Transcriptional network 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 an industrial natural yeast evolved from different hybridisation 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 or antagonistic 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% have 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 express preferentially one parental allele over the other, and that *S. eubayanus*-like alleles are significantly over-represented among the genes involved in cold acclimatisation. 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 in the hybrid are likely to be either exclusively chimeric or uni-specific, and that the redundancy is discouraged, a scenario which fits well with the stoichiometric balance-hypothesis. This study offers the first overview of the transcriptional pattern of *S. pastorianus* and provide a rationalisation for its unique industrial traits at expression level.

31 Introduction

32

33 *Saccharomyces pastorianus* is a natural evolved allopolyploid and sterile hybrid between the mesophilic

34 *Saccharomyces cerevisiae* and the cold-tolerant *Saccharomyces eubayanus*. *S. pastorianus* is used in 89% of brewed

35 beer worldwide (Gorter de Vries *et al.* 2019). This species has been isolated, domesticated and maintained by human

36 selection for colder brewing temperatures of 8-15°C (lager fermentation environment) (Nakao *et al.* 2009). Before

37 the discovery of *S. eubayanus* species, the non-*S. cerevisiae* portion of the genome of *S. pastorianus* was considered

38 as being *S. uvarum* and/or *S. bayanus* genome, that are closely related to *S. eubayanus* (Vaughan-Martini and Martini

39 1993). *S. cerevisiae* was isolated in Europe, while *S. eubayanus* have been isolated from Nothofagus trees in

40 Patagonia and in East Asia (Tibet) (Bing *et al.* 2014). The connection of Asia and Europe via the silk road could

41 explain how the hybridisation occurred between those two species. Strong evidence suggests that the ancestry of

42 *Saccharomyces sensu stricto* yeast underwent an event of whole genome duplication (WGD), that may have occurred

43 100 million years ago (Wolfe 2015). The doubling of an organism's genetic content leads immediately to a

44 reproductive barrier with their relative species and ancestors. However, in the yeasts, two ancestral species mated

45 before the WGD which restored its fertility. WGD is a rare evolutionary event with great consequences as new

46 paralogs are the basis of speciation, as they can sub-functionalise. The analysis of transposon sequence distribution

47 within sub-telomeric regions and recombination breakpoints in the genome of *S. pastorianus* strains suggested its

48 separation into two genomically distinct groups, which may have arisen from multiple hybridisation events

49 (Monerawela and Bond 2018). One hypothesis state that an initial spontaneous hybridisation event occurred between

50 a haploid *S. cerevisiae* strain and a diploid *S. eubayanus* strain (Bond *et al.* 2004). That event led to a progenitor of

51 the Group I strains, which evolved through further reduction of the *S. cerevisiae* genome content, to produce an

52 aneuploid and an approximate triploid genome. In parallel, the progenitor strain made a second hybridisation event

53 with a different *S. cerevisiae* strain and evolved to give strains with an approximate tetraploid genome, that are

54 classified in the Group II. A more recent theory supports that a single hybridisation event occurred between a diploid

55 *S. cerevisiae* and a diploid *S. eubayanus*, resulting in a tetraploid progenitor that evolved to give the Group II strains

56 (Okuno *et al.* 2016). In parallel, this progenitor underwent chromosomal deletions of the *S. cerevisiae* sub-genome,

57 leading to a progenitor approximately triploid, that evolved to give the Group I strains (Salazar *et al.* 2019, Alsammar

58 and Delneri 2020). Therefore, Group II strains possess about 2 to 3 times more *S. cerevisiae* genomic content than

59 the Group I strains.

60

61 The difference in the genome composition of Group I and Group II strains results in different brewing properties.

62 Group I strains appears to be more cold-tolerant (*i.e.*, *S. eubayanus* trait) while Group II strains show a better

63 maltotriose consumption (*i.e.*, *S. cerevisiae* trait) (Brouwers *et al.* 2019). Group I include Saaz-type and Carlsberg

64 type strains from Czech Republic and Denmark breweries, respectively. Group II, also referred to Froberg-type,

65 includes strains brewed in Canada, the Netherlands (Heineken, Oranjeboom and other breweries), and Denmark (non-

66 Carlsberg breweries) (Dunn and Sherlock 2008). The *S. cerevisiae* parental strain of the Group I yeasts is related to

67 those used for Ale beer production in Europe, while the one of the Group II contains, in addition to the Ale-like gene

68 content, Stout-like sub-genome. *S. eubayanus* parental strain possesses similar fermentation capacities to Saaz group

69 at cold temperature (10°C), and are more resistant to cold temperatures compared to Froberg group and *S. cerevisiae*
70 Ale strains (Gibson *et al.* 2013). *S. pastorianus* has also the ability to ferment under stressful conditions such as
71 anaerobiosis, high hydrostatic pressure and high gravity sugar solutions, producing complex metabolites leading to
72 unique flavours and aromas (Monerawela and Bond 2017a). The parental chromosomes of *S. pastorianus* underwent
73 homeologous recombination, which resulted in unique hybrid chromosomes (Monerawela and Bond 2017b).
74 Therefore, multiple copies of *S. cerevisiae*-like, *S. eubayanus*-like and hybrid gene alleles coexist in the genome.
75 Consequently, the presence of multiple protein isoforms can lead to agonistic or antagonist competition for substrates.
76 Protein redundancy can also broaden the possibilities of complex composition, that can be either uni-specific (*S.*
77 *cerevisiae*-like or *S. eubayanus*-like) or potentially chimeric (Piatkowska *et al.* 2013). This mixture of proteomes
78 results in flexible biochemical activities leading to a unique cellular metabolism. The *S. cerevisiae*-like and *S.*
79 *eubayanus*-like alleles of three *S. pastorianus* Group I strains (CBS 1513, CBS 1503 and CBS 1538) and one Group
80 II strain (WS34/70) have been predicted using HybridMine (Timouma *et al.* 2020), an open-source predictive tool
81 our group developed (available on GitHub, <https://github.com/Sookie-S/HybridMine>).

82
83 Here, we focused on the Group I *S. pastorianus* CBS 1513, also known as *S. pastorianus carlsbergensis*, which is
84 the first established lager yeast strain (Hewitt *et al.*, 2014; Walther *et al.* 2014). This bottom fermenting strain has
85 been used in breweries for lager-style production since its isolation by Emil Chr. Hansen in 1883 (Hansen, 1883).
86 The brewing and distilling industries are constantly looking to improve their production strains for specific properties
87 such as temperature tolerance, maltose utilisation, and balanced flavour profiles (Gorter de Vries *et al.* 2020). In
88 *Saccharomyces*, a large number of studies presents temperature as a factor that influences several traits, including
89 fermentation, cell division or protein synthesis (Hartwell and McLaughlin 1968, Ciani *et al.* 2016). At low
90 temperature (<15°C), the beer fermentation process is slowed down, which causes a positive impact on volatile
91 flavour production and retention, and increases the consumption of the wort sugars (García-Ríos *et al.* 2019).
92 However, low temperatures can also be responsible for the termination of the fermentation process. Hybrids of cryo-
93 and thermo-tolerant strains like *S. pastorianus* have a favourable genetic background to ferment at different
94 temperatures. Historically, temperature treatments on *Saccharomyces* yeast had been used for industrial purposes,
95 with beer brewing being one of the main applications. In this study, we analysed the transcriptome data of *S.*
96 *pastorianus* CBS 1513 at different environmental conditions to determine the genes involved in temperature stress
97 that are media-independent. We observed the presence of a skew towards the expression of one or the other parental
98 sub-genome (*S. cerevisiae* or *S. eubayanus*) in the different conditions. Using the genomic features and expression
99 data, we predicted the proportion of protein complexes that are more likely to be chimeric, uni-specific, fully or
100 partially redundant in *S. pastorianus* CBS 1513. We identified that the majority of the protein complexes tend to be
101 either chimeric or uni-specific even if both parental alleles are present in the genome. This study highlights that
102 functional redundancy is efficiently exploited by *S. pastorianus* by using preferentially one allele over the other to
103 adapt its environment. As this strain is still evolving, most of the redundant unused alleles could be predicted to be
104 eventually lost. Our study is the first to provide insights into the genetics of *S. pastorianus* CBS 1513 at gene
105 expression level and a rationalisation for its unique industrial traits.

106

107 Results and Discussion

108

109 1. Temperature variations induce larger transcriptional changes than nutritional media.

110 To determine the genes affected by temperature stress in different culture media, the expression profiles of the *S.*
 111 *pastorianus* CBS 1513 (Group I strain) at 13°C, 22°C and 30°C in SD media, wort (maltose rich medium), SD media
 112 with 6% ethanol, and SD media without leucine were analysed. These media were chosen given their relevance to
 113 industrial conditions, as wort is used during the beer fermentation, alcohol is produced during this process, and the
 114 leucine amino acid metabolism is responsible for important flavour compound production, such as the isoamyl-
 115 acetate production (fruity flavour) (Stewart 2017). The cells were grown aerobically and the yeast growth on these
 116 media and their comparison is reported in Figure S1, Tables S1 and S2. The expression of 6 *S. cerevisiae*-like and 6
 117 *S. eubayanus*-like alleles was validated by RT-qPCR (Figure S2).

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

128 To assess whether the type of culture media had an impact on the expression network at different temperatures, we
 129 compared the absolute number of differentially expressed (DE) genes (absolute fold change > 2 and p-adjusted value
 130 <0.05) obtained in each temperature condition, for each media. The absolute number of DE genes up- and down-
 131 regulated at different temperatures varied significantly according to the culture media (Figure 2). As expected, at
 132 extreme temperature conditions (13°C vs 30°C), changes in the transcriptome are higher in each culture media
 133 compared to the other temperatures (13°C vs 22°C and 22°C vs 30°C), with the exception of wort where the total
 134 number of DE genes is similar for all temperatures (Figure 2). Within the SD media, there are less DE genes at 22°C
 135 vs 30°C compared to the other temperatures. Given that *S. pastorianus* CBS 1513 optimal growth temperature is
 136 around 25°C (Fischer *et al.* 2016), it is somehow expected that a shift between two temperatures close to its optimal
 137 would not induce a drastic difference in the gene expression compared to a shift involving colder temperatures (13°C).
 138 Surprisingly, in SD w/o leucine and SD + 6% ethanol, the absolute number of genes differentially expressed is
 139 gradually increasing when the shift goes towards the warmest temperature of 30°C. Furthermore, the changes in *S.*
 140 *pastorianus* CBS 1513 transcriptome appeared to be very low in SD media w/o leucine between 13°C and 22°C. It
 141 has been shown in a recent study in *Staphylococcus aureus* that amino acid uptake and release is correlated to
 142 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 cause 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.

2. Temperature-dependent media-independent genes are primarily located in the cell wall, plasma membrane and mitochondria.

Given that media has an impact on temperature acclimatisation, we isolated and analysed the genes that are temperature-dependent and media-independent. When comparing the expression network at 13°C vs 30°C, 94 temperature-dependent genes were common for all media (Figure 3, Panel A). 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 di- carboxylic acids metabolic and catabolic processes, glutamine metabolic process, oxidoreductase activity, vitamin binding, protein folding and co-enzyme binding (Figure 3, Panel B). 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 *Saccharomyces kudriavzevii* (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 vs 22°C (Figure S3, Panel A) and 22°C vs 30°C (Figure S3, Panel C), 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 oxido-reductions. Seven out of the 13 genes at 13°C vs 22°C are associated with mitochondria subcellular localisation (Figure S3, Panel B and D). 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 temperature-dependent, media-independent genes is presented in Table S3.

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 non-growth related, such as *CURI* (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. Vitamin biosynthesis, lipid/fatty acid processes and oxido-reduction reactions have also been described in *S. cerevisiae* by Paget and co-workers (2014) to be highly affected by the cold condition. Our transcriptome data in *S. pastorianus* supports this scenario.

177 In complete SD media, the extracellular leucine uptake is increased at cold temperature as *OPT1* (encoding for an
178 oligopeptide transporter 1 of tetra- and pentapeptides including leucine) is up-regulated. Strikingly, 35% of the 94
179 core temperature-dependent genes have a unique and opposite behaviour in SD w/o leucine compared to all the other
180 media (Figure 3, Panel C). Such result suggests an impact of leucine uptake on temperature acclimatisation in *S.*
181 *pastorianus*. Among these genes, *GUT2* is highly up-regulated at 30°C in all media (*i.e.* 11, 10 and 4 folds in SD,
182 SD + 6% Ethanol, and Wort, respectively) with the exception of SD media w/o leucine. In this medium, *GUT2* shows
183 an opposite trend as it is 2.6 times less expressed at 30°C. Such differential expression has been validated via RT
184 qPCR for both parental alleles of *S. pastorianus*, and also measured for the parental strains *S. cerevisiae* 96.2 and *S.*
185 *eubayanus* PYCC 6148 type strains (Figure 3, panel D). *GUT2* expression in medium w/o leucine seems to be hybrid
186 specific as *S. cerevisiae* and *S. eubayanus* parental strains show a different trend: in *S. cerevisiae* 96.2, *GUT2* is up-
187 regulated at 30°C in both media and the *S. eubayanus* PYCC 6148 is not differentially expressed in SD and down-
188 regulated in SD w/o leucine (Figure 3, panel D). Both leucine and *GUT2* are involved in fatty acids regulation, by
189 acting as precursor of branched fatty acids and phospholipids, respectively (Kerkhoven *et al.* 2017, Ferreira *et al.*
190 2018). Temperature impacts on the physical state of membranes, that needs to stay in a lamellar liquid crystalline
191 phase to function properly. As adaptation mechanism, the yeasts avoid the formation of lamellar gel phase caused by
192 cold temperature by changing the membrane lipids composition, which alters its fluidity (Gunde-Cimerman *et al.*
193 2014). Short chain lengths and/or unsaturated fatty acids and triacyl-glycerides abundance increase at cold
194 temperatures, while the ratio phosphatidylcholine/phosphatidylethanolamine and the phosphatidic acid content
195 decreases (Martin *et al.* 2007, Redón *et al.* 2011). Here, it is possible that the lack of leucine is already sufficient to
196 limit the amount of phosphatidic acid produced without the need of *GUT2* down-regulation at cold, which would be
197 shifting free glycerol towards glycerone phosphate. In addition, phosphatidylcholine fatty acid is known to be
198 essential for efficient functioning of the mitochondrial Gut2p in *Saccharomyces cerevisiae* (Rijken *et al.* 2007). The
199 absence of extracellular leucine could lead to a decreased production of phosphatidylcholine, which negatively
200 impacts on Gut2p efficiency that is compensated by an upregulation at the transcriptome level at 13°C in SD media
201 w/o leucine compared to 30°C.

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

211 **3. Distribution of expression profile in parental alleles shows that *S. eubayanus*-like alleles are primarily**
212 **involved in temperature response.**

213 We broke down the contribution of the specific parental alleles to the expression of the core temperature-dependent
214 DE genes. We first analysed the pool of the 94 temperature-dependent media-independent core genes of which 24
215 have maintained both parental copies in the genome.

216 Out of the 53 *S. eubayanus*-like alleles and 33 *S. cerevisiae*-like alleles, there are 40 and 22 alleles that have lost the
217 *S. cerevisiae* and *S. eubayanus* copy, respectively (Table 1). There were also 8 *S. pastorianus*-specific genes (Table
218 1). Only 3 genes had both alleles DE in the same direction. One DE orthologous pair (*GLC3*) that have both alleles
219 down-regulated at 13°C, encodes for a 1,4-alpha-glucan-branching enzyme a protein involved in the pathway
220 glycogen biosynthesis, previously described to have been affected by temperature in the rainbow trout, human and
221 mouse (Seibert 1985, Naperalsky *et al.* 2010, Hanya and Katz 2018). The two other pairs, *CBF2* and *RTP1*, that
222 encode the centromere DNA-binding part of the protein complex CBF3, and RNA polymerase II assembly factor,
223 respectively, have all their alleles strongly up-regulated at 13°C. These are genes involved in cell division and
224 transcription processes, so it is possible that their change in expression could be due to the different growth rate of
225 the yeast at 13°C and 30°C, rather than the temperature itself. In the core genes involving cold temperature (13°C),
226 there is ~37% more *S. eubayanus* alleles which are DE, while there is a similar amount of *S. eubayanus*-like and *S.*
227 *cerevisiae*-like alleles for higher temperatures (22°C vs 30°C) (Table 1). These results suggest that *S. eubayanus*
228 alleles are those who are responding to large temperature shifts and are primarily involved in transcriptional changes
229 at cold temperatures.

230 We expanded this analysis to all DE genes detected in our transcription study to see whether a parental sub-genome
231 is more likely to be differentially expressed under temperature stress. *S. pastorianus* CBS 1513 has evolved from a
232 precursor that appeared after a hybridisation event between a haploid *S. cerevisiae* and a diploid *S. eubayanus*. There
233 are 5228 and 3751 *S. eubayanus*-like and *S. cerevisiae*-like alleles detected in *S. pastorianus* CBS 1513 genome
234 (Timouma *et al.* 2020). Therefore, if there is no allele bias, out of the total of the DE genes we would expect to
235 identify approximately 58% *S. eubayanus*-like alleles and 42% *S. cerevisiae*-like alleles. We compared the expected
236 and observed number of *S. cerevisiae*-like and *S. eubayanus*-like alleles that show a difference in expression (absolute
237 FC > 1.5 and p-adjusted value <0.05) in all the temperature conditions for each growth media (Table 2). We observed
238 a statistical over-representation of the *S. eubayanus*-like alleles in all the temperature conditions, for all the media
239 except wort for the temperature shifts to 30°C (confidence interval of 95%). In the case of wort there is not a
240 statistically significant difference between the two type of alleles, but there is *ca.* 7 % more DE *S. cerevisiae* alleles
241 than expected (rather than less *S. eubayanus*). This suggest that more *S. cerevisiae* alleles undergo transcriptional
242 changes in wort to help sugar fermentation and to cope with stresses present in this complex rich media.

243 4. Analysis of the expression of ortholog parental alleles reveals that the majority of the functionally 244 redundant alleles are not concomitantly expressed by the cell.

245 *S. pastorianus* CBS 1513 strain is maintained in cold temperature for lager beer production, a selective pressure that
246 may impact on its evolution. Group I strains are indeed still evolving, as they show high genomic plasticity and an
247 instable chromosome copy number (Gorter de Vries *et al.* 2020). The higher the loss of *S. cerevisiae* sub-genome in

248 *S. pastorianus*, the lower is the amount of functionally redundant genes (Timouma *et al.* 2020). Here, we investigated
 249 how cell response to cold temperatures has shaped the functional redundancy found in *S. pastorianus* CBS 1513.
 250 When both *S. cerevisiae*-like and *S. eubayanus*-like alleles are DE, they can either be concordant (both up or down
 251 regulated), with or without an allele playing as a major player, or discordant (one allele up the other down regulated).
 252 Strikingly, in 81% of the cases, only one of the parental alleles is differentially expressed across all the conditions
 253 (Table 3). Out of the remaining 19% of the genes where both parental alleles are DE, 82% have a concordant DE
 254 expression (both alleles up or down regulated) across all the conditions, with approximately half of them showing a
 255 predominant major player. Only in 3% of the cases the orthologs alleles have a discordant expression (Table 3).

256 The fact that the hybrid can modulate the expression of both types of alleles confers an advantage over the parental
 257 strains to achieve the best fitness in a given environment. However, functional redundancy can be costly (and
 258 eventually evolutionary unstable) and most of the redundant unused alleles are expected to be silenced or lost as the
 259 hybrid is adapting to its new niche. Recent transcription studies on laboratory hybrids *S. cerevisiae* x *S. uvarum*
 260 revealed a clear pattern of concerted allelic transcription between the parental alleles (Paget *et al.* 2014). Moreover,
 261 a temperature biased gene retention was observed with major loss at 15°C of alleles deriving from the mesophilic
 262 parent (Smukowski *et al.* 2019).

263 5. Analysis of the expression of orthologous members of protein complexes shows a trend which favour 264 the formation of either exclusively uni-specific or chimeric complexes.

266 The different nature of protein assemblies can act as an evolutionary force in the hybrid organisms where orthologous
 267 members can bind together forming uni-specific or chimeric protein complexes. Considering the presence/absence
 268 of parental alleles in *S. pastorianus*, the protein complexes can be either exclusively uni-specific with subunits
 269 coming only from one parent (and when all the subunits from the other parent are lost); or exclusively chimeric, with
 270 a mixture of subunits from both parents; or partially or fully redundant when a series of protein complexes with
 271 different orthologous members can be established because both alleles are present for some or all subunits,
 272 respectively. Moreover, according to which alleles are expressed, different scenarios of protein assemblies can occur
 273 even when both parental alleles are presents in the genome, specifically in the partially or fully redundant cases
 274 (Figure 4, Panel A).

276 Given the complexity of the protein-protein network which increases exponentially with the number of subunits, here
 277 we restricted our study to homo- and hetero-dimers and trimers (Figure S4, and Table S4). We determined that
 278 over the 607 protein complexes originally identified in *S. cerevisiae* are conserved in *S. pastorianus* CBS 1513, with
 279 at least one allele identified for each subunit (Table S5). Among these 575 protein complexes, 8 are homodimers,
 280 210 heterodimers and 117 trimers (Figure S4). For these complexes, the majority of orthologous alleles are present
 281 and therefore partially or fully redundant protein complexes can potentially be established (Table S6 and Figure 4,
 282 panel B). Using the absolute expression, we analysed for each gene which parental allele is the major player in all
 283 the environmental conditions studied. An allele has been considered a major player if the expression was at least two

284 times higher than the homologous allele. Such assessment allowed us to recognize which protein assemblies are more
285 prevalent in *S. pastorianus* CBS 1513 when both parental alleles are present (*i.e.* partially or fully redundant cases).
286

287 Firstly, we analysed the composition of the protein complexes in our standard condition, SD medium at 22°C. Out
288 the 8 homodimers, 3 are exclusively uni-specific as only one type of parental alleles is present in the genome, and 5
289 are fully redundant. The absolute allele expression showed that 3 out of 5 preferentially express the same parental
290 allele, suggesting that uni-specific protein complexes are more prevalent in these cases (Table S6). The remaining 2
291 have alleles which are similarly expressed and can therefore form every possible combinations of the complex. In
292 the 210 heterodimers, based on the presence/absence of parental alleles at the genome level, only 9 and 18 complexes
293 can form exclusively chimeric and uni-specific assemblies, respectively, with the remaining being either fully (89)
294 or partially (94) redundant. The absolute expression data for the 89 fully redundant heterodimers suggest that about
295 53% form predominantly either uni-specific or chimeric complexes (*i.e.* 31 uni-specific complexes and 14 chimeric)
296 (Figure 4 Panel B; Table S6). More strikingly, the analysis of the absolute expression of the 94 partially redundant
297 heterodimers, reveal that 70% of the cases are likely to form exclusively chimeric (31) or uni-specific (35) (Figure 4
298 Panel B, Table S6). In the 117 trimers, based on the genetic, there are no possibility of exclusive chimeric complexes
299 and only 2 cases where the same parental alleles are uniquely retained for all the members of the complex (uni-
300 specific combination). In fact, in the trimers the majority protein complexes are either fully (38) or partially (77)
301 redundant. Similarly, to the heterodimers, the absolute expression data for the 38 fully redundant trimers suggest that
302 50% forms either exclusively uni-specific or chimeric complexes (*i.e.* 10 uni-specific complexes and 9 chimeric)
303 (Figure 4, Panel B; Table S6). Out of the 77 partially redundant cases, 58.5% are more likely to be chimeric (23) and
304 uni-specific (22), respectively (Figure 4 Panel B, Table S6). The same analysis was extended to the other
305 environmental conditions and showed a consistency in this trend (Figure S5).
306

307 Overall, these results show that the majority of the protein complexes established in the hybrid are more likely to be
308 either exclusively chimeric or uni-specific, and that the redundancy is discouraged, which is concordant to our
309 previous conclusions at genome scale on the preferential expression of one allele locus (Table 3). This scenario also
310 fits well with the gene balance hypothesis (GBH), which states that the stoichiometric imbalances can alter protein
311 complexes function, due to the mode of assembly and kinetics (Birchler and Veitia 2010). The GBH predicts that
312 pairs of chromosomes should be co-gained or co-lost to avoid generating imbalanced protein complexes that causes
313 reduced proliferation rates, often observed in aneuploid cells (Chen *et al.* 2019). This could explain our observation
314 that compared to the fully redundant cases, partially redundant cases are more likely to express only one parental
315 allele per subunit, to form uni-specific or chimeric complexes. The level of transcription of orthologous genes/alleles
316 (*cis*-regulation) and/or mutations in up-stream regulatory factors (*trans* effects) could be a way to buffer the
317 concentration of a given subunit (Comai 2005, Tirosh *et al.* 2009). Moreover, as hybridization events bring together
318 diverged genomes within a same nucleus, it has been hypothesized that in addition to the novel epistatic interactions
319 in the hybrid genome, regulatory interferences between the parental sub-genomes may occur, which could lead to a
320 genomic shock, that induce transcriptional changes (Hovhannisyan et al. 2020).
321

322 Additionally, for the heterodimers, we investigated how the protein assemblies may change according to the different
323 conditions. In the case of fully redundant heterodimers, there is ca 33% of the cases where the protein complex is
324 preferentially formed with the same parental proteins across all conditions tested, such as, for example, CPX-1277
325 (Ino2p-Ino4p transcription activation complex) composed by an *S. eubayanus*-like Ino2p and an *S. cerevisiae*-like
326 Ino4p (Figure 5 Panel A). In the case of the 94 partially redundant heterodimers, the expression data shows that ca.
327 50% of the complexes prefer the same composition across all conditions (Figure 5 Panel B). Thus, it is possible that
328 in these cases the other unused alleles may soon pseudogenise and eventually be lost from the genome. However,
329 there are cases where the nature of the protein complex is different according to the environment, such as CPX-1661
330 (Spt4p-Spt5p transcription elongation factor complex). In SD medium, this complex that is likely be uni-specific (*S.*
331 *eubayanus*-like) at 13°C and chimeric (with Spt4 *S. cerevisiae*-like and Spt5p *S. eubayanus*-like) at 30°C. This
332 suggest that some protein complexes may swap parental subunits to adapt to different environmental conditions. A
333 previous study on hybrids of *S. cerevisiae*/*S. mikatae* and *S. cerevisiae*/*S. uvarum* concluded that protein complexes
334 are able to spontaneously exchange orthologous subunits, and that the different types of assemblies have an impact
335 on the phenotype in specific environments (Piatkowska *et al.* 2014).

336 337 **Conclusions**

338
339 *S. pastorianus*, interspecies hybrid of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus*, is a bottom
340 fermenting yeast that is maintained in cold temperature for lager beer production, a selective pressure that may impact
341 on its evolution. The hybridization event developed unique genetic characteristics for *S. pastorianus*. Here, we
342 analysed transcriptome data from *S. pastorianus* grown at low, ideal, and high temperature and under different media
343 conditions to identify the response of temperature and media acclimatisation at gene expression level. We also
344 identify the protein complex composition and plasticity according to the presence/absence of the parental alleles and
345 the transcriptome profile at different environmental conditions. The primary analysis of the samples clustering
346 suggested that temperature has a higher impact on the transcriptome variability compared to the media. However, the
347 culture media appeared to have an impact on temperature acclimatisation as the absolute number of DE genes at
348 different temperatures varies according to the media. We identified core temperature-dependent media-independent
349 genes. They are mainly involved in protein folding, vitamin biosynthesis, lipid/fatty acid processes and oxido-
350 reduction reactions. We found that extracellular leucine uptake is increased at cold temperature in SD media.
351 Furthermore, the expression of a part of the temperature-dependent genes, including *GUT2*, is affected by the
352 presence/absence of extracellular leucine. We showed that there is an interplay between extracellular leucine uptake
353 and temperature acclimatisation. We also analysed the distribution of expression profiles in parental alleles at the
354 genome level. We strongly predict that overdominance at gene expression level of *S. eubayanus*-like alleles plays a
355 pivotal role for temperature acclimatisation. Expression of ortholog parental alleles reveals that the majority of the
356 functionally redundant alleles are not simultaneously expressed by the cell. We investigated the impact on protein
357 composition when at least one subunit has both parental alleles conserved in the genome. According to our expression
358 data, there is a trend for the formation of either exclusively uni-specific or chimeric complexes. Our results also

support the notion that the protein complexes may swap parental subunits to adapt to different environmental conditions.

Material and Methods

Genome sequence and annotation

S. 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). The Yeast Genome Annotation Pipeline (YGAP) has been used to predict the potential ORFs in its genome (Proux-Wéra *et al.* 2012). HybridMine tool (<https://github.com/Sookie-S/HybridMine>) has been used to identify the parental allele content in this strain (Timouma *et al.* 2020). Among the 9728 potential ORFs of *S. pastorianus* CBS 1513, HybridMine predicted 5228 *S. eubayanus*-like alleles and 3751 *S. cerevisiae*-like alleles.

Media and yeast culture

S. pastorianus CBS 1513 cells are incubated overnight in Yeast Peptone Dextrose (2% glucose) at 25°C with shaking at 200 rpm. The pre-cultured yeast cells were inoculated in fresh in maltose-rich media (wort), synthetic dextrose (SD) with all amino acids, SD without leucine, SD with 6% ethanol 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 600nm). Growth was measured every 5 minutes as previously described by Naseeb and Delneri 2012, and recorded by a BMG FLUOstar OPTIMA Microplate Reader (OD 595nm), 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 (OD₆₀₀ 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. Optimised qPCR reactions contained 6ng of cDNA, 3pmol 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 seconds at 95°C; 30 seconds at 55°C; and 30 seconds 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/>). 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), and gene expression were counted with the featureCounts tool (Liao *et al.* 2014). Differential gene expression analysis has 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 differentially expressed. 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 (API) 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. When a parental allele is at least two times more expressed than the other, it was considered as major player.

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Conflicts of Interest

The authors declare no conflict of interest.

429 **Figures legend**

430

431 **Figure 1:** Multi-dimensional scaling (MDS) plot of RNA-seq expression profiles of *S. pastorianus* CBS 1513, in
432 two dimensions. For visual aid, samples cultured at 13°C, 22°C and 30°C are coloured in red, green and blue,
433 respectively. Samples cultured in the different media are represented with circles, triangles, squares and crosses
434 shapes for SD + 6% ethanol, SD with all amino acids, SD w/o leucine and wort media, respectively.

435

436 **Figure 2:** Volcano plots and histograms representing the differential expression and absolute number of DE genes
437 of *S. pastorianus* CBS 1513 genes in SD, SD +6% ethanol, SD w/o leucine and wort media, in each temperature
438 shifts (13°C vs 22°C, 22°C vs 30°C and 13°C vs 30°C). Up- and down-regulated genes are represented in green and
439 red, respectively (absolute fold change > 2 and adjusted p-value < 0.05). Genes with significant, but low expression
440 change (absolute fold change < 2 and adjusted p-value < 0.05) are coloured in grey, and the ones not significant in
441 black (adjusted p-value > 0.05).

442 **Figure 3:** Differentially expressed genes temperature-dependent media-independent. **Panel A:** Venn diagram of the
443 genes differentially expressed between growth at 13°C and 30°C in standard medium (SD; green), standard media
444 without leucine (SD-Leu; blue), standard media with 6% ethanol (SD ethanol; yellow) and maltose rich medium
445 (Wort; pink). The genes present in the intersection of all media conditions are considered temperature-dependent
446 media-independent. **Panel B:** Histogram representing the significance (-log₁₀(p-value)) of the GO terms enriched of
447 the core DE genes at 13°C vs 30°C. Molecular function, cellular component and biological process are coloured in
448 red, yellow and blue, respectively. **Panel C:** Heatmap showing individual expression of the 94 temperature-
449 dependent media-independent core genes at 13°C vs 30°C in SD-Leu, SD, SD+ 6% ethanol and wort. **Panel D:** *GUT2*
450 differential expression at 13°C vs 30°C, for both parental alleles of *S. pastorianus* CBS 1513 (*S. cerevisiae*-like and
451 *S. eubayanus*-like alleles), and for the type strains *S. cerevisiae* 96.2 and *S. eubayanus* PYCC 6148, in SD (red) and
452 SD w/o leucine (blue) media. Error bars denote standard deviations and p-values are indicated as: * $p < 0.05$ ** $p <$
453 0.01 *** $p < 0.001$ **** $p < 0.0001$; ns = no significant change.

454 **Figure 4 Panel A:** Protein complexes assembly diagram according to the presence/absence of parental alleles in the
455 hybrid genome. Here, an example of heterodimer is illustrated. After hybridization event, genes A (grey) and B
456 (green) inherited from *S. cerevisiae* and A' (light grey) and B' (light green) from *S. eubayanus* are present in the
457 hybrid genome, which evolves through reduction of genetic content (gene loss). When 2 parental alleles are lost, the
458 protein complexes can be either chimeric or uni-specific. When one parental is lost, it creates partially redundant
459 configurations. When all alleles are conserved, it is a fully redundant case. Based on the alleles expression, chimeric
460 and uni-specific can only form chimeric (red arrow) and uni-specific (beige arrow) protein complexes, respectively.
461 Partially redundant cases can form chimeric, uni-specific or partially redundant protein complexes (light blue arrow).
462 Fully redundant cases can form all the configurations (dark blue arrow). **Panel B:** Histogram representing the total
463 number of chimeric, uni-specific, partially and fully redundant configurations in the 210 heterodimers and 117 trimers
464 based on the presence/absence of parental alleles. The partially and fully redundant cases can form different
465 assemblies based on the expression. An example in SD media at 22°C is illustrated.

466 **Figure 5:** Heatmap showing the assembly of heterodimers (chimeric, uni-specific, partially or fully redundant) based
 467 on the absolute expression at 13°C, 22°C and 30°C (prefix “13”, “22”, “30” used in the naming of the conditions,
 468 respectively) in SD, SD w/o leucine, SD+ 6% ethanol and wort media (suffix “aa”, “leu”, “eth” and “w” used in the
 469 naming of the conditions, respectively). Protein 1 and protein 2 constituting the heterodimer are annotated P1 and P2
 470 in the legend. *S. cerevisiae*-like and *S. eubayanus*-like alleles are abbreviated with “Sc” and “Se” in the legend. **Panel**
 471 **A:** Assemblies of the 89 fully redundant protein complexes. **Panel B:** Assemblies of the 94 partially redundant protein
 472 complexes.
 473

Tables

Table 1: Number of *S. eubayanus*-like, *S. cerevisiae*-like and *S. pastorianus*-specific differentially expressed genes detected in the core genes (temperature-dependent, media-independent) for each temperature condition.

Condition	<i>S. eubayanus</i> -like allele	<i>S. cerevisiae</i> -like allele	<i>S. pastorianus</i> - 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*-like and *S. eubayanus*-like alleles differentially expressed (absolute fold change > 1.5, p-adjusted value < 0.05) per condition.

Condition	Media	Total	<i>S. cerevisiae</i> -like alleles DE (out of the 3751 <i>S. cerevisiae</i> -like alleles present in the genome)	<i>S. eubayanus</i> -like alleles DE (out of the 5228 <i>S. eubayanus</i> -like alleles present in the genome)	P-value	Outcome
13°C vs 30°C	SD	3885	1537	2348	0.0052	<i>S. eubayanus</i> -like alleles over represented
	SD+6%eth	4090	1628	2462	0.0106	<i>S. eubayanus</i> -like alleles over represented
	SD-leu	1838	715	1123	0.0125	<i>S. eubayanus</i> -like alleles over represented
	Wort	2019	802	1217	0.0615	No significant difference
13°C vs 22°C	SD	3357	1320	2037	0.0039	<i>S. eubayanus</i> -like alleles over represented
	SD+6%eth	2260	886	1374	0.0132	<i>S. eubayanus</i> -like alleles over represented
	SD-leu	245	84	161	0.0175	<i>S. eubayanus</i> -like alleles over represented
	Wort	1852	703	1149	0.0009	<i>S. eubayanus</i> -like alleles over represented
22°C vs 30°C	SD	1193	464	729	0.0436	<i>S. eubayanus</i> -like alleles over represented
	SD+6%eth	3009	1189	1820	0.0119	<i>S. eubayanus</i> -like alleles over represented
	SD-leu	1483	564	919	0.0035	<i>S. eubayanus</i> -like alleles over represented
	Wort	2161	864	1297	0.0909	No significant difference

481 **Table 3:** Expression of the orthologous *S. cerevisiae*-like and *S. eubayanus*-like alleles in the genes differentially expressed for each condition. *S. cerevisiae*-like and
482 *S. eubayanus*-like alleles are annotated “S. cer” and “S. eub”, respectively. Up- and down-regulation, are represented by “+” and “-”, respectively. An allele not DE
483 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
484 compared to the other allele, it is represented with “++” or “--” for up-regulation and down-regulation, respectively. Discordant expression is annotated with (+/- or
485 -/+).

			DE expression of only one parental allele				DE expression for both parental alleles							
			Up-regulation of one allele		Down-regulation of one allele		Concordant DE expression Up regulation			Concordant DE expression Down regulation			Discordant DE expression	
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

486

487 **Figure 1**

490 **Figure 2**

491

492

Figure 3

502 SUPPLEMENTARY FIGURES

503 **Figure S1:** Growth curve of *S. pastorianus* CBS 1513 cultured in wort, SD + 6% ethanol, SD media w/o leucine and
504 SD media with all amino acids, at 13°C (Panel A), 22°C (Panel B) and 30°C (Panel C).

505 **Figure S2:** Validation of DE genes obtained during RNA-seq by RT-qPCRs. Relative mRNA of **Panel A:** *REB1*,
506 **Panel B:** *PRE7*, **Panel C:** *YBR241C*, **Panel D:** *TDA10*, **Panel E:** *POT1* and **Panel F:** *GUT2* at 13°C vs 30°C in SD
507 medium, for both *S. cerevisiae*-like and *S. eubayanus*-like alleles of *S. pastorianus* CBS 1513. Error bars denote
508 standard deviations and *p*-values are indicated as: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$; and ns = no significant
509 change upon *t*-test.

510 **Figure S3: Panel A:** Venn diagram of the genes differentially expressed between growth at 13°C and 22°C in
511 standard medium (SD; green), standard media without leucine (SD-Leu; blue), standard media with 6% ethanol (SD
512 ethanol; yellow) and maltose rich medium (Wort; pink). The genes present in the intersection of all media conditions
513 are considered temperature-dependent media-independent. **Panel B:** Histogram representing the significance ($-\log_{10}(p\text{-value})$) of the GO terms enriched of the core DE genes at 13°C vs 22°C. Molecular function, cellular
514 component and biological process are coloured in red, yellow and blue, respectively. **Panel C:** Venn diagram of the
515 genes differentially expressed between growth at 22°C and 30°C in standard medium (SD; green), standard media
516 without leucine (SD-Leu; blue), standard media with 6% ethanol (SD ethanol; yellow) and maltose rich medium
517 (Wort; pink). The genes present in the intersection of all media conditions are considered temperature-dependent
518 media-independent. **Panel D:** Histogram representing the significance ($-\log_{10}(p\text{-value})$) of the GO terms enriched of
519 the core DE genes at 22°C vs 30°C. Molecular function, cellular component and biological process are coloured in
520 red, yellow and blue, respectively.
521

522 **Figure S4:** Histogram representing the total number of protein complex (Y axis) that have X different subunits (X=2*
523 represents homodimers and X=2 heterodimers).

524 **Figure S5:** Histograms representing the potential assemblies of the partially and fully redundant heterodimers and
525 trimers, based on the expression data obtained at 13°C, 22°C and 30°C, in wort, SD, SD w/o leucine and SD+ 6%
526 ethanol media. Chimeric, uni-specific, partially and fully redundant cases are coloured in red, beige, light blue and
527 dark blue, respectively.

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