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Thermo-adaptive evolution to generate improved *Saccharomyces cerevisiae* strains for cocoa pulp fermentations

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ABSTRACT

Cocoa pulp fermentation is a consequence of the succession of indigenous yeasts, lactic acid bacteria and acetic acid bacteria that not only produce a diversity of metabolites, but also cause the production of flavour precursors. However, as such spontaneous fermentations are less reproducible and contribute to produce variability, interest in a microbial starter culture is growing that could be used to inoculate cocoa pulp fermentations. This study aimed to generate robust *S. cerevisiae* strains by thermo-adaptive evolution that could be used in cocoa fermentation. We evolved a cocoa strain in a sugary defined medium at high temperature to improve both fermentation and growth capacity. Moreover, adaptive evolution at high temperature (40 °C) also enabled us to unveil the molecular basis underlying the improved phenotype by analysing the whole genome sequence of the evolved strain. Adaptation to high-temperature conditions occurred at different genomic levels, and promoted aneuploidies, segmental duplication, and SNVs in the evolved strain. The lipid profile analysis of the evolved strain also evidenced changes in the membrane composition that contribute to maintain an appropriate cell membrane state at high temperature. Our work demonstrates that experimental evolution is an effective approach to generate better-adapted yeast strains at high temperature for industrial processes.

1. Introduction

Thermotolerant microorganisms may be useful for industrial applications, such as a high-temperature growth yeast for bioethanol production (Mienda and Shamsir, 2013) or cocoa fermentation (Goddard, 2016). During these processes, cells have to face with high stress levels such as the temperature, which influences both growth and fermentation capacity (Morano et al., 2012). Industry spends a huge amount of energy cooling or heating fermentations to fine-tune temperature as closely as possible to the optimum growth temperature (Hamelinck et al., 2005; Stephen et al., 2012). In spite of this, this optimum temperature does not often very well match the final product's cost-effectiveness or quality. These problems can be avoided by providing better-adapted yeasts to ferment at non-optimal temperatures. However, we are far from either understanding the molecular and physiological mechanisms of adaptation at high temperatures or knowing what makes them thermotolerant.

Several genes have been related to thermotolerance in *S. cerevisiae*. Enzymes involved in membrane synthesis and composition have been linked to high thermotolerance, such as *ERG3* (Caspeta et al., 2014), a C-5 sterol desaturase; *ERG13* (Pinheiro et al., 2020), a protein involved in early ergosterol biosynthesis; chaperones like *HSP104* and *HSP12* (Sanchez et al., 1992); trehalose and glycogen genes *TPS1*, *TPS2*, *NTH1* (De Virgilio et al., 1994) and *GSY1* (Pinheiro et al., 2020); genes of RNA processing like *PRP42* and *SMD2* (Yang et al., 2013). Overexpression of *RSP5*, a ubiquitin ligase, also increases thermotolerance (Shahsavarani et al., 2012). Nevertheless, these genes have not yet been applied to genetically improve yeast strains for industrial processes. This could be because trade-offs occur with other properties that are important in industry, such as the fermentation, propagation, drying or storage of yeasts (Deparis et al., 2017; Matallana and Aranda, 2017; Walker et al., 2019).

Experimental evolution is an important tool for investigating adaptive shifts, clonal dynamics, competition and fitness, and the genetic

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underlying of complex traits. The culture of *S. cerevisiae* populations at long-term selective pressures results in different adaptive phenomena (Payen and Dunham, 2016). Such experiments also shed light on a bigger question about the molecular basis underlying the improved phenotype. Evolutionary engineering enables us to study adaptation by analysing changes in gene expression patterns, the genome structure and the whole genome sequence of the evolved strains (Payen and Dunham, 2016).

Cocoa beans (*Theobroma cacao*) are the main material for chocolate production and fermentation of beans is essential for developing chocolate flavour precursors (De Vuyst and Weckx, 2016; Ho et al., 2014). Cocoa pulp consists of 82–87% water, 10–15% sugar, 2–3% pentosans, 1–3% citric acid, and 1–1.5% pectin. Pulp fermentation is still an uncontrolled traditional process (de Melo Pereira et al., 2013) carried out by a mixture of indigenous yeasts, lactic acid bacteria and acetic acid bacteria (Lima et al., 2011; Schwan and Wheals, 2004). During cocoa bean fermentation, microorganisms grow in pulp and produce a diversity of metabolites like ethanol and organic acids, along with a rise in the fermenting mass' temperature, which can rise even further to 50 °C (Schwan and Wheals, 2004). The organic acids and heat diffuse into cocoa seeds, kill them and disrupt their cellular integrity (Voigt et al., 1994). The production of acids in pulp is important in cocoa fermentation because they diffuse into beans and subsequently induce important biochemical reactions that lead to well fermented cocoa beans. However, high levels of acid production in pulp is detrimental because it leads to excessive acid diffusion into beans that results in the production of acidic beans. In fact pH is expected to increase during fermentation, which is crucial for final bean quality (Afoakwa et al., 2013). Thus in cocoa fermentation, yeasts are subjected to different stress conditions, such as high temperature (~45–48 °C), elevated ethanol and low pH, which may impact the growth of strains and, subsequently, influence the production of pectinolytic enzymes, whose activity is needed for removing pulp form fresh cocoa beans (Lima et al., 2011).

This study had two main goals: to generate an improved thermotolerant strain that could be used in industrial cocoa fermentation processes; to provide a deeper understanding of the metabolic and genomic changes needed to occur in *S. cerevisiae* to become better-adapted at high temperature. To do so, we evolved a cocoa fermentation strain of *S. cerevisiae* in a defined medium at 40 °C. We selected three evolved clones after showing better fermentation performance than the original strain and selected the best one for genome sequencing. The genomic analysis of the evolved strain sheds new light on our understanding of what happens in *S. cerevisiae* during adaptation at high temperature. The lipid profile analysis of the evolved strain also evidenced changes in membrane composition to contribute to maintain the appropriate cell membrane state at high temperature. Finally, the evolved strain was tested during cocoa fermentation during a pilot-scale trial with extremely promising results.

2. Material and methods

2.1. Strains and culture conditions

The yeast strain used in this work was *S. cerevisiae* L5 (Strain collection of Lallemand Inc., France), an industrial strain isolated from cocoa fermentation. The strain that resulted after experimental laboratory evolution was named L5 EVO. All the lab experiments were performed on defined synthetic minimal medium (DSMM) containing 20 g L^{-1} glucose, 5 g L^{-1} $(NH_4)_2SO_4$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4.7H_2O$, 1 mL L^{-1} trace mineral solution and 1 mL L^{-1} vitamin solution, 1 mL L^{-1} of anaerobic factors (final concentration in the medium of 10 mg L^{-1} of ergosterol and 420 mg L^{-1} of Tween 80), as described by Verduyn et al. (1990). The pH of the medium was adjusted to 6.0. We modified the DSMM medium composition for the fermentation trials by increasing sugar content to 230 g L^{-1} (10 g L^{-1} glucose, +10 g L^{-1} fructose and 210 g L^{-1} of sucrose) to mimic an industrial medium with a higher sugar concentration.

The growth rate was monitored by determining optical density at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 4 days after 20 s of pre-shaking. Microplate wells were filled with the required volume of inoculum and 0.25 mL of medium to always ensure an initial OD of approximately 0.1 (inoculum level of about 10^6 cells mL^{-1}). Growth parameters were calculated from each treatment by directly fitting OD measurements *versus* time to the reparametrized Gompertz equation proposed by Zwietering et al. (1990):

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

where y = ln(ODt/OD0), OD0 is the initial OD and ODt is the OD at time t; D = ln(ODt/OD0) is the asymptotic maximum, μmax is the maximum specific growth rate (h^{-1}) and λ is the lag phase period (h) (Aguilera et al., 2007). The number of generations was calculated by this equation: n = (log Nt - log N_0)/log 2, where n is the number of generations, N_0 is the initial OD and Nt is the OD at time t. Hence the generation time (GT) was calculated by the equation GT = t/n.

2.2. Experimental laboratory evolution

Experimental evolution was based on batch serial dilutions. Batch cultures were prepared in 100 mL flasks filled with 60 mL of medium. The population inoculated in each flask had an OD of approximately 0.2. Batch selection was performed at 40 °C, with continuous orbital shaking at 100 rpm for 150 generations. Cultures were allowed to grow through a normal growth curve by weekly transferring a small volume (the volume required to inoculate at an OD of 0.2) of the expanded culture to 60 mL of fresh medium.

Batch cultures were plated on solid YPD at 0, 50, 100 and 150 generations, and 30 colonies of each sampling point were randomly selected and kept at −80 °C in 35% (v/v) glycerol for the genotyping and phenotyping analyses. Yeast typing was performed by delta element amplification from the genomic DNA (Legras and Karst, 2003) and mitochondrial DNA restriction (Querol et al., 1992) analyses. Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels (Fig. S1).

2.3. Genomic DNA extraction and whole genome sequencing

Genomic DNA was extracted from strains L5 and L5 EVO according to (Querol et al., 1992). Paired-end sequencing libraries of 150 bp with a mean insert size of 300 bp were prepared and run on an Illumina HiSeq2500 instrument. Raw reads were submitted to the NCBI Sequence Read Archive (SRA) as SRR10077336 and SRR10077335. Reads were trimmed with Sickle v1.2 (Joshi and Fass, 2011) with a minimum quality value per base of 28 at both ends and a minimum read length of 35 bp.

2.4. Genome mapping and variant calling

Sequencing reads were mapped against the reference *S. cerevisiae* S288C genome (version R64-2-1) using bowtie2 v2.3.0 (Langmead and Salzberg, 2012) with default parameters. The read depth (RD) or coverage "per base" was then obtained using bedtools v2.17.0 (Quinlan and Hall, 2010). The obtained coverage files were processed by a sliding windows strategy with a windows size of 1 kb moving by 1 kb. This permitted an average coverage value to be obtained every 1000 positions.

Single nucleotide variants (SNVs) and indels were called using Freebayes (v1.1.0–60- gc15b070) with the following options: -C (minimum count of observations supporting an alternate allele) 10, —F (minimum fraction of observation supporting an alternate allele) 0.25 and pool continuous for each one of the strains mapping files taking

S288c as a reference. Variants were then filtered to only retain those with a minimum base quality of 200 with VCFtools (v0.1.13) (Danecek et al., 2011). This minimum base quality is a phred-scaled quality score for the assertion made in calling a non-reference (alternative) position. A first approach was to create a new .vcf file, in which indels were eliminated, and the SNV frequency for each strain in relation to S288c was independently analysed. An SNV frequency representation per chromosome was obtained for each strain in comparison to S288c. In a second approach, the variant calling files, which contained all the SNV and indels for each strain, were used as input for VCFtools. The -gzdiff–diff-site option was used to compare changes between strains L5 EVO and L5. The obtained files were modified to keep only the variants present in the L5 EVO strain in relation to L5. The Ensembl Variant Effect Predictor was employed to characterise the functional effect of the mutations present in L5 EVO (McLaren et al., 2016). Missense variants in coding positions were retrieved and were manually confirmed by visual inspections in the Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

2.5. Determination of total fatty acids, squalene and sterols of yeast cells

Inocula were prepared by introducing three independent single colonies from each strain (L5 and L5 EVO) into 15 mL of DSMM medium. After overnight growth at 28 °C, the volume required to obtain a concentration of about 10^6 cells mL^{-1} was inoculated in 250 flasks with screw caps filled with 100 mL of fresh DSMM medium. Cultures were incubated at two temperatures (28 °C and 40 °C) with orbital shaking at 150 rpm. Culture growth was monitored by measuring absorbance at 600 nm. When cultures reached the stationary growth phase, sampling was done. Cultures corresponded to 30 h and 72 h for 28 °C and 40 °C, respectively. For lipid determination, the volume required for approximately 10^8 cells mL^{-1} was centrifuged at 4000 *g* min^{-1}. After centrifugation, the supernatant was discarded, and cell pellets were washed twice with precooled distilled water, frozen in liquid nitrogen and stored at −80 °C until further analyses.

The fatty acid methyl ester analysis was performed as reported by Borrull et al. (2015). Briefly from the yeast pellet (*ca* 10^8 cells), 1 mL of HCl 1.25 N in methanol and 10 μL of heptanoic acid (C7, 1 g L^{-1}) and heptadecanoic acid (C17, 4 g L-1) were added to the glass tubes containing cells. Samples were heated to 90 °C for 60 min before being cooled to room temperature. After cooling, 1 mL of NaCl 0.9% (w/v) in water and 300 μL of hexane were added. The extraction was repeated twice. Between each extraction phase, tubes were centrifuged at 3000 ×*g* for 5 min to allow the best phase separation. Analytical GC was carried out in an Agilent 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies). The extract (2 μL) was injected (splitless, 0.75 min) into an FFAP-HP column of 30 m × 250 μm × 0.25 μm phase thickness (Agilent Technologies) with an automatic injector (Agilent). Relative amounts of the given fatty acids were calculated from their respective chromatographic peak areas after normalisation with internal standards (C17).

Sterols and squalene were determined by the method reported by and squalene were determined using the method reported by Quail and Kelly (1996). Briefly, around 10^8 cells were re-suspended with 1.5 mL of methanol (MeOH), 1 mL of pyrogallol (0.5% (w/v) in MeOH), 1 mL of KOH solution (60% (w/v) KOH in distilled water) and 10 μL of α-cholestane (internal standard (IS), 1 mg mL^{-1} in hexane) in a glass tube and saponified at 90 °C for 2 h. Finally, sterols and squalene were extracted twice with 500 μL of hexane and the extract was dried in a speed vacuum system SC110 (Savant Instruments, USA). The dried residue was dissolved in 100 μL of hexane. From the collected organic phase, 2 μL were injected in the pulsed splitless mode (70 psi, 0.10 min) into a DB-5HT column (30 m × 0.25 mm × 0.1 μm, Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). Each compound was identified by comparing the mass fragmentation pattern of the peak with those of the injection of the available standard or that described by Quail and Kelly (1996). The relative abundance of each identified compound was calculated according to the respective chromatographic peak areas corrected in relation to the IS peak area. The results were expressed as individual percentage of the total sum of the identified sterols and squalene.

2.6. Lab-scale fermentations

Fermentations were performed at 40 °C with continuous orbital shaking at 100 rpm using 100 mL bottles filled with 80 mL of DSMM. Fermentations were monitored by the density of media (g L^{-1}) using a densitometer (Densito 30PX, Mettler Toledo, Switzerland) and were considered complete when density reached 995 g L^{-1}. Yeast cell growth was determined by absorbance at 600 nm and by plating on YPD.

2.7. Pilot-scale fermentations

The fresh cocoa beans used for this experiment came from French Guyana and were provided by CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement). Pods were frozen right after pod opening and the beans were transported on ice within 3 days after harvest. To perform cocoa bean fermentation, a specific bioreactor was designed to reproduce the best possible field conditions. The L5 strain was propagated in YPD medium (10 g L^{-1} Yeast Extract, 20 g L^{-1} Peptone and 20 g L^{-1} Glucose) for 24 h at 30 °C and 130 rpm. The pre-culture was first washed with PBS and enumerated by a Thoma chamber. A volume corresponding to 10^7 cells g^{-1} of beans was inoculated on fresh cocoa beans. pH was recorded by a specific probe (Polilyte Plus H ARC 120, Hamilton) that was calibrated beforehand. Bioreactors were filled with 1.3 kg of fresh cocoa beans. Temperature was controlled by a double envelope, and making 3 °C increments every 12 h. Cocoa beans were mixed daily and the end of fermentation was determined by assessing the cut test (Kadow et al., 2015).

Yeast viability was estimated by following the method described by Camu et al. (2007). Therefore, 100 mL of sterile peptone water was added to 10 g of beans in a sterile stomacher bag that was vigorously shaken for 10 min in a lab Stomacher® to obtain a uniform homogenate. The homogenate was coarsely filtered in a stomacher filter bag and the filtrate was used to prepare 10-fold dilutions in sterile peptone water, from which aliquots (0.1 mL) were plated on the selective yeast medium Sabouraud Chloramphenicol Agar (Biomérieux, France) and incubated for 48 h at 30 °C. Enumeration was done by recording the number of colony-forming units per gram of the whole weight of the beans (CFU g^{-1}). To assess the implantation percentage of the inoculated strain, 10 colonies were randomly selected and genotyped by Delta-PCR with the primers targeting inter-delta sequences (Legras and Karst, 2003). The DNA extraction of the selected colonies was performed by the KingFisher Flex System (Thermo Fisher Scientific).

2.8. Ploidy estimations by flow cytometry

The DNA content of both the parental (L5) and evolved (L5 EVO) strains was assessed by flow cytometry in a Beckman Coulter FC 500 (Beckman Coulter Inc., Brea, CA, USA) by the SYTOX Green dye method described in Haase and Reed (2002). The DNA content values were scored based on the fluorescence intensity compared to the haploid (S288c) and diploid (FY1679) *S. cerevisiae* references strains. The DNA content value reported for each strain was the result of two independent measures (Table 1).

2.9. Statistical analysis

All the lab experiments were carried out at least in triplicate. The physiological data were analysed by the Sigma Plot 12.5 software, and the results were expressed as mean and standard deviation. To evaluate

Table 1
DNA content of the parental and evolved strains.

Strains	DNA content
S288c	1 ± 0.05
FY1679	2.14 ± 0.13
L5	2.12 ± 0.01
Evolved L5	2.57 ± 0.03[a]

Values expressed as mean ± standard deviation.
[a] Indicates significantly different values (ANOVA and Tukey HSD test, α = 0.05, *n* = 2) compared to the parental strain.

statistical significance, two-tailed t-student tests were applied with a *p*-value of 0.05. *P*-values were corrected for multiple testing by the Bonferroni test. The phenotypical data were fitted to the reparametrized Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in v.3.6 of the R statistical software. The cytometry results were tested by a one-way ANOVA and a Tukey HSD test (α = 0.05, *n* = 2). The FAME and sterol data were submitted to a one-way ANOVA with a subsequent analysis using the Tukey HSD test (p-value ≤ 0.05) with XLSTAT, version 2019.1.2. (Addinsoft, Paris, France).

3. Results

3.1. Evolution experiment of an industrial strain in its growth capacity at 40 °C

The aim of this work was to improve the high-temperature growth capacity of an industrial *S. cerevisiae* strain and elucidate the adaptive mechanisms underlying this thermotolerance. Industrial strain L5 was grown in serial batch cultures at 40 °C during 150 generations. The growth improvement of cultures was evidenced every 50 generations by comparing the maximum specific growth rate at each time point. Fig. 1 shows the μ_{max} distribution of the evolved populations at three different time points, 50, 100 and 150 generations (μ_{max} values were obtained from the individual growth of 30 individual clones per population), compared to the parental strain. No significant differences were observed in the average μ_{max} value after 50 generations of evolution at high temperature. However, as the evolution process progressed, μ_{max} significantly increased and its initial value had doubled at 150 generations.

The three better-adapted clones in growth terms (μ_{max} and maximum OD) of the 30 assayed (C24, C27 and C29) were selected to perform further analyses. The growth values of the selected strains significant rose, mainly when the maximum population was reached (more than 2-fold higher; Fig. 2A). After taking these results into account, the fermentation capacity of the three evolved clones was analysed compared to the parental strain at 40 °C in a more demanding medium (230 g L^{-1} of sugars). Fig. 2B represents the fermentation activity of the three strains as the relative time to consume 5% and 50% of sugars (T5 and T50) *versus* parental strain L5 (value 1). In all cases, the evolved strains presented impaired fermentation performance at the start of the fermentation process (higher T5), which represented a longer lag phase in the evolved strains. Nevertheless, as fermentation progressed, the time needed by the evolved strains to consume 50% of sugars was cut by half compared to the parental strain. Both the evolved and parental strains were unable to consume the total amount of sugars present in

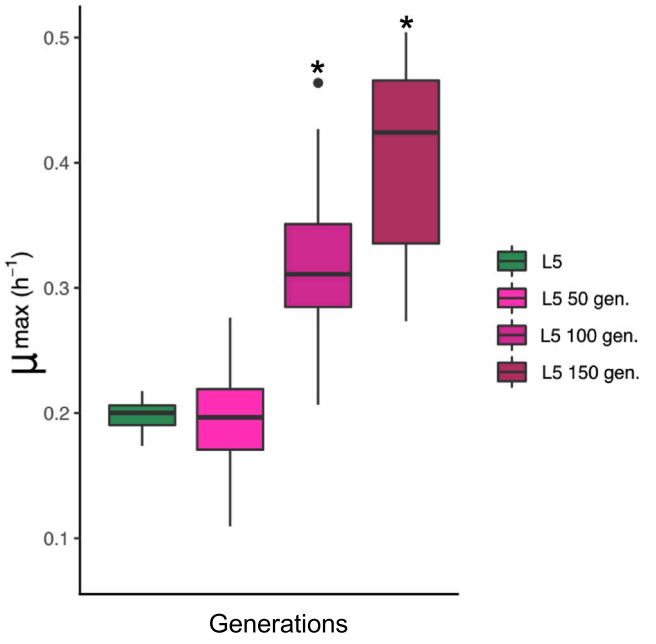

Fig. 1. Box plot representation of the μ_{max} distribution in the different evolved populations at 40 °C. The μ_{max} values were obtained from the growth curves of 30 individual clones per evolved population. *Significant differences ($p \leq 0.05$) compared to parental strain L5.

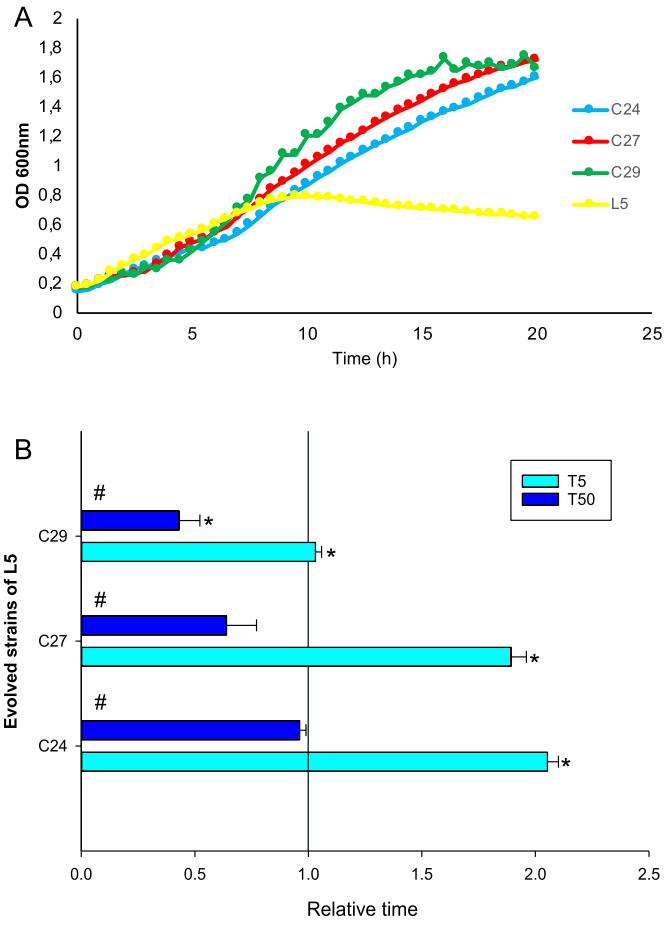

Fig. 2. (A) Growth kinetics in DSMM medium at 40 °C of the original industrial strain L5 (yellow) and the evolved strains: C24 (blue), C27 (red), C29 (green). (B) Fermentation kinetics of the evolved strains compared to parental strain L5. T50 and T5 were the time needed to consume 50% and 5%, respectively, of the sugars present in must at 40 °C. Both T50 and T5 were compared to the T values of parental strain L5 normalised as value 1. Values are expressed as mean ± standard deviation. *Significant differences (p ≤ 0.05) compared to parental strain L5. # Indicates stuck fermentation before the total sugar consumption (T100). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

must, which was likely due to the large amount of ethanol and/or high osmotic stress present in the medium. However, of the evolved clones, strain C29 left much less residual sugars than the parental strain and was selected for further experiments (L5 EVO).

3.2. Changes in the lipid composition of membranes

It is well-known that the plasma membrane is the primary target of non-optimal growth temperatures and its composition plays a paramount role in high-temperature yeast adaptation. Fatty acids and sterols, the main basic components of the plasma membrane, were analysed in both L5 and L5 EVO at 28 °C and 40 °C (Table S1). As expected, fatty acids and sterols were profoundly modified at high temperature regardless of the strain.

The most significant changes noted for the fatty acids at high temperature were an increase in palmitic acid (C16) and oleic acid (C18:1), and a marked reduction in palmitoleic acid (C16:1) (Table S1). These changes in fatty acid composition at 40 °C led the UFA/SFA and C16:1/C16 ratios to significantly lower (Fig. 3A). However, no significant differences were found in any of the analysed fatty acids between the parental and L5 EVO strains at the same temperature.

The most remarkable changes at high temperature for sterol composition were a drop in ergosterol content and an increase in many precursors, such as lanosterol, zymosterol and episterol at 40 °C (Fig. 3B). However for L5 EVO, ergosterol content was significantly higher and zymosterol content was lower than for the parental L5 at 40 °C. This higher ergosterol/zymosterol ratio could be crucial for better adaptation at high temperature in L5 EVO.

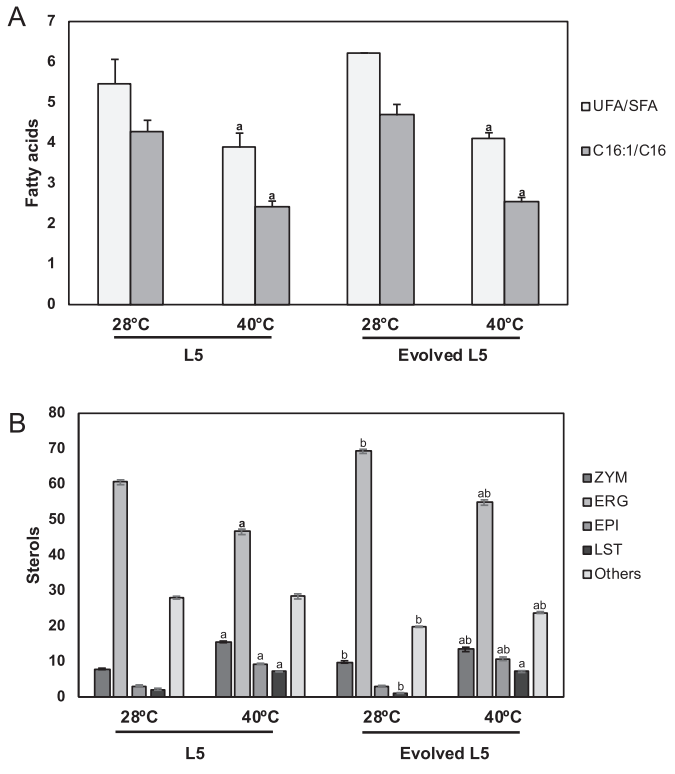

Fig. 3. Percentages of fatty acids (A) and sterols (B) at 28 and 40 °C in both strains. (A) Ratios of unsaturated fatty acids (C14:1, C16:1, C18:1)/Saturated fatty acids (C14, C16, C18), (UFA/SFA) and Palmitoleic acid (C16:1)/ Palmitic acid (C16) in strains L5 and L5-EVO at both 28 °C and 40 °C. (B) Percentages of Zymosterol (ZYM), Ergosterol (ERG), Episterol (EPI), Lanosterol (LST) and Others (Neoergosterol + Ignosterol + Ergosta-8-enol + Ergosta-7-enol + 4,4-dimethyl-8,24-cholestadienol + Ergosta-4,6,8(14),22-tetraen-3-one) in strains L5 and L5 EVO at both 28 °C and 40 °C. [(a)] Indicates significant differences in each strain when comparing 28 °C to 40 °C. [(b)] denotes significant differences at the same temperature when comparing L5 to strains L5 EVO. ($p < 0.05$) according to a Tukey *post hoc* comparison test.

3.3. Genomic differences analysis: aneuploidies, segmental duplication, and SNVs

Both L5 and L5 EVO were whole-genome sequenced to detect changes in the genome of L5 EVO to explain the better adaptation noted at 40 °C. The strategy used to study both genomes was based on sequenced reads mapping against S288c reference genome. This approximation is useful for SNPs, duplications and deletions detection, but assumes that the genomes of the analysed strains are collinear with S288c reference, thus the possible variations in genome structure (translocations, inversions) would not be detected. After sequencing strains L5 and L5-EVO, their estimated coverages values (against S288c reference) were 40.6× and 125.1×, respectively.

SNVs frequency analysis from the genome sequencing results by comparing to the S288c reference strain indicated that the L5 parental strain was a diploid strain with high heterozygosity level along the genome. However, a large region on the right arm of chromosomes VII, VIII and XII, and on the left arm of chromosomes X, XV and XVI, had undergone loss of heterozygosity (LOH) (Fig. 4A). LOH regions in L5 parental strain are supported by the coverage values files obtained after aligning the sequenced reads of L5 with S288c, showing the same coverage in the LOH regions that in the rest of the chromosome, while their SNV frequency was 0 instead of 0.5 (Fig. S2).

The sequence analysis (SNVs frequency analysis and average coverage read depth) of L5 EVO revealed marked changes in its genome in terms of single nucleotide variants (SNVs), intrachromosomal large duplications and aneuploidies (Fig. 4B). Bedtools was used to obtain coverage values per chromosome. If we compare chromosome II read depth in L5 strains and in L5-EVO strain by using the following equation: (chromosome II coverage L5 strain / total chromosome coverage in L5 strain) / (chromosome II coverage L5-EVO strain/ total chromosome coverage in L5-EVO strain) the obtained value is 1.427. In the rest of the chromosomes this value is similar to 1, with the exception of chromosome I and chromosome III (0.703 and 0.78), as they are small chromosome which suffer biases during the fragmentation that takes places during the sequencing process. Thus, L5 EVO gained an extra copy of chromosome II and showed segmental duplications in chromosomes IV, VI, XI and XIII (marked in red). The applied approximation used to infer the segmental duplications demonstrates that these segments are in fact duplicated in L5-EVO strain in comparison with the parental strain. In Fig. 4B the duplicated regions are represented in the original position of the duplicated fragments, but their exact location in L5-EVO genome cannot be assumed.

The evolved strain also underwent additional LOH on the right arm of chromosome XV. These results agree with the ploidy analysis by flow cytometry (Table 1): the L5 strain was a perfect 2n, while the evolved strain had a higher ploidy value between 2n and 3n.

Chromosome II was integrated by 456 ORFs involved in a variety of cellular processes (Table S2). Six of the 456 genes were previously identified as being involved in heat tolerance (*ENP1*, *HSP26*, *RER2*, *SSA3*, *SSE2*, *TIP1*) (Haslbeck et al., 1999; Kachroo et al., 2015; Sato et al., 1999; Sinha et al., 2008), and 70 others belonged to the functional categories of processes that could be related to heat tolerance, such as responses to other stresses or lipids (Table S2).

Genome sequencing also revealed SNVs (Table 2, Table S3) and different sized duplications (Table S4) present in the evolved strain. Fig. 5A shows the SNVs frequency distribution along the genome of L5 EVO compared to the S288c strain. Of the 48,970 identified variable positions, 30,898 positions were SNP variants in relation to S288c, and 18,072 were heterozygous positions in L5 EVO genome. As we can see, L5 EVO is a diploid and highly heterozygous strain (estimated SNV density of 1.5 SNV/kb). For the density distribution for each chromosome, values of 0 and 1 were retrieved as most SNVs were changes in the

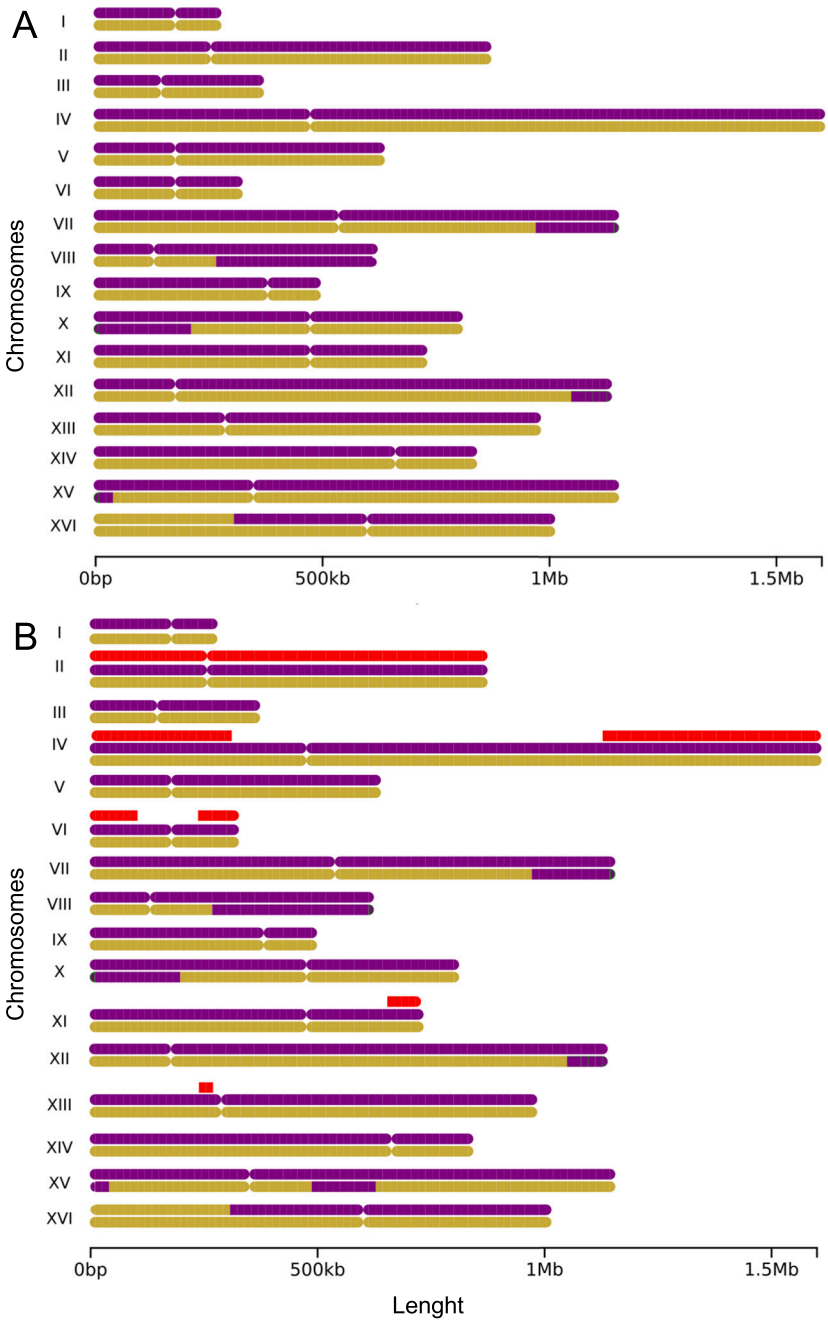

Fig. 4. Genome-wide representation of the chromosomes of L5 (A) and L5 EVO (B). The R package chromoMap was used to construct plots. Chromosomes' lengths are based on the S288c *S. cerevisiae* reference genomes, even though the real chromosome size of L5 and L5-EVO strains can be different from those ones shown. These representations are based on coverage, SNVs files and the ploidy of each strain. The pairs of homologous chromosomes of strain L5 diploid strain are represented in gold and purple. Loss of the heterozygosity (LOH) regions in chromosomes VII, VIII, X, XII, XV and XVI are represented in the same colour in the two pairs of homologous chromosomes. The L5 EVO aneuploidies are marked in red (chromosome II and regions of chromosomes IV, VI, XI and XIII) and a LOH region in chromosome XV is marked in the same colour for the two pairs of chromosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variant in our L5 EVO strain compared to S288c. Most chromosomes showed one peak around 0.5, which corresponded to heterozygous SNVs in strain L5 EVO. If one chromosome was present in three copies, which was the case of chromosome II, two peaks were observed at around 0.33 and 0.66. This distribution also profoundly changed in the large intra-chromosomal segmental duplications, which was the case of chromosomes IV and VI that had duplicated both extremes, with SNVs shown for each frequency (0.33, 0.50, 0.66) and the observed peaks were wider. Those results coincided with the data obtained from the coverage (Fig. 4B).

The analysis of the exclusive SNVs of L5 EVO, which were not present in L5 and were located in coding regions and had a quality value above 1000 (Table 2), resulted in 46 SNVs (Fig. 5B, Table S3). Fig. 5B shows the SNVs of L5 EVO (grey) with a quality value above 200, and SNVs in different colours according to their chromosome location, with 12 out of 46 that resulted in amino acid change (quality value above 1000). As a segmental duplication/deletion in a genome can sometimes lead to rapid adaptation (Adams et al., 1992; Koszul et al., 2004), we investigated the duplication/deletion events in our evolved strain. Six segmental duplications located in four different chromosomes (IV, VI, XI and XIII) were also identified (Fig. 4B, 5B, Table S4). The analysis of the duplicated genes belonging to the above four chromosomes resulted in a large number of significant ($p \leq 0.01$) functional categories (Table S5). Among these categories, it is noteworthy the catabolic process of leucine, tyrosine and branched amino acids, process related with DNA repair and cell cycle, carbohydrates metabolism and mitochondrial respiratory chain. No significant functional categories enrichment was found from the list of filtered genes (Table 2), although three out of 12 genes were previously related to heat response (*CYM1*, *REG1*, *NUP1*) (Belanger et al., 2005; Sinha et al., 2008).

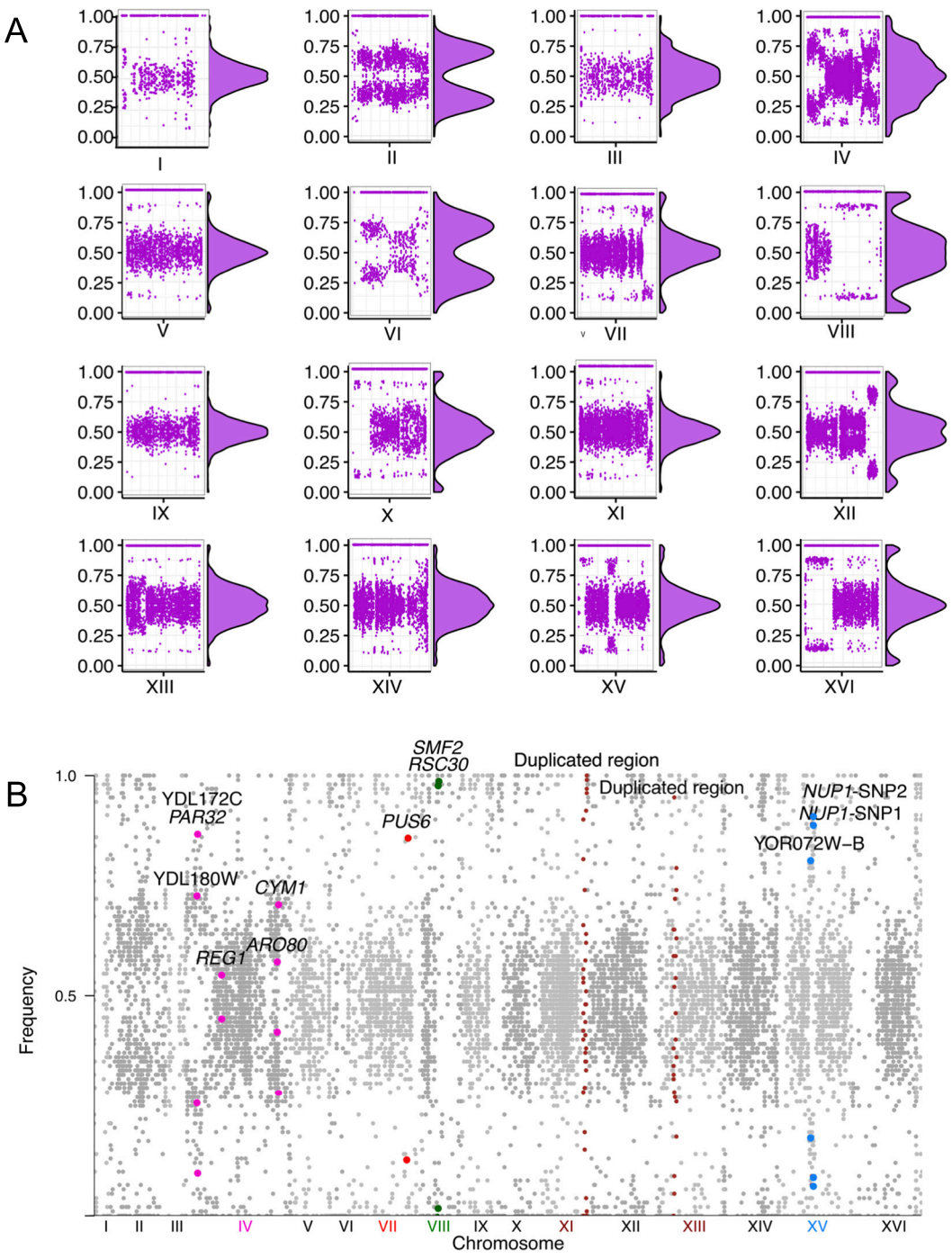

Fig. 5. SNVs frequency representation of strain L5 EVO compared to the S288c haploid strain (A) and SNVs frequency representation of strain L5 EVO compared to the L5 strain (B). The SNVs with quality > 200 and the density distribution of these SNVs for each chromosome, except values 0 and 1, are represented in purple (A). The SNVs with quality > 200 and are present in L5 EVO, but not in L5, are represented in grey (B). The curated SNVs with quality > 1000 whose change led to either an amino acid change or a stop codon in the coded proteins are annotated in B, as well as two small duplicated regions (chromosome XI, chromosome XIII), which are annotated based on SNV frequency changes. R packages ggplot2 and ggpubr were used to represent these plots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Pilot-scale experiment

To assess the fermentation performance of L5 EVO under industrial conditions, a bioreactor experiment was performed with natural cocoa beans from Guyana. The characteristics of L5 EVO were compared to those of spontaneous cocoa fermentation. Fig. 6A depicts the evolution of yeast populations (CFU g^{-1} of seed) of both experiments. As fermentation progressed, temperature rose, and the populations of both L5 EVO (green triangles) and indigenous yeasts (red squares) increased until the 48-hour time point, when their viable population started to diminish. L5 EVO presented higher CFU counts throughout the experiment compared to the indigenous microorganism, even when temperature reached 48 °C. During the pH follow-up of pulp (Fig. 6B), L5 EVO was able to first maintain pH, while pH during spontaneous fermentation dropped during the first 30 fermentation hours. Afterwards, an increase in pH was monitored during both fermentations, with higher

Table 2
Single Nucleotide Polymorphisms (SNVs) of the L5 evolved strain that were located in coding sequences with a quality value above 1000.

Chr position	L5	L5 EVO	Gene	Description	Amino acids
IV 1327272	A	G/A	YDR430C/*CYM1*	CYtosolic Metalloprotease	I/T
IV 148638	A/G	G	YDL172C		F/S
IV148638	A/G	G	YDL173W/*PAR32*	Phosphorylated After Rapamycin	N/D
IV 136793	G	A	YDL180W		E/K
IV 1312613	G	A/G	YDR421W/*ARO80*	AROmatic amino acid requiring	E/K
IV 500714	G	G/A	YDR028C/*REG1*	REsistance to Glucose repression	Q/*
VII 835722	C/T	T	YGR169C/*PUS6*	PseudoUridine Synthase	G/D
VIII 217411	C/T	T	YHR056C/*RSC30*	Remodel the Structure of Chromatin	V/I
VIII 209176	C/T	T	YHR050W/*SMF2*	Suppressor of Mitochondria import Function	P/L
XV 508682	T/C	C	YOR098C/*NUP1*	NUclear Pore	T/A
XV 508802	T/C	C	YOR098C/*NUP1*	NUclear Pore	N/D
XV 464502	A/T	T	YOR072W-B		R/W

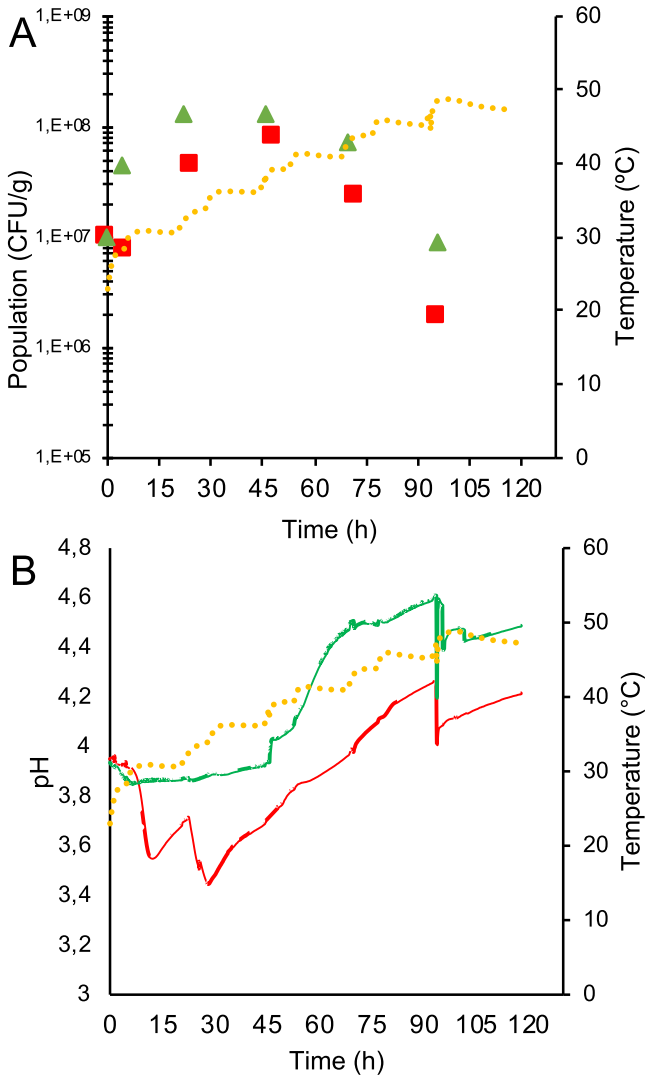

Fig. 6. Pilot-scale experiments. (A) Yeast viability of L5 EVO (green triangles) and the indigenous yeasts (red squares) (B) Pulp pH evolution in L5 EVO (green) and during the spontaneous (red) fermentations. Temperature increases are represented by the orange dotted line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values in the inoculated fermentation. We also assessed the percentage of the prevalence of the inoculated strain *versus* the indigenous contaminant strains, and 100% colonies showed the delta-PCR pattern of L5 EVO (data not shown).

4. Discussion

Cocoa pulp fermentation is one of the post-harvest processing stages that mostly governs ultimate product quality. It is a spontaneous process during which the natural microbiota present on cocoa farms is allowed to ferment the pulp surrounding cocoa beans. As such spontaneous fermentations are less reproducible and contribute to product variability, there is growing interest in a microbial starter culture to be used to inoculate cocoa pulp fermentations (Meersman et al., 2015). So it is interesting for industry to acquire appropriate starters cultures, which must be able to outcompete indigenous yeasts present in the environment that can contaminate fermenting pulp for commercial production. However, a vital trait of the selected starter for dominance in a cocoa pulp environment is thermotolerance because these fermentations are carried out at very high temperatures (≥40 °C) (Schwan and Wheals, 2004). So, our first aim was to genetically improve a native *S. cerevisiae* strain isolated from cocoa fermentation by an adaptive laboratory experiment (ALE). Serial cultivation for many generations under selective pressure to obtain fitter strains is a very appealing technique to improve industrial strains (Pérez-Torrado et al., 2015). Our results evidenced that long-term cultivation (150 generations) at supraoptimal temperature (40 °C) improved yeast strain ability to grow and ferment at high temperature. A similar strategy has already been followed by Caspeta et al. (2014) to improve thermotolerance in a lab strain. However, we went one step forward and tested the evolved strain during pilot-scale fermentation. To evaluate the evolved strain's competitiveness, the yeast counts of the inoculated fermentation were compared to a spontaneous fermentation of the same cocoa pulp, and the inoculated fermentation always showed higher counts than the spontaneous fermentation, mainly as temperature rose. Moreover, the implantation analysis evidenced that the inoculated strain outcompeted most indigenous yeast species, which proved its competitiveness in such a stressful environment. pH monitoring is a good indicator of fermentation state. Typically, at the start of fermentation, pH drops because of the production of organic acids by yeast and bacteria metabolism, mainly due to the conversion of ethanol into acetic acid by acetic acid bacteria. After the first 48 h, microbial consumption of citric acid, this being the main acid of cocoa pulp (1–3%), and evaporation of acetic acid produced in the first fermentation stage, as temperature rises, make pH increase to values around 5–6 (Lagunes Gálvez et al., 2007; Schwan and Wheals, 2004). We did not observe an initial drop in pH during our inoculated fermentation, which could be explained by the inoculated *S. cerevisiae* strain's rapid imposition. In the second fermentation stage, inoculated fermentation showed greater pulp alkalinisation, which correlated with good fermentation activity.

However, our second, but not least important, goal was to provide knowledge about the molecular and physiological mechanisms that occur in yeast cells during growth at 40 °C, which is a very stressful temperature. Our evolutionary process of 150 generations allowed a yeast strain to accumulate mutations and chromosomal rearrangements to alleviate fitness impairment at high temperature, and to finally

survive and grow at 40 °C. As an adaptation phenomenon, genome duplication has been observed before (Oud et al., 2013). Other works have also mentioned that diploidisation can be a faster way to adapt to a new environment (Gilchrist and Stelkens, 2019; Todd et al., 2017), and is an opportunity for heterozygous gene changes to occur (Turunen et al., 2009). The large number of aneuploidies described as a result of evolution experiments in yeast strains reinforces its role as an adaptation mechanism to harsh environments, including low nutrient availability (Gresham et al., 2008; Hong and Gresham, 2014; Selmecki et al., 2015; Sunshine et al., 2015), high ethanol concentrations (Lairón-Peris et al., 2020; Voordeckers et al., 2015), high temperature (Caspeta et al., 2014; Huang et al., 2018; Yona et al., 2012), low temperature (López-Malo et al., 2015), flocculation (Hope et al., 2017) and telomerase insufficiency (Millet et al., 2015), among others. We observed the complete duplication of chromosome II (trisomy) and four segmental duplications in chromosomes IV, VI, XI and XIII. The applied approximation to detect the segmental duplications does not provide evidence on their genomic location of L5-EVO strain, however the most plausible explanation would be that these segments are intrachromosomic tandem duplications or transpositions located in distant parts of the chromosomes (Achaz et al., 2000; Koszul et al., 2006, 2004). In this sense, it is important to remark that we have assumed that both L5 and L5-EVO genomes are collinear with S288c strain, and we cannot discard the possibility that large translocation or inversions were present in L5 original strain or that had occurred during the evolution process. The most suitable approach to detect if those genomic events had taken place would be the use of electrophoretic karyotyping combined with long-read sequencing technologies (Giordano et al., 2017; Hou et al., 2014).

Unlike our results, previous studies (Caspeta et al., 2014; Huang et al., 2018; Yona et al., 2012) of experimental evolution at 39 °C have found complete or segmental duplications of chromosome III. Duplication of chromosome II has also been described to be involved in copper adaptation in yeast (Covo et al., 2014; Gerstein et al., 2014). Aneuploidy is a complex beneficial adaptation with the potential to affect many genes simultaneously. However, its reversion rate is much higher than other types of mutations (Yona et al., 2012). Large-scale transitions in genome size from tetraploid or triploid to diploid as the predominant vegetative state of *S. cerevisiae* have been observed during long-term evolution experiments under stress conditions (Aguilera et al., 2010; Gerstein et al., 2008, 2006; Voordeckers et al., 2015). According to Yona et al. (2012), aneuploidy is a temporary adaptation mechanism to high temperature but, after long-term exposure (600 generations), euploidy was restored and novel beneficial mutations were fixed to compensate loss of the extra chromosome. In our study, we did not reach this number of generations, so it would be plausible that the aneuploidies present in L5 EVO were the initial evolution process stages. When stress disappear from the environment and aneuploidies are no longer an advantage, they are removed from the population, conferring the genomic rearrangements and SNVs more flexibility (Mangado et al., 2018; Sipiczki, 2011).

Extra chromosome copies have been related to a higher expression of the genes in this chromosome (Morard et al., 2019; Torres et al., 2007) and could be a way to change the dose of important genes present in chromosome II. A vast number of genes is present in chromosome II, some of which are directly related to heat stress (*ENP1*, *HSP26*, *RER2*, *SSA3*, *SSE2*, *TIP1*), but many others from the total could potentially improve the strain's thermotolerance. Previous works (Caspeta et al., 2016, 2014; Huang et al., 2018; Oud et al., 2013; Pinheiro et al., 2020; Yona et al., 2012) have shown that several biological processes are involved in heat adaptation, such as lipid and amino acid metabolism, protein folding and cell wall remodelling. We identified several genes that could potentially influence heat tolerance. *CDS1* is a CDP-diglyceride synthetase that catalyses a critical step in the synthesis of all major yeast phospholipids (Shen et al., 1996); *URA7* is involved in both phospholipids and the *de novo* biosynthesis of pyrimidines (Ozierkalogeropoulos et al., 1991); *TPS1* synthesises the storage of carbohydrate trehalose, which the yeast accumulates in response to certain stress conditions like heat stress (Eleutherio et al., 1993). Yeast strains lacking *TPS1* exhibit defects in both thermotolerance and acquired thermotolerance compared to the wild type, and these defects are not rescued with the addition of trehalose (De Virgilio et al., 1994; Gibney et al., 2013). The increase in their expression according to their copy number is consistent with our results, but further work is needed to identify the genes in chromosome II responsible for the evolved phenotype.

High temperature stress, as one general stress, involves a wide spectrum of cellular regulatory pathways, such as detoxification of misfolded proteins or regulation of general stress responsive genes. For the genes with non-synonymous mutations, we found a small overlap with those genes previously identified as being essential for growth in heat (Caspeta et al., 2016, 2014; Huang et al., 2018; Oud et al., 2013; Yona et al., 2012). We also observed some mutations in genes that could potentially lead to better adaptation at high temperature. *NUP1* is localised in the nucleoplasmic face of the Nuclear Pore Complex (NPC), where it is associated transiently with the NPC basket. Nup1 deficiencies provoke failures in the export of poly(A) mRNA or the import of cNLS-containing cargoes at high temperature (Belanger et al., 2005; Fischer et al., 2002). L5 EVO presented two non-synonymous mutations in this gene that could change the conformational state of the protein to avoid failures in the export of poly(A) mRNA. *REG1* encodes the regulatory subunit of phosphatase Glc7p and is involved in both the negative regulation of glucose-repressible genes and the regulation of the nucleocytoplasmic shuttling of Hxk2p. The L5 EVO strain presented a stop codon in the *REG1* gene which may lead to a higher respiratory metabolism rate (no glucose repression) (Entian and Zimmermann, 1980). Caspeta et al. (2014) also reported a higher oxygen uptake rate, tricarboxylic acid cycle activity and increased flux through the respiratory chain in their evolved thermotolerant strain. They explained this increased respiratory metabolic flux as a strategy that enhances ATP production, supports faster growth and generates more biomass.

We also aimed to correlate all these genomic changes with specific metabolic modifications in the evolved strain. In spite of not finding specific changes in the genes involved in lipid metabolism, we expected to find major differences in the main lipid membrane composition. Biological membranes are the first barrier between the inner part of the cell and its environment, and are a primary target for damage during temperature stress. *S. cerevisiae* can develop several strategies to maintain the appropriate membrane fluidity according to the environmental temperature. Our results mainly revealed adaptation at high temperature by a reduction in the unsaturation degree (mainly represented by a drop in the C16:1/C16 ratio) and a reduction in ergosterol, the final product of the sterol synthesis pathway, to benefit other intermediates (mainly lanosterol, zymosterol and episterol). Sterols contribute to maintain adequate lipid membrane fluidity and the lipid rafts function, which are important for many cellular processes, including cellular sorting, cytoskeleton organisation, and mating (Lingwood and Simons, 2010; Simons and Gerl, 2010). Membrane fluidity is also affected by the ratio, composition, and structure of the sterols found in membranes. Caspeta et al. (2014) observed "bended sterol" fecosterol accumulation in the evolved strain. They proposed that the increased presence of "bent" sterols (as opposed to the "flat" sterols normally present in membranes) could confer thermotolerance to the evolved *S. cerevisiae* strains. Curiously instead of fecosterol, we observed the accumulation of episterol, which is the next intermediate in the sterol pathway and the substrate of *ERG3*, a gene with a point mutation in the evolved strain obtained by Caspeta et al. (2014) that definitely determined its thermotolerance. However, in our study the only significant difference between the evolved strain and the wild type was an increase in the ratio between ergosterol and the other intermediates (Fig. 3 and Table S1). Thus, we can assume that this modified ratio in the sterol composition would also improve adaptation at high temperature.

5. Conclusions

Although some studies have previously dealt with the commonest genomic changes that occur in an evolved lab strain at high temperature (Caspeta et al., 2016, 2014; Huang et al., 2018; Oud et al., 2013), to the best of our knowledge, this is the first report about conducting adaptive evolution (ALE) in a diploid industrial strain. Moreover, we preliminarily tested the strain of improved thermotolerance on the pilot plant scale. The use of a cocoa strain could explain the genomic and physiological differences observed in the adaptation of our strain compared to these previous studies. Our data provide clear evidence for the genetic improvement to the L5 EVO strain's thermotolerance. We evolved a cocoa strain in a defined medium at high temperature to improve both fermentation and growth capacity by showing several molecular and physiological changes compared to the parental strain. The aneuploidy of chromosome II and the segmental chromosomic duplications, together with its capacity to remodel its membrane composition, may be the main adaptive responses of the evolved strain. Our work demonstrates that experimental evolution is an effective approach to generate better-adapted yeast strains at high temperature under industrial conditions. As this experimental evolution approach is not considered a GMO, the evolved strains can be quickly transferred to the industry.

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Declaration of competing interest

The author declares that there are no conflict of interest regarding the publication of this paper.

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CRediT authorship contribution statement

EGR conducted the experiments, analysed the data, participated in the study design and wrote the manuscript. MLP analysed the data. SMC, JMH, AOJ, PP and NR conducted the experiments. JMG and AQ conceived the study, participated in the study design and wrote the manuscript. All the authors read and approved the final manuscript.

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