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Lysine analogue-driven adaptive evolution produces lysine-overproducing wine yeast strains

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ABSTRACT

Lysine, though a poor nitrogen source for *Saccharomyces cerevisiae*, is rapidly consumed during wine fermentation and has been linked to enhanced oxidative and sulfur dioxide resistance. To investigate and harness this phenotype, the industrial wine strain P14 was subjected to adaptive laboratory evolution under increasing concentrations of the toxic lysine analogue S-aminoethyl cysteine (AEC). The resulting evolved isolates accumulated up to tenfold more intracellular lysine, displayed elevated reduced glutathione, and exhibited substantially decreased ROS levels while maintaining robust fermentation performance. Whole-genome sequencing revealed extensive genomic rearrangements and recurrent mutations associated with lysine biosynthesis. Among these, the *LYS21*^{V127F} mutation emerged as the major driver of lysine overproduction and AEC resistance. CRISPR/Cas9 reconstruction of this allele in multiple wine yeasts confirmed its causal role and conferred a competitive advantage during fermentation. These findings demonstrate the effectiveness of AEC-driven evolution for generating lysine-overproducing wine yeasts and highlight *LYS21* feedback-resistance as a key metabolic innovation.

1. Introduction

Nitrogen availability in grape must is a key parameter for the wine fermentation progress as it acts at many levels. It regulates the formation of yeast biomass and, in turn, the fermentation rate and the time to completion of fermentation (Crépin et al., 2012; Gutiérrez et al., 2012; Su et al., 2019). Moreover, the catabolism of nitrogen sources, together with central carbon metabolism, is involved in the synthesis of fermentative aroma precursors in wine yeasts (Barbosa et al., 2012; Su et al., 2020). Grape musts contain a wide range of yeast assimilable nitrogen (YAN) sources, defined as the amino acids (excluding proline) and ammonium available for yeast metabolism, including not only amino acids and ammonium, but also urea, citrulline, ornithine and small peptides (Kevvai et al., 2016). However, these nitrogen sources do

not all have the same effect on yeast growth, fermentation rate or aroma production during wine fermentation. Nitrogen source quality, in terms of the capacity to support yeast growth and fermentation activity, has been mainly evaluated in *Saccharomyces cerevisiae* (Godard et al., 2007; Gutiérrez et al., 2013), but also in non-*Saccharomyces* species (Su et al., 2020), with major differences between poor nitrogen sources, such as tryptophan, tyrosine, threonine or methionine, and rich nitrogen sources like ammonium, glutamine or asparagine.

Yeast preferentially uses substrates that allow the best growth through a regulation mechanism called nitrogen catabolite repression (NCR). This mechanism operates at various levels to prevent the uptake of the poorest nitrogen sources if better sources are available (Crépin et al., 2012; Gutiérrez et al., 2012; Magasanik & Kaiser, 2002). However, there is one notable exception to the supposed correlation between

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good nitrogen sources and early uptake: lysine. Previous studies that have explored the nitrogen source uptake order have shown that lysine was practically the first amino acid taken up both in *S. cerevisiae* (Crépin et al., 2012; Su et al., 2019) and in non-*Saccharomyces* (Rollero et al., 2018; Su et al., 2019). Moreover, this avidity of yeast cells to rapidly acquire lysine from the growth medium was independent of the initial concentration (Crépin et al., 2012; Su et al., 2019). Unlike to other amino acids, lysine was rapidly exhausted regardless its concentration in the medium (Su et al., 2019). This result is highly unexpected considering that *S. cerevisiae* is unable to grow on lysine as a sole nitrogen source because this species lacks the metabolic machinery to catabolise this amino acid and to synthesise the rest of the proteinogenic amino acids. Therefore, as shown in previous studies, lysine did not support either good growth or fermentation activity (Su et al., 2019) and should be considered a poor nitrogen source for *S. cerevisiae*. Thus, the question raised by this result is why lysine is rapidly depleted from the grape-must medium, regardless of its initial concentration, whether it cannot be channelled directly to biomass production or to enhance fermentation activity.

A possible explanation to this question can be provided by the study of Olin-Sandoval et al. (2019) in which they link lysine metabolism with an antioxidant stress response. Extracellular lysine is taken up by cells to reach concentrations up to one hundred times higher than those required for growth. Such extensive harvest is not observed for the other amino acids, is dependent on the polyamine pathway and triggers a

reprogramming of redox metabolism. As a result, NADPH, which would otherwise be required for lysine biosynthesis, is channelled into glutathione metabolism, leading to a large increase in glutathione concentrations, lower levels of reactive oxygen species (ROS) and increased oxidant tolerance (Olin-sandoval et al., 2019). Another interesting result is the connection of lysine metabolism with sulfur dioxide (SO₂) resistance during wine fermentation (Lage et al., 2019). It was demonstrated that lysine supplementation to the grape must can alleviate the toxic effects of this antimicrobial, widely used in winemaking, in *S. cerevisiae*. The explanation provided by Lage et al. (2019) was that SO₂-induced stress can lead to a depletion of yeast intracellular lysine and this lysine addition supplements the intracellular shortage of this amino acid, increasing yeast tolerance along vinification. Nonetheless, the demonstration that lysine supplementation can alleviate the toxic effects of SO₂ in *S. cerevisiae* represents an important finding since it can be easily explored by winemakers to improve performance of yeast strains along vinification, especially those that are more susceptible to the preservative action of SO₂. Novo et al. (2013) further highlighted the need for lysine supplements for winemaking, as they detected a short supply of this amino acid in some natural grape musts, which negatively affected yeast fitness during wine fermentation.

All these evidences support the importance of getting new insights about lysine metabolism in wine yeasts. To this end, we have obtained several mutant strains that overproduce intracellular lysine by ALE in presence of the toxic lysine analogue S-aminoethyl cysteine (AEC). We

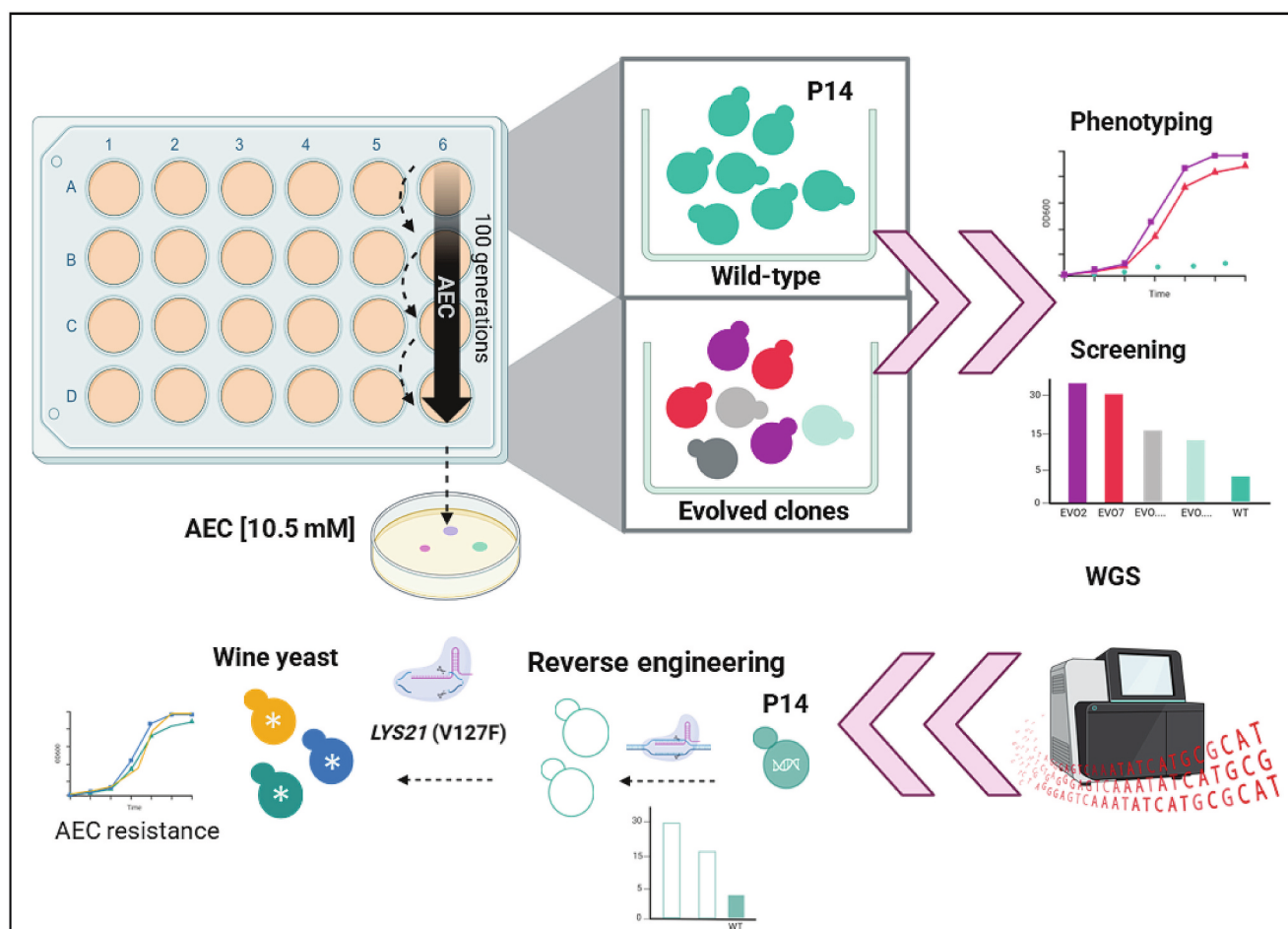


Fig. 1. Schematic representation of the experimental design (created with BioRender.com). Adaptive laboratory evolution of wine yeast strain P14 was performed by serial batch culture transfers and selection for AEC resistance by increasing concentration of AEC. Individual colonies isolated from the evolved populations after 100 generations were isolated and subjected to phenotyping, genotyping, and screening for lysine production. The two most promising colonies (2 and 7) were selected for further analysis and whole-genome sequenced. The selected mutations identified were reverse-engineered using CRISPR-Cas9 on the wild-type parental strain P14. The mutation *LYS21* (V127F) was introduced using CRISPR-Cas9 into the genome of different wine yeast and the resistance to AEC was assessed.

have phenotyped these evolved strains in their capacity to antioxidant resistance, fermentation performance, correlation with intracellular glutathione increase, etc. We have also genotyped these over-accumulating strains by analysing their whole genome in comparison with the parental wine strain to detect and validate genomic changes responsible of the overproduction of lysine. The overall experimental design is schematized in Fig. 1. These over-accumulating strains could be fitter for winemaking processes as active dry yeasts (ADY); however, their industrial production can be also used as a nutrient supplement of yeasts during wine fermentation in the form of Inactive Dry Yeasts (IDY). IDY is a product derived from yeast strains cultured on sugar-enriched mediums in aerobic condition, and subjected to thermal or enzymatic inactivation, which are enriched in different metabolites such as amino acids or glutathione (López-Solís et al., 2016). It is a widespread practice in oenology the use of these IDY products to improve either technological processes or the sensory characteristics of wines. However, to date, there is no lysine-enriched IDY available for improving the stress resistance of wine yeast under the harsh conditions of wine fermentation.

2. Material and methods

2.1. Strains and media

The strains used in this work were the industrial strains P14, P23, P6 and P4, all of them belonging to Lallemand Inc. (Canada), and the laboratory strains BY4741 and BY4743. The ALE was done with the P14 industrial strain. The complete list of strains used in this work is available in Table S1.

Yeast strains were grown in synthetic defined (SD) media (0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate, 2% (w/v) glucose). For ALE experiments, a modified SD medium containing 0.1% arginine as the sole nitrogen source (SDA), instead of ammonium sulphate, was used (Gasent-Ramírez & Benítez, 1997). We also designed the SD60 as a nitrogen-limiting medium that contains 60 mg/L of nitrogen in ammonium chloride form. When necessary, 2% agar was added to solidify the medium. Synthetic grape must (SM) was prepared as described by Bely et al. (1990). The SM composition included 200 g/L of sugars as a mixture of 50% glucose, 50% fructose. The concentrations of organic acids, minerals and vitamins were the same as those described by Su et al. (2019). Nitrogen sources were supplied as a mixture of 40% ammonium chloride and 60% amino acids. The composition of amino acids in the 1-L stock was 1.5 g/L tyrosine, 13.4 g/L tryptophan, 2.5 g/L isoleucine, 3.4 g/L aspartic acid, 9.2 g/L glutamic acid, 28.3 g/L arginine, 3.7 g/L leucine, 5.8 g/L threonine, 1.4 g/L glycine, 38.4 g/L glutamine, 11.2 g/L alanine, 3.4 g/L valine, 2.4 g/L methionine, 2.9 g/L phenylalanine, 6 g/L serine, 2.6 g/L histidine, 1.3 g/L lysine, 1.5 g/L cysteine and 46.1 g/L proline, which corresponded to 22.15 g/L of YAN (YAN is the sum of the amino acids and ammonium concentrations, except proline). The final concentration of the nitrogen in SM was adjusted to 140 mg/L of YAN. The final pH of the SM was adjusted to 3.3 with sodium hydroxide. Synthetic musts were sterilised by filtration through 0.22 µm pore-sized Nalgene sterile vacuum membrane filters (Thermo scientific, MA, USA).

2.2. AEC resistance phenotype previous to adaptive evolution

The resistance of the parental strain P14 to AEC was determined by growing them in SDA medium supplemented with 0, 0.01, 0.02, 0.05, 0.07, 0.1, 0.5 and 1 mM of AEC. Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were taken every 30 min for 5 days after 30-s pre-shaking for all the experiments. The experiments were done in triplicate in a 96-well plate. The maximum specific growth rate (μ_{max}) was estimated from the growth curve by directly fitting OD₆₀₀ measurements versus time as previously described (García-Ríos et al., 2017). The overall yeast growth

was estimated as the area under the OD vs. time curve (AUC 120 h). This parameter was calculated by integration, using the OriginPro 2023 software (OriginLab Corporation, Northampton, MA, USA).

2.3. Adaptive laboratory evolution experiment

P14 strain was subjected to a laboratory adaptive evolution in SDA medium supplemented with increasing concentrations of AEC at 28 °C through serial batch cultivation. The adaptive evolution experiment was performed in 24-well plates. For this purpose, at the start of the experiment, six individual colonies were inoculated in 1 mL SD medium and incubated overnight at 28 °C in an orbital shaker under 150 rpm. Then, 1×10^6 cells/mL (OD₆₀₀ = 0.1) were subcultured into SDA medium supplemented with 0.07 mM of AEC medium and incubated at 28 °C in an orbital shaker under 250 rpm. When this population reached the stationary phase, 50 µL were transferred into a new well containing 1 mL fresh SDA medium supplemented with increased concentrations of AEC and cultivated as described above. This process was repeated using AEC concentrations from 0.07 up to 10.5 mM. The number of generations was calculated by the equation: $n = (\log N_t - \log N_0) / \log 2$, where n is the number of generations, N_0 is the initial OD₆₀₀ and N_t is the OD at time t . Thus, the generation time (GT) was calculated by the equation $GT = t/n$. During the course of this adaptive evolution process, samples of the evolving populations were taken approximately every 25 generations and maintained in a glycerol stock (35% glycerol) at −80 °C for further phenotypic analysis. Additionally, each transfer was plated on SD agar to check viability and for yeast typing which was performed by delta element amplification from genomic DNA as previously described (López-Malo et al., 2015).

2.4. Extraction of intracellular lysine

For measuring lysine levels, yeast cells were pre-cultured overnight in 5 mL of SD, and main cultures were initiated by diluting to OD₆₀₀ = 0.1–0.2 in 20 mL of SD media with or without lysine supplementation. Cultures were grown until they reached different sampling points that cover all the growth stages. Cells were collected by centrifugation and then washed twice with sterile water. Harvested cells were frozen in dry ice and stored at −80 °C until intracellular amino acid extraction was performed. Collected cells were resuspended in 1 mL of sterile water and intracellular amino acids were extracted by boiling water at 100 °C for 10 min. After centrifugation (15,000 ×g, 5 min, 4 °C), the supernatant was transferred to a new tube and the pellet was subjected to a new process of extraction with another 1 mL of sterile water. The same protocol was applied for the determination of the intracellular content of the rest of amino acids and ammonium.

2.5. Nitrogen determination by HPLC

The concentration of the different amino acids and ammonium in the different samples taken during the fermentations were analysed on an Ultimate 3000 (Thermo Scientific) high-performance liquid chromatography (HPLC) system equipped with a UV–visible detector (Thermo Scientific, MA). The HPLC analysis method was based on Gómez-Alonso et al. (2007) with some modifications (García-Ríos et al., 2024). The samples were derivatized by a reacting mixture that included 430 µL of borate buffer 1 M (pH 10.2), 300 µL of methanol, 400 µL of the sample and 12 µL of diethyl ethoxymethylenemalonate (DEEMM) followed by vortex mixing. The sample was then heated at 70 °C for 2 h to allow complete degradation of excess DEEMM and reagent by-products. Chromatography separation was performed in a C18 Accucore™ C18 column (Thermo Scientific) at 30 °C. A total volume of 5 µL was injected. The content of lysine and other amino acids was represented as mg/g of dry cell weight (DCW), obtained by removing the water content from the yeast biomass.

2.6. Determination of intracellular glutathione

Oxidized (GSSG) and total glutathione levels were determined by the enzymatic glutathione reductase recycling method (DTNB/GR/NADPH), as described in Picazo et al. (2018) with some modifications. This method is based on the reduction of GSSG to GSH in the presence of glutathione reductase and NADPH, and formation of the colored product which is formed by the reaction of GSH with DTNB (5,5'-dithio-bis (2-nitrobenzoic acid)), followed by its absorbance at 412 nm (Griffith, 1980). Briefly, an equivalent of 25 OD₆₀₀ units of cells were collected and resuspended in 1 mL ice-cold HCl 8 mM, 1.3% (w/v) 5-sulphosalicylic acid. Cells were broken by vortexing at 4 °C with glass beads in four series of 1 min alternated with 1 min incubation on ice. Cell debris and proteins were pelleted by centrifugation for 15 min (13,000 rpm at 4 °C), and extracted supernatant extracts were used for glutathione determination. Total glutathione was determined using 200 µL of the supernatant, while 4 µL of 1 M 2-vinylpyridine were added to determine GSSG content. The reduced glutathione content was calculated as the difference between the total and oxidized glutathione. Glutathione levels were expressed as ng/mg of dry cells (DW).

2.7. ROS quantification by dihydrorhodamine staining

ROS quantification was determined as previously described by Olín-Sandoval et al. (2019) with some modifications. WT P14 and evolved strains 7 and 2 were precultured in SD medium until the mid-log phase in presence or absence of lysine (1 mM) and melatonin (0.1 mM). Then, cells were incubated with menadione (1.5 mM) for 1 h. Subsequently, cells were collected, washed three times in SM medium and incubated for 90 min in the dark at 30 °C with 15 µM dihydrorhodamine 123 (DHR 123). Cells were washed once with media and stained for 5 min with propidium iodide (500 µg/mL) to exclude dead cells, and subsequently washed three times with PBS. Cells were analysed using flow cytometer MACSQuant® Analyzer (Miltenyi Biotec, Madrid, Spain).

2.8. Phenotyping assay

Growth of the parental strain P14 and the evolved AEC-resistant colonies 2 and 7 was evaluated using quantitative growth curves. The selected stress conditions included exposure to SO₂ (50 mg/L), high sugar content (260 g/L glucose), ethanol (12% v/v), low temperature (16 °C), and high temperature (37 °C). All stress conditions were tested in SD medium. Additionally, SO₂ was evaluated in both SD and SM media adjusted to pH 3.5. Each condition was assessed in the presence or absence of 1 mM lysine.

Single colonies of each strain were inoculated into SD or SM medium and grown overnight. Cultures were initiated by inoculating 250 µL to an initial OD₆₀₀ of 0.1 in 96-well clear flat-bottom microtiter plates (Corning, Glendale, AZ, USA). Plates were incubated at 28 °C with 80% humidity, and OD₆₀₀ was recorded every 30 min using a SPECTROstar Omega plate reader equipped with a Microplate Stacker (BMG LabTech, Offenburg, Germany). For the temperature-comparison experiments, cultures were monitored using a SPECTROstar Omega microplate reader operated without the stacker. In both setups, 30 s of shaking at 300 rpm was performed prior to each reading. The AUC at 24 h was calculated for each strain using OriginPro 2023 (OriginLab Corporation, Northampton, MA, USA).

2.9. Spot assays

To test SO₂ resistance, yeast cells were pre-cultured overnight in 12 mL tubes containing 1 mL of SD medium (at pH 3.5) with or without lysine (1 mM) at 28 °C with orbital agitation (250 rpm). Next day, 3.5 µL of cells were spotted onto SD (at pH 3.5) with or without lysine agar plates on 10-fold serial dilutions starting at an OD₆₀₀ of 0.1, and incubated at 28 °C for 3 days.

To test AEC resistance, yeast cells were pre-cultured overnight in 12 mL tubes containing 1 mL of SD medium at 28 °C with orbital agitation (250 rpm). Next day, 3.5 µL of cells were spotted onto SDA with 2 mM of AEC on 10-fold serial dilutions starting at an OD₆₀₀ of 0.1 and incubated at 28 °C for 3 days.

2.10. Fermentation conditions

Fermentations were carried out in 100 mL bottles fitted with closures that enabled the carbon dioxide to escape, containing 80 mL of the SM. After yeast inoculation (2x10⁶ cells/mL), the bottles were incubated at 28 °C with continuous orbital shaking at 120 rpm and fermentation kinetics was followed by weighing the bottles twice a day and calculating the weight loss of each culture. The fermentations were considered finished when bottle weight stopped lowering and density reached 995 g/L. Density was measured with a portable densitometer (Densito 30PX, Mettler Toledo, Switzerland).

2.11. Competitive growth during fermentation

Cell populations were monitored at middle and end of fermentation by plating each time the appropriate dilution onto YPD agar plates and incubated for 2–3 days at 28 °C. From three biological replicate fermentations (*n* = 3), 12 single colonies were randomly selected and inoculated into 96-well plate containing 200 µL of liquid SDA medium and precultured overnight at 28 °C with shaking at 300 rpm. Next day, cells were pinned into 200 µL fresh SDA medium supplemented with 2 mM AEC. Plates were incubated at 28 °C for 20 h and OD₆₀₀ was measured using a SPECTROstar Omega Plate Reader (BMG LabTech). To assess which strain imposed during fermentation, OD₆₀₀ measurements were classified using a cut-off of 1.0. Cultures with OD₆₀₀ < 1.0 were considered non-growing or minimally growing, whereas OD₆₀₀ > 1.0 indicated clear growth.

2.12. Chemical characterization of the final wines

To quantify the basic oenological compounds of the final wines (glucose, fructose, glycerol, ethanol and organic acids), fermentation samples were filtered through a 0.22 µm nylon filter and measured using HPLC. The HPLC system was equipped with RI and UV detectors and a HyperREZ XP Carbohydrate H+ column and HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific-ES, Madrid, Spain). The system was operated using 1.5 mM of sulfuric acid as mobile phase with a flow rate of 0.6 mL/min and column temperature of 50 °C. Quantification of glucose, fructose, glycerol and ethanol was performed using a refractive index detector (RID), while organics acids (acetic, citric, tartaric, malic, lactic and succinic acids) were quantified using a UV/VIS detector at 210 nm. The concentration (g/L) of each compound was determined with calibration curves using the corresponding standard compound.

2.13. Genomic DNA extraction and genome sequencing and analysis of the parental and evolved strains

Genomic DNA was extracted from the parental strain P14 and the evolved selected strains, 2 and 7, as described in Querol et al. (1992). DNA Libraries were generated using Illumina Truseq Nano DNA library Preparation following manufacturer's recommendations. Libraries were sequenced on an MiSeq instrument and 15 M 2 × 250 bp pair-end reads generated. Raw sequencing reads in FASTQ format were subjected to initial quality control and preprocessing. First, fastp v0.23.2 (Chen et al., 2018), was used for adapter removal and filtering of low-quality reads. Processed data quality was then assessed using FastQC v0.11.9 (Andrews, 2010), generating individual quality reports. Genomes sequences are deposited at NCBI under BioProject number PRJNA1504605 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA1504605>).

Cleaned reads were aligned to the *S. cerevisiae* S288c reference

genome (release R64–1-1) using BWA v0.7.17 (Li, 2013). The resulting SAM files were converted and sorted into BAM format. Alignment quality and genome coverage were evaluated with Qualimap v2.2.1 (Okonechnikov et al., 2016), and duplicate reads were identified and marked using MarkDuplicates from Picard Tools v2.25.7 (<http://picard.sourceforge.net>).

Variant detection was performed using the Genome Analysis Toolkit (GATK) v4.5.0.0 (McKenna et al., 2010), following the Best Practices workflow of the Broad Institute and a strategy similar to that described by Bigey et al. (2021). The analysis pipeline consisted of four consecutive stages: read alignment to the reference genome, refinement of mapped reads, identification of SNPs and indels through variant calling, and hard filtering of the detected variants to obtain a high-confidence variant set. SNPs and indels were identified independently for each sample using GATK HaplotypeCaller in BP_RESOLUTION mode, generating genomic variant call format (GVCF) files containing genotype likelihood information for every genomic position. The resulting per-sample GVCFs were subsequently subjected to joint genotyping using GenotypeGVCFs to produce a multi-sample VCF callset. Because both evolved strains displayed partial polyploidization, ploidy was estimated using nQuire (Weiß et al., 2018) prior to variant calling. The resulting variants were subjected to hard filtering using predefined thresholds based on the following quality metrics: Quality by Depth (QD), RMS Mapping Quality (MQ), Fisher Strand (FS), Mapping Quality Rank Sum (MQRankSum), Strand Odds Ratio (SOR), and Read Position Rank Sum (ReadPosRankSum). Variants that did not meet the established criteria were excluded, resulting in a high-confidence set of SNPs and indels for downstream analyses.

Variant calling was performed independently for the parental strain (P14) and the two evolved isolates using the same analytical pipeline. Although the S288c genome was used as the common mapping reference and coordinate system, the identification of mutations acquired during adaptive laboratory evolution was based on direct comparison of the parental and evolved variant call sets using bcftools (Danecek et al., 2021). Variants detected in the parental strain P14 were considered pre-existing polymorphisms, whereas variants identified exclusively in the evolved isolates were classified as mutations acquired during adaptive laboratory evolution. Shared and strain-specific variants were identified by direct comparison of the three variant call sets.

Finally, variants were annotated using the Ensembl Variant Effect Predictor (VEP) (McLaren et al., 2016), which was used to predict the potential functional consequences of each variant on genes and proteins.

2.14. Ploidy estimations by flow cytometry

The DNA content of both the parental (P14) and the evolved strains (2 and 7) 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 & Reed, 2002). The DNA content values were scored based on the fluorescence intensity compared to the haploid (BY4741) and diploid (BY4743) *S. cerevisiae* reference strains. The DNA content value reported for each strain was the result of three independent measures.

2.15. Plasmid construction and genetic manipulations

CRISPR/Cas9-mediated genomic editing was performed to validate the different mutations observed in genes related to lysine metabolism in both evolved strains. To do so, the corresponding parental strain was transformed with both, a guide RNA (gRNA)-Cas9 expressing plasmid and a repair fragment containing the desired mutation to facilitate homology-directed repair at the double-strand break. Briefly, two oligonucleotides encoding the gRNA were annealed in vitro and integrated into the plasmid pWS174 or pWS175 using a BsmBI Golden Gate assembly. Plasmids pWS174 and pWS175 were a gift from Tom Ellis (Addgene plasmid # 90961 and # 90962, respectively). The specific

gRNA sequences were designed using Benchling (<https://www.benchling.com>) and the constructed plasmids and primers used can be found in Tables S2 and S3, respectively. Donor DNA containing the punctual mutation (Table S4) was transformed into yeast cells together with CRISPR gRNA-Cas9 expressing plasmids. Yeast transformation was carried out by the lithium acetate method (Gietz & Woods, 2002). For each transformation, 250 ng of CRISPR plasmid and around 1.5 µg of donor DNA were used. The successful introduction of each CRISPR/Cas9-mediated mutation was verified by colony PCR followed by Sanger sequencing prior to phenotypic characterization. Only sequence-confirmed edited strains were used for subsequent analyses of AEC resistance, intracellular lysine accumulation, and other phenotypic assays. Primer pairs used for PCR validation are listed in Table S3.

To introduce the point mutation in *URA3* for P14 and 7 strains, primers pair 953/954 were used to generate the gRNA target whereas oligonucleotides 1134/1135 were used to generate the donor DNA containing a mutation that causes an early STOP codon in the *URA3* gene. Primers 1136/1137 were used to validate the introduction of the mutation by colony PCR.

To integrate the gene *gapN* from *Streptococcus mutans* into the yeast genome, the codon-optimised sequence was synthesised by Twist Bioscience (San Francisco, USA). This codon-optimised sequence is shown in Table S5. The *gapN* coding sequence was amplified using primers 892 and 893. PCR product and p423GPD vector was digested with *Bam*HI and *Xho*I enzymes, gel purified and then ligated to obtain p423GPD-*gapN* plasmid. *E. coli* positive transformants were selected and plasmids sequenced with primers 538 and 539. The integrative cassette bearing *gapN* under the promoter GPD was amplified using primer pair 729/1129. These amplifications were carried out with Phusion U Hot Start polymerase (Thermo Scientific). In parallel, the vector pCfB2988 from the EasyCloneMulty vector set (Maury et al., 2016) was prepared by sequential treatment with enzymes *Asi*SI (*Sfa*AI) (Thermo Fisher Scientific) and *Bsm*I (New England Biolabs). After purification, the prepared vector and PCR products were mixed and treated with the USER™ enzyme mix (New England Biolabs). The corresponding mixture was directly used for bacterial transformation. The successful cloning of different vectors was identified by PCR in *E. coli* colonies using the 690 and 691 primers and verified by Sanger sequencing (EUROFINS genomics, Ebersberg, Germany). Prior to yeast transformation, the resulting integrative vector pCfB2988-*gapN* was linearized by FastDigest *Not*I (Thermo Fisher Scientific).

2.16. Docking simulation of the Lys21 WT and Lys21^{V127F} with α-KG and L-lysine

Two homology models of the wild type and mutant variant of mitochondrial homocitrate synthase Lys21 from *S. cerevisiae* were created using the server SWISS-MODEL (Bienert et al., 2017; Waterhouse et al., 2018). The template protein used for construction of both models was the crystal structure of homocitrate synthase from *Thermus thermophilus* (TtHCS; 54.32% sequence identity to Lys21 [PDB ID no. 3A9I]). The model matches the template protein closely at the N-terminal TIM barrel domain with the active site.

Docking was carried out using SwissDock and AutoDock Vina (Bugnon et al., 2024; Grosdidier et al., 2011). The ligands α-KG and L-lysine were downloaded from the PDB structures 3A9I and 2ZTJ, respectively. The center of search space was set on the TIM barrel domain (31, 24, 9 Å), with a box size of 30 Å. Sampling exhaustivity was defined at 9. The docking calculations showed that approximately 35 clusters of ligand-protein orientations were returned. Each cluster contained up to eight similar poses. Some clusters contained the ligand bound within one of the active sites of the dimer, while other clusters contained the ligand bound on the protein surface and were ignored. The binding energies of each pose within the clusters placing the ligand in the active site were averaged, and the standard deviation of these energies was calculated.

2.17. Statistical analysis

Experiments were performed using at least three biological replicates. For each experiment, detailed information on respective numbers of biological replicates (n), mean and standard deviation (s.d.) are reported in the figure legends.

For two groups' comparison of data, statistical significance was tested with two-tailed Student's *t*-test assuming equal variance with a *p*-value of 0.05. Data analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Generation of lysine over-accumulating strains by adaptive laboratory evolution (ALE)

A conventional improvement strategy has traditionally been used to obtain yeast strains that overproduce lysine, involving the screening of mutants resistant to the toxic lysine analogue S-aminoethyl cysteine (AEC) (Feller et al., 1999; Garrigós et al., 2024; Gasent-Ramírez & Benítez, 1997). In this study, we used an ALE approach to develop non-recombinant *Saccharomyces cerevisiae* wine strains that overaccumulate lysine. The strain selected for this study was P14, which is marketed by Lallemend Inc., and widely used as a wine fermentation starter worldwide.

AEC resistance of P14 strain was firstly determined in SDA medium supplemented with increasing concentrations of AEC. The wild type P14 (WT) was unable to grow at 1.1 mM and its growth rate ($\mu_{\max}$) was reduced to half at a concentration of 0.07 mM (Fig. S1). Once we determined the inhibitory concentration for the wild type, we carried out a parallel ALE process in which six independent populations of the wine strain P14 were grown during 100 generations in SDA with continuous increase concentrations of AEC starting from 0.07 mM up to 10.5 mM. This concentration is 10-fold higher than the MIC determined for the wild strain. The resulted evolved strains were genotyped by delta elements and all of them presented the same delta profile that the wild type P14 (data not shown).

The individual evolved colonies with the highest tolerance to AEC were used to determine their intracellular concentration of lysine in comparison with the wild strain (Fig. 2A). As shown, P14 (green triangle) produced approximately 3 mg of lysine per 1 g of dry weight (DW), whereas some of the evolved colonies were able to produce up to 39 mg/g of DW. This increase represented more than 10-fold in comparison with the parental strain. The evolved 7 and 2 strains, originated from two different ALE lineages, had the highest intracellular lysine content of all the colonies, so they were selected for further experiments. Recently, Isogai et al. (2021) constructed a high lysine-producer *S. cerevisiae* strain by introducing punctual mutations in the homocitrate synthase (HCS), first enzyme of the lysine biosynthesis pathway and encoded by *LYS20*. The HCS activity is tightly regulated via feedback inhibition by the end product lysine. However, the amino acid changes rationally introduced by these authors in the HCS converted to this key enzyme in insensitive to feedback inhibition, resulting in high-level lysine production in yeast cells. Nonetheless, these authors found that the lysine content in yeast cells expressing the best accumulating mutation was 4.62-fold higher than the cells expressing the wild-type HCS, yet still substantially lower than any of our selected evolved strains. Thus, our results showed that selection of lysine-overproducing mutants using cultures with progressively increasing concentrations of a toxic amino acid analogue is highly effective.

These results were obtained during the exponential growth of the strains in SD medium. However, we aimed to assess the kinetics of lysine accumulation during a complete growth curve and to figure out whether this biosynthesised lysine was kept intracellularly or was released into the culture medium. The results of this kinetic of intra- and extracellular lysine production are shown in Fig. 2B and C, respectively. As it can be

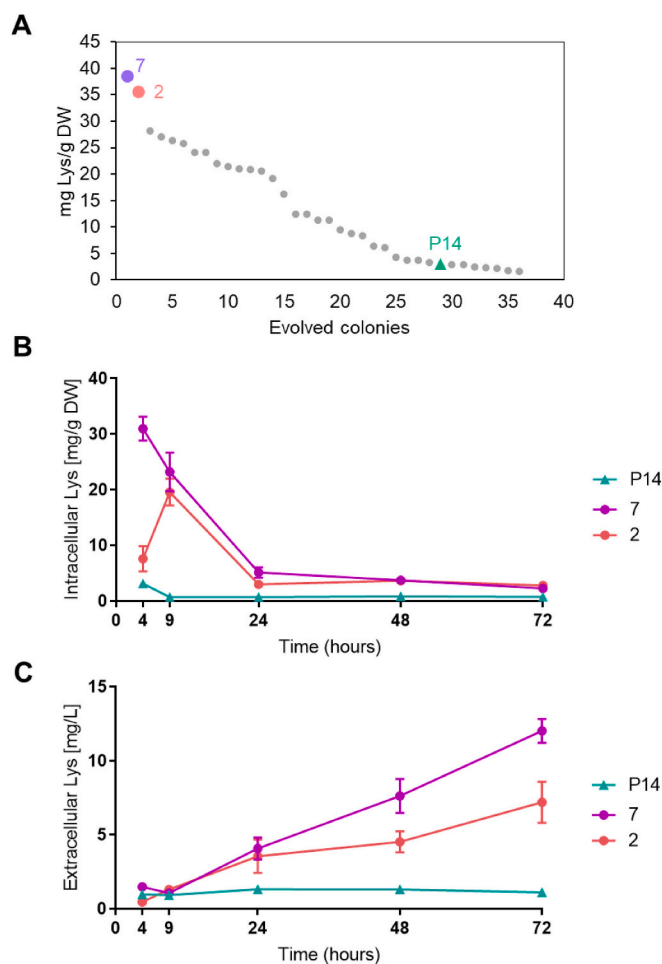


Fig. 2. Lysine production profile of individual colonies from AEC-resistant evolved population. **A** Screening for intracellular lysine production in SD medium from individual colonies of the AEC evolved population. Cultures were grown until the mid-log phase (≈ 0.6 OD₆₀₀) and 5 OD₆₀₀ were used for intracellular lysine extraction and quantification with HPLC-UV. **B** Time-course for intracellular and **C** extracellular lysine production of the wildtype strain P14 in comparison to the two evolved mutants for AEC resistance selected from screening in A. Cultures were initiated with an OD₆₀₀ of 0.2 and grown for 72 h. Samples were taken at different time points, and 5 OD₆₀₀ were collected for intracellular lysine extraction, and the supernatant was used for extracellular lysine quantification. Lysine content is represented as mg per g of dry weight and mg/L for intracellular and extracellular concentrations, respectively. Mean $\pm$ s.d.; *n* = 3 biologically independent cultivations.

seen, maximum intracellular lysine accumulation of the evolved strains corresponded with this initial mid-exponential phase (around 4–9 h of culture). After this point, intracellular accumulation was decreasing along with a continuous increase in the extracellular medium. This result evidenced a release of the lysine synthesised into the culture medium.

In addition to analysing the intracellular lysine content, we also examined the kinetics of the intracellular accumulation of all amino acids and ammonium (Fig. S2). Compared to the WT P14 strain, the evolved strain 7 exhibited greater accumulation of total amino acids, particularly at the start of the exponential phase (4.5 h), when the total amount of intracellular amino acids doubled. This increase can be explained by the accumulation of amino acids alongside lysine, particularly the metabolically related ones, such as glutamate, glutamine and ornithine (see Fig. S2B). Strain 2 also exhibited higher concentrations of certain amino acids, including aspartate, glutamate, glutamine, methionine, phenylalanine, tryptophan and valine, at certain sampling points,

albeit not as significantly (Fig. S2C). In both evolved strains, arginine accumulation was diminished compared to WT P14. This suggests that the over-synthesis of lysine also led to the accumulation of other amino acids. Recently, Garrigós et al. (2024), using an ALE strategy similar to our work, have obtained AEC-resistant mutants with an overactivated Retrograde Response Pathway (RRP). The RRP is a signalling pathway that communicates mitochondrial dysfunction to the nucleus, whose activation triggers the induction of a wide range of target genes. RRP targets include genes encoding mitochondrial and peroxisomal enzymes that divert cytosolic pyruvate and acetyl-CoA to citrate and then to α -ketoglutarate, a precursor for glutamate/glutamine and lysine biosynthesis (reviewed in Jazwinski, 2013). Furthermore, *S. cerevisiae* synthesizes ornithine from glutamate, via intermediates such as *N*-acetyl L-glutamate (NAG) and *N*-acetyl glutamyl-5-phosphate in mitochondria (Ohashi et al., 2022). Ornithine is also synthesised as a result of arginine catabolism and it is recognised as a stress protector. Over accumulating yeast strains of several amino acids including lysine and/or ornithine would be of great commercial value to be used in agriculture or wineries, among others.

3.2. Glutathione metabolic rewiring and oxidative stress resistance of the over-accumulating strains

Recently, it has been reported that lysine harvesting of the growth medium triggers a reprogramming of redox metabolism (Olin-sandoval et al., 2019). As a result, NADPH, which would otherwise be required for lysine biosynthesis, is channelled into glutathione metabolism, leading to a large increase in glutathione concentrations and lower levels of reactive oxygen species (ROS). To determine whether or not a reprogramming of redox metabolism leading to a large increase in glutathione concentrations and lower levels of reactive oxygen species was occurring as a consequence of over accumulation of lysine (Olin-sandoval et al., 2019), we determined the total and reduced intracellular glutathione levels (Fig. 3). All of the analysed strains exhibited a higher glutathione content (mainly in the form of GSH) during the exponential phase than during the stationary phase. In general, the evolved strains exhibited higher concentrations of both total and reduced glutathione compared to the WT P14. Notably, strain 7 stood out with the highest accumulation of total (4700 ng/mg DW) and reduced (3400 ng/mg DW) glutathione, as well as the highest percentage of the reduced form (72.7%). These results were unexpected, as the lysine accumulation in the developed strains was derived from biosynthesis, which could have been expected to deplete NADPH and reduce glutathione synthesis. However, strain 7 proved particularly interesting as it not only accumulated lysine but also enhanced levels of reduced glutathione which is a feature of great industrial interest.

To further investigate the correlation between NADPH availability and glutathione synthesis, we cloned the bacterial gene *gapN* from *Streptococcus mutans* in both the parental strain P14 and the best evolved strain 7. Lysine biosynthesis needs a large amount of NADPH, and thus an improvement of the NADPH regeneration system may also be required (Takeno et al., 2010). Gene *gapN* catalyses the reaction of glyceraldehyde-3-phosphate to 3-phosphoglycerate, reducing NADP^+ to NADPH in the process. This generates more NADPH from glycolysis (Boyd et al., 1995). Our results confirmed that the presence of this gene in the parental strain P14 increased total glutathione levels by more than 30% during exponential growth phase, mainly in the form of reduced glutathione (Fig. 3B). However, in evolved strain 7, this increase was particularly significant during the stationary phase, with a substantial rise in total glutathione levels, though it decreased during the exponential growth phase, especially the reduced form. Interestingly, the increased availability of NADPH appeared to have little effect on the lysine-accumulating strain during exponential growth. However, introducing the bacterial *gapN* gene into wine yeast could be a highly effective strategy to boost intracellular glutathione, particularly in its more beneficial form, GSH. Though, this would result in the strain being

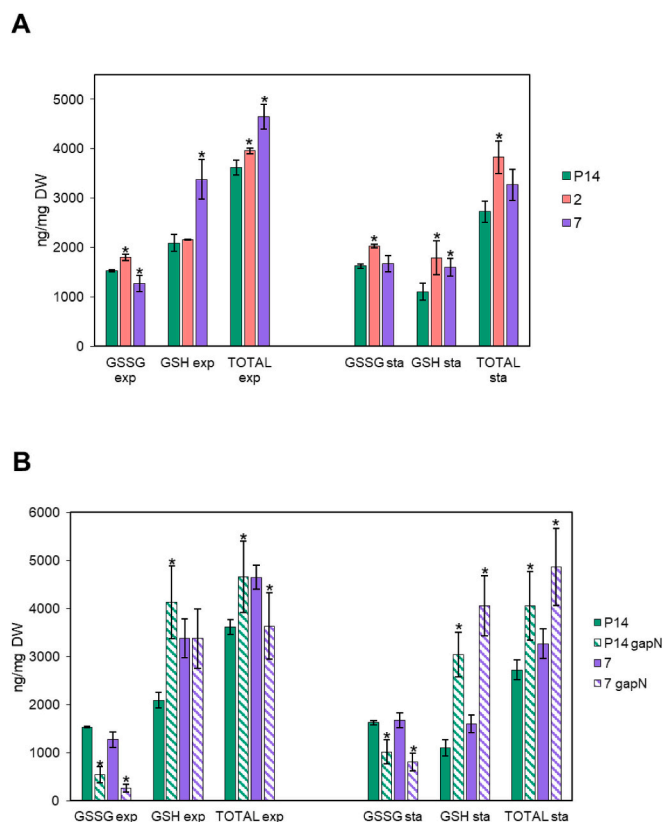


Fig. 3. Glutathione levels vary in evolved AEC-resistant colonies and *gapN*-expressing strains. Oxidized (GSSG) and total glutathione levels were determined by an enzymatic method (as described in material and methods section) for the evolved AEC-resistant colonies 2 and 7 as well as the parental strain P14 (solid colored bars) and for the strain 7 and P14 overexpressing *gapN* (striped pattern bars). Glutathione measurements were done from 25 OD₆₀₀ equivalent cells during exponential (exp) and stationary (sta) phases. Mean $\pm$ s. d.; $n = 3$ biologically independent cultivations. Asterisks (*) indicate significant differences ($p \leq 0.05$) between the WT P14 and the evolved strains, and between the strain expressing *gapN* and the corresponding control strain without *gapN* overexpression.

classified as a GMO.

Finally, based on the hypothesis that proposes that strains that accumulate higher levels of glutathione are expected to exhibit greater tolerance to oxidative stress (Olin-Sandoval et al., 2019), ROS concentration was measured by flow cytometry in the parental and in the over-accumulating strain 7 (Fig. S3). The experiment involved exposing the cells to a potent oxidant, such as menadione, which induces a variety of oxidative stresses and can trigger either necrosis or apoptosis (Farrugia & Balzan, 2012; Yamashoji et al., 2020). To test the effect of lysine taken up from the medium, we also incubated the cells in the presence of lysine. As a positive control for an antioxidant molecule, we used melatonin, as we have previously verified its antioxidant effect and ability to reduce intracellular ROS (Bisquert et al., 2018). As expected, menadione increases dramatically the levels of ROS in yeast cells compared to the control condition of no exposure to the oxidant molecule (Fig. S3).

Looking at the bars corresponding to cells exposed to menadione but without any antioxidant molecules (lysine or melatonin; wo), we observed that strain 7 has a lower percentage of oxidized cells compared to the parental strain. Referring back to the intracellular glutathione results, it becomes clear that higher glutathione levels correlate with lower ROS in the cells (Olin-sandoval et al., 2019). When lysine is present in the medium, its uptake has a significant antioxidant effect, reducing the percentage of ROS-positive cells across all strains, even

surpassing the effect of melatonin. Thus, as proposed by [Olin-Sandoval et al. \(2019\)](#), extracellular lysine availability appears to be a promising strategy for improving oxidative stress tolerance. Interestingly, the evolved strain that accumulates lysine through biosynthesis demonstrates greater tolerance to oxidative stress than the parental strain.

3.3. Stress resistance and fermentation activity of the over-accumulating strains: harvesting vs. production

To determine whether the evolved strains would have higher tolerance to stresses during wine fermentation due to lysine accumulation, the parental strain and the two evolved strains (2 and 7) were grown in SD under conditions that hinder yeast growth, such as osmotic stress (250 g/L of sugars), high ethanol content (12%), low pH, SO₂, low (15

°C) and high temperature (37 °C). The same conditions were also tested by supplementing the growth media with lysine in order to compare the phenotypic effect of imported lysine from the growth medium (harvesting) versus the lysine produced by the metabolic activity of the different strains (production).

The AUC was calculated and compared between strains and conditions and it is represented as a heatmap in [Fig. 4A](#). Unexpectedly, the evolved strains mostly showed worse growth (lower AUC) than the WT P14 in most of the stresses assayed (SD pH 3.5, SD 250, SD 12% EtOH and SD 15 °C). However, there was one significant exception: the evolved strains showed better growth at high temperatures (SD 37 °C), regardless of whether lysine was present in the medium ([Fig. 4B](#)). The higher accumulation of glutathione in these strains may explain this better growth because it is known that oxidative stress increase at high

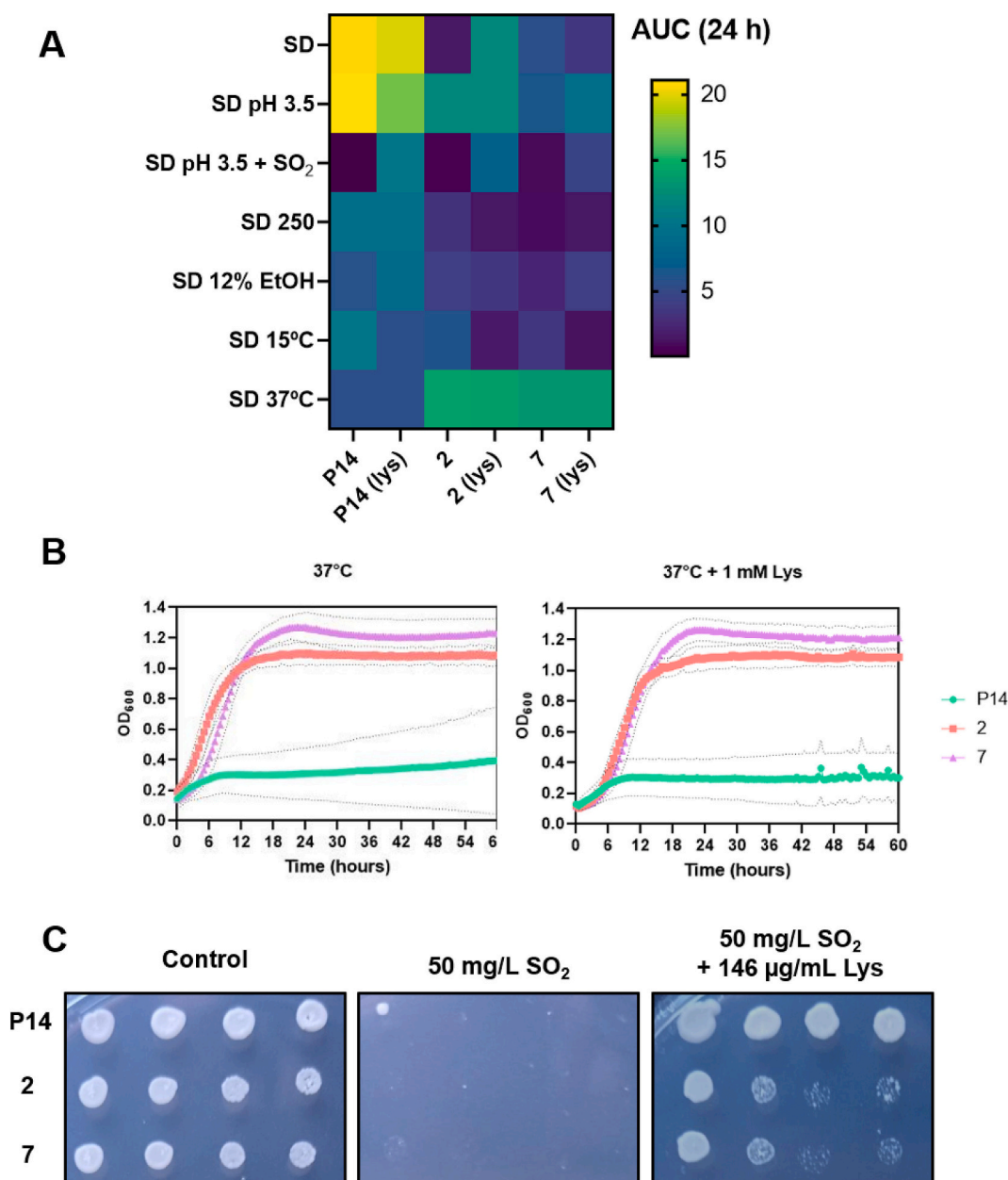


Fig. 4. Phenotypic characterization of the parental strain P14 and evolved colonies 2 and 7, across diverse environmental and stress conditions in the presence and absence of 1 mM lysine. **A** AUC values at 24 h derived from 96-well growth assays in SD medium under multiple stress conditions: acidic pH 3.5, 50 mg/L SO₂, 250 g/L glucose, 12% ethanol, 15 °C, and 37 °C. Values are expressed as mean, $n = 3$ biologically independent cultivations. **B** Representative curves for growth at 37 °C from. Mean $\pm$ s.d.; $n = 8$ biologically independent cultivations. The error is shown as a faint dotted line. **C** Representative images from the SO₂ drop test assay.

temperature (Davidson & Schiestl, 2001; Morano et al., 2012; Olin-Sandoval et al., 2019). Our results evidence that the intracellular accumulation of lysine may exert a protective effect against heat stress. This phenomenon was also reported in *E. coli* lysine-overproducing strains exhibiting higher tolerance to high-temperature (42.5 °C) stress than the WT strain (Isogai & Takagi, 2021), which could indicate a common mechanism across different microbial species.

Another exception is relative to SO₂ tolerance. None of the strains was practically able to grow in the medium SD pH 3.5 + SO₂. However, lysine supplementation in the growth medium led to a notable improvement in growth across all strains, including the evolved ones (Fig. 4A). Since SO₂ tolerance is critical in winemaking, we conducted a drop test under the same conditions and strains (Fig. 4C). The results were similar to those obtained in a liquid medium. SO₂ tolerance was rescued primarily by extracellular lysine supplementation rather than by the evolved strains' own intracellular lysine biosynthesis, indeed, the evolved strains were slightly more sensitive to SO₂ than the parental strain in the absence of exogenous lysine. This indicates that lysine uptake from the medium, rather than biosynthetic overproduction, is the main driver of SO₂ protection. This result is in concordance with other reports in which addition of lysine to the medium could alleviate the toxicity of this compound (Lage et al., 2019). These authors suggest that one of the features of SO₂ exposure is the induction of oxidative stress, and we have already reported a better response to oxidative stress when the cells are cultivated in the presence of lysine (Fig. S3). However, the molecular basis of this protection remain unclear and further research is needed in this regard.

In order to evaluate possible trade-offs during wine fermentation, over-accumulating strains and the parental strain P14 were inoculated in SM and fermentation activity was monitored by weight loss. The fermentation kinetics and the final chemical composition of the wines

were very similar for all strains in the three conditions, with 2 being the strain with the fastest fermentation rates (Fig. 5A, Fig. S4). Anyway, all the strains were able to finish the fermentation in a similar time.

Trade-off effects are commonly observed in ALE populations subjected to prolonged selection pressure (Barrick & Lenski, 2013; Lenski, 2017). These populations often become highly specialized for the selective environment but tend to show decreased fitness compared to their ancestors when placed in alternative niches, under different propagation methods, or nutrient conditions (Caspeta & Nielsen, 2015; Hong & Nielsen, 2013; Jacobus et al., 2024; Lindsay et al., 2024; López-Malo et al., 2015; Menegon et al., 2022). As mentioned above, our evolved strains present some trade-offs for some of the stresses assayed in the phenotyping experiment. However, surprisingly, we did not detect that any of these trade-offs affected to the fermentation kinetics, which it is a complex metabolic process in which many of the stresses tested are acting against the yeast cells.

We also determined the intracellular lysine content of strains 7 and P14 at the same time points during fermentation. Strain 7 accumulated significantly more lysine (around 25 mg/g DW) than WT P14 (approximately 1 mg/g DW) growing in SM. However, this amount is slightly lower than that observed in lab medium SD. This lysine was subsequently released into the external medium throughout the fermentation process (Fig. 5B).

Consequently, since the evolved strains demonstrate good fermentation performance, they could, in principle, be used as active dry yeast (ADY). However, our primary goal is to develop them as inactive dry yeast (IDY) to enrich natural grape must with lysine, which is a limiting amino acid in must, as well as other amino acids and reduced glutathione. These strains also accumulate these substances in excess.

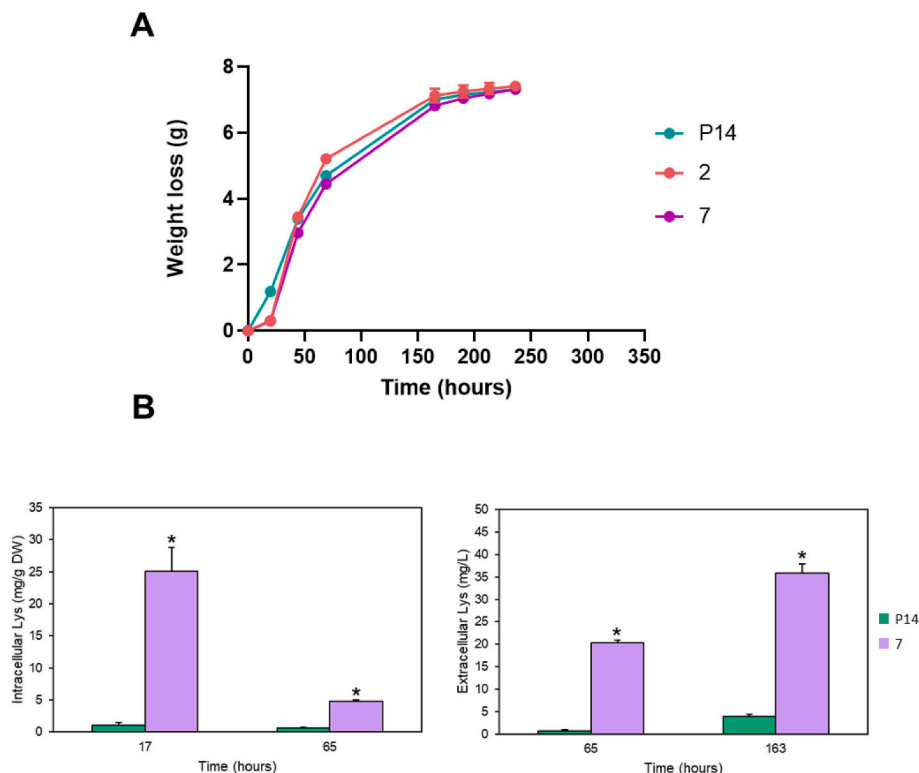


Fig. 5. AEC-resistant evolved colonies fermented similarly to the parental strain in synthetic must but altered intracellular lysine production. **A** Fermentation kinetics of the parental strain P14 and the evolved colonies 2 and 7 followed as weight loss (g). Data points represent the mean of three independent fermentations. Error bars (smaller than the symbols) indicate SD. **B** Intracellular (left) and extracellular (right) lysine content produced by the P14 and the evolved clone 7. Values are expressed as mean $\pm$ standard deviation, $n = 3$ biologically independent cultivations. *Significant differences ($p \leq 0.05$) compared to parental strain P14.

3.4. AEC adaptation is associated with extensive copy-number alterations and sequence variation

To identify beneficial mutations for the observed increased lysine accumulation in the evolved strains, we sequenced the parental strain P14 and the evolved colonies 2 and 7. The approach for analysing both genomes involved mapping the sequenced reads to the S288c reference genome. This method is effective for detecting SNPs, duplications, and deletions, but it assumes that the genomes of the strains being studied are collinear with the S288c reference. As a result, potential structural variations, such as translocations and inversions, may go undetected. After sequencing the three strains, their estimated coverage values relative to the S288c reference genome were $81.2\times$, $101.5\times$ and $72.1\times$, in P14, 2 and 7 strains, respectively.

Analysis of SNV frequency from genome sequencing data (Fig. 6), compared to the S288c reference strain, revealed that the P14 parental (green) strain is diploid with a high level of heterozygosity across the genome. However, large regions, including the left arms of chromosomes VI, IX and X, as well as the right arm of chromosome XV, exhibited loss of heterozygosity (LOH). The presence of LOH regions in the P14 parental strain is further supported by coverage values obtained from aligning P14's sequenced reads (green) to the S288c reference, showing consistent coverage in LOH regions and the rest of the chromosome (Fig. S5).

The sequence analysis of the evolved strains 2 and 7, including single nucleotide variants (SNVs) frequency and average coverage read depth,

revealed genomic alterations consistent with chromosome copy-number changes, segmental duplications inferred from read-depth analysis and numerous SNPs and indels (Fig. 6). Notably, strain 7 acquired additional copies of chromosomes II, IV, V, VII, XII, XIII, and XVI, along with segmental duplications in chromosome XI. In evolved strain 2, extra copies of chromosomes II, V, VII, XV, and XVI were observed, along with segmental duplications in chromosomes IV and XI. Curiously, most of the aneuploidies were shared between both independently evolved strains.

The approach used to infer segmental duplications confirmed that these regions are indeed duplicated in the evolved strains compared to the P14 parental strain. Although Fig. 6 shows the duplicated regions in their original chromosomal positions, their precise locations in the evolved genomes remain uncertain. These results agree with the ploidy analysis by flow cytometry (Table 1) where the P14 strain was a perfect $2n$, while the evolved strains had a higher ploidy value between $2n$ and $3n$.

Although consequences of aneuploidy are usually detrimental for strain growth (Mangado et al., 2018), previous studies have suggested that increase ploidy levels can be a faster method for adapting to new environments (Gilchrist & Stelkens, 2019; Todd et al., 2017) and provides an opportunity for heterozygous gene changes (Turunen et al., 2009). The numerous aneuploidies reported in yeast evolution experiments further highlight their role as an adaptation mechanism in harsh conditions, such as low nutrient availability (Gresham et al., 2008; Hong & Gresham, 2014; Selmecki et al., 2015; Sunshine et al., 2015), high

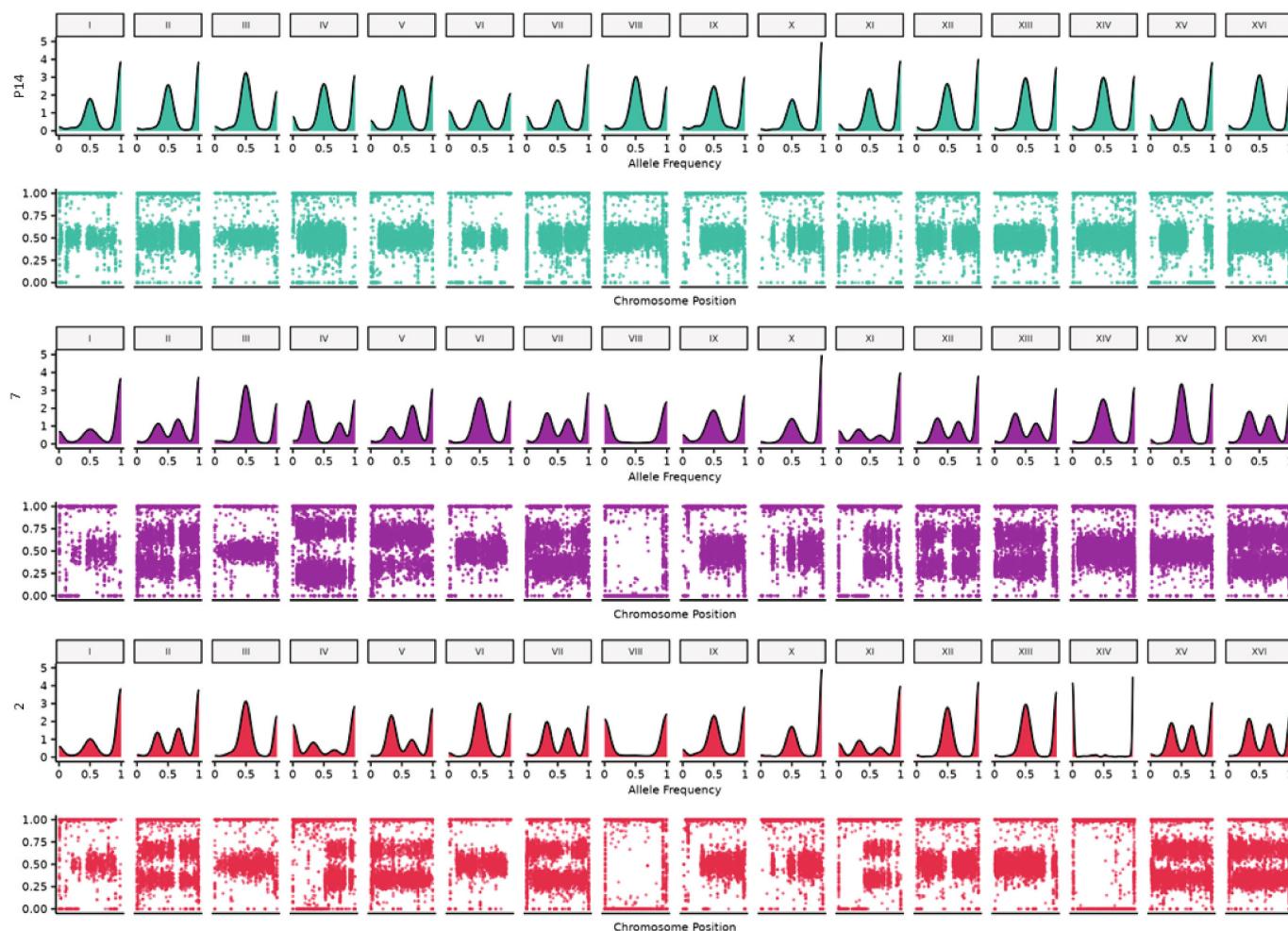


Fig. 6. Genome structure of the strains, P14 (green), 7 (purple) and 2 (red). SNP frequencies in each chromosome for the parental and evolved strains. Frequency distribution shows the different ploidies and aneuploidies present. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

DNA content of the parental and evolved strains.

Strains	DNA content
BY4741	1.07 ± 0.09
BY4743	2.18 ± 0.05
P14	2.14 ± 0.11
Evolved 2	2.73 ± 0.05*
Evolved 7	2.69 ± 0.10*

Values expressed as mean ± standard deviation. *

Indicates significantly different values (ANOVA and Tukey HSD test, $\alpha = 0.05$, $n = 2$) compared to the parental strain.

ethanol concentrations (Lairón-Peris et al., 2020; Morard et al., 2019; Voordeckers et al., 2015), extreme temperatures (Caspeta et al., 2014; García-Ríos et al., 2021; Huang et al., 2018; López-Malo et al., 2015; Yona et al., 2012), flocculation (Hope et al., 2017), and telomerase insufficiency (Millet et al., 2015). In our study, we observed a complete duplication of several complete chromosomes (trisomy) and some segmental duplications (or complete duplications+LOH) in the evolved strains, that probably are intrachromosomal tandem duplications or transpositions located in distant parts of the chromosomes (Achaz et al., 2000; Koszul et al., 2006).

After independent variant calling against the S288c reference genome, the three variant datasets were directly compared. Variants present in P14 were considered part of the parental genetic background, whereas variants detected exclusively in the evolved isolates were classified as mutations acquired during adaptive evolution. Genome sequencing analysis also revealed a huge number of SNVs in the evolved strains compared to the parental strain P14 (Table S6). The observed 5436 SNVs and 1450 indels in the genome of the evolved strains together with the great number of aneuploidies give us an idea of the mutagenic power of the lysine analogue AEC. However, strikingly enough was that most of the mutations were shared by both evolved strains. 4201 of the observed SNVs were shared among both evolved strains and only 1336 and 1349 changes were exclusive in strains 2 and 7, respectively (Fig. S6). When we look at the nature of these common changes, especially in SNVs, we observe a high proportion (16.21%) of synonymous changes with a potential low impact (synonymous variant). In contrast, 29.39% of the changes do represent a change of amino acid in proteins (Fig. S6). There are also other changes with a high impact in a smaller percentage (Table S6 and Fig. S6), such as insertions and deletions, which represent a change in the gene reading pattern (frameshift variant) or truncated proteins due to the production of a stop codon (stop gained). Regarding the changes that are exclusive of each evolved strain, 6.96 and 8.00% were synonymous variants in the strains 2 and 7, respectively, while 5.91 and 5.55% represent an amino acid change in

the strains 2 and 7, respectively.

Even more interesting for us, if we look in more detail at the changes affecting lysine metabolism genes, we observed that 14 out of 15 SNVs detected that involved a change in amino acid in the protein (missense variant) were shared between both evolved strains (Table 2). The high proportion of shared variants suggests that the independently evolved isolates converged on a similar genomic configuration under AEC selection. However, because short-read sequencing cannot resolve the precise evolutionary history of these variants, these observations should not be interpreted as definitive evidence of parallel acquisition of identical mutations.

3.5. Genome sequencing identified a key mutation in *LYS21* gene

Nonetheless, beyond the genomic general landscape, we were interested in the changes in lysine metabolism genes and those 14 non-synonymous mutations observed in both evolved strains, which impacted in the amino acid composition of 9 genes (Table 2). To unveil the impact of these SNVs in the accumulation of lysine, we selected the mutations observed in key genes of the lysine biosynthesis, *LYS1*, *LYS2*, *LYS21*, and *LYS80* (*MKS1*) (Fig. 7A), to be introduced in the parental strain P14 by the genome-editing tool CRISPR-Cas9. Selected mutations identified by whole-genome sequencing were independently validated by PCR amplification of the corresponding genomic regions followed by Sanger sequencing of genomic DNA from the evolved isolates. A total of six non-synonymous SNVs were individually tested in the parental P14 in terms of AEC tolerance (indirect phenotyping of lysine accumulation) (Table 2). Of all the SNPs analysed, point mutation *LYS21* (V127F) was the only change that showed a clear tolerance to toxic concentrations of AEC (Fig. 7B and Fig. S7). This tolerance was slightly weaker than that of the evolved strains 7 and 2, but was still clearly significant in comparison with WT P14. The rest of the punctual changes analysed showed the same phenotype as WT P14. Furthermore, determination of intracellular lysine and intracellular amino acids in the strain with the *LYS21* change (V127F) (Fig. 7C) showed a higher accumulation than the parental strain P14, although lower than the evolved strain 7, so the higher accumulation in the evolved strain indicated that this lysine accumulation phenotype must be the product of a combination of other unvalidated mutations acting synergistically with the change in the *LYS21* gene (V127F). Nevertheless, *LYS21*^{V127F} should be considered the main contributor to this phenotype, but not the sole or primary cause of lysine overproduction.

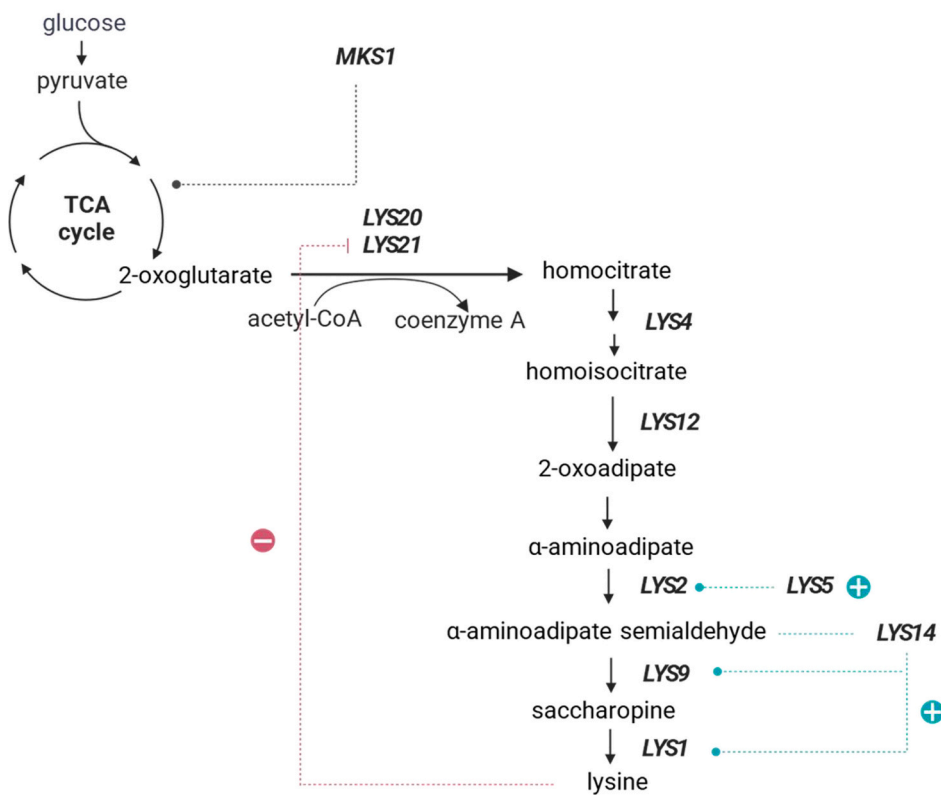
Yeasts use the α -amino adipate (AAA) pathway for lysine biosynthesis that involves eight enzyme-catalysed steps (Zabriskie & Jackson, 2000). In *S. cerevisiae* and other fungi, this pathway is regulated both genetically and biochemically (Ramos et al., 1988). Homocitrate synthase (HCS), the first enzyme in the AAA pathway, catalyses the transfer

Table 2

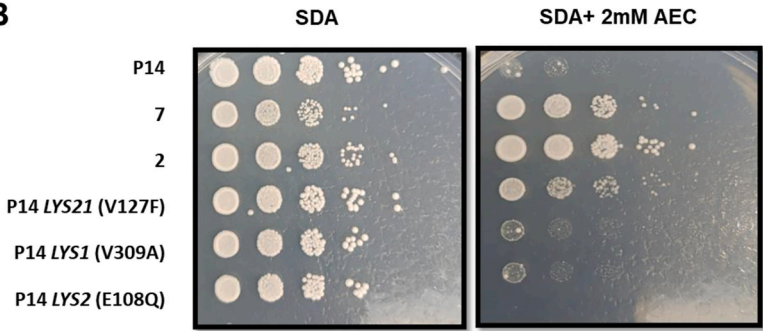
Single Nucleotide Polymorphisms (SNVs) of the 2 and 7 evolved strains that were located in coding sequences of the lysine metabolism.

Strains	Chromosome	Position	WT	Evolved	Gene name	Systematic name	Amino acid change	Evaluated in this study
2, 7	II	473,605	G	C	<i>LYS2</i>	YBR115C	E108Q	Yes
2, 7	IV	227,771	G	T	<i>LYS21</i>	YDL131W	V127F	Yes
2, 7	IV	228,626	C	T	<i>LYS21</i>	YDL131W	F412L	Yes
2, 7	IX	419,811	G	A	<i>LYS1</i>	YIR034C	A309V	Yes
2, 7	XIV	484,786	C	T	<i>MKS1</i>	YNL076W	N237F	Yes
2, 7	XIV	485,281	C	T	<i>MKS1</i>	YNL076W	R756C	Yes
2, 7	VII	215,394	C	T	<i>LYS5</i>	YGL154C	V/I	No
2, 7	VII	215,826	C	T	<i>LYS5</i>	YGL154C	D/N	No
2, 7	VII	264,137	A	G	<i>SNT2</i>	YGL131C	F/L	No
2, 7	VII	264,746	C	T	<i>SNT2</i>	YGL131C	G/S	No
2, 7	VII	265,689	C	G	<i>SNT2</i>	YGL131C	R/S	No
2, 7	XIII	138,066	G	A	<i>ITT1</i>	YML068W	V/M	No
2, 7	XIII	735,372	G	A	<i>PEP5</i>	YMR231W	G/S	No
2, 7	XV	800,337	A	G	<i>APC5</i>	YOR249C	M/T	No
7	IV	429,269	G	A	<i>SLX5</i>	YDL013W	R/K	No

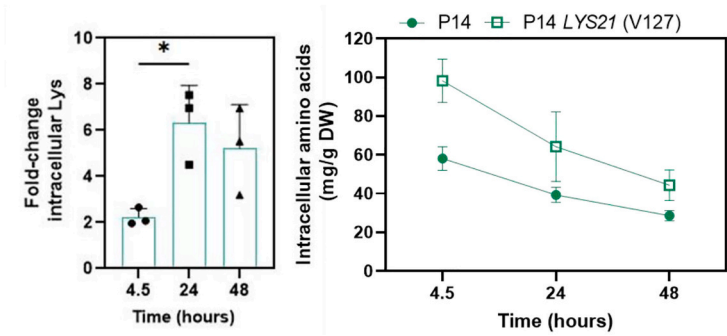
A



B



C



(caption on next page)

Fig. 7. **A** Overview of the lysine biosynthetic pathway in yeast *S. cerevisiae*. Regulatory features highlighted in this figure include feedback inhibition of homocitrate synthase (Lys21p and Lys20p) by lysine shown in red dotted lines. Induction of Lys14p by α -aminoadipate semialdehyde, which activates the expression of lysine biosynthetic genes, and conversion of the inactive apo-form of α -aminoadipate reductase (Lys2p) into its active form via post-translational phosphopantetheinylation by Lys5p shown in cyan dotted lines. Mks1p, indicated by grey dotted lines, indirectly modulates central carbon metabolism through its effects on the 2-oxoglutarate pool, thereby influencing lysine biosynthetic flux. **B** Evaluation of the AEC resistance phenotype for the parental strain P14, the evolved clones 7 and 2, and the reverse engineered mutations *LYS21* (V127F), *LYS1* (V309A) and *LYS2* (E108Q) in the P14 parental strain. OD₆₀₀ of each strain were adjusted to 1 and then serially diluted culture (from 10⁻¹ to 10⁻⁴). A volume of 2 μ L was spotted on SD (untreated) or SD containing 2 mM AEC plates which were incubated at 30 °C for 48 h. **C** Fold-change in intracellular lysine content for the reverse engineered P14 *LYS21* (V127F) compared to the parental strain P14 at different time points of growth in SD (left) and intracellular amino acids content for the reverse engineered P14 *LYS21* (V127F) compared to the parental strain P14 (right). For amino acid extraction an equivalent of 5 units of OD₆₀₀ were used and the amount of total amino acids is represented as mg per g of dry weight. Values are expressed as mean $\pm$ standard deviation, $n = 3$ biologically independent cultivations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of an acetyl group from acetyl coenzyme A to 2-oxoglutarate (2-OG), producing homocitrate. This reaction is the rate-limiting step in lysine

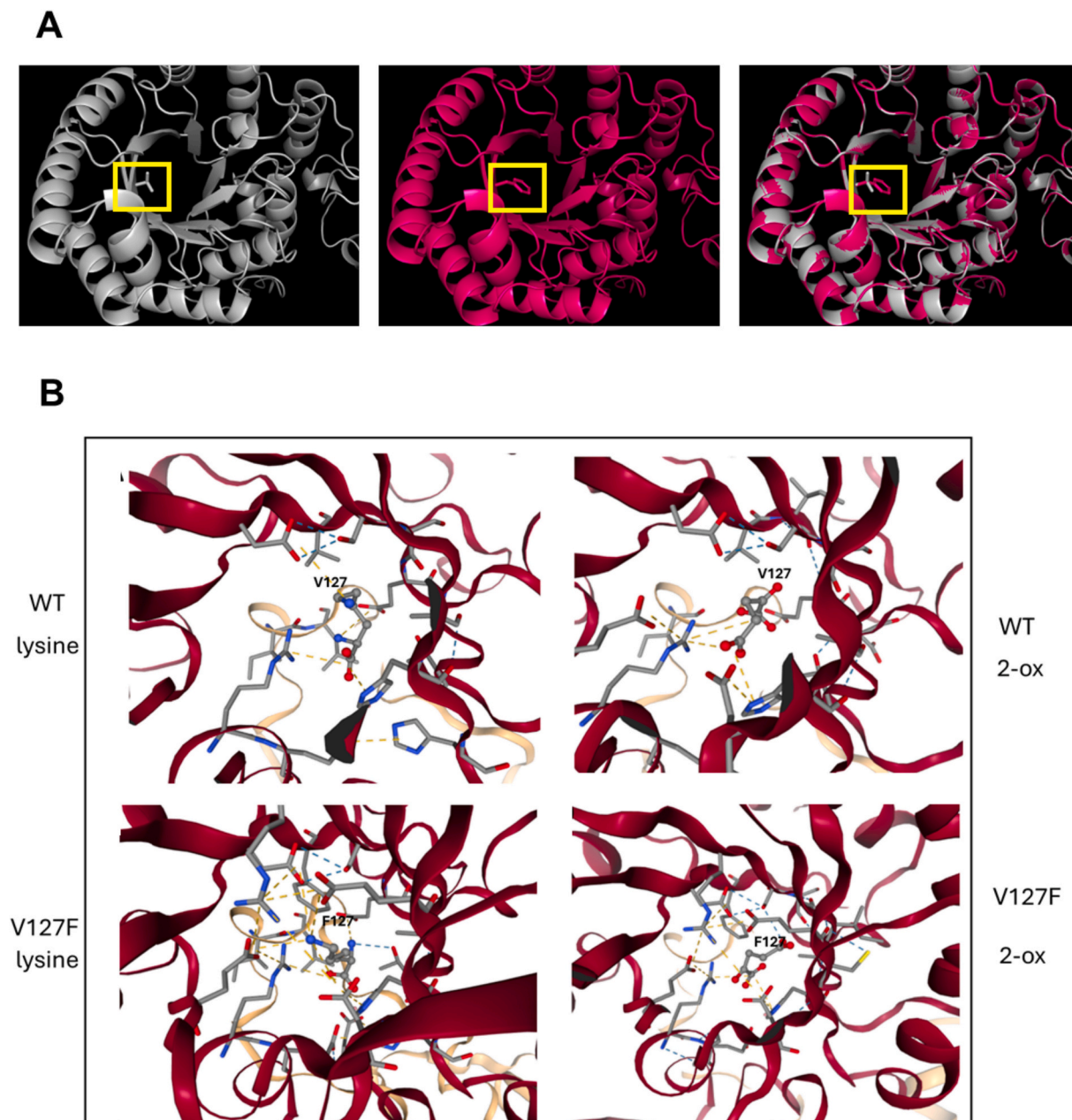


Fig. 8. **Structural modeling and docking analysis of Lys21p WT and V127F variant.** **A** Predicted three-dimensional structure of homocitrate synthase Lys21p in its wild-type form (WT, grey) and the evolved variant (V127F, pink), generated by homology modeling using SWISS-MODEL. The yellow box highlights the region containing the V127F mutation. **B** Catalytic pocket showing molecular docking results for wild type (WT) and *LYS21* V127F proteins in complex with lysine (left) or 2-oxoglutarate (2-ox) (right). Interactions between ligands and active-site residues are depicted, highlighting the position of residue 127. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biosynthesis in *S. cerevisiae*, with lysine acting as a feedback inhibitor of HCS activity (Isogai et al., 2021). In *S. cerevisiae*, two homologs of HCS, *LYS20* and *LYS21*, are encoded in the genome and are similarly inhibited by lysine. Although these homologs share over 95% amino acid sequence identity, their K_i values for lysine differ significantly (550 μ M for *LYS20* and 53 μ M for *LYS21*), indicating that *LYS21* is more sensitive to lysine feedback inhibition than *LYS20* (Quezada et al., 2008).

Fig. 8A shows the predicted structure for Lys21p, WT (A, grey) and evolved (B, pink), whose structure has been modelled using SwissModel. The substrate recognition mechanism of homocitrate synthase has been extensively studied in different organisms (Okada et al., 2010). By aligning the sequences of Homocitrate Synthase from *Thermus thermophilus* and Lys21, we observe that the amino acids involved in the binding of α -KG or lysine in yeast are: His226, Arg45, Arg165, Glu46, Thr199, His228, Ser167, Ala197, Glu224. The substitution of valine with phenylalanine at position 127 of *LYS21* may lead to the desensitization of feedback inhibition by lysine likely due to an increase in the affinity of 2-OG. To test this hypothesis, the tertiary structures of WT *LYS21* and *LYS21*^{V127F} variant were predicted using a homology model of *LYS21* (bound to lysine) as a template through the SWISS-MODEL repository (Waterhouse et al., 2018). We used AutoDock Vina via the SwissDock server to perform docking of both structures (Bugnon et al., 2024). Table 3 shows the average calculated affinities of both substrates, 2-OG and lysine, for each of the WT *LYS21* and *LYS21*^{V127F} variant models. Regarding the binding of lysine, we observe that the calculated affinity is lower in the case of WT *LYS21* (-5.181 ± 0.1045 kcal/mol) than for its mutated version *LYS21*^{V127F} (-4.651 ± 0.107 kcal/mol), indicating a lower affinity for lysine. In the case of 2-OG, we also observe a similar effect in the affinity predictions, showing lower values for the WT *LYS21* (-4.946 ± 0.085) than for the variant *LYS21*^{V127F} (-4.424 ± 0.109 kcal/mol). In both cases, the affinity of 2-OG is lower than that of lysine, which does not allow us to explain the inhibition of feedback based on a change in substrate affinities. Fig. 8B represents the structure of each protein with its respective ligand, indicating that the docking prediction is made in the appropriate catalytic pocket and how affect the mutation V127F to the catalytic pocket. It is important to note that docking-derived binding affinities are computational estimates that do not capture enzyme kinetics, conformational dynamics, or allosteric effects, and therefore cannot by themselves establish changes in enzymatic activity or in lysine-mediated feedback inhibition. The analysis presented should accordingly be interpreted as a preliminary structural hypothesis rather than as mechanistic evidence.

Previous works have also found lysine-overproducing strains derived from the AEC-resistant mutants, and amino acid changes have been identified in both *LYS20* and *LYS21* of these mutants (Feller et al., 1999; Garrigós et al., 2024; Gasent-Ramírez & Benítez, 1997). Nevertheless, this is the first time that this specific mutation has been identified as the primary cause of intracellular lysine accumulation. Moreover, in order to check the universality of this change in other genetic backgrounds, we introduced the change *LYS21*^{V127F} in other three commercial wine strains: P23, P6 and P4 (Lallemend Inc.). As a result of the genomic modification, the wine strains, which initially could not grow on AEC, became resistant to the lysine analogue and exhibited robust growth across various concentrations of the analogue (Fig. S8). Additionally, the three modified strains accumulated higher levels of lysine, as well as other amino acids such as glutamate, glutamine, and ornithine (Fig. 9 B, C), in comparison to the parental strain without V127F change.

Table 3

Calculated affinity values (kcal/mol) for each ligand (α -KG or lysine) for each enzyme (WT Lys21p and Lys21p^{V127F}).

	WT Lys21p	Lys21p ^{V127F}
Lysine	-5.181 ± 0.104	-4.651 ± 0.107
2-OG	-4.946 ± 0.085	-4.424 ± 0.109

Remarkably spectacular was the accumulation of lysine in strain P6, which reached 30 mg/g dry weight. Again, however, none of them reached the amount accumulated by the evolved strain 7, where different mutations together with the genetic background may be acting synergistically to accumulate lysine and other amino acids. To assess the competitiveness of the modified allele in wine fermentation, both *LYS21* versions (WT and V127F) were co-inoculated in SM to perform a competition assay. As can be seen in Fig. S9, the engineered versions of the wine yeast tested were able to outcompete their WT from middle fermentation to the end of the process, with a total displacement of the WT strain. This result highlights as a punctual mutation in a key gene of yeast metabolism can lead to an improved phenotype for industrial applications. Moreover, this CRISPR-engineered strain did not show some of the trade-offs previously observed in the AEC-evolved strains (data not shown).

4. Conclusions

Lysine is an essential amino acid for humans and protects various organisms from multiple stresses, such as freezing, oxidation and high temperatures combined with dryness (Isogai et al., 2021). Industrially produced lysine is widely used as a feed additive to ensure livestock receive sufficient lysine, promoting good growth, improving meat quality, and boosting animal immunity. Moreover, we evidenced an unexpected role of lysine for yeast metabolism during wine fermentation (Su et al., 2019), a limited-amino acid in grape must (Novo et al., 2013). Therefore, the availability of these essential microorganisms during fermentation processes can be very useful for improving the stress resistance of wine yeast in a harsh environment.

Previous genetic and metabolic engineering strategies have successfully increased lysine production in *S. cerevisiae*, with approaches falling into two categories: rational enzyme design and selection-based mutagenesis using toxic lysine analogues. In this study, we successfully generated non-recombinant *S. cerevisiae* wine strains that were able to accumulate lysine at higher levels through adaptive laboratory evolution, involving the use of increasing concentrations of the toxic analogue AEC. The evolved strains displayed intracellular lysine levels over ten times higher than the parental strain, alongside the concurrent accumulation of related amino acids.

Furthermore, this lysine accumulation has other metabolic consequences, including significantly increased levels of total and reduced glutathione, which correlate with enhanced tolerance to oxidative stress. Our results somehow showed that lysine supplementation alone provides strong antioxidant protection, which reinforces its role as a key modulator of cellular redox balance (Olin-Sandoval et al., 2019). Of particular interest was the finding that lysine uptake from the medium was also critical for SO₂ tolerance, emphasising that extracellular lysine may play a more decisive role than intracellular biosynthesis in certain stress responses.

Genome sequencing revealed extensive mutational landscapes in the evolved strains, comprising widespread single nucleotide variants (SNVs), insertions and deletions (indels), segmental duplications, and multiple aneuploidies. Notably, the majority of these genomic alterations, including those affecting lysine metabolic genes, were shared between independently evolved lines, indicating robust parallel evolutionary trajectories under AEC selection. The *LYS21*^{V127F} mutation, which emerged as a major determinant of lysine overproduction and AEC tolerance, was among these changes. Its functional relevance was validated by CRISPR editing in the parental strain, as well as by its transferability to other commercial wine strains. This punctual change consequently improved their lysine, and other amino acids, accumulation capacity and competitive fitness in the four wine strains tested.

Overall, our results demonstrate that ALE using AEC is an effective approach for producing yeast strains that exhibit enhanced lysine biosynthesis and altered amino acid metabolism, as well as increased redox resilience. Our results also identify *LYS21*^{V127F} as a key

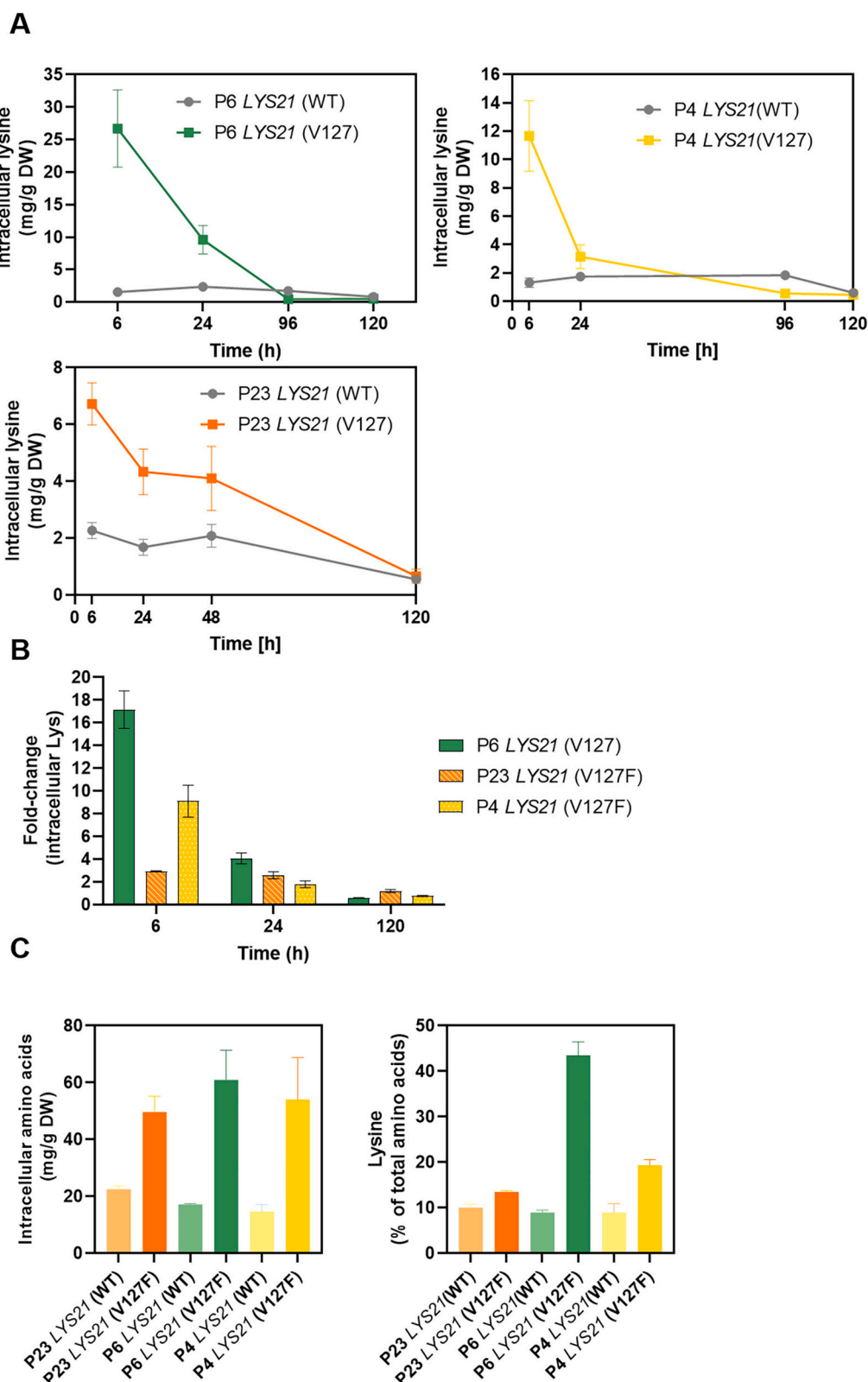


Fig. 9. A Intracellular lysine levels measured at multiple time points in the wine strains P6 (green), P23 (orange), and P4 (yellow). For each strain, the WT *LYS21* is represented by circles and the *LYS21* (V127) mutant by squares. All cultures were grown in SD medium, and 5 OD_{600s} were used for intracellular lysine extraction. Lysine content is represented as mg/g of dry weight. Values are expressed as mean $\pm$ standard deviation, $n = 3$ biologically independent cultivations. Where not visible, error bars are smaller than the symbols. B Fold-change levels on intracellular lysine concentration for *LYS21* (V127F) in the wine yeast strains P23, P6 and P4 in comparison for the respectively parental strains with *LYS21* WT. Values are expressed as mean $\pm$ standard deviation, $n = 3$ biologically independent cultivations C Intracellular amino acids concentration (left) and percentage of lysine from the total of amino acids (right) for the wine yeast strains P23, P6 and P4 with WT *LYS21* or *LYS21* (V127F) at 6 h. Values are expressed as mean $\pm$ standard deviation, $n = 3$ biologically independent cultivations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evolutionary target with broad applicability across industrial *S. cerevisiae* backgrounds. These findings open up new possibilities for developing strains for use in winemaking and the biotechnological industry. However, further validation and scaling up processes are needed to ensure their future industrial application.

CRediT authorship contribution statement

Sara Muñiz-Calvo: Writing – original draft, Visualization, Methodology, Investigation. **Elena Valera-García:** Methodology, Investigation. **Judit Pardo:** Methodology. **María Vázquez:** Investigation, Formal analysis. **Andrés Planells-Cárcel:** Validation, Methodology. **Javier Alonso-del-Real:** Data curation. **Ying Su:** Methodology, Investigation. **José María Heras:** Project administration, Funding acquisition. **Anne Ortiz-Julien:** Visualization, Project administration, Funding acquisition. **Estéfani García-Ríos:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **José M. Guillamón:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2026.120791>.

Data availability

The primary datasets used in the preparation of this manuscript and their analyses are openly available in Digital CSIC (<http://hdl.handle.net/10261/403811>)

References

- Achaz, G., Coissac, E., Viari, A., & Netter, P. (2000). Analysis of intrachromosomal duplications in yeast *Saccharomyces cerevisiae*: A possible model for their origin. *Molecular Biology and Evolution*, 17, 1268–1275. <https://doi.org/10.1093/oxfordjournals.molbev.a026410>
- Andrews, S. (2010). *FastQC: A quality control tool for high throughput sequence data*. <https://www.Bioinformatics.Babraham.Ac.Uk/Projects/Fastqc/>.
- Barbosa, C., Mendes-Faia, A., & Mendes-Ferreira, A. (2012). The nitrogen source impacts major volatile compounds released by *Saccharomyces cerevisiae* during alcoholic fermentation. *International Journal of Food Microbiology*, 160, 87–93. <https://doi.org/10.1016/j.ijfoodmicro.2012.10.003>
- Barrick, J. E., & Lenski, R. E. (2013). Genome dynamics during experimental evolution. *Nature Reviews Genetics*, 14, 827–839. <https://doi.org/10.1038/nrg3564>
- Bely, M., Sablayrolles, J.-M., & Barre, P. (1990). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *Journal of Fermentation and Bioengineering*, 70, 246–252. Agenbach, W. A., Proc. South Afr. Soc. Enol. Vitic.
- Bienert, S., Waterhouse, A., De Beer, T. A. P., Tauriello, G., Studer, G., Bordoli, L., & Schwede, T. (2017). The SWISS-MODEL repository-new features and functionality. *Nucleic Acids Research*, 45, D313–D319. <https://doi.org/10.1093/nar/gkw1132>
- Bigey, F., Segond, D., Friedrich, A., Guezennec, S., Bourgaïs, L., ... Sicard, D. (2021). Evidence for two Main domestication trajectories in *Saccharomyces cerevisiae* linked to distinct bread-making processes. *Current Biology*, 31, 722–732. e5. <https://doi.org/10.1016/j.cub.2020.11.016>
- Bisquert, R., Muñiz-Calvo, S., & Guillamón, J. M. (2018). Protective role of intracellular melatonin against oxidative stress and UV radiation in *Saccharomyces cerevisiae*. *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.00318>
- Boyd, D. A., Cvitkovitch, D. G., & Hamilton, I. R. (1995). Sequence, expression, and function of the gene for the nonphosphorylating, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus mutans*. *Journal of Bacteriology*, 177, 2622–2627. <https://doi.org/10.1128/jb.177.10.2622-2627.1995>
- Bugnon, M., Röhrig, U. F., Goullieux, M., Perez, M. A. S., Daina, A., Michielin, O., & Zoete, V. (2024). SwissDock 2024: Major enhancements for small-molecule docking with attracting cavities and autoDock vina. *Nucleic Acids Research*, 52, W324–W332. <https://doi.org/10.1093/nar/gkac300>
- Caspeta, L., Chen, Y., Ghiaci, P., Feizi, A., Baskov, S., Hallström, B. M., ... Nielsen, J. (2014). Altered sterol composition renders yeast thermotolerant. *Science*, 346, 75–78. <https://doi.org/10.1126/science.1258137>
- Caspeta, L., & Nielsen, J. (2015). Thermotolerant yeast strains adapted by laboratory evolution show trade-off at ancestral temperatures and preadaptation to other stresses. *mBio*, 6, Article e00431–15. <https://doi.org/10.1128/mBio.00431-15>
- Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, 34, i884–i890. <https://doi.org/10.1093/bioinformatics/bty560>
- Crépin, L., Nidelet, T., Sanchez, I., Dequin, S., & Camarasa, C. (2012). Sequential use of nitrogen compounds by *Saccharomyces cerevisiae* during wine fermentation: A model based on kinetic and regulation characteristics of nitrogen permeases. *Applied and Environmental Microbiology*, 78, 8102–8111. <https://doi.org/10.1128/AEM.02294-12>
- Danecek, P., Bonfield, J. K., Liddle, J., Marshall, J., Ohan, V., Pollard, M. O., ... Davies, R. M. (2021). Twelve years of SAMtools and BCFtools. *GigaScience*, 10. <https://doi.org/10.1093/gigascience/giab008>
- Davidson, J. F., & Schiestl, R. H. (2001). Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 21, 8483–8489. <https://doi.org/10.1128/mcb.21.24.8483-8489.2001>
- Farrugia, G., & Balzan, R. (2012). Oxidative stress and programmed cell death in yeast. *Frontiers in Oncology*, 2 JUN. <https://doi.org/10.3389/fonc.2012.00064>
- Feller, A., Ramos, F., Piérard, A., & Dubois, E. (1999). In *Saccharomyces cerevisiae*, feedback inhibition of homocitrate synthase isoenzymes by lysine modulates the activation of LYS gene expression by Lys14p. *European Journal of Biochemistry*, 261, 163–170. <https://doi.org/10.1046/j.1432-1327.1999.00262.x>
- García-Ríos, E., Lairón-Peris, M., Muñiz-Calvo, S., Heras, J. M., Ortiz-Julien, A., Poirot, P., ... Guillamón, J. M. (2021). Thermo-adaptive evolution to generate improved *Saccharomyces cerevisiae* strains for cocoa pulp fermentations. *International Journal of Food Microbiology*, 342, Article 109077. <https://doi.org/10.1016/j.ijfoodmicro.2021.109077>
- García-Ríos, E., Morard, M., Parts, L., Liti, G., & Guillamón, J. M. (2017). The genetic architecture of low-temperature adaptation in the wine yeast *Saccharomyces cerevisiae*. *BMC Genomics*, 18, 159. <https://doi.org/10.1186/s12864-017-3572-2>
- García-Ríos, E., Pardo, J., Su, Y., & Guillamón, J. M. (2024). Different nitrogen consumption patterns in low temperature fermentations in the wine yeast *Saccharomyces cerevisiae*. *Foods*, 13, 2522. <https://doi.org/10.3390/foods13162522>
- Garrigós, V., Picazo, C., Matallana, E., & Aranda, A. (2024). Activation of the yeast retrograde response pathway by adaptive laboratory evolution with S-(2-aminoethyl)-L-cysteine reduces ethanol and increases glycerol during winemaking. *Microbial Cell Factories*, 23(1), 1–16. <https://doi.org/10.1186/s12934-024-02504-z>
- Gasent-Ramírez, J. M., & Benítez, T. (1997). Lysine-overproducing mutants of *Saccharomyces cerevisiae* baker's yeast isolated in continuous culture. *Applied and Environmental Microbiology*, 63, 4800–4806. <https://doi.org/10.1128/aem.63.12.4800-4806.1997>
- Gietz, R. D., & Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in Enzymology*, 350 (2001), 87–96. [https://doi.org/10.1016/S0076-6879\(02\)50957-5](https://doi.org/10.1016/S0076-6879(02)50957-5)
- Gilchrist, C., & Stelkens, R. (2019). Aneuploidy in yeast: Segregation error or adaptation mechanism? *Yeast*, 36, 525–539. <https://doi.org/10.1002/yea.3427>
- Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J., & André, B. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 27, 3065–3086. <https://doi.org/10.1128/mcb.01084-06>
- Gómez-Alonso, S., Hermosín-Gutiérrez, I., & García-Romero, E. (2007). Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoone derivatives in wine and beer samples. *Journal of Agricultural and Food Chemistry*, 55, 608–613. <https://doi.org/10.1021/jf062820m>
- Gresham, D., Desai, M. M., Tucker, C. M., Jenq, H. T., Pai, D. A., Ward, A., ... Dunham, M. J. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genetics*, 4(12). <https://doi.org/10.1371/journal.pgen.1000303>
- Griffith, O. W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Analytical Biochemistry*, 106.
- Grosdidier, A., Zoete, V., & Michielin, O. (2011). SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic Acids Research*, 39(Suppl. 2). <https://doi.org/10.1093/nar/gkr366>

- Gutiérrez, A., Chiva, R., Beltran, G., Mas, A., & Guillamon, J. M. (2013). Biomarkers for detecting nitrogen deficiency during alcoholic fermentation in different commercial wine yeast strains. *Food Microbiology*, 34, 227–237. <https://doi.org/10.1016/j.fm.2012.12.004>
- Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F. N., & Guillamon, J. M. (2012). Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. *Food Microbiology*, 31, 25–32. <https://doi.org/10.1016/j.fm.2012.02.012>
- Haase, S. B., & Reed, S. I. (2002). Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle*, 1, 117–121. <https://doi.org/10.4161/cc.1.2.114>
- Hong, J., & Gresham, D. (2014). Molecular specificity, convergence and constraint shape adaptive evolution in nutrient-poor environments. *PLoS Genetics*, 10(1). <https://doi.org/10.1371/journal.pgen.1004041>
- Hong, K. K., & Nielsen, J. (2013). Adaptively evolved yeast mutants on galactose show trade-offs in carbon utilization on glucose. *Metabolic Engineering*, 16, 78–86. <https://doi.org/10.1016/j.ymben.2013.01.007>
- Hope, E. A., Amorosi, C. J., Miller, A. W., Dang, K., Heil, C. S., & Dunham, M. J. (2017). Experimental evolution reveals favored adaptive routes to cell aggregation in yeast. *Genetics*, 206, 1153–1167. <https://doi.org/10.1534/genetics.116.198895>
- Huang, C., Lu, M., Chang, Y., & Li, W. (2018). Experimental evolution of yeast for high temperature tolerance. *Molecular Biology and Evolution*, 1–17. <https://doi.org/10.1093/molbev/msy077/4976544> (April 2018).
- Isogai, S., Matsushita, T., Imanishi, H., Koonthongkaew, J., Toyokawa, Y., Nishimura, A., Yi, X., Kazlauskas, R., & Takagi, H. (2021). High-level production of lysine in the yeast *Saccharomyces cerevisiae* by rational design of homocitrate synthase. *Applied and Environmental Microbiology*, 87, 1–12. <https://doi.org/10.1128/AEM.00600-21>
- Isogai, S., & Takagi, H. (2021). Enhancement of lysine biosynthesis confers high-temperature stress tolerance to *Escherichia coli* cells. *Applied Microbiology and Biotechnology*, 105, 6899–6908. <https://doi.org/10.1007/s00253-021-11519-0>
- Jacobus, A. P., Cavassana, S. D., de Oliveira, I. I., Barreto, J. A., Rohwedder, E., Frazzon, J., ... Gross, J. (2024). Optimal trade-off between boosted tolerance and growth fitness during adaptive evolution of yeast to ethanol shocks. *Biotechnology for Biofuels and Bioproducts*, 17, 63. <https://doi.org/10.1186/s13068-024-02503-7>
- Jazwinski, S. M. (2013). The retrograde response: When mitochondrial quality control is not enough. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1833(2), 400–409. Elsevier B.V. <https://doi.org/10.1016/j.bbamcr.2012.02.010>
- Kevvai, K., Kütt, M. L., Nisamedtinov, I., & Paalme, T. (2016). Simultaneous utilization of ammonia, free amino acids and peptides during fermentative growth of *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing*, 122, 110–115. <https://doi.org/10.1002/jib.298>
- Kozul, R., Dujon, B., & Fischer, G. (2006). Stability of large segmental duplications in the yeast genome. *Genetics*, 172, 2211–2222. <https://doi.org/10.1534/genetics.105.048058>
- Lage, P., Sampaio-Marques, B., Ludovico, P., Mira, N. P., & Mendes-Ferreira, A. (2019). Transcriptomic and chemogenomic analyses unveil the essential role of Com2-regulon in response and tolerance of *Saccharomyces cerevisiae* to stress induced by sulfur dioxide. *Microbial Cell*, 6, 509–523. <https://doi.org/10.15698/mic2019.11.697>
- Lairón-Peris, M., Pérez-Través, L., Muñiz-Calvo, S., Guillamón, J. M., Heras, J. M., Barrio, E., & Querol, A. (2020). Differential contribution of the parental genomes to a *S. cerevisiae* × *S. uvarum* hybrid, inferred by phenomic, genomic, and transcriptomic analyses, at different industrial stress conditions. *Frontiers in Bioengineering and Biotechnology*, 8, 1–20. <https://doi.org/10.3389/fbioe.2020.00129>
- Lenski, R. E. (2017). Experimental evolution and the dynamics of adaptation and genome evolution in microbial populations. *The ISME Journal*, 11, 2181–2194. <https://doi.org/10.1038/ismej.2017.69>
- Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv: Genomics, 1303.3997v2. doi: <https://doi.org/10.6084/M9.FIGSHARE.963153.V1>
- Lindsay, R. J., Holder, P. J., Hewlett, M., & Gudeli, I. (2024). Experimental evolution of yeast shows that public-goods upregulation can evolve despite challenges from exploitative non-producers. *Nature Communications*, 15, 7810. <https://doi.org/10.1038/s41467-024-52043-9>
- López-Malo, M., García-Rios, E., Melgar, B., Sanchez, M. R., Dunham, M. J., & Guillamón, J. M. (2015). Evolutionary engineering of a wine yeast strain revealed a key role of inositol and mannoprotein metabolism during low-temperature fermentation. *BMC Genomics*, 16, 537. <https://doi.org/10.1186/s12864-015-1755-2>
- Magasanik, B., & Kaiser, C. A. (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene*, 290, 1–18. [https://doi.org/10.1016/S0378-1119\(02\)00558-9](https://doi.org/10.1016/S0378-1119(02)00558-9)
- Mangado, A., Morales, P., Gonzalez, R., & Tronchoni, J. (2018). Evolution of a yeast with industrial background under winemaking conditions leads to diploidization and chromosomal copy number variation. *Frontiers in Microbiology*, 9, 1–12. <https://doi.org/10.3389/fmicb.2018.01816>
- Maurly, J., Germann, S. M., Baallal Jacobsen, S. A., Jensen, N. B., Kildegaard, K. R., Herrgård, M. J., ... Borodina, I. (2016). EasyCloneMulti: A set of vectors for simultaneous and multiple genomic integrations in *Saccharomyces cerevisiae*. *PLoS One*, 11. <https://doi.org/10.1371/journal.pone.0150394>
- McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernysky, A., ... DePristo, M. A. (2010). The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research*. <https://doi.org/10.1101/gr.107524.110>
- McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R. S., Thormann, A., ... Cunningham, F. (2016). The Ensembl variant effect predictor. *Genome Biology*, 17. <https://doi.org/10.1186/s13059-016-0974-4>
- Menegon, Y. A., Gross, J., & Jacobus, A. P. (2022). How adaptive laboratory evolution can boost yeast tolerance to lignocellulosic hydrolyses. *Current Genetics*, 68, 319–342. <https://doi.org/10.1007/s00294-022-01237-z>
- Millet, C., Ausiannikava, D., Le Bihan, T., Granneman, S., & Makovets, S. (2015). Cell populations can use aneuploidy to survive telomerase insufficiency. *Nature Communications*, 6. <https://doi.org/10.1038/ncomms9664>
- Morano, K. A., Grant, C. M., & Moye-Rowley, W. S. (2012). The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics*, 190, 1157–1195. <https://doi.org/10.1534/genetics.111.128033>
- Morard, M., Macías, L. G., Adam, A. C., Lairón-Peris, M., Pérez-Torrado, R., Toft, C., & Barrio, E. (2019). Aneuploidy and ethanol tolerance in *Saccharomyces cerevisiae*. *Frontiers in Genetics*, 10, 82. <https://doi.org/10.3389/FGENE.2019.00082>
- Novo, M., Mangado, A., Quirós, M., Morales, P., Salvadó, Z., & Gonzalez, R. (2013). Genome-wide study of the adaptation of *Saccharomyces cerevisiae* to the early stages of wine fermentation. *PLoS One*, 8. <https://doi.org/10.1371/journal.pone.0074086>
- Ohashi, M., Isogai, S., & Takagi, H. (2022). Functional analysis of feedback inhibition-insensitive variants of N-acetyl glutamate kinase found in sake yeast mutants with ornithine overproduction. *Microbiology Spectrum*, 10. <https://doi.org/10.1128/spectrum.00822-22>
- Okada, T., Tomita, T., Wulandari, A. P., Kuzuyama, T., & Nishiyama, M. (2010). Mechanism of substrate recognition and insight into feedback inhibition of homocitrate synthase from *Thermus thermophilus*. *Journal of Biological Chemistry*, 285, 4195–4205. <https://doi.org/10.1074/jbc.M109.086330>
- Okonechnikov, K., Conesa, A., & García-Alcalde, F. (2016). Qualimap 2: Advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics*, 32, 292–294. <https://doi.org/10.1093/bioinformatics/btv566>
- Olin-Sandoval, V., Yu, J. S. L., Miller-Fleming, L., Alam, M. T., Kamrad, S., Correia-Melo, C., ... Ralser, M. (2019). Lysine harvesting is an antioxidant strategy and triggers underground polyamine metabolism. *Nature*, 572, 249–253. <https://doi.org/10.1038/s41586-019-1442-6>
- Picazo, C., Matallana, E., & Aranda, A. (2018). Yeast thioredoxin reductase Trp1p controls TORC1-regulated processes. *Scientific Reports*, 8. <https://doi.org/10.1038/s41598-018-34908-4>
- Querol, A., Barrio, E., & Ramón, D. (1992). A comparative study of different methods of yeast strain characterization. *Systematic and Applied Microbiology*, 15, 439–446. [https://doi.org/10.1016/S0723-2020\(11\)80219-5](https://doi.org/10.1016/S0723-2020(11)80219-5)
- Quezada, H., Aranda, C., DeLuna, A., Hernández, H., Calcagno, M. L., Marín-Hernández, Á., & González, A. (2008). Specialization of the paralogue *LYS21* determines lysine biosynthesis under respiratory metabolism in *Saccharomyces cerevisiae*. *Microbiology*, 154, 1656–1667. <https://doi.org/10.1099/mic.0.2008/017103-0>
- Ramos, F., Dubois, E., & Piérard, A. (1988). Control of enzyme synthesis in the lysine biosynthetic pathway of *Saccharomyces cerevisiae*. *European Journal of Biochemistry*, 171, 171–176. <https://doi.org/10.1111/j.1432-1033.1988.tb13773.x>
- Rollero, S., Bloem, A., Ortiz-Julien, A., Camarasa, C., & Divol, B. (2018). Altered fermentation performances, growth, and metabolic footprints reveal competition for nutrients between yeast species inoculated in synthetic grape juice-like medium. *Frontiers in Microbiology*, 9. <https://doi.org/10.3389/fmicb.2018.00196>
- Selmecki, A. M., Maruvka, Y. E., Richmond, P. A., Guillet, M., Shores, N., Sorenson, A. L., ... Pellman, D. (2015). Polyploidy can drive rapid adaptation in yeast. *Nature*, 519, 349–352. <https://doi.org/10.1038/nature14187>
- Su, Y., Origone, A. C., Rodríguez, M. E., Querol, A., Guillamón, J. M., & Lopes, C. A. (2019). Fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions. *International Journal of Food Microbiology*, 291, 111–120. <https://doi.org/10.1016/j.jfoodmicro.2018.11.020>
- Su, Y., Seguinot, P., Sanchez, I., Ortiz-Julien, A., Heras, J. M., Querol, A., ... Guillamón, J. M. (2020). Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions. *Food Microbiology*, 85, Article 103287. <https://doi.org/10.1016/j.fm.2019.103287>
- Sunshine, A. B., Payen, C., Ong, G. T., Liachko, I., Tan, K. M., & Dunham, M. J. (2015). The fitness consequences of aneuploidy are driven by condition-dependent gene effects. *PLoS Biology*, 13, 1–34. <https://doi.org/10.1371/journal.pbio.1002155>
- Takeno, S., Murata, R., Kobayashi, R., Mitsuhashi, S., & Ikeda, M. (2010). Engineering of *Corynebacterium glutamicum* with an NADPH-generating glycolytic pathway for l-lysine production. *Applied and Environmental Microbiology*, 76, 7154–7160. <https://doi.org/10.1128/AEM.01464-10>
- Todd, R. T., Forche, A., & Selmecki, A. (2017). Ploidy variation in fungi: Polyploidy, aneuploidy, and genome evolution. *Microbiology Spectrum*, 5, Article FUNK-0051-2016. <https://doi.org/10.1128/microbiolspec.funk-0051-2016>
- Turunen, O., Seelke, R., & Macosko, J. (2009). In silico evidence for functional specialization after genome duplication in yeast. *FEMS Yeast Research*, 9, 16–31. <https://doi.org/10.1111/j.1567-1364.2008.00451.x>
- Voordeckers, K., Kominek, J., Das, A., Espinosa-Cantú, A., De Maeyer, D., Arslan, A., ... Verstrepen, K. J. (2015). Adaptation to high ethanol reveals complex evolutionary pathways. *PLoS Genetics*, 11, Article e1005635. <https://doi.org/10.1371/journal.pgen.1005635>
- Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., ... Schwede, T. (2018). SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Research*, 46, W296–W303. <https://doi.org/10.1093/nar/gky427>
- Weiß, C. L., Pais, M., Cano, L. M., Kamoun, S., & Burbano, H. A. (2018). nQuire: A statistical framework for ploidy estimation using next generation sequencing. *BMC Bioinformatics*, 19. <https://doi.org/10.1186/s12859-018-2128-z>
- Yamashoji, S., Al Mamun, A., & Bari, I. (2020). Cytotoxic effects of menadione on normal and cytochrome C-deficient yeast cells cultivated aerobically or anaerobically.

- Biochemistry and Biophysics Reports, 24. <https://doi.org/10.1016/j.bbrep.2020.100823>
- Yona, A. H., Manor, Y. S., Herbst, R. H., Romano, G. H., Mitchell, A., Kupiec, M., ... Dahan, O. (2012). Chromosomal duplication is a transient evolutionary solution to stress. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 21010–21015. <https://doi.org/10.1073/pnas.1211150109>
- Zabriskie, T. M., & Jackson, M. D. (2000). Lysine biosynthesis and metabolism in fungi. *Natural Product Reports*, 17, 85–97. <https://doi.org/10.1039/a801345d>