *S. eubayanus*-like alleles). This

Table 1. Number of *S. eubayanus*-Like, *S. cerevisiae*-Like, and *S. pastorianus*-Specific DE Genes Detected in the Core Genes (temperature-dependent, media-independent) for Each Temperature Condition.

Condition	*S. eubayanus*-Like Allele	*S. cerevisiae*-Like Allele	*S. pastorianus*-Specific Gene	Total
13 °C vs. 30 °C	53	33	8	94
13 °C vs. 22 °C	9	3	1	13
22 °C vs. 30 °C	13	14	2	29

Table 2. Chi-Square Test on the Total Number of *S. cerevisiae* and *S. eubayanus*-Like Alleles DE (absolute fold change > 1.5, *P*-adjusted value < 0.05) per Condition.

Condition	Media	Total	*Saccharomyces cerevisiae*-Like Alleles DE (out of the 3,751 *S. cerevisiae*-like alleles present in the genome)	*Saccharomyces eubayanus*-Like Alleles DE (out of the 5,228 *S. eubayanus*-like alleles present in the genome)	*P* value	Outcome
13 °C vs. 30 °C	SD	3,885	1,537	2,348	0.0052	*S. eubayanus*-like alleles over-represented
	SD + 6% eth	4,090	1,628	2,462	0.0106	*S. eubayanus*-like alleles over-represented
	SD-leu	1,838	715	1,123	0.0125	*S. eubayanus*-like alleles over-represented
	Wort	2,019	802	1,217	0.0615	No significative difference
13 °C vs. 22 °C	SD	3,357	1,320	2,037	0.0039	*S. eubayanus*-like alleles over-represented
	SD + 6% eth	2,260	886	1,374	0.0132	*S. eubayanus*-like alleles over-represented
	SD-leu	245	84	161	0.0175	*S. eubayanus*-like alleles over-represented
	Wort	1,852	703	1,149	0.0009	*S. eubayanus*-like alleles over-represented
22 °C vs. 30 °C	SD	1,193	464	729	0.0436	*S. eubayanus*-like alleles over-represented
	SD + 6% eth	3,009	1,189	1,820	0.0119	*S. eubayanus*-like alleles over-represented
	SD-leu	1,483	564	919	0.0035	*S. eubayanus*-like alleles over-represented
	Wort	2,161	864	1,297	0.0909	No significative difference

suggests that more *S. cerevisiae* alleles undergo transcriptional changes in wort to help sugar fermentation and to cope with stresses present in this complex rich media.

Analysis of the Expression of Ortholog Parental Alleles Reveals That the Majority of the Functionally Redundant Alleles Are Not Concomitantly Expressed by the Cell

Saccharomyces pastorianus CBS 1513 strain is maintained in cold temperature for lager beer production, a selective pressure that may impact on its evolution. Group I strains are indeed still evolving, as they show high genomic plasticity and an instable chromosome copy number (Gorter de Vries et al. 2020). The higher the loss of *S. cerevisiae* subgenome in *S. pastorianus*, the lower is the amount of functionally redundant genes (Timouma et al. 2020). Here, we investigated how cell response to cold temperatures has shaped the functional redundancy found in *S. pastorianus* CBS 1513. When both *S. cerevisiae*-like and *S. eubayanus*-like alleles are DE, they can either be concordant (both up- or downregulated), with or without an allele playing as a major player, or discordant (one allele up the other downregulated). Strikingly, in 81% of the cases, only one of the parental alleles is DE across all the conditions (table 3). Out of the remaining 19% of the genes where both parental alleles are DE, 82% have a concordant DE expression (both alleles up- or down-regulated) across all the conditions, with approximately half of them showing a predominant major player. Only in 3% of the cases the orthologs alleles have a discordant expression (table 3).

The fact that the hybrid can modulate the expression of both types of alleles confers an advantage over the parental strains to achieve the best fitness in a given environment. However, functional redundancy can be costly (and eventually evolutionary unstable) and most of the redundant alleles are expected to be silenced or lost as the hybrid is adapting to its new niche. In fact, a temperature biased gene retention was observed in hybrids *S. cerevisiae*/*S. uvarum* with major loss at 15 °C of alleles deriving from the mesophilic parent (Smukowski Heil et al. 2019). Additionally, due to its hybrid nature, *S. pastorianus* CBS 1513 may encounter incompatibilities between nuclear and mtDNA (Lee et al. 2008; Chou et al. 2010); therefore, it is possible that this phenomenon also triggers the loss of the redundant incompatible alleles. It has also been shown in *S. cerevisiae*/*S. uvarum* synthetic hybrids that the different parental mtDNAs have an effect on the nuclear gene expression and that the transcriptional regulation was nearly exclusively retained within the same parental type of alleles for specific biological functions (Hewitt et al. 2020). This phenomenon could be due to the divergence of *cis*-regulatory elements in the hybrids (Borneman et al. 2007; Li and Fay, 2017; Hovhannisyan et al. 2020) and their interaction with the mitochondrial genome.

Table 3. Expression of the Orthologous *S. cerevisiae*-Like and *S. eubayanus*-Like Alleles in the Genes DE for Each Condition.

			DE Expression of Only One Parental Allele				DE Expression for Both Parental Alleles							
			Upregulation of one allele		Downregulation of one allele		Concordant DE expression			Concordant DE expression			Discordant DE expression	
							Upregulation			Downregulation				
Conditions	Media	Total number of pair of othologs with at least one DE allele	S. cer + S. eub NDE	S. cer NDE S. eub +	S. cer − S. eub NDE	S. cer NDE S. eub −	S. cer ++ S. eub +	S. cer + S. eub ++	S. cer + S. eub +	S. cer − − S.eub −	S. cer − S. eub − −	S. cer − S. eub −	S. cer + S. eub −	S. cer − S. eub +
13 °C vs. 30 °C	SD	721	131	173	137	100	17	25	32	13	7	44	23	19
	SD + 6% ethanol	740	147	201	116	103	25	35	39	9	7	29	9	20
	SD—Leucine	279	60	78	51	45	0	4	13	6	2	17	0	3
	Wort	290	38	49	78	88	2	3	8	5	3	12	3	1
13 °C vs. 22 °C	SD	575	125	143	108	88	12	15	28	9	5	18	12	12
	SD + 6% ethanol	316	78	83	56	51	6	14	15	0	0	3	4	6
	SD—Leucine	21	3	2	11	4	0	0	0	0	0	1	0	0
	Wort	264	35	39	74	79	1	0	9	4	5	15	2	1
22 °C vs. 30 °C	SD	148	40	45	26	23	2	1	2	0	1	6	0	2
	SD + 6% ethanol	456	88	147	68	60	12	8	35	7	4	11	8	8
	SD—Leucine	179	43	47	30	40	2	1	3	4	0	8	0	1
	Wort	281	69	86	43	34	4	12	17	2	2	4	3	5

NOTES.—In the table, *S. cerevisiae*-like and *S. eubayanus*-like alleles are annotated "S. cer" and "S. eub", respectively. Up- and downregulation are represented by "+" and "−," respectively. An allele not DE is annotated "NDE." Concordant DE expression similar on both alleles is annotated with (+/+ or −/−). If an allele is a major player with more than twice the expression compared with the other allele, it is represented with "++" or "− −" for upregulation and downregulation, respectively. Discordant expression is annotated with (+/− or −/+).

Analysis of the Expression of Orthologous Members of Protein Complexes Shows a Trend Which Favor the Formation of Either Exclusively Unispecific or Chimeric Complexes

The different nature of protein complexes can act as an evolutionary force in the hybrid organisms where orthologous members can bind together forming unispecific or chimeric protein complexes. Considering the presence/absence of parental alleles in *S. pastorianus*, the protein complexes can be either exclusively unispecific with subunits coming only from one parent; or exclusively chimeric, with a mixture of subunits from both parents; or partially or fully redundant when a series of protein complexes with different orthologous members can be established because both alleles are present for some or all subunits, respectively. Moreover, according to which alleles are expressed, different scenarios of protein assemblies can occur even when both parental alleles are presents in the genome, specifically in the partially or fully redundant cases (fig. 4*A*). Partially and fully redundant cases can form one and all combination(s) of chimeric and unispecific complexes, respectively.

Given that the number of isoforms of protein complexes increases exponentially with number of subunits, here we restricted our study to homo- and heterodimers and trimers (supplementary fig. S4 and table S5, Supplementary Material online). We determined that 575 over the 607 protein complexes originally identified in *S. cerevisiae* are conserved in *S. pastorianus* CBS 1513, with at least one allele identified for each subunit (supplementary table S6, Supplementary Material online). Among these 575 protein complexes, 8 are homodimers, 210 heterodimers, and 117 trimers (supplementary fig. S4, Supplementary Material online). For these complexes, the majority of orthologous alleles are present and therefore partially or fully redundant protein complexes can potentially be established (supplementary table S7, Supplementary Material online and fig. 4*B*). Protein abundance depends on several factors, such as transcription rates, mRNA half-lives, translation rate constants, and protein half-live (Hausser et al. 2019). However, as protein abundance and mRNA levels show a reasonable correlation in all organisms (Buccitelli and Selbach 2020), the expression of orthologous members of protein complexes has been compared with assess whether or not a parental subunit is more likely to form the complex. Using the absolute expression, we analyzed for each gene which parental allele is the major player in all the environmental conditions studied. An allele has been considered a major player if the expression was at least two times higher than the homologous allele. Such assessment allowed us to recognize which protein assemblies are more prevalent in *S. pastorianus* CBS 1513 when both parental alleles are present (*i.e.* partially or fully redundant cases).

Firstly, we analyzed the composition of the protein complexes in our standard condition, SD medium at 22 °C. Out of the eight homodimers, three are exclusively unispecific as only one type of parental alleles in present in the genome and five are fully redundant. The absolute allele expression showed that three out of five preferentially express the same parental allele, suggesting that unispecific protein complexes are more prevalent in these cases (supplementary table S7, Supplementary Material online). The remaining two have alleles that are similarly expressed and can therefore form every possible combination of the complex. In the 210 heterodimers, based on the presence/absence of parental alleles at the genome level, only 9 and 18 complexes can form exclusively chimeric and unispecific assemblies, respectively, with the remaining being either fully (89) or partially (94) redundant. The absolute expression data for the 89 fully redundant heterodimers suggest that about 53% form predominantly either unispecific or chimeric complexes (*i.e.* 30 unispecific complexes and 15 chimeric; fig. 4*B* and supplementary table S7, Supplementary Material online). More strikingly, the analysis of the absolute expression of the 94 partially redundant heterodimers reveals that 70% of the cases are likely to form exclusively chimeric (31) or unispecific (35) (fig. 4*B* and supplementary table S7, Supplementary Material online). In the 117 trimers, based on the genetic, there is no possibility of exclusive chimeric complexes and only two cases where the same parental alleles are uniquely retained for all the members of the complex (unispecific combination). In fact, in the trimers, the majority of protein complexes are either fully (38) or partially (77) redundant. Similarly to the heterodimers, the absolute expression data for the 38 fully redundant trimers suggest that 44.7% forms either exclusively unispecific or chimeric complexes (*i.e.* ten unispecific complexes and seven chimeric) (fig. 4*B* and supplementary table S7, Supplementary Material online). Out of the 77 partially redundant cases, 57% are more likely to be chimeric (22) and unispecific (22), respectively (fig. 4*B* and supplementary table S7, Supplementary Material online). The same analysis was extended to the other environmental conditions and showed a consistency in this trend (supplementary fig. S5, Supplementary Material online).

Overall, these results show that the majority of the protein complexes established in the hybrid are more likely to be either exclusively chimeric or unispecific and that the redundancy is discouraged, which is concordant to our previous conclusions at genome scale on the preferential expression of one allele locus (table 3). This scenario also fits well with the gene balance hypothesis (GBH), which states that the stoichiometric imbalances can alter protein complex's function, due to the mode of assembly and kinetics (Birchler and Veitia 2010). The GBH predicts that pairs of chromosomes should be cogained or colost to avoid generating imbalanced protein complexes that causes reduced proliferation rates, often observed in aneuploid cells (Chen et al. 2019). This could explain our observation that compared with the fully redundant cases, partially redundant cases are more likely to express only one parental allele per subunit, to form unispecific or chimeric complexes. The level of transcription of orthologous genes/alleles (*cis*-regulation) and/or mutations in upstream regulatory factors (*trans*-effects) could be a way to buffer the concentration of a given subunit (Comai 2005; Tirosh et al. 2009). Moreover, as hybridization events bring together diverged genomes within a same nucleus, it has been hypothesized that in addition to the novel epistatic interactions in

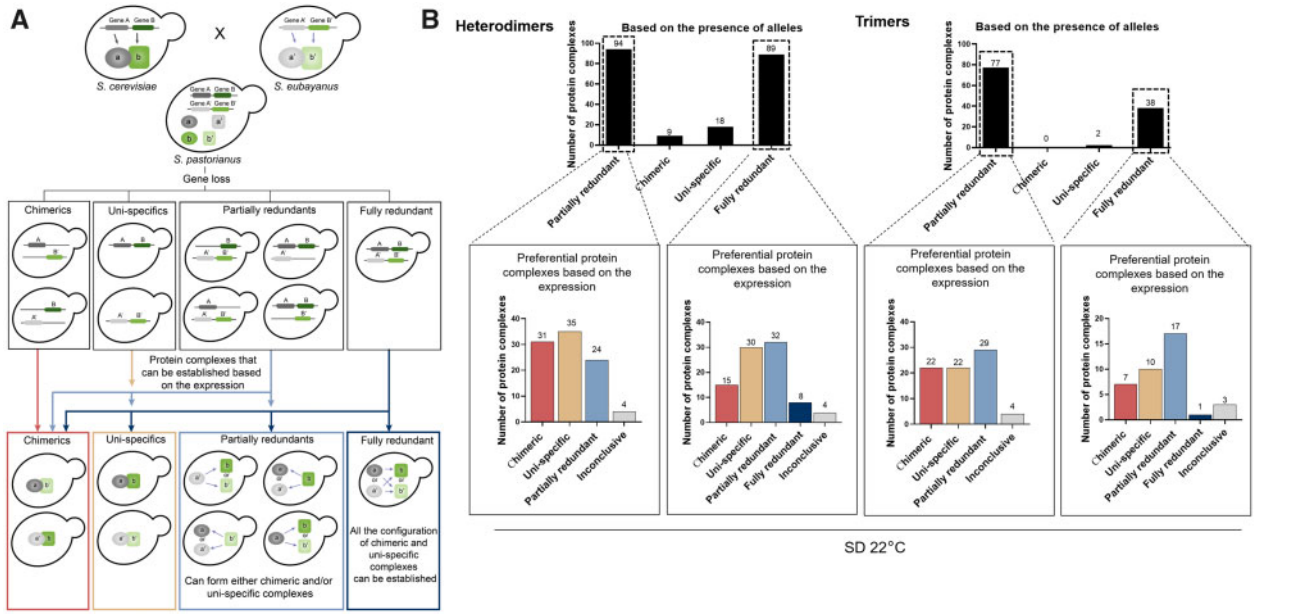

Fig. 4. (*A*) Protein complexes assembly diagram according to the presence/absence of parental alleles in the hybrid genome. Here, an example of heterodimer is illustrated. After hybridization event, genes A (gray) and B (green) inherited from *S. cerevisiae* and A' (light gray) and B' (light green) from *S. eubayanus* are present in the hybrid genome, which evolves through reduction of genetic content (gene loss). When two parental alleles are lost, the protein complexes can be either chimeric or unispecific. When one parental allele is lost, it creates partially redundant configurations. When all alleles are conserved, it is a fully redundant case. Based on the allele's expression, chimeric and unispecific can only form chimeric (red arrow) and unispecific (beige arrow) protein complexes, respectively. Partially redundant cases can form chimeric, unispecific, or partially redundant protein complexes (light blue arrow). Fully redundant cases can form all the configurations (dark blue arrow). (*B*) Histogram representing the total number of chimeric, unispecific, partially, and fully redundant configurations in the 210 heterodimers and 117 trimers based on the presence/absence of parental alleles. The partially and fully redundant cases can form different assemblies based on the expression (chimeric, unispecific, partially, and fully redundant are colored in red, beige, light blue, and dark blue, respectively). Inconclusive configuration (represented in gray) is the cases where the expression of at least one allele is below ten reads, and therefore no conclusion can be drawn. An example in SD media at 22 °C is illustrated.

the hybrid genome, regulatory interferences between the parental subgenomes may occur, which could lead to a genomic shock, that induce transcriptional changes (Hovhannisyan et al. 2020).

Additionally, for the heterodimers, we investigated how the protein assemblies may change according to the different conditions. In the case of fully redundant heterodimers, there is ca. 18% of the cases where the protein complex is preferentially formed with the same parental proteins across all conditions tested, such as, for example, CPX-1022 (DNF2p-LEM3p P4-ATPase complex) composed by an *S. eubayanus*-like DNF2p and an *S. cerevisiae*-like LEM3p (fig. 5*A*). In the case of the 94 partially redundant heterodimers, the expression data show that ca. 44% of the complexes prefer the same composition (unispecific or chimeric) across all conditions (fig. 5*B*). Thus, it is possible that in these cases, the other alleles may soon pseudogenize and eventually be lost from the genome. However, there are cases where the nature of the protein complex is potentially different according to the environment, such as CPX-1661 (Spt4p-Spt5p transcription elongation factor complex). In SD medium, this complex that is likely be unispecific (*S. eubayanus*-like) at 13 °C and chimeric (with Spt4 *S. cerevisiae*-like and Spt5p *S. eubayanus*-like) at 30 °C. This suggests that some protein complexes may swap parental subunits to adapt to different environmental conditions. Seven complexes CPX-1661, CPX-1905, CPX-1732, CPX-1674, CPX-417, CPX-3078, and CPX-1341 appeared to be more likely to swap parental subunits according to the environmental condition (supplementary table S8, Supplementary Material online). No significant GO term enrichment and no pattern in the composition of the complexes have been found either with media or temperature that could lead to a strong conclusion. However, interestingly, five out of seven complexes are involved in protein trafficking (supplementary table S8, Supplementary Material online). It has been shown in Delneri et al. (2008) that protein trafficking is the cellular category most affected by gene dosage. Here, it is possible that the gene coding for the proteins involved in these complexes is also haplo-insufficient in *S. pastorianus* background. In fact, all of these protein complexes except for CPX-1905 (Pex7-Pex18 receptor complex) have at least one gene that has been shown to be haplo-insufficient in *S. cerevisiae* (Delneri et al. 2008). The need of an optimal expression of these alleles could provide a rationale for the different combinations of parental subunits detected in the conditions tested. A previous study on hybrids of *S. cerevisiae*/*S. mikatae* and *S. cerevisiae*/*S. uvarum* concluded that protein complexes are able to spontaneously

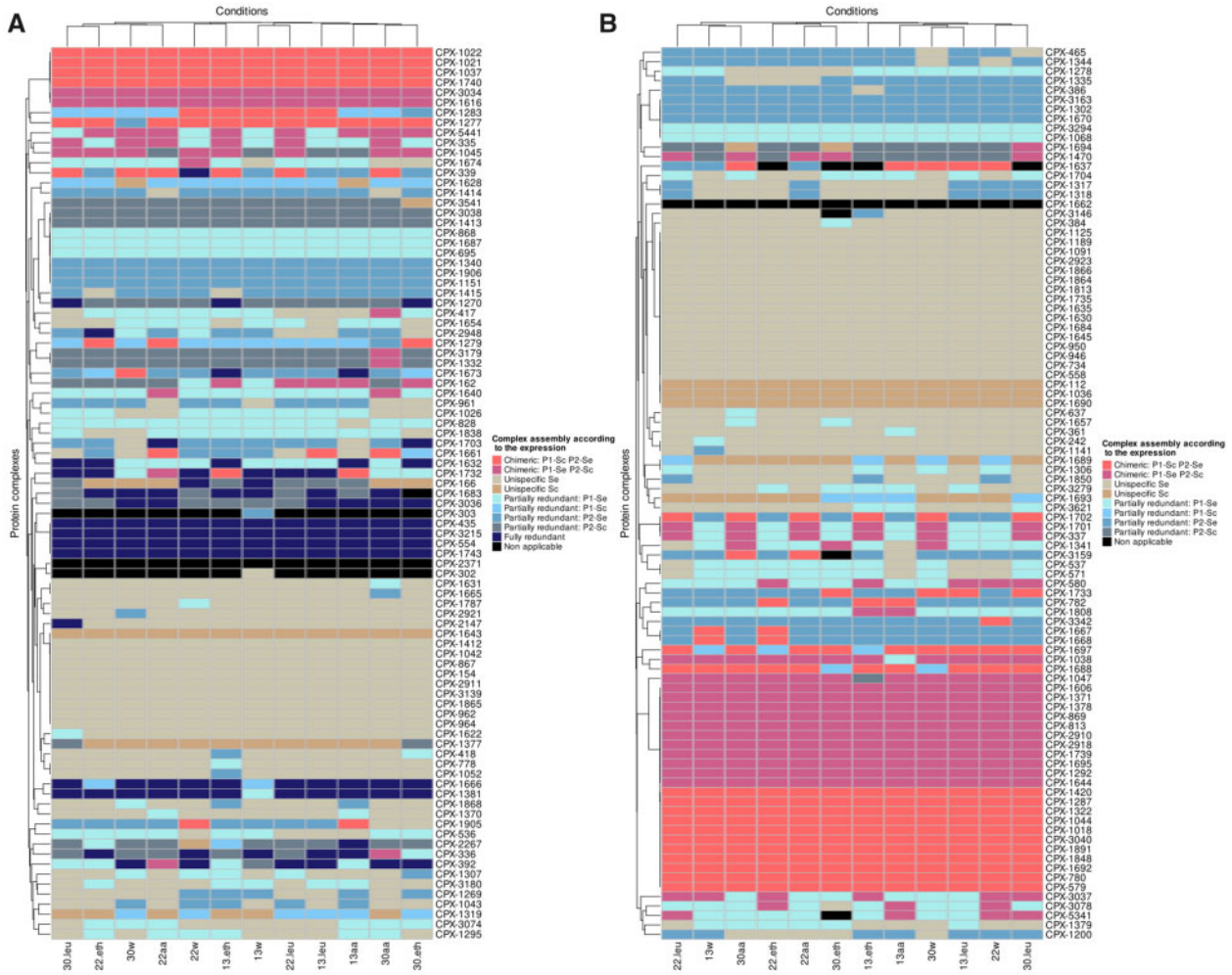

Fig. 5. Heatmap showing the assembly of heterodimers (chimeric, unispecific, partially, or fully redundant) based on the absolute expression at 13 °C, 22 °C, and 30 °C (prefix "13," "22," "30" used in the naming of the conditions, respectively) in SD, SD w/o leucine, SD+ 6% ethanol, and wort media (suffix "aa," "leu," "eth," and "w" used in the naming of the conditions, respectively). Inconclusive cases are colored in black. Protein 1 and protein 2 constituting the heterodimer are annotated P1 and P2 in the legend. *Saccharomyces cerevisiae*-like and *S. eubayanus*-like alleles are abbreviated with "Sc" and "Se" in the legend. (*A*) Assemblies of the 89 fully redundant protein complexes. (*B*) Assemblies of the 94 partially redundant protein complexes.

exchange orthologous subunits and that the different types of assemblies have an impact on the phenotype in specific environments (Piatkowska et al. 2013).

Conclusions

Saccharomyces pastorianus, interspecies hybrid of *S. cerevisiae* and *S. eubayanus*, is a bottom fermenting yeast that is maintained in cold temperature for lager beer production, a selective pressure that may impact on its evolution. The hybridization event developed unique genetic characteristics for *S. pastorianus*. Here, we analyzed transcriptome data from *S. pastorianus* grown at low, ideal, and high temperature and under different media conditions to identify the response of temperature and media acclimatization at gene expression level. We also identify the protein complex composition and plasticity according to the presence/absence of the parental alleles and the transcriptome profile at different environmental conditions. The primary analysis of the samples clustering suggested that temperature has a higher impact on the transcriptome variability compared with the media. However, the culture media appeared to have an impact on temperature acclimatization as the absolute number of DE genes at different temperatures varies according to the media. We identified core temperature-dependent media-independent genes. They are mainly involved in protein folding, vitamin biosynthesis, lipid/fatty acid processes and oxido-reduction reactions. We found that extracellular leucine uptake is increased at cold temperature in SD media. Furthermore, the expression of a part of the temperature-dependent genes, including *GUT2*, is affected by the presence/absence of extracellular leucine. We showed that there is an interplay between extracellular leucine uptake and temperature acclimatization. We also analyzed the distribution of expression profiles in parental alleles at the genome level. We strongly predict that overdominance at gene expression level of *S. eubayanus*-like alleles plays a pivotal role for temperature acclimatization.

Expression of ortholog parental alleles reveals that the majority of the functionally redundant alleles are not simultaneously expressed by the cell. We investigated the impact on protein composition when at least one subunit has both parental alleles conserved in the genome. According to our expression data, there is a trend for the formation of either exclusively unispecific or chimeric complexes. Our results also support the notion that the protein complexes may swap parental subunits to adapt to different environmental conditions.

Materials and Methods

Genome Sequence and Annotation

Saccharomyces pastorianus CBS 1513 strain has been sequenced and assembled (Hewitt et al. 2014; Okuno et al. 2016). Its genome sequence is available from the National Center for Biotechnology Information (NCBI: txid1073566). The Yeast Genome Annotation Pipeline (YGAP) has been used to predict the potential Open Reading Frames (ORFs) in its genome (Proux-Wéra et al. 2012). HybridMine tool (https://github.com/Sookie-S/HybridMine, v4.0) has been used to identify the parental allele content in this strain (Timouma et al. 2020). Among the 9,728 potential ORFs of *S. pastorianus* CBS 1513, HybridMine predicted 5,228 *S. eubayanus*-like alleles and 3,751 *S. cerevisiae*-like alleles.

Media and Yeast Culture

Saccharomyces pastorianus CBS 1513 cells are incubated overnight in Yeast Peptone Dextrose (2% glucose) at 25 °C with shaking at 200 rpm. The precultured yeast cells were inoculated in fresh industrial wort from Brouwland (according to Giannakou et al. 2021), SD with all amino acids +2% glucose (complete), SD without leucine, SD with 6% ethanol media at 13 °C, 22 °C, and 30 °C until mid-phase of exponential growth. Liquid fitness assays have been performed for expression validation, three technical replicates for three biological replicates. Cells were grown at 30 °C from an optical density of 0.1 (measured at a wavelength of 600 nm). Growth was measured every 5 minutes as previously described by Naseeb and Delneri (2012) and recorded by a BMG FLUOstar OPTIMA Microplate Reader (OD 595 nm), for up to 55 hours incubation time. Growth parameters were calculated using "Growthcurver" R package.

Total RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from three biological replicates of cells collected at mid-log phase (OD_{600} 0.4–0.6) using the RNeasy Mini Kit (QIAGEN, Germany). The lysis was performed by enzymatic digestion of cell wall followed by lysis of spheroplasts. To eliminate genomic DNA contamination, an additional DNAse treatment was performed with RNAse-free DNase set (QIAGEN, Germany) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (QIAGEN, Germany) according to the manufacturer's protocol. Optimized qPCR reactions contained 6 ng of cDNA, 3 pmol each primer, and 5 µl of iTAq Universal SYBR Green super Mix 2X in a final volume of 10 µl. Reactions were cycled on a Roche Light Cycler real-time System for 35 cycles of: 15 s at 95 °C; 30 s at 55 °C; and 30 s at 72 °C.

RNA Sequencing and Data Analysis

Total RNA was sequenced using HiSeq4000 Illumina Platform (Genomic Technologies Core Facility, University of Manchester). Quality and integrity of the RNA samples were assessed using a 2200 TapeStation (Agilent Technologies) and then libraries generated using the TruSeq Stranded mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. A quality control using FastQC was performed on the reads (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, v0.11.9). Trimming and filtering were done using Trimmomatic 0.36 (Bolger et al. 2014). Reads were mapped to the annotated genome using STAR aligner 2.5.3a (Dobin et al. 2013). For the genes having several identical copy numbers the reads have been mapped (STAR aligner 2.5.3a) to one single copy. The gene expression was counted with the featureCounts tool (Liao et al. 2014). Data normalization and differential gene expression analysis have been done using the DESeq2 package (Love et al. 2014) on R version 3.4.4. We applied a false discovery rate (FDR) criterion proposed by Benjamini and Hochberg (Reiner-Benaim 2007) for multiple testing corrections of the raw *P* value. The threshold of DEGs was set as FDR < 0.05. The genes showing a statistically significant difference in expression between each pair of conditions, and greater than 2-fold change (FC) in expression, were considered as DE. Data mining has been performed to collect annotation stored in UniProtKB on the *S. pastorianus* CBS 1513 parental alleles identified by HybridMine. A Python 3.6 script that uses the REST application programming interface of UniProt has been developed to query and access its data (Nightingale et al. 2017). Gene enrichment has been performed using YeastMine (Balakrishnan et al. 2012).

Protein Complexes Analysis

Protein complexes present in *S. cerevisiae* has been retrieved from the IntAct Molecular Interaction Database of the EMBL-EBI (Orchard et al. 2014). Their presence in *S. pastorianus* CBS 1513 has been detected using an in-house python script (Python version 3.6.9) that searched for each protein in each complex the presence/absence of *S. cerevisiae*-like and *S. eubayanus*-like alleles and their expression in different environmental conditions. The script is available in GitHub at https://github.com/Sookie-S/Protein_complexes_analysis_S_pastorianus_CBS1513 (released June 25, 2021). The different configurations that can occur in homodimers, heterodimers, and trimers in terms of presence/absence of alleles are presented in supplementary table S7, Supplementary Material online. Based on the normalized reads and the median of three biological replicates, when one allele is consistently expressed at least twice more than the other allele, it has been classified as major player. When an allele is poorly expressed or twice as less abundant as the other, it was not considered as absent. The presence/absence of the parental alleles was determined at the genomic level (Timouma et al.

2020). However, when less than ten reads were counted per allele, no preferential protein complex configuration was concluded (*i.e.* fully and partially redundant, unispecific and chimeric) as it this expression could be noise rather than real low expression.

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Data Availability

The RNAseq data presented in this article are available in the European Nucleotide Archive Array Express (https://www.ebi.ac.uk/arrayexpress/) under the accession number E-MTAB-11009. All the other data in this study are included in the article and/or in its Supplementary Material online.

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