Effect of deletion and overexpression of tryptophan metabolism genes on growth and fermentation capacity at low temperature in wine yeast

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Abstract

Low-temperature fermentations produce wines with greater aromatic complexity, but the success of these fermentations greatly depends on the adaptation of yeast cells to cold. Tryptophan has been previously reported to be a limiting amino acid during *S. cerevisiae* growth at low temperature. The objective of this study was to determine the influence of the tryptophan metabolism on growth and fermentation performance during low-temperature wine fermentation. To this end, we constructed the deletion mutants of the *TRP1* and *TAT2* genes in a derivative haploid of a commercial wine strain, and the *TAT2* gene was overexpressed in the prototroph and auxotroph (*Δtrp1*) backgrounds. Then we characterized growth and fermentation activity during wine fermentation at low and optimum temperatures. Our results partially support the role of this amino acid in cold yeast growth. Although deletion of *TRP1* impaired amino acid uptake and the growth rate at low temperature in synthetic must, this growth impairment did not affect the fermentation rate. Deletion of *TAT2* endorsed this strain with the highest nitrogen consumption capacity and the greatest fermentation activity at low temperature. Our results also evidenced reduced ammonium consumption in all the strains at low temperature.

Keywords: wine, cold, yeast, nitrogen, mutant, overexpressing strains, *TAT2*

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Introduction

Many winemakers prefer low-temperature fermentation (10-15ºC) for white and "rosé" wine production because it improves the characteristics of taste and aroma. This improved aroma profile can be attributed not only to the prevention of volatilization of primary aromas, but also to the increased synthesis of secondary aromas. Thus the final wine product has greater terpenes retention, a reduction in higher alcohols, and an increased proportion of ethyl and acetate esters in the total volatile compounds. [1-3] Another positive aspect is that low temperatures diminish the growth of acetic and lactic bacteria, which makes alcoholic fermentation control easier. [4] However, low temperatures lower the growth rate and prolong the lag phase, which lead to longer fermentations and increase the risk of producing sluggish, stuck fermentations.[5] Therefore, the quality of wines produced at low temperature depends on the yeast's ability to adapt to cold.

The nitrogen fraction in grape must consists of organic (amino acids) and inorganic (ammonium) nitrogen. *Saccharomyces cerevisiae* is able to regulate nitrogen uptake by the so-called nitrogen catabolite repression (NCR) mechanism.[6] Recently, Crepin *et al.*[7] determined the consumption kinetics of the amino acids and ammonium present in a synthetic grape must. These authors found that the assimilation sequence of the nitrogen compounds during fermentation is largely determined by the kinetic characteristics and regulation of the transporters of amino acids and ammonium. Most late consumed amino acids (i.e., arginine and alanine) are transported by permeases under NCR, except valine, tryptophan and tyrosine, which are transported by SPS-regulated permeases.[7] Quiros *et al.*[8] mostly confirmed the order of consumption of the different nitrogen sources reported previously by Crepin *et al.*[7] These authors also observed that the consumption of amino acids is also affected by both sugar concentration and

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55 temperature. Beltran *et al*.[9] showed that cells growing at low temperature had a less
56 effective NCR, which resulted in a higher uptake of two of these late consumed amino
57 acids, arginine and tryptophan, and that the consumption of glutamine, one of the earlier
58 consumed and more preferred amino acids, was lower. These authors[9] also reported that
59 the consumption of ammonium in cells growing at low temperature significantly
60 diminished.

61 It has been reported that tryptophan uptake at low temperature is a rate-limiting step for
62 *S. cerevisiae* growth.[10] Broad-range permease (Gap1p), or specific amino acid
63 permeases Tat1p and Tat2p, are able to transport tryptophan into the cell. Tat2p
64 mediates the high-affinity uptake of tryptophan, and Tat1p mediates the low-affinity
65 uptake of this amino acid.[11] Low temperature increases the rigidity of the plasma
66 membrane[12], which results in the slower lateral diffusion of membrane proteins, less
67 active membrane-associated enzymes, and a major reduction in membrane transport.[13]
68 Membrane permeases are highly temperature-dependent because changes in temperature
69 can cause conformational changes in their structure[14]. Sensitivity of tryptophan uptake
70 at low temperature has been related to a dramatic conformational change in Tat2p. Abe
71 and Horikoshi[15] demonstrated that the overexpression of *TAT2* in a laboratory *S.*
72 *cerevisiae* strain confers yeast cells good capacity to grow at low temperature.
73 Moreover, enhancement of the gene expression for tryptophan biosynthesis or uptake
74 has also been related to ethanol stress tolerance.[16] Recently in a metabolic comparison
75 between a wine strain of *S. cerevisiae* and a cryotolerant strain of *S. bayanus,*[17] we
76 detected more active tryptophan biosynthesis in the cryotolerant strain via the shikimate
77 pathway, which partially explains its improved growth at low temperature.

78 The aim of this study was to analyze the importance of tryptophan biosynthesis and
79 uptake in a context which mimics wine fermentation conditions. First we deleted the

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80 *TRP1* gene, which is involved in tryptophan biosynthesis, and the *TAT2* gene, which is
81 related to tryptophan uptake, in the genetic background of a derivative haploid of the
82 commercial wine strain QA23. We also constructed strains which overexpressed the
83 *TAT2* gene in the background of the wine strain and in its auxotroph for tryptophan
84 (Δ*trp1*). Then we analyzed the effect of deletions and overexpression on growth and
85 fermentation activity during wine fermentations at low and optimum temperatures.
86

87 **Materials and Methods**

88 *Construction of the mutant and overexpressing strains*

89 The *TAT2* and *TRP1* genes were deleted on the derivative haploid of the commercial
90 wine strain QA23 (Lallemand S.A., Canada) *ho*QA23.[18] The primers used to construct
91 the mutant and overexpressing strains are listed in Table 1. The *TAT2* and *TRP1* genes
92 were deleted using the short flanking homology (SFH) method[19] based on the *KanMX4*
93 and the *NatMX4* deletion cassette, respectively, which provided geneticin (G418) and
94 nourseothricin resistance. Given the high GC content of the *NAT1* gene encoding
95 nourseothricin-resistance, the PCR reaction mixture contained 5% of DMSO, as
96 previously described.[20] The deletion cassettes were used to transform the haploid
97 *ho*QA23 strain by the lithium acetate procedure.[21] After selecting the transformants in
98 YPD with geneticin (G418) or noursethricin (clonNAT), the correct insertion of the
99 deletion cassettes was also verified by PCR using the primers upstream and downstream
100 of the deleted region, combined with the primers of the *KanMX4* or *NatMX4* genes
101 (Table 1).

102 The *TAT2* gene was overexpressed by cloning it into the centromeric plasmid
103 pGREG505 in strains *ho*QA23 and *ho*QA23 (Δ*trp1)*, as described in Jansen *et al.*[22]
104 *TAT2* was amplified from approximately 600 nucleotides upstream of the start codon to

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400 nucleotides downstream of the stop codon in order to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94ºC (2 min), followed by 30 cycles of 10 s at 94ºC, 30 s at 52ºC and 3 min at 72ºC. The last cycle was followed by a final extension step of 10 min at 72ºC. PCR fragments were generated with oligonucleotides containing short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp). The plasmid was linearized by *Sal*I digestion and digested with *Asc*I to avoid sticky ends and to make the recombination process easier.[22] Derivative wine yeasts *ho*QA23 and *ho*QA23 (Δ*trp1)* were co-transformed with the digested pGREG505 plasmid, together with the PCR-amplified target gene that was flanked by recombination sequences homologous to the plasmid ends. This co-transformation promotes an *in vivo* homologous recombination between both fragments. This recombination process also deleted the *GAL1* promoter of the plasmid (genes were cloned with their own promoters). Transformants were selected by geneticin resistance, which is encoded by the *KanMX4* gene in the plasmid. Correct integration of the gene into the vector was verified by plasmid DNA isolation using a modified version of the protocol described by Robzyk and Kassir[23], and further PCR amplification was performed with the primers specified for sequences rec5 and rec2 (Table 1).

Growth media

Strains were cultured in the synthetic grape must (SM) (pH 3.3) described by Riou *et al*.[24], but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors.[25] The following were utilized: organic acids, malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L; mineral salts KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$

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3 130 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$
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5 131 1 mg/L; vitamins myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid
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7 132 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and
8
9 133 biotin 0.003 mg/L. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L
10
11 134 as ammonium and 180 mg N/L in the amino acid form). Tryptophan represented
12
13 135 approximately 6.5% of the total assimilable organic nitrogen (12 mg N/L).
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15 136 The population inoculated in the synthetic grape must came from an overnight culture in
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17 137 YPD (1% yeast extract, 2% peptone, 2% glucose) at 30ºC. After counting
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19 138 microscopically, the appropriate dilution of the overnight culture was transferred to SM
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21 139 to achieve an initial cell concentration of 2 x 10^6 cells/mL.
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25 141 *Gene expression analysis by real-time quantitative PCR*
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27 142 The total RNA of 10^8 cell/mL was isolated from the different samples, as described by
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29 143 Sierkstra *et al.*[26], and was resuspended in 50 µL of DEPC-treated water. Total RNA
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31 144 suspensions were purified using the High Pure Isolation kit (Roche Applied Science,
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33 145 Germany), following the manufacturer's instructions. RNA concentrations were
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35 146 determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,
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37 147 USA), and RNA quality was verified electrophoretically in 0.8% agarose gel. Solutions
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39 148 and equipment were treated to leave them RNase-free, as outlined in Sambrook *et al.*[27]
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41 149 Total RNA was reverse-transcribed with Superscript™ II RNase H^- Reverse
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43 150 Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems,
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45 151 USA). The reaction contained 0.5 µg of Oligo $(dT)_{12\text{-}18}$ primer (Invitrogen, USA) and
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47 152 0.8 µg of total RNA as a template in a total reaction volume of 20 µL. Following the
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49 153 manufacturer's guidelines, cDNA was synthesized at 42ºC for 50 min after denaturation
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51 154 at 70ºC for 10 min. Then the reaction was inactivated at 70ºC for 15 min.
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155 The primers of the *TAT2* gene were designed according to the *Saccharomyces* Genome
156 Database. The primers of housekeeping gene *ACT1* have been previously described by
157 Beltran *et al.*[25] (Table 1). Amplicons were shorter than 100 bp, which ensured maximal
158 PCR efficiency and the most precise quantification. Real-time quantitative PCR was
159 performed using the LightCycler® 480 SYBR Green I Master (Roche, Germany). The
160 SYBR PCR reactions contained 2.5µM of each PCR primer, 5 µL of cDNA and 10 µL
161 of SYBR Green I Master (Roche, Germany) in a 20-µL reaction.
162 All the PCR reactions were mixed in a LightCycler® 480 Multiwell Plate 96 (Roche,
163 Germany) and cycled in a LightCycler® 480 Instrument II, 96-well thermal cycler
164 (Roche, Germany) under the following conditions: 95ºC for 5 min, and 45 cycles at
165 95ºC for 10 sec, at 60ºC for 10 sec and 72ºC 10 sec. Each sample had two controls that
166 were run in the same PCR: no amplification control (sample without reverse
167 transcriptase reaction) to avoid interference by contaminant genomic DNA, and no
168 template control (sample without RNA template) to avoid interference by primer-dimer
169 formation. All the samples were analyzed in triplicate with the LightCycler® 480
170 Software, version 1.5 (Roche, Germany), and the expression values were averaged. The
171 gene expression levels of *TAT2* in the overexpressing strains are shown as a relative
172 value in comparison to their control strains, *ho*QA23-pGREG and pGREG (Δ*trp1)*.
173 Housekeeping gene *ACT1* was used as an endogenous reference gene to normalize input
174 amounts. This endogenous *ACT1* gene showed excellent uniformity in the expression
175 levels of all the study samples.
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177 *Generation time (GT)*

178 Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG
179 Labtech, Germany) at 12ºC and 28ºC. Measurements were taken after pre-shaking the

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microplate for 20 sec every 30 min for 3 days. At 12ºC, the microplate had to be incubated outside the SPECTROstar spectrophotometer and was then moved inside to take measurements every 8 hours in the lag phase and every 3 hours in the exponential phase. Microplate wells were filled with 0.25 mL of SM medium to reach an initial OD of approximately 0.2 (inoculum level of 2 x 10^6 CFU/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and to consequently subtract, the noise signal. All the experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering *et al.*[28]:

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

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD, OD_t is the OD at time t, $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h).[29] Generation time (GT) was calculated using the $GT=\ln 2/\mu_{max}$ equation.

Fermentations

Fermentations were performed at 28ºC and 12ºC with continuous orbital shaking at 100 rpm, using 100-mL bottles filled with 60 mL of SM fitted with caps that enabled carbon dioxide to escape and samples to be removed. The population inoculated in each flask was 2 x 10^6 cells/mL from an overnight culture in YPD. Fermentation was monitored by measuring media specific density with a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Fermentation was considered complete when density was below 0.998. Yeast cell growth was also determined by absorbance at 600 nm and by plating samples

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at the end of fermentation on YPD medium in serial decimal dilutions, and they were incubated at 28ºC for 2 days.

Nitrogen content analysis

Samples were taken at 74 h and 240 h at 28ºC and 12ºC, respectively (around 1.020 of specific density), during fermentation**.** The individual amino acids present in the media were analyzed by OPA and FMOC derivatizations with the Agilent 1100 Series HPLC, as described in Beltran *et al*.[25] Each amino acid concentration was calculated using external and internal standards, and was expressed as mg N/L. The Agilent ChemSation Plus software was used (Agilent Technologies, Germany).

Statistical data processing

All the experiments were repeated at least 3 times and data were reported as the mean value ± SD. Significant differences among the control strain, the mutant and the overexpressing strains were determined by *t*-tests (the SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$.

A principal component analysis (PCA) was done using the *vegan* package (the *rda* function) of the R v.2.15 statistical software (R Development Core Team, 2010).[30]

Results

Gene expression analysis of overexpressing strains by real-time quantitative PCR

After constructing the overexpressing strains, we aimed to validate and quantify the gene expression in these strains. Samples were taken at time 0 h (before inoculation), 6 and 18 h after inoculation in the synthetic grape must (SM) at low temperature (12ºC).

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229 The relative expression values of *TAT2* are shown in Figure 1. The two overexpressing
230 strains showed a significant overexpression of *TAT2* at time 0 h, which was 4-fold
231 greater than the control for the pGREG *TAT2* strain and 27-fold greater than the control
232 for the pGREG *TAT2* (Δ*trp1)* strain. Moreover, the pGREG *TAT2* (Δ*trp1)* strain also
233 showed overexpression at 6 h and 18 h after inoculation (20- and 5.5-fold more than the
234 control, respectively).
235

236 *Phenotype effect of the mutant and overexpressing strains*

237 *Determination of generation time (GT)*

238 In order to determine the effect of the *TRP1* and *TAT2* deletions on growth, and the
239 overexpression of *TAT2,* in two different genetic backgrounds (tryptophan prototroph
240 and auxotroph), the GT of the mutant and overexpressing strains at 12ºC and 28ºC in
241 the SM were calculated (Table 2). No significant differences in the growth rate of the
242 *TAT2* mutant and the overexpressing strains were found, and only the Δ*trp1* strain
243 showed lower growth rate than its control strain, *ho*QA23, at both temperatures.

244 *Fermentation activity*

245 The fermentation activity of the mutant and overexpressing strains was estimated by
246 calculating the time required to ferment 5% (T5), 50% (T50) and 100% (T100) of the
247 sugars in the SM (Fig. 2). T5, T50 and T100 approximately matched the beginning (lag
248 phase), middle (end of exponential phase) and end of fermentation, respectively. At the
249 beginning of fermentation (T5), no differences were observed at low temperature. The
250 Δ*tat2* strain displayed greater fermentation activity at low temperature, and required a
251 considerably shorter time to consume 50% and 100% of the sugars (17 h and 60 h less,
252 respectively) if compared to the fermentation of the control *ho*QA23 strain at 12ºC.
253 Conversely this strain finished later than the control for the fermentation at 28ºC.

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254 pGREG *TAT2* also finished earlier at 12ºC if compared to its control (*ho*QA23-
255 pGREG), but took longer to finish the fermentation at 28ºC. pGREG *TAT2* (Δ*trp1)* was
256 able to finish fermentation at 28ºC before its control pGREG (Δ*trp1)*, but no differences
257 were found at 12ºC. These differences in fermentation activity cannot be ascribed to
258 biomass production because non significant differences were observed in the viable
259 population at the end of the fermentations among the different strains and temperatures,
260 except for the Δ*trp1* strain, which showed a larger population size at 28ºC (Table 3).
261

262 *Nitrogen consumption of mutant and overexpressing strains*

263 Different nitrogen requirements in yeast fermenting were observed at both temperatures.
264 At the same fermentation point (around 1.020 of specific density, which matches the
265 end of the exponential phase), nitrogen consumption by yeast was generally lower at
266 12ºC than at 28ºC (Table 4). These differences between both temperatures were greater
267 for ammonium consumption. Major differences in the amino acids present in the SM
268 between the temperatures were observed in the tryptophan auxotroph strains (Δ*trp1* and
269 pGREG (Δ*trp1*)). Conversely mutations and overexpressions did not significantly
270 modify the uptake of ammonium and amino acids at the same temperature. Only
271 pGREG *TAT2* (Δ*trp1)* at 28ºC consumed more amino acids and ammonium than its
272 control pGREG (Δ*trp1)*.

273 In order to explore the effect of deletion/overexpression and temperature fermentation
274 on ammonium and amino acid preferences, a PCA was carried out (Fig. 3). This PCA
275 was performed on the seven strains using the residual concentration (mg N/L) of 20
276 amino acids and ammonium measured in the SM at the analyzed fermentation time
277 point (1.020 of specific density) (Table S1). The first two components were retained and
278 explained 86.7% of total variance. The first component (PC1) explained 68.8% of

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3 279 variation, and was marked by a high positive component loading for histidine (+0.366)
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5 280 and high negative loadings for glutamine (-0.755) and ammonium (-0.509). The second
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7 281 component explained 17.9% of variation, and was marked by a high positive component
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9 282 loading for glutamine (+0.495) and a high negative loading for ammonium (-0.841).
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11 283 This result revealed the different nitrogen preferences at both temperatures. The most
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13 284 evident result was that control strain *ho*QA23 and its mutants consumed more histidine
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15 285 at the low temperature, and more glutamine and ammonium at the optimum
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17 286 temperature.
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23 288 **Discussion**
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25 289 Low temperature produces several effects on biochemical and physiological properties
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27 290 in yeast cells. Recently in their metabolomic study on wine fermentations at low
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29 291 temperature, López-Malo *et al.*[17] reported that the main differences in the metabolic
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31 292 profiling of the *S. cerevisiae* industrial strain QA23 growing at 12ºC and 28ºC were
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33 293 observed in the lipid and amino acid metabolisms. These differences in metabolites
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35 294 might be related to the previously reported different nitrogen consumption pattern at
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37 295 low temperature,[8,9] or with the greater demand of some specific amino acids at low
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39 296 temperature, such as tryptophan.[8,10] In this study, most strains also showed higher
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41 297 nitrogen consumption at the optimum temperature than at 12ºC. As already reported by
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43 298 Beltran *et al.*[9], the main differences in nitrogen use at both temperatures are accounted
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45 299 for by ammonium consumption. This result supports the idea that there is a problem
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47 300 with the uptake or metabolization of one of the main nitrogen sources at low
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49 301 temperature.[9] As previously reported[31], low temperature exerts strong nutritional stress,
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51 302 similarly to growth under nitrogen-limiting conditions. Regarding amino acid
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53 303 consumption, only the tryptophan auxotroph strains (Δ*trp1* and pGREG (Δ*trp1*))
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3 304 exhibited impaired amino acids uptake at low temperature, which was not affected at the
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5 305 optimum temperature. This lower amino acid consumption resulted in a growth defect
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7 306 in the Δ*trp1* strain, which showed a longer GT at 12ºC in comparison with the wild
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9 307 type, as previously reported.[32] Nevertheless, we also observed impaired growth at the
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11 308 optimal temperature. It is important to consider that our experimental approach differs
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13 309 substantially from those previously reported using laboratory strains and growth
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15 310 conditions in standard laboratory media. We used an industrial wine strain to mimic
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17 311 wine fermentation conditions. Similar non fitting results were also obtained from the
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19 312 fermentation activity of this Δ*trp1* strain. Some authors have revealed that the deletion
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21 313 mutants of tryptophan biosynthesis show ethanol-sensitive growth at the optimal
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23 314 temperature.[16] In comparison with the control strain however, no significant differences
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25 315 were found with the Δ*trp1* strain during the time required to complete fermentation.
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27 316 The cold-sensitive phenotype of the Δ*trp1* strain has been related with impaired
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29 317 tryptophan uptake. Abe and Horikoshi[15] postulated that the increased rigidity of the
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31 318 plasma membrane at low temperature induces conformational changes in permease
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33 319 Tat2p and impairs tryptophan transport. In the present study, no differences were found
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35 320 in the growth rate of Δ*tat2* at both temperatures if compared to *ho*QA23. Thus in this
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37 321 industrial strain, tryptophan uptake must be compensated by the action of other
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39 322 permeases, such as low-affinity tryptophan permease, Tat1p or the general amino acid
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41 323 permease Gap1p. Although it was not statistically significant, this strain consumed more
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43 324 amino acids and ammonium than its control strain *ho*QA23 at low temperature.
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45 325 However one unexpected result revealed that this strain also displayed better
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47 326 fermentation performance at low temperature. Although this direct correlation between
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49 327 *TAT2* deletion and fermentation improvement at low temperature should be confirmed
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51 328 with other industrial strains, our working hypothesis is that the greater nitrogen uptake
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329 of this strain produces a fermentation activity stimulus. Regulation of glycolytic
330 enzymes by ammonium and amino acids uptake has been widely reported.[33,34]
331 Abe and Horikoshi[15] demonstrated that the cells expressing Tat2p at high levels are
332 endowed with the ability to grow under low-temperature conditions in a tryptophan
333 auxotroph laboratory strain. However, the present study evidences no growth
334 improvement with the overexpression of *TAT2* in either the prototroph or the auxotroph
335 background, and this result highlights the importance of the genetic background. As
336 with the mutant strain, the overexpression of *TAT2* also significantly reduced
337 fermentation time.
338 Unexpectedly tryptophan uptake showed few differences between the different strains
339 and temperature. This amino acid was practically consumed completely under all the
340 fermentation conditions (Table S1). The PCA only revealed lower ammonium and
341 glutamine consumption, as previously reported,[9] and higher histidine consumption at
342 low temperature, which has not been reported to date.
343

344 Conclusions

345 One conclusion drawn from this study is the need to test the phenotypes observed in the
346 mutant laboratory strain in the genetic background of industrial strains under conditions
347 that mimic the industrial process. Our experience has revealed that many of these
348 phenotypes are not reproduced when transferred to more complex, robust and polyploid
349 industrial strains.[35,36] Tryptophan has been previously reported to be a limiting amino
350 acid during *S. cerevisiae* growth at low temperature, and the results of the present study
351 partially support this assessment. Deletion of *TRP1*, a key gene of tryptophan
352 biosynthesis, impaired the growth rate at low temperature in synthetic must. However, it
353 was also affected at 28ºC. Thus the cold sensitivity of this strain in this genetic

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background was not absolutely conclusive. Moreover this growth impairment did not affect the fermentation rate. A more evident phenotype in Δ*trp1* was the lowest amino acid consumption noted at low temperature, which might explain its lower growth rate. Strangely enough, the overexpression of *TAT2* in the Δ*trp1* strain did not reveal that nitrogen consumption recovered sufficiently, whereas deletion of *TAT2* (Δ*tat2*) endorsed this strain with the highest nitrogen consumption capacity at low temperature. This strain underwent significant reduction during the time required to complete fermentation at low temperature. Although this result was unexpected, it may prove promising to meet the objective of obtaining industrial strains with improved fermentation capacity at low temperature through the selection of proper strains or the genetic modification of current industrial yeast.

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3 471 **Figure legends**

472 **Figure 1.** Relative expression of *TAT2* during SM fermentation at 12ºC. Changes in
473 gene expression in the overexpressing strains are shown as compared to their controls,
474 *ho*QA23-pGREG and pGREG (Δ*trp1)*, at the same fermentation point (0 h, 6 h and 18
475 h), set as value 1. Values higher than 1 indicate a higher gene expression than the
476 control, whereas those lower than 1 denote a lower gene expression than the control.
477 *Statistically significant differences (P-value ≤ 0.05) versus their control strains.

478 **Figure 2.** Determination of the time required by the mutant and overexpressing strains
479 to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a
480 synthetic must at 12ºC (gray bars) and 28ºC (black bars). Positive and negative values
481 represent the increases and decreases in time (h) of the mutant and overexpressing
482 strains if compared to the control strains (normalized as value 0). The fermentation
483 times of the control strains were: ***ho*QA23** at 12ºC T5 = 62.75 h ± 2.29 h, T50 = 176.75
484 h ± 3.77 h T100 = 371.75 h ± 8.66 h; at 28ºC T5 = 17.94 h ± 2.26 h, T50 = 50.12 h ±
485 3.99 h, T100 = 133.84 h ± 8.66 h and ***ho*QA23-pGREG** at 12ºC T5 = 81.75 h ± 5.20 h,
486 T50 = 176.75 h ± 16.75 h, T100 = 361.75 h ± 1.73 h; at 28ºC T5 = 21.85 h ± 1.13 h,
487 T50 = 53.82 h ± 2.10 h, T100 = 136.34 h ± 13.56 h. **pGREG (Δ*trp1)*** at 12ºC T5 =
488 47.75 h ± 1.73 h, T50 = 151.75 h ± 7.40 h, T100 = 341.75 h ± 4.82 h; at 28ºC T5 =
489 20.12 h ± 1.36 h, T50 = 53.38 h ± 1.36 h, T100 = 148.41 h ± 1.85 h. *Statistically
490 significant differences (P-value ≤ 0.05) as compared with their control strains.

491 **Figure 3.** Biplot of the first two PCA components according to the residual amino acids
492 present in synthetic must at a specific density of 1.020 in the 28ºC and 12ºC
493 fermentations. Variables are represented in gray and samples are denoted in black.

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Table 1. List of the DNA sequences of the oligonucleotides used in this study.

Primer	SEQUENCE (5′→ 3′)	Use
TRP1-K1[a]	ATGTCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGCGTACGCTGCAGGTCGAC	Deletion *TRP1*
TRP1-K2[a]	ATTGTTTTATCGTTTAAAGCAGTTTTTACGATTCTTTATCATCGATGAATTCGAGCTCG	Deletion *TRP1*
cmTRP1F	AAAGACATGGAGGGCGTTAT	Verification deletion *TRP1*
cmTRP1R	GCACTCCTGATTCCGCTAATA	Verification deletion *TRP1*
TAT2- S1[b]	GCAAACAATTACCCGGAAATAGGGCTTAAATCAAGAATAAACCCCTAATTCGTACGCTGC	Deletion *TAT2*
TAT2-C2[b]	GAAGTCCTCATCCACGTATTCAAATGATAATTTTTCAAAATGTCAGGTTTACTAGTGGAT	Deletion *TAT2*
TAT2-ovF	CATGTCCGATAGCGGGAACTGA	Verification deletion *TAT2*
TAT2-ovR	ATTATCGCATGCAAACCAACTTGGCTACCG	Verification deletion *TAT2*
TAT2rec5F[c]	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCCATGTCCGATAGCGGGAACTGA	Overexpression *TAT2*
TAT2rec2R[c]	GCGTGACATAACTAATTACATGACTCGAGGTCGACATTATCGCATGCAAACCAACTTGGCTACCG	Overexpression *TAT2*
rec5F[c]	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCC	Verification overexpression *TAT2*
rec2R[c]	GCGTGACATAACTAATTACATGACTCGAGGTCGAC	Verification overexpression TAT2
TAT2F	CTGGCCACGTGCATTGTCT	Real-time quantitative PCR *TAT2*
TAT2R	GCCTTCATCGCCAGTCTAAATC	Real-time quantitative PCR *TAT2*
ACT1F	TGGATTCCGGTGATGGTGTT	Real-time quantitative PCR *ACT1*
ACT1R	CGGCCAAATCGATTCTCAA	Real-time quantitative PCR *ACT1*

[a] The sequence with homology to the plasmid pAG25 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.
[b] The sequence with homology to the plasmid pUG6 is underlined. The remainder of the primer sequences is homologous to the flanking region of the deleted ORF.
[c] The recombination sequences homologous to the plasmid pGREG 505 ends are underlined.

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Table 2. Generation time (GT) of the strains.

Strain	**12ºC**	**28ºC**
*ho*QA23	22.73 ± 0.48	4.82 ± 0.15
Δtat2	24.20 ± 1.35	4.76 ± 0.10
Δtrp1	25.56± 1.26 *	6.00 ± 0.12 *
*ho*QA23 pGREG	25.55 ± 1.16	4.78 ± 0.14
pGREG *TAT2*	24.47 ± 0.72	4.99 ± 0.11
pGREG (*Δtrp1)*	26.00 ± 2.62	4.71 ± 0.13
pGREG *TAT2* (*Δtrp1)*	24.11 ± 0.18	4.81 ± 0.09

* Statistically significant differences (P-value ≤ 0.05) as compared with their control strain at the same temperature.

Table 3. Viable cells (CFU/mL (10^7) at the end of fermentation at 12ºC and 28ºC.

	Viable cells (CFU/mL (10^7))	
Strain	12ºC	28ºC
*ho*QA23	4.40 ± 1.04	7.53 ± 1.72
Δtat2	5.20 ± 1.76	5.37 ± 0.42
Δtrp1	2.80 ± 0.14	13.83 ± 1.20*
*ho*QA23 pGREG	7.97 ± 2.76	5.20 ± 2.43
pGREG *TAT2*	5.70 ± 2.91	6.57 ± 2.37
pGREG (*Δtrp1)*	4.60 ± 2.34	4.43 ± 1.82
pGREG *TAT2* (*Δtrp1)*	4.37 ± 1.80	4.50 ± 0.44

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Table 4. Residual Yeast Assimilable Nitrogen (YAN) in the synthetic must (SM) at 1.020 density of fermentation at 28ºC and 12ºC, expressed as amino acid (YANaa) and ammonia (YAN NH_4) (mg N /L)

Strain	*ho*QA23	*Δtat2*	*Δtrp1*	*ho*QA23 pGREG	pGREG *TAT2*	pGREG (*Δtrp1)*	pGREG *TAT2* (*Δtrp1)*
28ºC							
YANaa	85.67 ± 11.34	90.12 ± 2.16	68.18 ± 9.67	102.32 ± 4.50	102.85 ± 13.20	87.57 ± 0.73	78.75 ± 3.90$_a$
YAN NH_4	2.01 ± 0.43	3.58 ± 2.72	1.56 ± 0.18	1.73 ± 0.07	10.03 ± 7.61	1.62 ± 0.01	1.52 ± 0.03$_a$
Total YAN	90.80 ± 16.77	93.71 ± 4.88	69.73 ± 9.80	104.11± 3.94	112.87 ± 19.43	89.20 ± 0.50	80.27 ± 3.93
12ºC							
YANaa	102.24 ± 7.92	76.27 ± 15.84	114.33± 8.19$_b$	109.64 ± 0.88	118.96 ± 6.90	120.47 ± 11.10$_b$	105.62 ± 18.93
YAN NH_4	38.24 ± 5.73$_b$	11.29 ± 9.83	20.62 ± 3.05$_b$	22.88 ± 2.98$_b$	17.72 ± 4.77	14.28 ± 10.19$_b$	14.41± 13.79
Total YAN	140.48 ± 13.65$_b$	87.55 ± 25.67	134.96 ± 11.25$_b$	132.52 ± 2.09$_b$	136.68 ± 5.33	134.75 ± 21.28	120.04 ± 32.00

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.

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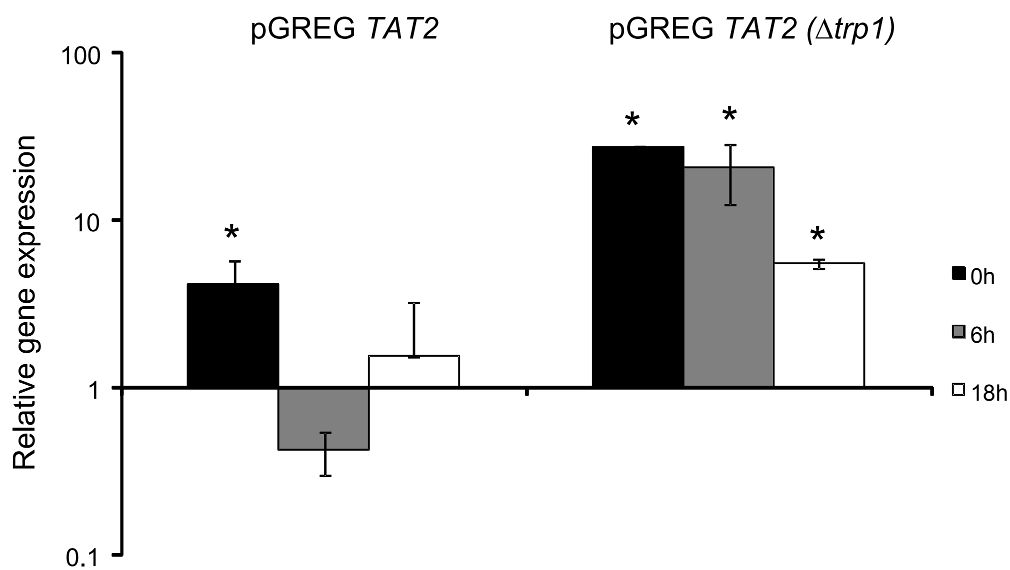
Figure 1



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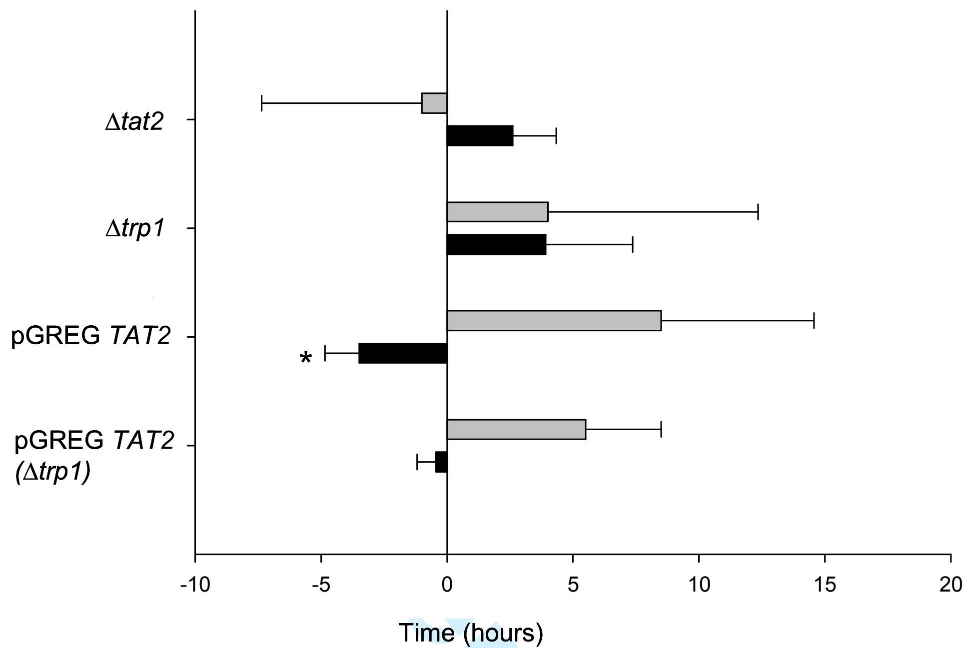

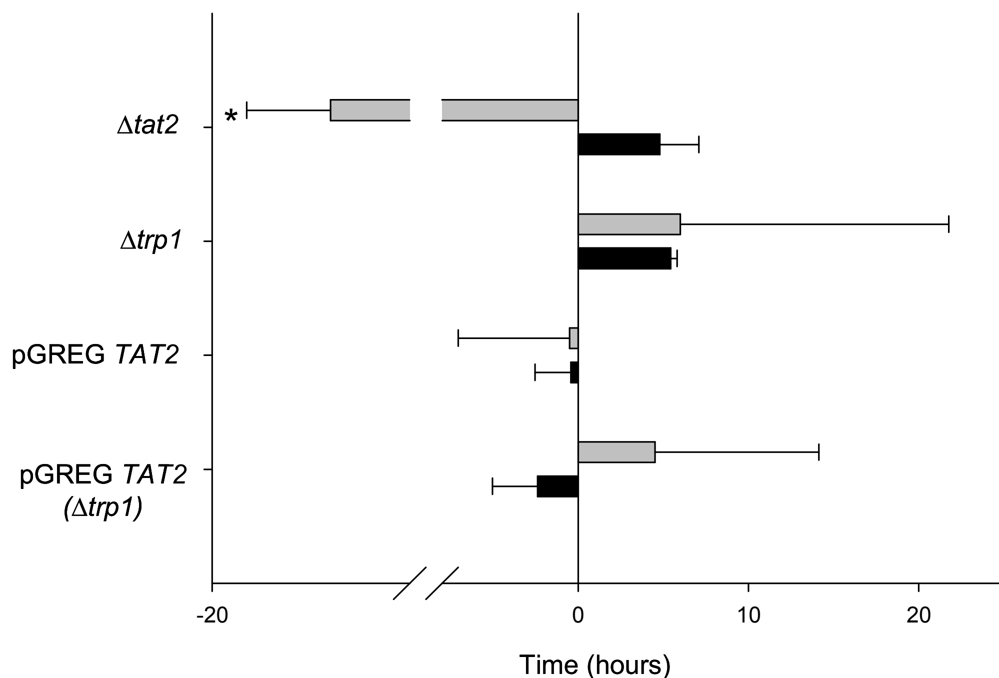

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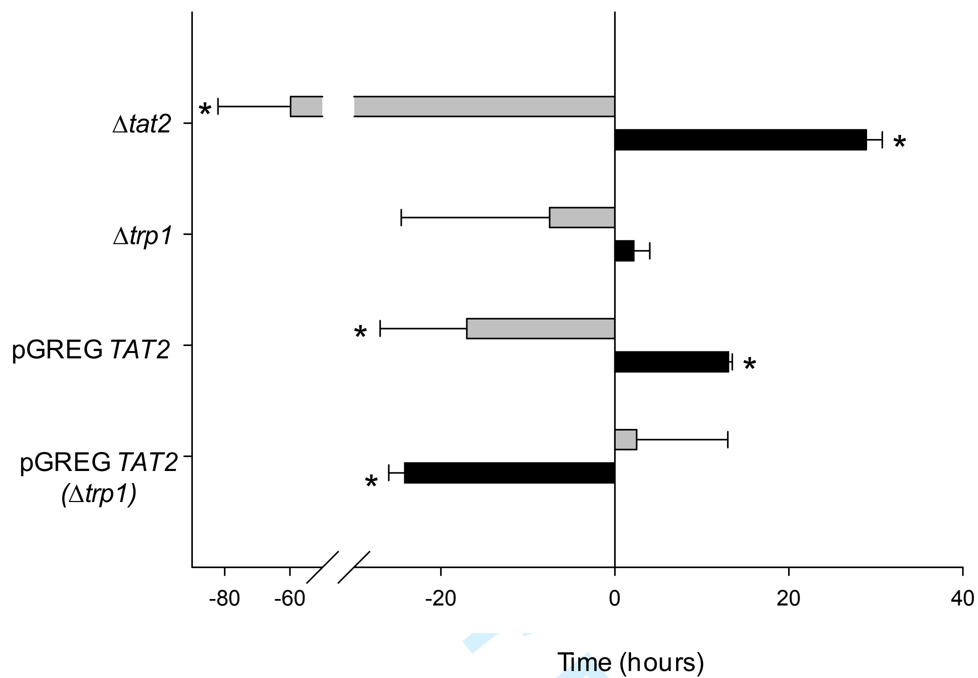

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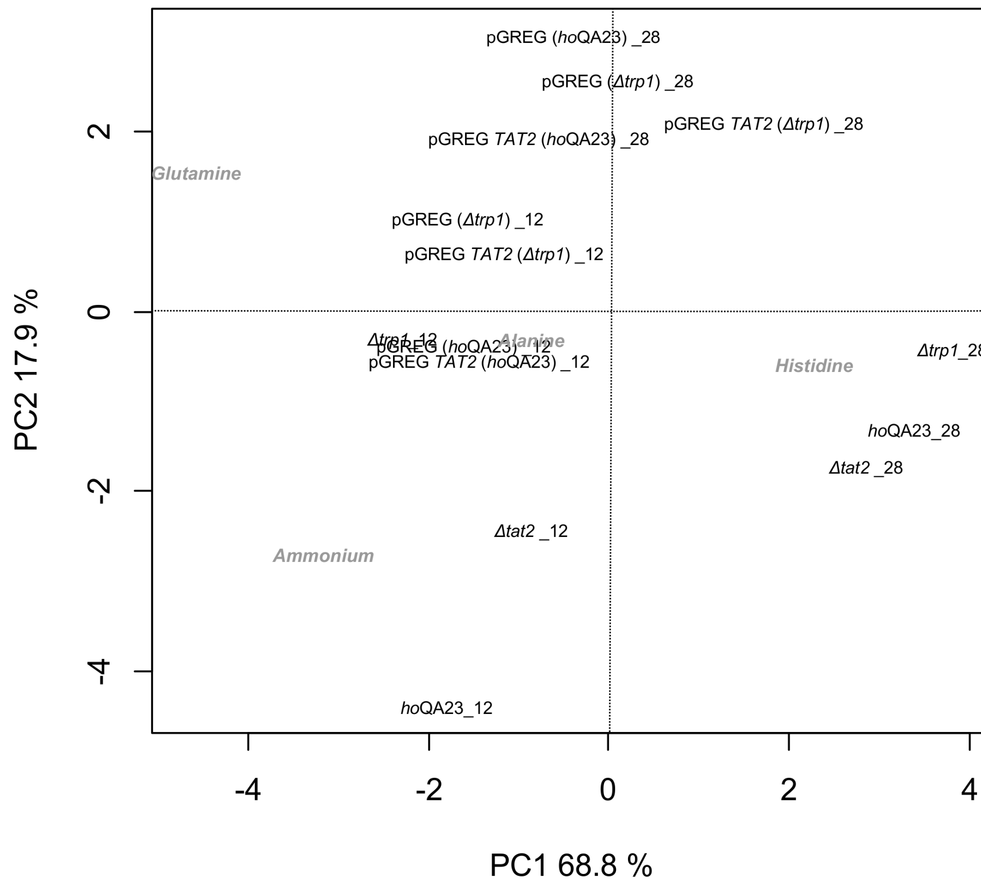

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Table S1: Residual amino acids present in synthetic must at a specific density of 1.020 in the fermentations at 28ºC and 12ºC, expressed as the mean ± SEM (standard error of the mean) of the amino acid concentration (mg N /L).

	Aspartic	Glutamic	Asparagine	Serine	Glutamine	Histidine	Glycine	Threonine	Arginine	Alanine
28 ºC										
*ho*QA23	0.57 ± 0.09	1.37 ± 0.48	0.1 ± 0.18	0.84 ± 1.00	4.27 ± 3.81	3 ± 0.51	3.53 ± 0.18	1.56 ± 0.44	24.92 ± 2.39	10.57 ± 2.04
Δ*tat2*	0.68 ± 0.07	1.82 ± 0.28	-	0.27 ± 0.01	6.11 ± 0.46	3 ± 0.15_b	3.29 ± 0.16	1.8 ± 0.11	25.43 ± 0.33	12.78 ± 0.67
Δ*trp1*	0.31 ± 0.15	0.95 ± 0.35	-	0.21 ± 0.04	1.94 ± 1.10	2.04 ± 0.50_b	2.8 ± 0.17_a	0.7 ± 1.32	23.28 ± 2.07	6.12 ± 2.00
*ho*QA23 pGREG	-	1.63 ± 0.11	-	2.7 ± 0.20	28.74 ± 3.26	-	3.33 ± 0.53	1.83 ± 0.17	24.21 ± 3.13	11.18 ± 0.75
pGREG *TAT2*	-	2.08 ± 0.33	-	3 ± 0.52	28.57 ± 3.51	-	3.59 ± 0.46	2.03 ± 0.38	26.13 ± 4.35	11.55 ± 1.46
pGREG (Δ*trp1*)	-	1.62 ± 0.06	-	2.23 ± 0.50	25.44 ± 0.53	-	3.85 ± 0.98	1.5 ± 0.31	21.98 ± 1.20	9.26 ± 1.19
pGREG *TAT2 (Δtrp1)*	-	1.07 ± 0.12_a	-	1.46 ± 0.27	22.51 ± 2.14	-	3.29 ± 0.07	1.04 ± 0.17	21.52 ± 0.38	7.11 ± 0.61
12 ºC										
*ho*QA23	1.31 ± 0.18_b	2.85 ± 0.24_b	0.87 ± 0.01_b	2.23 ± 0.48	27.31 ± 4.07_b	-	3.14 ± 0.01	1.54 ± 0.38	24.39 ± 0.89	14.43 ± 0.92
Δ*tat2*	0.56 ± 0.34	1.33 ± 0.78	0.77 ± 0.29	0.75 ± 0.63	15.79 ± 7.71	-	3.33 ± 0.01_a	0.53 ± 0.41	22.68 ± 0.43_b	8.68 ± 4.61
Δ*trp1*	1.49 ± 0.11_b	2.88 ± 0.10_b	1 ± 0.08_b	2.71 ± 0.40_b	32.98 ± 3.41_b	-	3.4 ± 0.18_b	1.77 ± 0.25_b	26.79 ± 1.47	15.37 ± 1.05_b
*ho*QA23 pGREG	1.28 ± 0.04_b	2.9 ± 0.02_b	0.82 ± 0.20_b	1.98 ± 0.05_b	31.5 ± 1.56	-	3.3 ± 0.26	1.49 ± 0.16	25.89 ± 1.62	15.15 ± 0.71_b
pGREG *TAT2*	1.06 ± 0.15_b	2.37 ± 0.20_a	0.83 ± 0.08	1.57 ± 0.31_b	27.6 ± 3.18	-	2.65 ± 1.17	1.22 ± 0.23_b	23.75 ± 2.15	14.26 ± 0.59_b
pGREG (Δ*trp1*)	1.3 ± 0.38_b	2.4 ± 0.58	0.63 ± 0.56	2.39 ± 0.96	30.6 ± 6.35	-	3.84 ± 0.30	1.58 ± 0.63	29 ± 1.73_b	14.93 ± 2.22_b
pGREG *TAT2 (Δtrp1)*	0.88 ± 0.78	2.25 ± 0.47_b	0.89 ± 0.13_b	1.76 ± 0.95	28.18 ± 6.04	-	3.97 ± 0.42	1.17 ± 0.55	26.90 ± 3.20	12.73 ± 3.25

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.

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	Tyrosine	Valine	Methionine	Cysteine	Isoleucine	Tryptophan	Leucine	Phenylalanine	Ornithine	Lysine
28ºC										
*ho*QA23	0.03 ± 0.01	-	0.67 ± 0.09	1.06 ± 0.1	1.19 ± 0.10$_b$	-	0.97 ± 0.15	-	0.18 ± 0.07	0.15 ± 0.02
Δ*tat2*	0.04 ± 0	-	0.75 ± 0.09	1.17 ± 0.02	-	0.25 ± 0.02$_a$	1.24 ± 0.11	-	0.23 ± 0.12	0.14 ± 0.01
Δ*trp1*	0.03 ± 0.1	-	0.68 ± 0.11	1.07 ± 0.05	-	0.29 ± 0.01$_a$	1.18 ± 0.13	-	0.26 ± 0.06	0.16 ± 0.05
*ho*QA23 pGREG	-	-	0.65 ± 0.04	0.86 ± 0.14$_b$	-	0.27 ± 0.05$_b$	1.27 ± 0.05$_b$	-	0.28 ± 0.05$_b$	-
pGREG *TAT2*	-	-	1.08 ± 0.55	1.14 ± 0.20$_b$	-	0.28 ± 0.02	1.46 ± 0.25	-	0.23 ± 0.02	-
pGREG (Δ*trp1*)	-	-	0.63 ± 0.04	0.81 ± 0.09$_b$	-	0.27 ± 0.05	1.14 ± 0.16$_b$	-	0.22 ± 0.01	-
pGREG *TAT2 (*Δ*trp1)*	-	-	0.58 ± 0.02	0.9 ± 0.04	-	0.32 ± 0	0.86 ± 0.04$_b$	-	0.19 ± 0.08	-
12ºC										
*ho*QA23	0.04 ± 0	-	0.66 ± 0.03	1.07 ± 0	-	0.1 ± 0$_b$	1.73 ± 0.06$_b$	-	0.17 ± 0.06	0.11 ± 0.01
Δ*tat2*	0.03 ± 0.01	-	0.52 ± 0.08	0.98 ± 0.16	-	0.15 ± 0$_{ab}$	1.14 ± 0.41	-	0.12 ± 0.01	0.05 ± 0.07
Δ*trp1*	0.05 ± 0	-	0.75 ± 0.08	1 ± 0.06	0.27 ± 0.38	0.49 ± 0.54	0.81 ± 1.14	-	0.2 ± 0.01	0.1 ± 0.11
*ho*QA23 pGREG	0.05 ± 0.01$_b$	0.83 ± 0.03$_b$	0.5 ± 0.02$_b$	-	-	-	-	0.14 ± 0.20	-	2.28 ± 0.19$_b$
pGREG *TAT2*	-	0.82 ± 0.19$_b$	0.71 ± 0.11	-	2.4 ± 1.29$_b$	0.19 ± 0.17	1.11 ± 1.93	11.17 ± 17.20	7.51 ± 11.36	-
pGREG (Δ*trp1*)	0.03 ± 0.03	0.79 ± 0.23$_b$	0.38 ± 0.33	-	3.36 ± 0.45$_b$	0.16 ± 0.27	-	1.08 ± 1.43	1.44 ± 0.03$_b$	2.46 ± 0.19$_b$
pGREG *TAT2 (*Δ*trp1)*	0.02 ± 0.02	0.31 ± 0.53	0.69 ± 0.13	0.61 ± 0.53	1.28 ± 1.28	0.16 ± 0.28	-	0.12 ± 0.21	0.12 ± 0.13$_a$	1.23 ± 1.00

a Significant differences (P-value ≤ 0.05) as compared to their control at the same temperature.
b Significant differences (P-value ≤ 0.05) due to temperature.
(-) not detected

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