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Adaptive laboratory evolution for acetic acid-tolerance matches sourdough challenges with yeast phenotypes

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ABSTRACT

Acetic acid tolerance of *Saccharomyces cerevisiae* is an important trait in sourdough fermentation processes, where the accumulation of acid by the growth of lactic acid bacteria reduces the yeast metabolic activity. In this work, we have carried out adaptive laboratory evolution (ALE) experiments in two sourdough isolates of *S. cerevisiae* exposed to acetic acid, or alternatively to acetic acid and myriocin, an inhibitor of sphingolipid biosynthesis that sped-up the evolutionary adaptation. Evolution approaches resulted in acetic tolerance, and surprisingly, increased lactic susceptibility. Four evolved clones, one from each parental strain and evolutionary scheme, were selected on the basis of their potential for CO_2 production in sourdough conditions. Among them, two showed phenotypic instability characterized by strong lactic sensitivity after several rounds of growth under unstressed conditions, while two others, displayed increased constitutive acetic tolerance with no loss of growth in lactic medium. Genome sequencing and ploidy level analysis of all strains revealed aneuploidies, which could account for phenotypic heterogeneity. In addition, copy number variations (CNVs), affecting specially to genes involved in ion transport or flocculation, and single nucleotide polymorphisms (SNPs) were identified. Mutations in several genes, *ARG82*, *KEX1*, *CTK1*, *SPT20*, *IRA2*, *ASG1* or *GIS4*, were confirmed as involved in acetic and/or lactic tolerance, and new determinants of these phenotypes, *MSN5* and *PSP2*, identified.

1. Introduction

Sourdough-baking technology is experiencing an increased demand because it provides products with improved bread flavor and texture (De Vuyst et al., 2021), as well as reduced phytate, gluten and glycemic index (Gobbetti et al., 2019; Ribet et al., 2023). These properties rely mostly on the sourdough microbiota composed by stable associations of lactic acid bacteria (LAB) and yeasts.

Up to 40 different yeast species have been identified in sourdoughs collected worldwide (De Vuyst et al., 2017), although a given sourdough usually contains one prevailing yeast (Comasio et al., 2020), quite often *Kazachstania humilis* or *Saccharomyces cerevisiae*. It is assumed that the yeast species diversity is influenced by its interaction with LABs based on mutual relationships. A well-known example is the nutritional mutualism of *Fructilactobacillus sanfranciscensis* and *K. humilis* (Sieuwerts et al., 2018). Nevertheless, the nature of these interactions is influenced by a variety of factors, among them, the stress intrinsic tolerance of each species (De Vuyst et al., 2017). The weak acids produced by LABs, but also by acetic acid bacteria (AABs), reduce dough pH and generate toxicity promoting the exclusion in the mature sourdough of less-adapted community members (Carbonetto et al., 2020). For example, *K. humilis* strains appear to differ from *S. cerevisiae* strains by displaying higher sensitivity to low pH, but higher tolerance to acetate (Carbonetto et al., 2020). Indeed, CO_2 production rate measurements in flour-basis dough model systems indicate that the presence of acetic acid impairs drastically the leavening activity of *S. cerevisiae* but has no major effect on *K. humilis* gas performance (Sánchez-Adriá et al., 2023). Hence, acetic-tolerance of baker's yeast strains of *S. cerevisiae* is a trait that needs to be improved in order to ensure a high counting of this species in mature sourdoughs and their leavening activity in the baking process.

It is generally accepted that the toxicity of different weak acids relies on their undissociated form, which diffuses across the plasma membrane

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due to their high solubility in the phospholipids (PLs) fraction (Lindgren and Dobrogosz, 1990). This explains why reducing plasma membrane permeability by increasing lipid saturation provides tolerance to weak acids (Lindberg et al., 2013). In full agreement, the complex sphingolipids (SLs) content in the acetic-tolerant *Zygosaccharomyces bailii* is higher than in *S. cerevisiae* (Lindberg et al., 2013). In addition, the synthesis of these lipids increases in acetic acid exposed cells of both species (Guerreiro et al., 2016; Lindahl et al., 2016). Likewise, several sterol biosynthesis pathway genes have been identify as acetic acid-tolerance determinants and/or with increased transcript levels in response to acetic (Mira et al., 2010). Hence, strong evidence supports a causal relationship between lipid saturation and acid tolerance (Lindberg et al., 2013).

Here, we have induced genomic changes in two sourdough isolates of *S. cerevisiae* (S06 and S13) by adaptive laboratory evolution (ALE). The ALE approach allows developing highly specialized yeasts in technologically important properties on the basis of the natural selection of beneficial mutations under specified growth conditions. In addition, the technique is ease to implement, does not require any prior knowledge and provides basic information on molecular aspects of evolution and genotype–phenotype interconnections (Mavrommati et al., 2022; Fernandes et al., 2023). In our study, ALE was based on successive culture of yeast cells in medium with or without increased concentrations of acetic acid, a strategy that induces constitutive tolerance to different stresses (González-Ramos et al., 2016). In parallel, evolved clones were obtained by alternating cultivation cycles in the presence of acetic acid or myriocin, an effective ALE-driver to obtain evolved clones with increased SLs abundance (Randez-Gil et al., 2020). Our results demonstrate the powerful of our ALE strategies to induce acetic tolerance and to generate novel strains with higher performance in the sourdough environment. The evolved yeast populations have been characterised in deep and the results are discussed in terms of the mechanisms that govern the yeast response to acetic acid.

2. Materials and methods

2.1. Strains, media and culture conditions

Two *S. cerevisiae* strains, S06 and S13, isolated from two industrial sourdoughs of the baking company Europastry, S.A. (St. Cugat del Vallès, Barcelona, Spain) and characterized by their high leavening performance and stress tolerance (Sánchez-Adriá et al., 2023) were used as parental strains. In some experiments, the commercial *S. cerevisiae* strain L'Hirondelle (LH), produced by the Lesaffre Group (Lille, France), was used as control.

Previously described standard methods were followed for media preparation (Guthrie and Fink, 1991). Yeast cells were cultured at 30 °C and 200 rpm in YPD or SCD supplemented with the appropriate amino acid drop out (ForMedium). Myriocin at 1.5–3.0 μM final concentration was added from a stock solution of 2 mM (ethanol:DMSO; 80:20, v-v). Media containing increased concentrations of acetic acid were prepared by mixing the appropriate volume of filter-sterilized SCD and SCD containing 120 mM acetic acid, both adjusted to pH 4.0. Solid media contained 2% agar.

For plate phenotype experiments, cells were grown to the mid-exponential phase at 30 ºC (OD_{600} ~ 0.5). Then, 10-fold serial dilutions were spotted (3 μl) onto SCD agar plates lacking or containing lactic, acetic or a combination of both as indicated. Myriocin tolerance was checked in YPD plates. Colony growth was inspected after 2–4 days of incubation at 30 ºC.

2.2. Adaptive laboratory evolution

Evolution experiments were conducted using batch culture techniques in 250 ml Erlenmeyer flasks as previously described (Aguilera et al., 2010). SCD-grown pre-cultures of the two parental strains under study were used to inoculate 50 ml of liquid SCD medium containing 60 mM acetic acid (pH 4) at initial OD_{600} ~ 0.05. Cultivations were carried out at 30 ºC and 200 rpm for 24 h, after which, cells were washed with water (González-Ramos et al., 2016), transferred to the same medium lacking acetic acid at OD_{600} of 0.05, and grown for 24 h under the same conditions. This scheme of alternating cultivation cycles in the presence and absence of acetic acid was followed until the end of the evolution experiment. After a few cycles, as specify in Fig. 1A, the acid concentration was increased, first from 60 to 90 mM and then to 120 mM, when a consistent final OD_{600} of 4–5 (~ 5–6 generations) was obtained. When growth was delayed, at the highest acid concentrations, cultures were extended for 48 h in order to obtain the mentioned increase in cell density. Acetic acid/Myriocin-driven ALE (A/M) was conducted similarly except that cells were grown by alternating cultivation in SCD and YPD containing acetic acid and myriocin, respectively. Myriocin concentration was 1.5 μM (final concentration) at the beginning and was further increased to 3.0 μM (Fig. 1A) and maintained over the course of the experiment. Evolution experiments involved a total of at least 34–37 cultivations in acid-containing media, which allow attaining 200 generations evolved populations when the experiment was ended. Cells from 50, 100 and 150 generations evolved cultures were also sampled in order to characterize the gradual acquisition of stress tolerance and to select the best phenotypes. The number of generations in each cultivation was inferred from the equation n = 3.3 x log (final OD_{600} / initial OD_{600}), and the total number in evolved populations was calculated by summing generations in acid media. Samples from the evolved populations were taken, maintained as frozen (−80 °C) glycerol stocks and then rescued in YPD agar plates at 30 °C for 24 h before further analysis.

2.3. Selection of evolved clones

Cell populations of each yeast background and evolutionary line attained after 100, 150 and 200 generations were compared for acid tolerance and gassing rate under sourdough conditions with their parental counterparts, and those showing the best performance, $S06A_{100}$, $S06A/M_{100}$, $S13A_{150}$ and $S13A/M_{100}$ were chosen for screening individual clones. For this, cells of each population were cultivated in solid YPD, SCD-75 mM acetic acid or SCD-175 mM lactic acid plus 30 mM acetic acid, and 5 colonies from each medium, marked with subscripts 01–05 (YPD), 06–10 (acetic acid) and 11–15 (lactic plus acetic), were randomly picked. Cells from these colonies (60 in total; S06A01–15; S06A/M01–15; S13A01–15 and S13A/M01–15) were grown overnight in YPD and then inoculated into two replicates of 4.5 ml of SCD-4% glucose with 175 mM lactic and 30 mM acetic acid (pH 4.0). The initial weight of each tube was recorded and cultivation was carried out at 80 rpm and 30 ºC for 24 h after which the tubes were open and the final weight was measured. The weight difference was used as a gross estimation of the gassing production of each isolate under this cultivation conditions. From this assay, the best two evolved clones from each set were selected for further characterization of gas production (see below), and finally, the top clone of each parental strain and evolution line was analysed for genomic changes.

2.4. Gas production

Fermentative capacity was determined by measuring gas production in both liquid dough (LD) (Panadero et al., 2005), lacking or containing lactic and/or acetic acid (sour LD), or flour-based dough (FBD). Molasses-grown cells (Hernandez-Lopez et al., 2003) were collected, washed, resuspended in distilled water (4 ºC) containing 16 g l^{-1} of NaCl, and 15 ml of the yeast suspension [30 mg (dry weight) per ml] was diluted with the same volume of a 30 ºC-prewarmed LD solution (Panadero et al., 2005). Then, the suspension was incubated at 30 ºC with low shaking (80 rpm), and the amount of CO_2 evolved recorded at 20 min intervals in a Fermograph equipment (ATTO Corporation, Tokyo, Japan). In all cases, values are expressed as ml of CO_2 per mg of

yeast cells (dry weight), and represent the mean ± SD of at least three independent experiments.

2.5. Sequencing and bioinformatics analysis

Whole-genome sequencing and bioinformatics analysis were performed at the Genomics service of the Valencia University (Valencia, Spain). Briefly, Illumina sequencing libraries from S06, S06A_{15}, S06A/M_{13}, S13, S13A_{14} and S13A/M_{14} strains were constructed using the Truseq nano DNA library preparation kit (Illumina Inc., San Diego, CA). The number of raw pair-end 251 bp reads collected were in average 2795,074 ± 689,772. Raw reads were quality trimming and filtering using AfterQC (Chen et al., 2017), with filter of minimum phred-quality score 15 and minimum read size as 50. Raw and processed reads quality control was made with FastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk) and AfterQC tools (Table S1). Resulting reads were aligned to the *S. cerevisiae* R64–1–1 reference strain using the bowtie2 mapping tool (Langmead and Salzberg, 2012). Samtools 1.19 and Picard 2.18 (Li et al., 2009) were used for mapping post-processing and remove duplicates. Only proper paired reads with a mapping quality score above 30 were retained from the alignment. Indel realignment and depth of coverage calculation was performed with GATK-3.6.

Alignment data files were quality check with Qualimap v2.2.1 (García-Alcalde et al., 2012; Okonechnikov et al., 2016). For SNP-calling (SNPs and indels detection) and filtering we use VarScan (v2.3.9; min-avg-qual 20 –min-var-frequation 0.3 –min-coverage 24 –min-reads 25 –min-freq-for-hom 0.70 –p-value 0.05). MiModD 0.1.9 tool (http://doi.org/10.5281/zenodo.2582000) was used for variant post-processing, including genotype filtering and annotation with SnpEff v 4.3 t (Cingolani et al., 2012) settled on approach that required

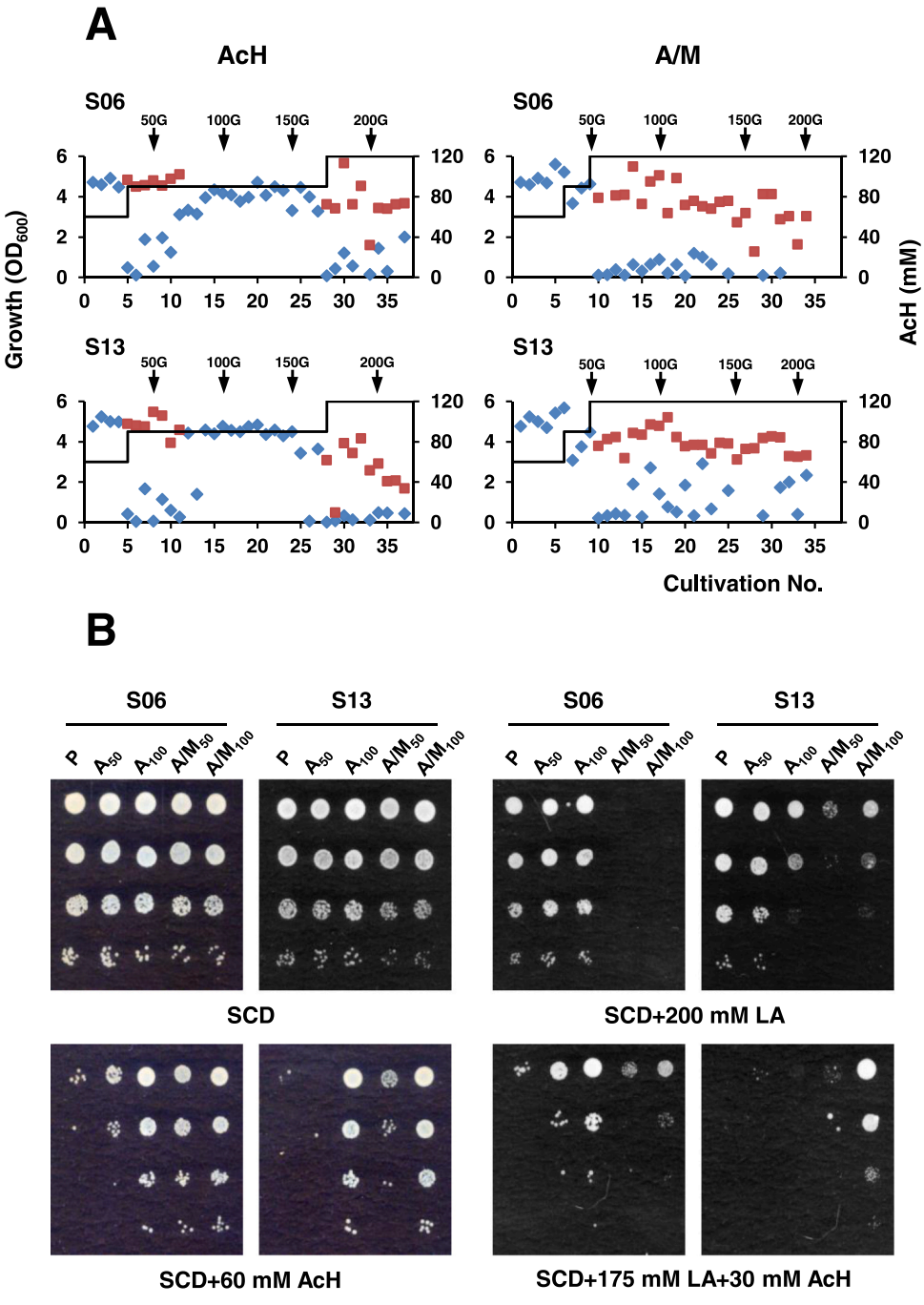

Fig. 1. Characterization of evolved populations of sourdough strains in acetic acid-containing media. (A) The graphs show the growth, measured as OD_{600}, of the yeast strains S06 and S13 after 24 (blue diamonds) and/or 48 h (red squares) of culture in the successive acetic-stressed cycles of the two evolutionary lines, referred to as AcH and A/M. Starting from 60 mM acetic acid and initial OD_{600} of 0.05, cultivations took 24 h in order to obtain a significant increase in cell density, but when the selective pressure increased and growth was impaired, they were extended to 48 h. Growth in media lacking acetic acid is not shown. The black line indicates the concentration of acetic acid (AcH) and the black arrows the sampling of 50-, 100-, 150- and 200-generations evolved populations. In the A/M evolution line, the myriocin concentration increased from the initial 1.5–3.0 μM after 8 cycles of cultivation and was longer maintained until the experiment was ended. (B) Cell samples of the parental (P) S06 and S13 strains and their corresponding 50- and 100-generations evolved populations in each evolutionary line, AcH (A_{50}, A_{100}) and A/M (A/M_{50}, A/M_{100}) were examined for acid tolerance in SCD solid medium lacking or containing acetic acid (AcH) and/or lactic acid (LA) at the indicated concentrations. Colony growth was inspected after 48 h or 4–5 days of incubation at 30 °C for control (SCD) or acid-containing medium, respectively. For more details see the Materials and Methods section.

> 10% base-call supporting a SNP in the evolved genomes and < 2% base-call supporting the same base in the parental genome data and genotype quality above 20. The variant calling files (.vcf) were used to compare changes between parental strains and their corresponding evolved clones and to select those that appear specifically during evolution. Indel, missense variants or frame shift in coding gene regions were kept after manually confirmed by visual inspection in the JBrowse (Diesh et al., 2023) and position of early STOP codon.

To detect chromosome copy-number changes we use the nQuire tool (Weiß et al., 2018) with mapping quality and minimum coverage filters set to 10 and applying lrdmodel to assess ploidy level.

2.6. Statistical analysis

Sample averages were compared by a student's t-test with the Excel software (Microsoft). The samples denoted with * were significantly different ($p < 0.05$). The kernel density graphic was performed with the platform www.bioinformatics.com.cn/en.

3. Results and discussion

3.1. Myriocin-driven evolution speed-up the acquisition of acetic acid-tolerance

We used two *S. cerevisiae* strains isolated from industrial sourdoughs, S06 and S13 (Sánchez-Adriá et al., 2023), as parental strains to carry out evolution experiments addressed to endow them with increased acetic acid tolerance and performance under sourdough conditions. Two evolutionary lines, referred as AcH and A/M, were conducted. In the first, yeast cells were cultivated in the presence or absence of acetic acid, which promotes the acquisition of constitutive rather adaptive tolerance (González-Ramos et al., 2016). In the second strategy (A/M), the acetic acid-stressed SCD cultures were alternated with refreshments in YPD containing myriocin, a drug that affects the lipid profile (Randez-Gil et al., 2020).

Fig. 1A shows the growth of the yeast strains S06 and S13 after 24 h of culture in the successive acetic-stressed cycles of the two evolutionary schemes. In general, the two strains exhibited improved acetic tolerance after around 100 generations of exposure to increased concentrations of acid (pH 4.0). By comparing, the presence of myriocin in alternation with acetic resulted in a more rapid acquisition of growth capacity in the presence of acid, a result that reinforces the idea of a connexion between lipid saturation and acid tolerance (Lindberg et al., 2013). Nevertheless, at concentrations of 120 mM acetic acid, no apparent further improvement was obtained. Neither the AcH-evolutionary experiments rendered cells adapted to this acid concentration (Fig. 1A). This observation likely reflects the existence of drawbacks and genetic constraints that preclude additional improvements (Meijnen et al., 2016).

3.2. Phenotypic characterization of evolved populations

We investigated more in deep how the evolutionary experiments affected the growth of S06 and S13 under different conditions. Fig. 1B shows the growth on solid media of parental, 50- and 100-generation evolved populations under non-stressed and acid-stressed conditions. As expected, the evolved populations showed a gradual increase in acetic acid tolerance that was first observed in cells of both strains evolved in the presence of myriocin (Fig. 1B, SCD+60 mM AcH; compare p.e. S06, A_{50} and A/M_{50}). However, no additional phenotypic change could be observed for the yeast populations evolved from 100- to 200-generations (data not shown) in good correspondence with the OD_{600} data displayed in Fig. 1A. A/M-evolved populations exhibited a high resistance to myriocin as compared with their parental (Fig. S1), although a slight tolerance was also evident in AcH-evolved populations that were not previously exposed to the drug (Fig. S1; S06 and S13, A_{50} and A_{100}).

Then, we examined the growth of the evolved cultures on a medium containing lactic acid. Lactic acid tolerance is an important trait to consider as this is the major weak acid in sourdough. To our surprise, the acquisition of acetic acid tolerance caused a loss of growth in lactic acid-containing medium that was especially pronounced in the presence of myriocin, likely due to their effects on the evolutionary process, and in the S06 strain (Fig. 1B; SCD+200 mM LA). To our knowledge, this is the first time that such a negative effect on lactic tolerance has been reported when yeast cells are adapted to acetic acid. The microbial inhibition by hydrophilic weak organic acids, such as acetic and lactic, has been traditionally related with their common ability to cause acidification and reduction of intracellular pH (Ullah et al., 2012; Stratford et al., 2013; Palma et al., 2018; Peetermans et al., 2021). However, it seems that these *S. cerevisiae* strains activated on the course of their evolution response mechanisms unrelated with low pH or that these acids may not act in the same manner on the yeast cell. In this line, different effects on glucose uptake and ethanol production by yeast cells have also been reported in response to acetic and lactic acid stress (Narendranath et al., 2001; Thomas et al., 2002). Earlier studies also reported a strong induction by acetic acid stress of the plasma membrane H^+-ATPase Pma1 (Piper et al., 2001). In addition, *PMA1* overexpression confers acetic tolerance (Lee et al., 2017). Unlike this, a decrease instead of an increase in Pma1 activity has been reported in response to lactic acid stress (Narendranath et al., 2001a). Neither *PMA1* has been found to be upregulated in evolved *S. cerevisiae* strains with enhanced tolerance to lactic acid (Fletcher et al., 2017). Hence, the evolutionary changes that were useful for improving acetic acid tolerance may have overridden the intrinsic high resistance to lactic of the strains analysed.

The phenotypic interaction between acetic and lactic acid was also examined (Fig. 1B; SCD+175 mM LA+30 mM AcH). Strong synergistic toxicity between lactic and acetic acid have been described resulting in a much stronger growth inhibition and reduction in metabolic activities (Narendranath et al., 2001b). Nevertheless, A_{100} and A/M_{100} evolved populations of S06 and S13, respectively, were still able to show increased tolerance in a medium containing both lactic and acetic acid (Fig. 1B). Our results reveal the strong background dependency of acid stress tolerance in natural strains and how the particular evolutionary conditions determine the phenotype of the evolved populations.

Finally, we checked if the acquisition of acetic tolerance in our experimental populations was achieved at the expense of their fermentative capacity. CO_2 production measurements were carried out in liquid dough (LD) model system lacking (control) or containing 175 mM lactic plus 30 mM acetic acid. Yeast populations of S06 and S13 evolved for 100, 150 and 200-generations through the two evolutionary lines were tested. As shown in Fig. S2, evolved cells of both strains showed a slight reduction in their fermentative activity under control conditions (Fig. S2). This result likely reflects the redistribution of energy resources toward acid resistance activities (Zakrzewska et al., 2011). However, this energy investment had no major cost in terms of gassing power in cells exposed to acid stress (Fig. S2).

3.3. Isolation, fermentative and phenotypic characterization of individual clones

Individual clones were isolated from the evolved populations $S06A_{100}$, $S06A/M_{100}$, $S13A_{150}$ and $S13A/M_{100}$, which showed the best fermentation performance in fermentation trials within each evolutionary scheme and parental strain (Fig. S2). The screening included 15 individual clones from each experimental population, 5 grown in YPD (subscript 1–5), 5 in acetic-YPD (subscript 6–10) and 5 in lactic plus acetic-YPD (subscript 11–15; see the Experimental section). Thus, a total of 60 colonies were randomly selected and tested subsequently for weight losses during fermentation, which allow for a gross evaluation of the CO_2 released by the fermentative activity of yeasts. As an example, Fig. S3 shows the results of the initial characterisation of clones 1–15 from the A/M_{100} population of the S13 strain. Based on these results, the

two top performing clones from each population: S06A_{12} - S06A_{15}; S06A/M_6 - S06A/M_{15}; S13A_{13} - S13A_{14}; and S13A/M_{11} - S13A/M_{14}, were chosen for a more detailed characterization of gassing production in LD medium and LD containing lactic plus acetic acid (Fig. 2A). The analysis included the evolved heterogeneous populations from which the clones were isolated and the strain L'Hirondelle as example of commercial baker's yeast. In general, the evolved clones showed a similar behaviour than their corresponding ancestor in control LD, and increased gassing power in acid-containing medium (sour LD), a result that was especially pronounced when compared with the commercial strain (Fig. 2A).

Then, we examined the phenotypic stability of four clones, S06A_{15}, S06A/M_{13}, S13A_{14} and S13A/M_{14}, one form each background and evolutionary line, selected as the best performers in the fermentation trials (Fig. 2A). Cells were grown for several generations in YPD and then a drop test was conducted in the presence of acetic and/or lactic acid. As shown in Fig. 2B, clones S06A/M_{13} and S13 A_{14} exhibited lactic sensitivity, a phenotype that was already observed in some evolved heterogeneous populations (Fig. 1B). In addition, the two clones hardly differed from their parental in acetic tolerance, suggesting genome alterations (see later). On the contrary, clones S06A_{15} and S13A/M_{14} still exhibited strong acetic tolerance as compared with their ancestors combined with no loss of growth in lactic medium (Fig. 2B). The results highlight the important heterogeneity in the response of genetically homogeneous *S. cerevisiae* cultures to acetic acid stress as it has been previously reported (Swinnen et al., 2014; Franco-Duarte et al., 2015; Palma et al., 2018).

3.4. Genome sequencing and ploidy level

We sought to identify genomic alterations and casual mutations in the evolved clones than could explain their phenotypic changes. The four clones, S06A_{15}, S06A/M_{13}, S13A_{14} and S13A/M_{14}, and their parental counterparts were sequenced and compared to the laboratory *S. cerevisiae* reference strain S288C. First, we carried out DNA content analysis by flow cytometry. As many yeast isolates from natural environments (Bigey et al., 2021), the parental strains showed near two copies of their genomes, 1.88 ± 0.12 and 1.87 ± 0.14 for S06 and S13 respectively. Comparing with this, the evolved clones showed values in a similar range, 1.80 ± 0.30 and 2.01 ± 0.05 for S06A_{15} and S06A/M_{13}, respectively, and 2.40 ± 0.30 for S13A/M_{14}. Only the clone S13A_{14} displayed a noticeable increase in its ploidy level, 3.30 ± 0.30, suggesting a stress-driven large-scale genomic amplification.

We also examined the changes in heterozygous SNPs frequency compared to the overall genome frequency distribution (Fig. S4) and the read-depth between chromosomes (Fig. 3A), to estimate ploidy levels (Morard et al., 2019). Both methods confirmed the 2 n ploidy level in both unevolved parental strains. However, aneuploidies were evident in some evolved clones (Fig. 3). Strains S06A/M_{13}, S13 A_{14} and S 13 A/M_{14} showed monosomy of chromosome I. Aneuploidies in this chromosome have been reported frequently in *S. cerevisiae* strains, a change that could be associated to its small size, and consequently weak effect on strain robustness (Peter et al., 2018; Morard et al., 2019). Remarkably, additional changes were found in the lowest lactic-tolerant strains. Strain S06A/M_{13} showed an extra copy of chromosome XV (Fig. 3A and B), while strain S13A_{14} appeared to display an extra copy of chromosome XII (Fig. 3B). Nevertheless, we observe a decrease of read depth on this chromosome (Fig. 3A). Given the ploidy level of this strain (3.30 ± 0.30), the results suggest a gross change in the chromosome dosage of this strain, including likely overall duplication of chromosomes, after dropping one copy of chromosome I, and a later loss of a copy of chromosome XII. Weather these changes are responsible of the phenotypic variations of these clones remains unclear. Distinct chromosomal instability patterns have been reported in cells exposed to different stress conditions (Mendes et al., 2017; Shen et al., 2020). In addition, ploidy changes and aneuploidies cause gene copy number imbalances, which

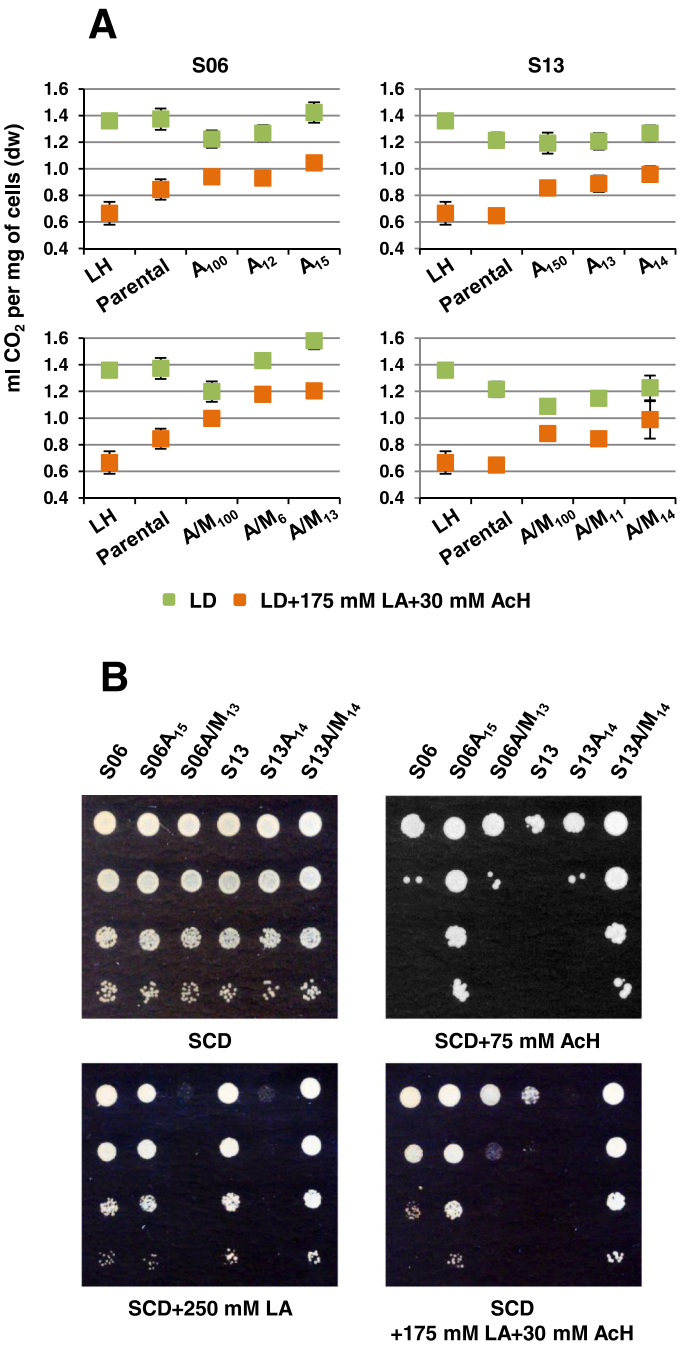

Fig. 2. Technological traits of evolved clones. (A) The parental strains S06 and S13, the evolved populations selected from each evolutionary line, AcH (S06A_{100} and S13A_{150}) and A/M (S06A/M_{100} and S13A/M_{100}), and the two individual clones isolated from each of them, A_{12} - A_{15}, A/M_6 - A/M_{13}, A_{13} - A_{14} and A/M_{11} – A/M_{14}, respectively, were assayed for CO_2 production in liquid dough model system (LD) and LD containing lactic (LA) plus acetic acid (AcH) at the indicated concentration. The analysis also included the strain L'Hirondelle (LH) as example of commercial baker's yeast. In all cases, values are expressed as ml of total CO_2 released per mg of yeast cells (dry weight) after 480 min of fermentation, and represent the mean ± SD of at least three independent experiments. (B) The acid-phenotypic stability of four clones, S06A_{15}, S06A/M_{13}, S13A_{14} and S13A/M_{14}, one from each background and evolutionary line, selected as the best performers in the fermentation trials (panel A), was examined after several rounds of culture in the absence of acetic acid (YPD medium). A drop test assay was used to evaluate the tolerance of yeast cells to acetic (AcH) and/or lactic (LA) acid at the indicated concentration. Colony growth was inspected after 2–4 days of incubation at 30 ºC. For more details see the Materials and Methods section.

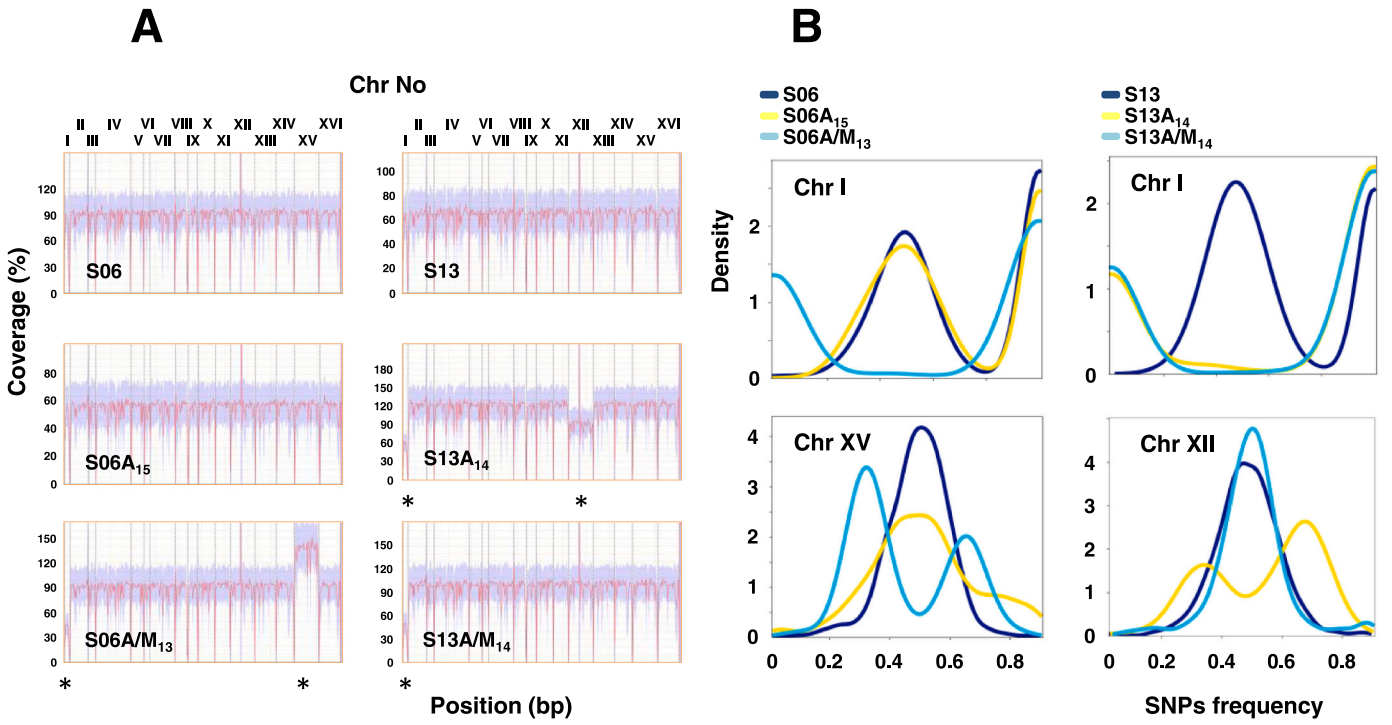

Fig. 3. Genome composition of sourdough strains and evolved clones. (A) Sequencing coverage across the reference *S. cerevisiae* S288c genome of parental sourdough strains S06 and S13 and their evolved clones $S06A_{15}$, $S06A/M_{13}$, $S13A_{14}$ and $S13A/M_{14}$. Deviations from the average of the genome are labelled with * . (B) Frequency SNPs density distribution in chromosomes of evolved clones showing aneuploidies (I, XII and XV). Each graph shows the SNPs distribution for the parental strain and the corresponding evolved clones. The graphics were created with the programme bioinformatics.com.cn/en.

results in non-genetic phenotypic variability (Beach et al., 2017).

3.5. Copy number variations

Small local variations in the genome referred as copy number variations (CNVs), including deletions and amplifications, were also detected across all sequenced sourdough strains (Fig. 4). As the genome from other industrial strains, S06 and S13 strains displayed significantly less transposable element and the telomeres regions were underrepresented in comparison to the reference strain S288C (Fig. 4). These features have been considered as a sign of selection in a man-made environment (Argueso et al., 2009; Babrzadeh et al., 2012; Gallone et al., 2016) like sourdough, and are related with important phenotypic characteristics that determine the technological potential of yeast strains (Franco--Duarte et al., 2016, 2022). Comparison of the CNVs resulted in the identification of 143 genes, mostly underrepresented, that showed altered copy number in the two backgrounds (Table S2), a result that is consistent with the common origin of the parental strains. The results agree well with previous reports that point out genes involved in ion transport or flocculation, including *FLO1*, *FLO9*, *FLO5*, *FLO11* or *FLO10* as the most heavily influenced by CNVs (Dunn et al., 2012; Bergström et al., 2014; Gallone et al., 2016). The list of underrepresented CNVs also contained respiration-, redox-, and ion balance-encoding genes found to provide protection against acetic acid stress (Henriques et al., 2017), like *PAU* genes, which encodes yeast cell wall mannoproteins (Marguet et al., 1988; Rachidi et al., 2000), the YRF/uncharacterized module of yeast helicases and the *CUP* gene family (Kang et al., 2019). Finally, some CNVs that have been reported to be niche-linked (Gallone et al., 2016), as *IMA5*, *MAL31* or *MAL33*, involved in maltose metabolism (Bigey et al., 2021), were found to be amplified (Table S2). Overall, the results are consistent with the high intrinsic acid tolerance of the parental S06 and S13 strains and the presence of maltose as main carbon source in sourdough.

3.6. Single nucleotide polymorphisms

Single nucleotide polymorphism (SNP) analysis identified a total of 148,857 variants between the reference strain S288C and the parental and evolved strains under study. In general, SNPs were present in similar frequency in both homozygosity and heterozygosity, and uniformly present along the genome. We wonder if this accumulation of SNPs could be driven by adaptation to the acid-environment of the sourdough. SNPs are the most abundant form of sequence variation (Bergström et al., 2014). Thus, we first analysed the accumulation of SNPs in candidate genes that have been previously associated to acetic acid tolerance in different studies. Only genes identified by different approaches or phenotypically confirmed were considered. Table S3 shows, as a heatmap, the number of SNPs found in each selected gene and strain analysed. As can be seen, most of the examined genes accumulated a big number of homozygous or heterozygous variants in both the parental and the evolved clones of the two strains analysed. This suggests that the continuous selective pressure due to the acid conditions during the sourdough fermentation, conformed a genetically separated yeast population. On the other hand, only eight genes, four in the S06 (*SRB6, COS9, CWP1, ISO1*), two in the S13 (*TPO2, YPT7*) and one in both strains (*ADY2*), were found to be unaltered as compared with the reference S288C strain (Table S3). The absence of SNPs in *ADY2* is remarkable as this gene encodes an acetate transporter, which deletion has been reported to improve growth and fermentation under acetic acid stress in the laboratory BY4741 strain (Zhang et al., 2017). Interestingly, *ADY2* alleles harbouring different SNPs have been reported to swap their function from acetate to lactate transporters in *S. cerevisiae* (de Kok et al., 2012). Hence, the lack of SNPs in *ADY2* could be the result of a balance between acetic and lactic acid tolerance in sourdough conditions.

Then in a second approach, the SNPs list was filtered (see the Materials and Methods section), in order to search for nucleotide variants and indels that introduce a non-conserved or a missense change into the coding region, which rendered a catalogue of 75 genes (Table S4). From

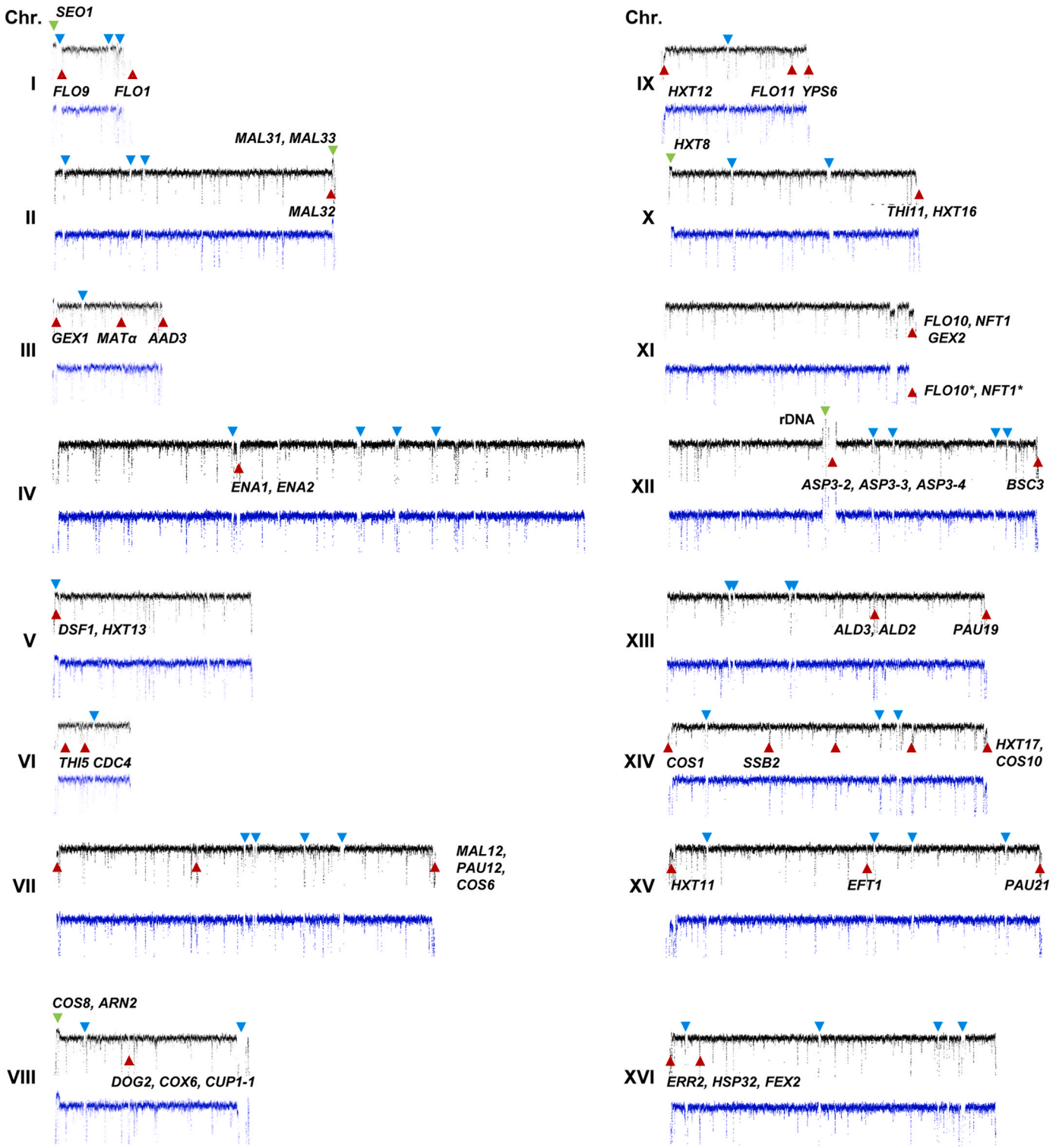

Fig. 4. Sourdough strains show a large amount of copy number variations (CNVs) events. Relative gene dosage plots through mapping S06 (black lines) and S13 (blue lines) sequencing reads onto S288c chromosome sequences. Genomic regions overrepresented and underrepresented are indicated with green and red triangles, respectively. Representative *S. cerevisiae* genes in these regions are named. Blue triangles denote Ty transposons or δ elements.

them, we first paid attention to genes, 43 in total, with SNPs that arose prior to the start of the laboratory evolution experiments. As it is shown, most of the mutations were strain-dependent, present in homozygous and did not vary in response to the environmental conditions imposed by the ALE (Table S4 and Fig. 5A; upper Venn diagram). Among the mutated genes, only *ARG82*, *PSP2* and *JJJ2* were shared by the two parental backgrounds. In addition, 11 genes showed loss of homozygous or heterozygous SNPs (Fig. 5A; lower Venn diagram) and 8 included new stop codons or changes in the frame shift. We evaluated the effect on acid tolerance of the deletion of all these genes in the BY4741 laboratory strain. Only the knock-out of 8 genes altered the growth of the wild-type strain in the presence of acetic and/or lactic acid (Fig. 5B). Deletion of *JJJ2* and *SGF73* had a weak effect, but absence of Arg82, the inositol polyphosphate multikinase 2 (Ipk2; Saiardi et al., 1999; Odom et al.,

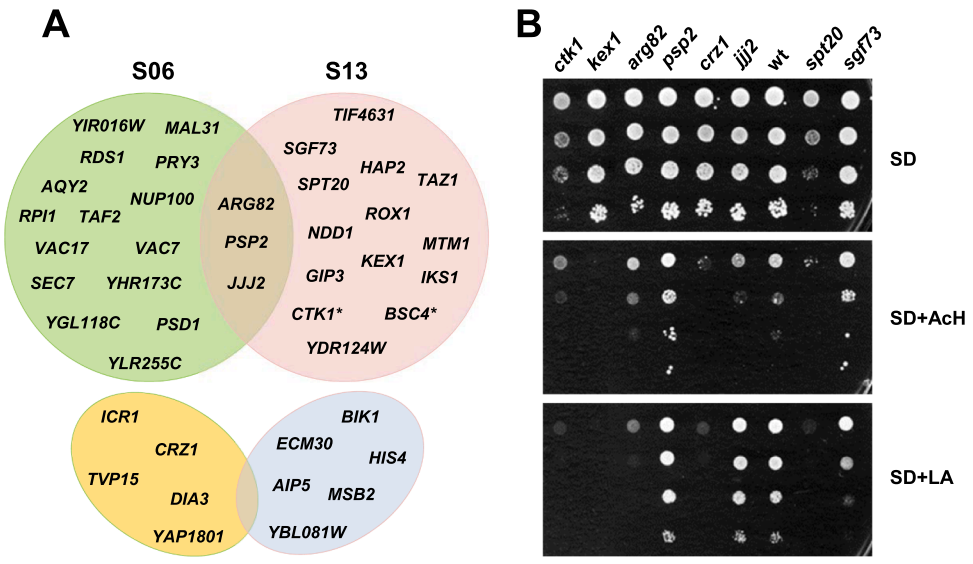

Fig. 5. Identification of genes involved in acetic acid tolerance. (A) Venn diagrams showing genes containing filtered SNPs in the parental strains S06 and S13, relative to the reference S288C strain, and their adaptation pattern in the evolved clones analyzed. The upper circles illustrate genes containing homozygotic variants in the parental strains that did not vary in the two evolved clones. (*) denotes genes containing heterozygotic SNPs that change to homozygotic in at least one evolved clone. The intersection illustrates the set of genes shared in the two backgrounds. Lower circles display genes containing homozygotic variants that change to heterozygotic in at least one evolved clone. SNPs have been detailed in Tabl3 S4. (B) BY4741 mutants carrying deletions in genes shown in panel A were analyzed for growth under acid-stress conditions. Only those showing a differential phenotype relative to the wild-type strain are shown. Cells were grown to the mid-exponential phase at 30 ºC (OD_{600} ~ 0.5). Then, 10-fold serial dilutions were spotted (3 µl) onto SCD agar plates lacking or containing lactic (LA) or acetic acid (AcH). Colony growth was inspected after 2–4 days of incubation at 30 ºC. For more details see the Materials and Methods section.

2000), Crz1, a calcineurin-dependent transcription factor (Stathopoulos and Cyert, 1997) and the cell-death protease Kex1 (Carmona-Gutierrez et al., 2013), resulted in a strong growth defect in lactic-containing medium, indicating that their function contributes to lactic tolerance. In addition, *crz1* and *kex1* mutants showed acetic-sensitivity (Fig. 5B). Knock-out of *SPT20*, a subunit of the SAGA transcriptional regulatory complex (Grant et al., 1998) and *CTK1*, cyclin-dependent protein kinase (Lee and Greenleaf, 1991), decreased acid tolerance, but these mutants already showed a growth defect under unstressed control conditions (Fig. 5B). Previous results reported the implication of Arg82, Kex1, Ctk1 and Spt20 in low-pH tolerance, which could explain the phenotype of these mutants (Kawahata et al., 2006). Crz1 and calcineurin have been also reported to play a role in the inorganic acid stress adaptation (de Lucena et al., 2012). On the other hand, we also found new determinants

GENE	$S6A_{15}$	$S6A/M_{13}$	PHENOTYPE		BIOLOGICAL PROCESS
			Acetic acid 60 mM	Lactic acid 200 mM	
HSP30			Ø	Ø	Regulation of ATP-dependent activity
MRX14			Ø	Ø	Mitochondrial translation
MSN5			+	-	Protein export from nucleus
DNF1			Ø	Ø	Stablishment or maintenance of cell polarity
LAM4			Ø	Ø	Sterol transport
GAR1			inviable		snRNA pseudouridine synthesis
MLP2			Ø	Ø	Spindle pole body organization
BBC1			Ø	Ø	Actin cytoskeleton organization
APL2			Ø	Ø	Golgi to vacuole transport
MIF2			inviable		Kinetochore assembly
YEF3			inviable		Translation elongation
GIM5			Ø	Ø	Regulation of transcription elongation
YNL319w			Ø	Ø	Hexose transmembrane transport
GAS4			Ø	Ø	Ascospore wall assembly
KIN4			Ø	Ø	Spindle pole body organization
MEC1			inviable		Signal transduction. DNA damage
OSW7			Ø	Ø	Ascospore wall assembly
GIS4			++	Ø	Intracellular signal transduction
IRA2			-	-	Regulation of Ras protein
MDM20			Ø	Ø	Actin cytoskeleton organization
RAD53			inviable		DNA repair
TFC1			inviable		Transcription by RNA pol III
YKR011c			Ø	Ø	Unknown

GENE	$S13A_{14}$	$S13A/M_{14}$	PHENOTYPE		BIOLOGICAL PROCESS
			Acetic acid 60 mM	Lactic acid 200 mM	
GIS4			++	Ø	Intracellular signal transduction
FCP1			inviable		Transcription by RNA pol II
APC1			inviable		Protein ubiquitination
YBL081w			Ø	Ø	Unknown
YER152c			Ø	Ø	Unknown
OSW7			Ø	Ø	Ascospore wall assembly
ASG1			++	Ø	Unknown
BBC1			Ø	Ø	Actin cytoskeleton organization
SMC5			inviable		Mitotic chromosome condensation
SAM4					Homocysteine metabolic process
CAM1					Transcription by RNA pol II

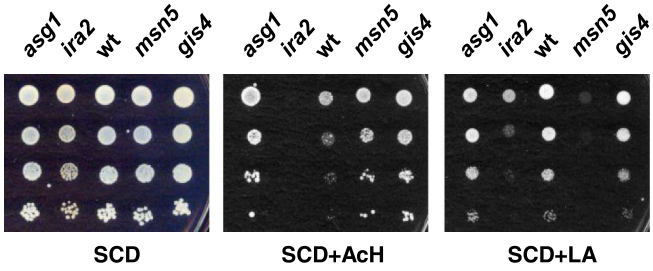

Fig. 6. Identification of genes showing specific SNPs variants in evolved clones and their phenotypic characterization in BY4741 deletion mutants. The tables display those genes with SNPs present exclusively in any of the evolved clones (marked in green). The sequence variation appears in heterozygosis in all cases, except *MDM20* in $S06A_{15}$ with both alleles switched. Neutral (Ø), positive (+) and negative (-) phenotypes relative to the wild type BY4741 strain are shown. Inviable mutants and the biological process terms for each gene are also indicated. A drop test assay in SCD medium lacking or containing 45 mM acetic acid (SCD+AcH) or 200 mM lactic acid (SCD+LA) for BY4741 wild-type (wt) and their corresponding deletion mutants, *asg1*, *ira2*, *msn5* and *gis4* is shown. Cells were grown and cultures were diluted, spotted and incubated as described in Fig. 5B.

of acetic tolerance (Fig. 5B). In particular, deletion of *PSP2*, which encodes a protein with RGG motifs that regulates autophagy (Yin et al., 2019), conferred protection against acetic acid stress (Fig. 5B), a phenotype not previously reported (Mira et al., 2010). Although the mutation found in *PSP2* (insertion of a Q in a Q-rich zone; Table S4) is expected to have no major impact in the Psp2 function, the finding that it was shared between all the strains analysed, suggests it might contribute together with other genetic changes to the phenotype of the sourdough strains.

Finally, we focused on those genes containing SNPs that arose during the evolution experiment (Fig. 6). As can be seen, the identified genes were strain- and evolutionary line-dependent. Remarkably, most of the genes, 22 out of 23, harbouring filtered SNPs acquired through the evolutionary procedures in the S06 background were found in the acetic-tolerant $S06A_{15}$ strain. The S13 strains presented a lower number of mutated genes, 11 in total, with only three, *GIS4*, *OSW7* and *BBC1*, shared between the acetic-tolerant strains, $S06A_{15}$ and $S13A/M_{14}$ (Fig. 6). As above, mutants of all these genes from the EUROSCARF haploid mutant collection were screened for susceptibility phenotypes to acetic and lactic acids. From them, knock-out of *GIS4*, *ASG1* and *MSN5* improved the acetic acid tolerance, while absence of Ira2 caused increased susceptibility to both acetic and lactic (Fig. 6). *IRA2*, which encodes a GTPase-activating protein (Tanaka et al., 1990), has been previously connected w ith acetic acid tolerance (Stojiljkovic et al., 2020). Previous reports following laboratory evolution approaches have also identified mutations in a group of four genes, between them *GIS4* and *ASG1*, as causal for the acquisition of constitutive acetic acid tolerance (González-Ramos et al., 2016). *ASG1* encodes a zinc cluster protein that regulates the utilization of fatty acids and accumulation of lipids in response to stress (Jansuriyakul et al., 2016), while Gis4, has been proposed to play a role in ion homeostasis (Ye et al., 2006) and glucose regulation (La Rue et al., 2005). Remarkably, mutation of *GIS4*, which results in an early stop codon, was exclusively found in the two acetic tolerant strains, $S06A_{15}$ and $S13A/M_{14}$ (Table S4). On the other hand, the enhanced acetic-tolerance of the *msn5* mutant is intriguing, as this phenotype was accompanied by a loss of protection against lactic acid. The exportin Msn5 is involved in nuclear export of Haa1, the transcription factor in charge of the transcriptional induction of most acetic- and lactic acid-tolerance genes (Abbott et al., 2008; Mira et al., 2010). Disruption of *MSN5* leads to the accumulation of Haa1 in the nucleus (Sugiyama et al., 2014). This unusual constitutive nuclear localization of Haa1 could affect its turnover and activity. Absence of Msn5 could also destabilize the nuclear export of other factors, as the exportin has been implicated in a variety of signalling systems (Alepuz et al., 1999). For example, *msn5* mutant cells block the nuclear export of Msn2, which reduces its protein levels and causes delayed growth under chronic stress conditions (Durchschlag et al., 2004). More work is required to shed light to the underlying mechanisms operating in the phenotypic response of *msn5* cells to acid conditions.

4. Conclusions

This work has evidenced the powerful of our targeted ALE strategies to induce acetic tolerance in *S. cerevisiae* and to generate novel strains with higher performance in the sourdough environment. Phenotypic characterization of evolved populations demonstrated that acetic acid tolerance acquisition was quite often accompanied by a loss of protection against lactic acid, indicating that these weak acids may not act in the same manner on the yeast cell. In addition, we have confirmed the involvement of several genes as *ARG82*, *KEX1*, *CTK1*, *SPT20*, *IRA2*, *ASG1* or *GIS4*, and identified new determinants, *MSN5* and *PSP2*, in acetic and/or lactic tolerance. Several of these genes presented weak impact mutations suggesting a combination of many genetic changes as causative of the tolerance phenotype. Nevertheless, the observation that SNPs in *GIS4* were shared by the superior evolved strains, underline the importance of this mutation for acetic acid tolerance.

CRediT authorship contribution statement

Isabel E. Sánchez-Adriá: Investigation, Validation, Visualization, Formal analysis. **Gemma Sanmartín**: Investigation, Visualization, Formal analysis. **Jose A. Prieto, Francisca Randez-Gil**: Supervision, Methodology. **Jose A. Prieto**: Writing – original draft. **Francisco Estruch**: Formal analysis. **Estefanía Fortis, Francisco Estruch**: Reviewed and edited the manuscript. **Francisca Randez-Gil**: Funding acquisition.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127487.

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