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Enhanced flavour profiles through radicicol induced genomic variation in the lager yeasts, *Saccharomyces pastorianus*

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Abstract

The yeasts, *Saccharomyces pastorianus*, are hybrids of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* and have acquired traits from the combined parental genomes such as ability to ferment a range of sugars at low temperatures and to produce aromatic flavour compounds, allowing for the production of lager beers with crisp, clean flavours. The polyploid strains are sterile and have reached an evolutionary bottleneck for genetic variation. Here we describe an accelerated evolution approach to obtain lager yeasts with enhanced flavour profiles. As the relative expression of orthologous alleles is a significant contributor to the transcriptome during fermentation, we aimed to induce genetic variation by altering the *S. cerevisiae* to *S. eubayanus* chromosome ratio. Aneuploidy was induced through the temporary inhibition of the cell's stress response and strains with increased production of aromatic amino acids via the Shikimate pathway were selected by resistance to amino acid analogues. Genomic changes such as gross chromosomal rearrangements, chromosome loss and chromosome gain were detected in the characterised mutants, as were single-nucleotide polymorphisms in *ARO4*, encoding for DAHP synthase, the catalytic enzyme in the first step of the Shikimate pathway. Transcriptome analysis confirmed the upregulation of genes encoding enzymes in the Ehrlich pathway and the concomitant increase in the production of higher alcohols and esters such as 2-phenylethanol, 2-phenylethyl acetate, tryptophol, and tyrosol. We propose that the polyploid nature of *S. pastorianus* genomes is an advantageous trait supporting opportunities for genetic alteration in otherwise sterile strains.

KEYWORDS

accelerated evolution, amino acid analogues, Ehrlich pathway, HSP90, radicicol, *S. pastorianus*

1 | INTRODUCTION

The lager yeasts, *Saccharomyces pastorianus*, are natural hybrids of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* that emerged during the Middle Ages in Central Europe (Monerawela & Bond, 2017). The hybridisation events converged the high fermentative rates of *S. cerevisiae* with the cryotolerance properties of *S. eubayanus*. The evolution of *S. pastorianus* has been influenced by human laws and practices (Baker et al., 2015). In modern day breweries, fermentations with *S. pastorianus* are conducted at temperatures between 7°C and 13°C, creating a beer with crisp, clean flavours that is the preferred beverage of most customers (Lin et al., 2021).

The complex polyploid genomes of *S. pastorianus* strains underpin the physiological outcome of the fermentation. The strains are classified into two broad groups, Group I and Group II that differ in the relative proportion of *S. cerevisiae* and *S. eubayanus* DNA content. Group I, or *Saaz* strains, are typically triploid in DNA content, retaining all the parental *S. eubayanus* chromosomes but have lost many *S. cerevisiae* chromosomes. The Group II, or *Frohberg* strains, are mainly tetraploid in DNA content, containing approximately 2n *S. cerevisiae* and 2n *S. eubayanus* genome content (Dunn & Sherlock, 2008; Okuno et al., 2016; Salazar et al., 2019). Both groups display chromosomal aneuploidy with chromosome numbers ranging from one to six (Monerawela & Bond, 2018; Salazar et al., 2019). In addition to the parental chromosomes, *S. pastorianus* strains contain several hybrid chromosomes containing both *S. cerevisiae* and *S. eubayanus* genes that resulted from recombination, at precise locations, between the parental chromosomes. Some of the recombination breakpoints are located within coding regions, creating a set of hybrid genes unique to lager yeasts (Bond et al., 2004; Dunn & Sherlock, 2008; Hewitt et al., 2014; Monerawela & Bond, 2017).

The presence of orthologous alleles, emanating from different parental chromosomes, creates a complex network of gene transcription. Trans-regulation by transcription activators encoded by the two subgenomes has been observed (Brouwers et al., 2019; Vidgren & Gibson, 2018). Superimposed on the presence of orthologous alleles are complexities of gene dosage due to the aneuploid nature of the genomes. The resultant steady state pool of mRNAs has the potential to create a complex proteome with the potential to affect the stoichiometry of *S. cerevisiae* and *S. eubayanus* proteins within protein complexes (Piatkowska et al., 2013) and to influence the final cellular physiology and the fermentation properties of *S. pastorianus* strains (Timouma et al., 2021).

We recently analysed the patterns of gene expression under fermentation conditions in representative Group I and Group II lager yeasts and showed that the steady state levels of *S. cerevisiae* and *S. eubayanus* orthologous alleles are directly correlated with the copy number of the genes (De la Cerda Garcia-Caro et al., 2022). Furthermore, we observed that *S. cerevisiae* and *S. eubayanus* alleles differentially contribute to metabolic processes in the cell, and in some cases, contribute exclusively to specific gene ontologies.

Take-away

- Radicicol was used to induce genetic diversity in interspecific *Saccharomyces pastorianus* hybrids for improved flavour profiles. This drug is known to inhibit the chaperone Hsp90. Amino acid analogues of phenylalanine were used to select strains with improved production of 2-phenylethanol and 2-phenylethyl acetate.
- Two *S. pastorianus* mutants were selected based on their overproduction of higher alcohols and esters derived from the secondary metabolism of aromatic amino acids; 2-phenylethanol and 2-phenylethyl acetate, together with tryptophol and tyrosol.
- Genomic analysis showed that Radicicol induced gross chromosomal rearrangements, chromosome gain and chromosome loss in *S. pastorianus* mutants. Furthermore, single-nucleotide polymorphisms in *ARO4* were found in both strains.
- Transcriptome analysis shows upregulated genes of the Ehrlich pathway and unique expression patterns in mutant strains under fermentative conditions.
- This study proposes radicicol as a non-GMO mutagenesis method to induce changes in chromosomal architecture of polyploid strains and generate new strains with improved traits for different biotechnological processes.

Flavour and aroma profiles impart unique characteristics to lager beers and arise from the production of higher alcohols and acetate esters as secondary metabolites from the catabolism of aromatic, branched-chain, and sulphur-containing amino acids via the Ehrlich pathway (Dzialo et al., 2017; Lin et al., 2021). Additionally, fatty-acid esters produced from the esterification of Acyl-CoA with ethanol add to the final organoleptic profile of the fermentation.

S. pastorianus hybrids are generally sterile, rarely producing spores and what spores are produced have low viability. Hybrid sterility is most likely a consequence of a lack of normal mitotic recombination or meiotic cross-over events (Bozdag et al., 2021). Increasing genetic diversity is challenging, but several approaches such as laboratory evolution, traditional mutagenesis and interspecific hybridisation have been used to generate new strains with phenotypes relevant to brewing (Gorter de Vries et al., 2019; Hebly et al., 2015; Krogerus et al., 2017, 2021; Mertens et al., 2015; Naseeb et al., 2017; Strejc et al., 2013). Genetic variation within hybrids can also be manifested through chromosomal recombination and changes in aneuploidy (Chen et al., 2012; Gorter de Vries et al., 2020; James et al., 2008).

Here we have used an accelerated evolution approach to generate novel strains of *S. pastorianus* strains with varied flavour profiles. As the relative expression of orthologous alleles was shown to be a significant contributor to the final gene expression pattern

during fermentation, we aimed to alter the *S. cerevisiae* to *S. eubayanus* chromosome ratio in lager yeast strains. We induced chromosomal aneuploidy and gross rearrangements by exposing cells to Heat Shock Thermal Stress (HSTS) or to the macrocyclic antibiotic radicicol, an inhibitor of Hsp90p (Chen et al., 2012; James et al., 2008). By coupling the stress treatment to the ability of mutants to grow in the presence of amino acid analogues of phenylalanine, we selected for variants with increased production of higher alcohols and esters produced via the Ehrlich pathway.

2 | MATERIALS AND METHODS

2.1 | Yeast strains and growth conditions

CBS1538, was obtained from the Collection de levures d'intérêt biotechnologique, Paris, France and Weihenstephan 34/70 (W34/70) was kindly supplied by Dr. Jurgen Wendland, Geisenheim Hoch Universitat, Germany. Strains were grown in YPDM (1% [w/v] yeast extract, 2% [w/v] peptone and 1%–2% [w/v]) of both dextrose and maltose at 20–25°C or in minimal medium (0.17% Yeast Nitrogen Base [YNB]) without amino acids and ammonium salts supplemented with 1% of dextrose and 1% of maltose, and 0.5% $(NH_4)_2SO_4$ as a nitrogen source at 20°C.

Small-scale (50 ml) and large-scale (2 L) fermentations were carried out in 12% wort containing 1 mM $ZnSO_4$. Cells were propagated in 4% YPDM at 25°C, washed in water, and then added to the wort solution at a cell density of 1.5×10^7 ml^{-1}. The specific gravity of the wort/cell suspension was measured (original specific gravity) using a refractometer. For the small-scale fermentations, the suspended cells were divided into 10 ml aliquots and transferred into 15 ml glass test tubes. The tubes were fitted with a water trap airlock attached to a bung. Individual tubes were used at time intervals to measure the specific gravity of the fermentation and to retrieve cells for RNA extraction. Large-scale fermentations were carried out in 3 L tall tubes. Small and large-scale fermentations were conducted at 13°C with the tubes at a 45° angle without shaking and carried out in triplicate or duplicate, respectively.

For the radicicol treatment, synthetic complete medium (SC; 1% of dextrose, 1% of maltose, 0.5% of $(NH_4)_2SO_4$, 0.17% of YNB) without amino acids and ammonium salts and 0.2% of a mix of amino acids was used.

2.2 | Mutagenesis

For the heat shock thermal stress (HSTS), 5 ml of cultures at 1×10^7 cells ml^{-1} in 2% YPDM were heated at 45–55°C for 10–15 min. After a recovery of 3–5 h at 25°C, the cells were washed with water and aliquots of 100 µl were plated onto minimal media agar plates containing amino acid analogues β-(2-thienyl)-DL-alanine (B2TA) or *p*-fluorophenylalanine (PFPA) at concentrations 75 or 200 µg ml^{-1} respectively.

For radicicol treatment, a modification of the method proposed by (Chen et al., 2012) was followed. Cells at a concentration of 1×10^7 cells ml^{-1} were inoculated in SC medium containing a final concentration of radicicol of 20, 40 or 100 µg ml^{-1} and incubated at 25°C for 24 or 48 h. After the treatment, aliquots of 100 µl were plated onto minimal medium agar plates containing 75µg ml^{-1} of B2TA or 200 µg ml^{-1} of PFPA. To ensure that mutants retained the phenotype of resistance to amino acid analogues, the colonies were re-streaked onto agar plates of minimum media containing the amino acid analogues at least three times. Resistant colonies were designated by a code based on the mutagenesis treatment and conditions (Supporting Information: Table S1). Mutants were further characterised by growth in B2TA or PFPA at different concentrations, and in different growth media (minimal medium, minimal medium with 20 mM phenylalanine as the sole nitrogen source or YPDM. Growth rates were calculated from the area under the curve. A Student *t*-test was used to identify mutants with growth rates that were significantly different to the parental strains.

2.3 | Gas chromatography–mass spectrometry (GC-MS) analysis

Analysis of volatile compounds in wort at the end of fermentation were conducted as previously described (Škrab et al., 2021) with the following modifications. Briefly, 0.5 ml of the samples in 20 ml vials were supplemented with sodium chloride to a final concentration of 1 g mL^{-1} and 25 µl of the 2-octanol as the internal standard (final concentration 200 µg L^{-1}). All samples were incubated for 10 min at 40°C, then the volatile compounds were collected on a divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB-CAR-PDMS) coating 50/30, and 2-cm length SPME fibre purchased from Supelco (Sigma-Aldrich) for 40 min. GC analysis was performed on a Trace GC Ultra gas chromatograph coupled with a TSQ Quantum Tandem mass spectrometer (Thermo Electron Corporation). Identification of compounds was based on comparison with a mass spectral database (NIST version 2.0) and with the retention time of the reference standards. For the small-scale fermentations, semi-quantitative results of the volatile organic compounds (VOCs) were calculated. The relative amount of each volatile was expressed as µg L^{-1} of 2-octanol. For the large-scale fermentations, VOCs were quantified. Calibration curves were measured with standards dissolved in simulated beer solution (7% ethanol in water, v/v).

2.4 | DNA extraction, de novo genome sequencing

DNA extraction was carried out as described by Querol et al. (1992). De novo genome sequencing was carried out by Novogene (www.novogene.com) with Illumina technology on paired-end reads (150 bp). The genome sequences were deposited into the SRA database at the National Centre for Biotechnology Information https://www.ncbi.nlm.nih.gov/bioproject/PRJNA844949 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA844975.

After trimming paired-end reads, the reads were mapped with bowtie2 (version 2.4.2) (Langmead & Salzberg, 2012) to the parental genomes of *S. pastorianus* CBS1483 or to a combined genome of *S. cerevisiae* and *S. eubayanus* derived from *S. cerevisiae* S288C, assembly R64 and *S. eubayanus* (SEU3.0). The annotation of the *S. eubayanus* genome was updated by blasting against the *S. cerevisiae* annotation in assembly R64. High-quality matches sharing >50% identity and >75% of the coverage of the *S. eubayanus* protein length was accepted as orthologs and renamed as alleles in the annotation. Of the 805 orthologs identified, 672 had >70% identity.

2.5 | Quantitative polymerase chain reaction (qPCR)

To confirm copy number variations, qPCR using specific primers to discriminate between subgenomes (Supporting Information: Table S9) was conducted as previously described (Beggs et al., 2012).

2.6 | Copy number variation and single-nucleotide polymorphism (SNP) analysis

Reads from de novo DNA sequencing, mapped against the combined genomes of the reference strains of *S. cerevisiae* and *S. eubayanus* were transformed into sorted BAM files using Samtools and the data was extracted as reads/500 bp and normalised by the size of the library (total number of reads) to determine chromosome copy number.

Samtools were used to call SNP/InDel from BAM files. ANNOVAR was used to annotate variants (Wang et al., 2010). A gene-based annotation file was generated using the genome of CBS1483 and a file containing the transcriptome of the same strain using the ANNOVAR tools. Synonymous, nonsynonymous, stop gain and stop loss SNPs common to both the wildtype and mutant strains were called. Nonsynonymous SNPs located in exonic regions and unique to the mutant strains were selected and any with a quality score of <100 and a depth read coverage score of <50, and with an incorrect allele frequency according to the gene copy number were discarded. The remainder were manually verified using Integrated Genome Viewer (IGV_2.11.0) (Thorvaldsdottir et al., 2013).

2.7 | RNA extraction, sequencing and analysis

RNA extraction was carried out from cells grown in minimal medium or from fermentations in wort on Day 2 and Day 4 as described by De la Cerda Garcia-Caro et al. (2022). RNA sequencing was conducted on cDNA libraries using the HiSeq. 4000 Illumina Platform at the Genomic Technologies Core Facility at the University of Manchester. The libraries were generated using the TruSeq Stranded mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. The RNAseq data set was deposited to the GEO database at NCBI https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197654 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE205364.

Read counts from the RNA mapping were uploaded into iDEP9.1 (Ge et al., 2018) and analysed as previously described De la Cerda Garcia-Caro et al. (2022). Differentially expressed genes (DEG) were calculated using DESeq. 2 with a false discovery rate (FDR) cutoff of 0.05 and a minimum $\log_2$ fold change of ≥1 or ≤−1. RNA from 2 cultures of the mutant strain 9.7 were omitted from further analysis due to the observation of a recombination event on chromosome VIII between *XRN1* and *ZUO1*. DEGs were manually removed if they had zero coverage in either strain or had read counts <20. Gene ontology enrichment was carried out using ClueGO (Bindea et al., 2009) with the following parameters, p value < 0.05, minimum of three genes per pathway, and a κ score of 0.4. A two-sided hypergeometric test and Bonferroni step-down pV correction were applied.

3 | RESULTS

3.1 | Mutant selection by heat stress and radicicol treatment

Two representative *S. pastorianus* strains, the Group I strain CBS1538, and the Group II strain W34/70 were chosen for analysis. The strains display similar organoleptic volatile profiles at the end of fermentation, yet each displays unique flavour profiles, with CBS1538 producing higher levels of ethyl butyrate and ethyl hexanoate that impart tropical fruit flavours and W34/70 producing higher levels of methionol, which produces a 'worty' note in fresh beers (Supporting Information: Figure S1).

With the aim of introducing genetic variation, the strains were exposed to varying temperatures or concentrations of radicicol (Supporting Information: Table S1) and following the treatment were plated onto minimal media agar plates containing amino acid analogues of phenylalanine, either β-2-thienylalanine (B2TA) or ρ-fluorophenylalanine (PFPA). Such amino acid analogues select strains with impaired negative feedback inhibition on the committed step in the Shikimate pathway, increasing the biosynthesis of aromatic amino acids and the flux through the Ehrlich pathway towards the production of higher alcohols and esters. A total of 96 mutants were isolated.

Heat shock treatment followed by selection on B2TA produced only clones from strain W34/70 (Supporting Information: Table S1). The isolation rate for W34/70 mutants was 1.86×10^{-7}. Similarly, radicicol treatment followed by B2TA selection produced only clones of W34/70. Heat shock treatment and PFPA selection produced clones from both CBS1538 and W34/70. The isolation rate of mutants following heat shock treatment and PFPA selection were 1.86×10^{-7} and 1.08×10^{-7} for W34/70 and CBS1538, respectively. Isolation rates for radicicol treatment followed by PFPA selection were 7.66×10^{-7} and 7.33×10^{-7} for W34/70 and CBS1538, respectively. No clones were obtained if the parental strains were plated on minimal medium with amino acid analogues without any treatment.

3.2 | Characterisation of mutants

Twenty-two random mutants resistant to B2TA or PFPA were selected for growth characterisation and aromatic profile analysis in a small-scale fermentation. The mutants were coded according to the mutagenesis treatment and conditions. Thus, mutant 9.7 was one of nine clones (9.1–9.9) derived from Radicicol treatment (40 µg/ml) for 24 h (Supporting Information: Table S1). The mutants were grown in minimum medium containing different concentrations of the amino acid analogues (Figure 1a) and under different growth conditions (Figure 1b). The mutant strains grew at rates similar to the parental strains (normalised to 1.0) in minimal medium (average mutant growth rate = 0.93 ± 0.08) and in rich medium at 20°C (0.96 ± 0.09) and 30°C (0.91 ± 0.06). Some mutant strains displayed growth rates that were significantly different from the parental strains (Supporting Information: Table S2), for example, the mutant strains W34/70 8.4, 9.1 displayed increased growth at 20°C (mean = 1.15, p values 3×10^{-4} and 1.6×10^{-3} respectively (Figure 1b). In minimal medium containing phenylalanine as the sole nitrogen source, almost all the strains exhibited lower growth rates than their respective parental strain (0.8 ± 0.16) (Supporting Information: Table S2), with the mutants, WS 7.3 and WS 8.1 displaying the lowest growth (mean = 0.49 and 0.32 and p values of 1.6×10^{-4} and 4.1×10^{-5}, respectively (Figure 1b and Supporting Information: Table S2).

Strains selected for resistance to PFPA displayed cross resistance to B2TA (Figure 1c). Likewise, B2TA mutants displayed resistance to PFPA, however the growth rate of such mutants was significantly lower than the PFPA mutants (Figure 1c).

To compare the aroma profiles of the mutants, small-scale fermentations were carried out and the volatile profiles were examined by semi-quantitative GC-MS analysis at the end of the fermentation (Figure 2), with a specific aim to identify mutants with increased production of higher alcohols and esters produced by the amino acid phenylalanine. Overall, the aromatic profiles produced by the mutants are similar to the parental strains, however some mutants showed distinctive changes. Eight strains (CBS1538 1.2, 11.1, W34/70 1.3, 2.2, 3.1, 5.5, 9.1, and 9.7 produced higher levels of 2-phenylethanol and seven strains (CBS1538 11.1, W34/70 1.3, 2.2,

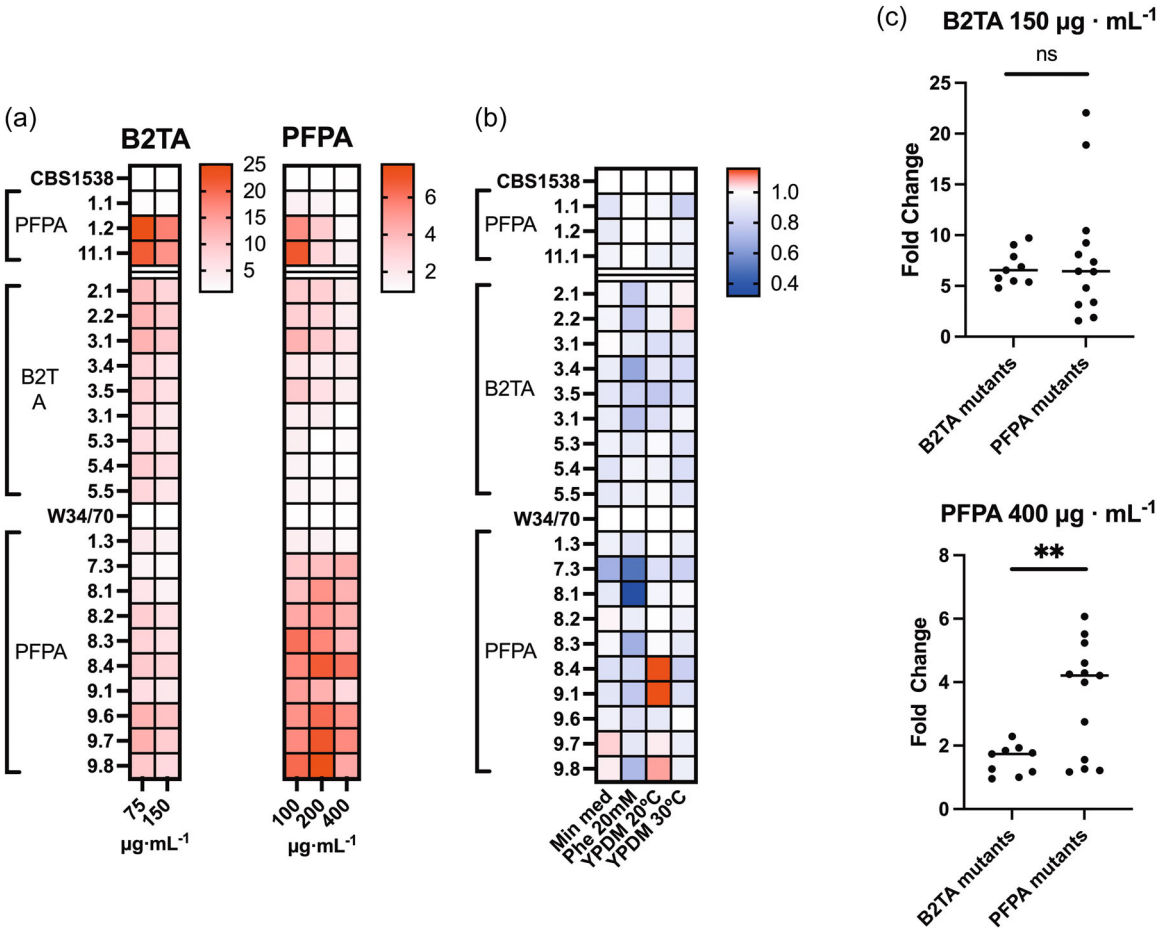

FIGURE 1 Characterisation of the mutant and parental strains in different concentration of amino acid analogues (a) and under different physiological conditions (b). Values correspond to the area under the curve (AUC) during 68 h of growth and normalised to the respective parental strain which was set at a nominal value of 1.0 (white box). The growth of mutants relative to the wildtype strains are shown in the heat map legends. (c) Range and median AUC of mutants selected in 150 mg ml^{-1} B2TA (top) and 400 mg ml^{-1} PFPA (bottom). ns, no significant difference. $^{**}p < 0.05$.

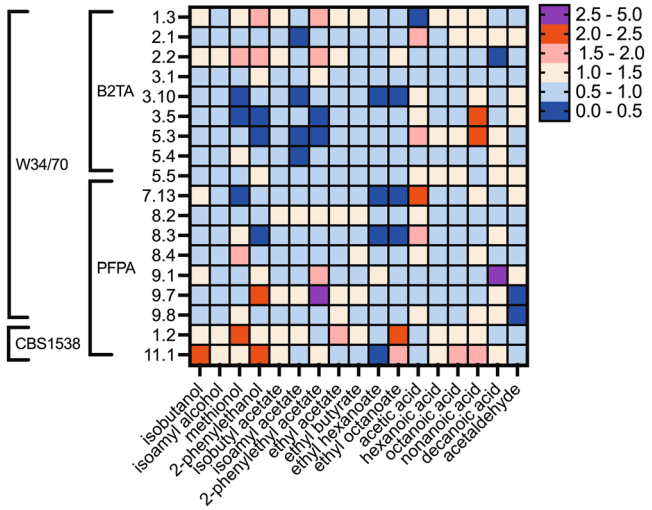

FIGURE 2 Characterisation of the flavour profile of the mutants in small-scale fermentations. Values are normalised to the values of the respective parental strain which was set at 1.0. Colour legend is to the right of heat map.

3.1, 8.2, 9.1, and 9.7) produced higher levels of 2-phenylethyl acetate compared with the parental strains. Based on the combined data for resistance to amino acid analogues, growth characteristics and flavour profiles, two strains, namely CBS1538 11.1 and W34/70 9.7, both obtained from radicicol treatment and selected on PFPA, were selected for further analysis.

3.3 | Confirmation of the phenotypes of the mutant strains

Growth in liquid minimal medium in the presence of the amino acid analogues confirmed the resistance of the mutants to the amino acid analogues. Mutant 9.7 was resistant up to a concentration of 400 μg ml^{-1} of PFPA while mutant 11.1 is more sensitive displaying a maximum resistance at 100 μg ml^{-1} (Figure 3a).

To quantify and compare the volatile compounds produced in the mutants and the WT strains, large-scale fermentations were carried out (Figure 3b). The Group II strain W34/70 fermented faster and reached a greater attenuation than the CBS1538 strain. Surprisingly, the mutant 9.7 fermented faster and consumed more sugars compared with its WT strain (Figure 3b) while the mutant 11.1 fermented slightly slower than its WT strain.

Quantitative GC/MS analysis of the volatile profiles of the mutant and WT strains confirmed the increased production of 2-phenylethanol and 2-phenylethyl acetate in the mutants 9.7 and 11.1 (Figure 3c). The increase in these compounds in mutant 11.1 was not deemed statistically different than the parent in this experiment with just duplicate samples. Mutant 11.1 displayed significantly increased levels of tyrosol while tryptophol levels were significantly increased in mutant 9.7. Both higher alcohols are derived from the aromatic amino acids tyrosine and tryptophan, respectively, via the Ehrlich pathway. Mutant 9.7 also showed increased levels of methionol (Figure 3c).

3.4 | Chromosomal changes in the mutant strains

To identify any genetic changes in the mutant strains, both the mutant and parental strains were sequenced de novo and mapped to the annotated and fully assembled reference genome *S. pastorianus* 1483 (Group II strain) as well as to a newly generated *S. pastorianus* 'combined' genome, assembled from the parental reference genomes *S. cerevisiae* and *S. eubayanus*. Both approaches yielded comparable results, however, since the *S. pastorianus* 1483 genome lacked some information for *S. cerevisiae* genes on chromosomes III and VII, the data from mapping to the combined parental genomes was used to identify any changes in copy number in mutant strains (Figure 4a).

Mutant 9.7 experienced a greater degree of radicicol-induced aneuploidy compared with mutant 11.1 (Figure 4a). We observed chromosome loss or gain in both mutants: mutant 11.1 displayed a loss of *Se* chromosomes I and VIII/XV (Figure 4b), while mutant 9.7 has reduced copy number of chromosomes *Se* II/IV, III, IV/II, V, VII, XI, and XIV as well as *Sc* chromosomes VIII, IX and XV. There was also a gain in copy number in *Se* I in mutant 9.7 and a gain of one type of hybrid chromosome *Se* V with a concomitant loss of the second type of hybrid *Se* V in mutant 11.1 (Figure 4b). Finally, we observed the formation of a new chromosomal translocation between chromosomes *Se* XIII and *Se* XVI (Figure 4b). Alignment of the translocation regions shows that both chromosomes contain a region with high similarity in an intergenic region containing a long terminal repeat similar to Tsu4. The newly created chromosome *Se* XIII/*Se* XVI contains approximately 367 kb of *Se*-like chromosome XIII and 350 kb of *Se*-like chromosome XVI. In total, there is a net loss of 9 *Se* and 3 *Sc* chromosomes, a net gain of 1 *Se* chromosome as well as a new translocation in mutant 9.7, while in mutant 11.1 there is a net loss of 2 *Se* chromosomes as well as a copy number change in Hybrid *Se* V. The copy number changes in selected chromosomes in mutant 9.7 were confirmed by qPCR of genomic DNA (Supporting Information: Figure S3).

3.5 | Analysis of allelic variants in W34/70

We also analysed the genomes to identify any SNPs in the mutant strains using the annotated genome of the Group II strain CBS1483 as the reference strain. The SNP analysis confirmed the presence of two different *S. cerevisiae* subgenomes in W34/70 strain, referred here as *Sc1* and *Sc2* (Okuno et al., 2016). Analysis of allelic frequencies between mutant 9.7 and W34/70 uncovered changes in chromosome copy number in *Sc1* and *Sc2* that were not detected by the chromosome copy number analysis. Specifically, we detected differences in allelic frequencies between mutant 9.7 and W34/70 on *Sc* chromosomes II, IV and XV. Two types of changes in allelic frequencies were observed, namely, changes in the ratio of allelic

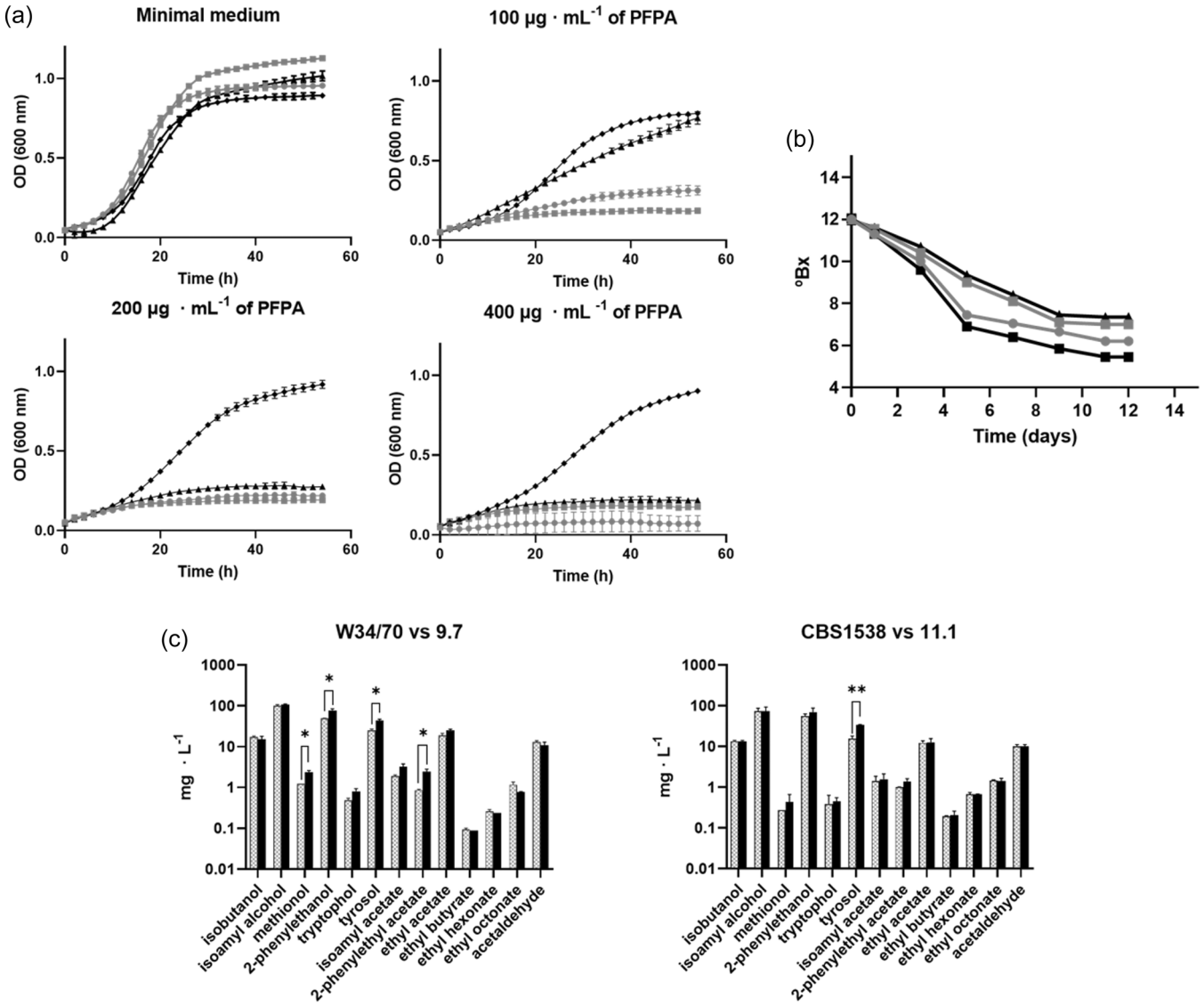

FIGURE 3 Selected mutants are resistant to the amino acid analogue PFPA and show different fermentative qualities. (a) Growth characterisation of mutant and WT strains in different concentrations of PFPA. (b) Fermentation profiles of the selected strains in 12°Bx wort at 13°C in 3 L cylindrical tubes. For (a) and (b), CBS1538: grey line with grey squares, 11.1: black line with triangles, W34/70: grey line with circles, and 9.7: black line with diamonds. (c) Aromatic profiles of the selected strains. Parental strains, W34/70 and CBS1538, Hatched columns, mutants 9.7 and 11.1, black columns. Error bars represent the standard deviations from the mean of duplicate fermentations. Compounds showing statistical differences between the mutants and the parental strains are indicated by connecting lines, $^{**}p \leq 0.01$ $^{*}p \leq 0.05$.

variants and loss of heterozygosity (LOH) (Supporting Information: Figure S3).

We also analysed the genomes to identify any SNPs that introduced nonsynonymous or stop gain codon changes into protein encoding genes in the mutant strains. Surprisingly, only a small number of SNPs were unique to the mutant strains (Supporting Information: Table S3). Just 25 SNPs, which passed all filters and were deemed unambiguous based on chromosome copy numbers were detected in mutant 9.7 (Supporting Information: Table S3). Of interest here is *ScARO4*, where two of the three copies of the gene have a nonsynonymous SNP (S195F). Mutant 11.1 had just two nonsynonymous SNPs, including a SNP in *SeARO4* (D22Y).

3.6 | Gene expression changes in the mutant strains

We examined the gene expression patterns in the mutants 9.7 and 11.1 and the parental strains in three experimental conditions, namely, growth in minimal medium and fermentation in wort on Day 2 and Day 4 (Supporting Information: Table S4). Mutant 9.7 has lost its only copy of *Se* chromosome XI but contains a hybrid copy of chromosome XI (Figure 4b), leaving a total of 166 *Se* genes missing from the mutant. This gene set was excluded from the analysis. The majority of DEGs in the mutant 9.7 are condition-specific with growth in minimal medium displaying the greatest number of genes

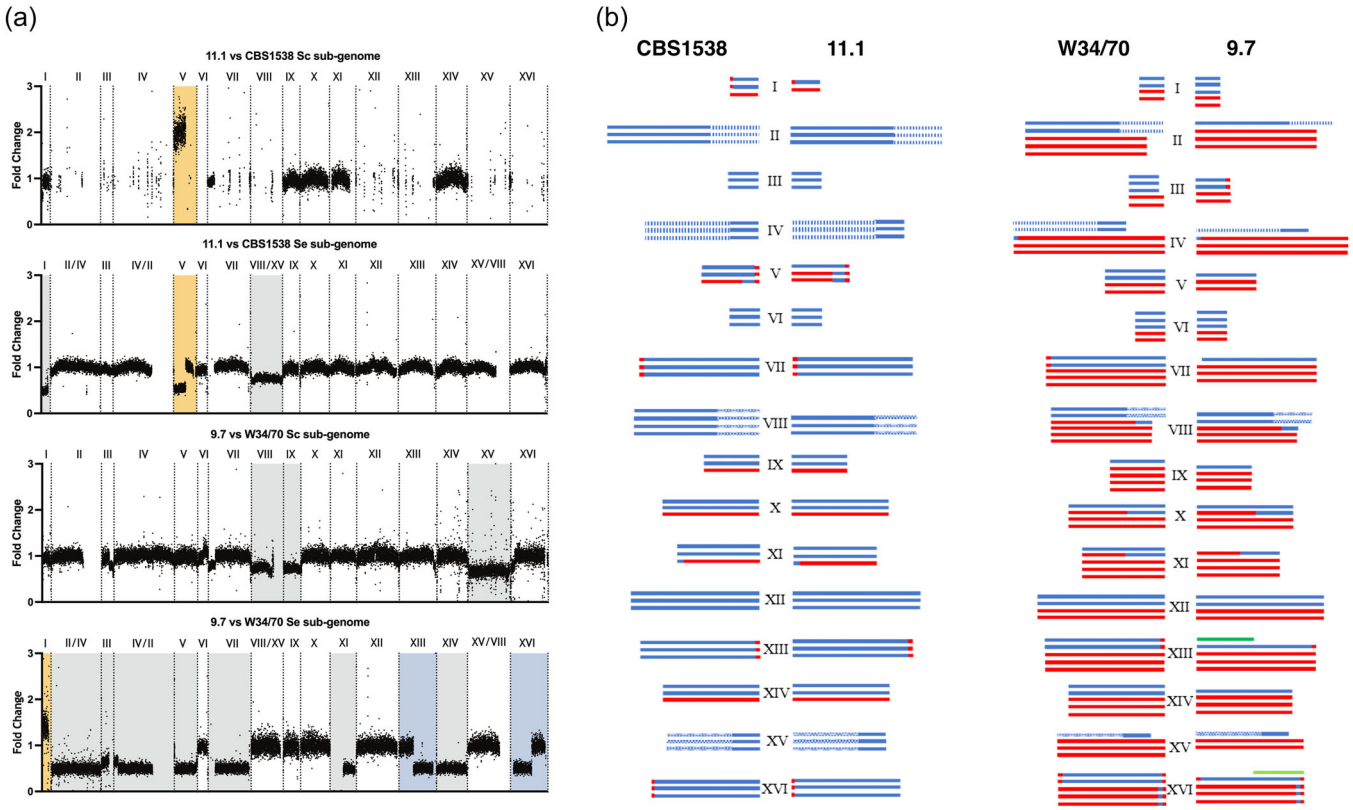

FIGURE 4 Chromosome maps of the parental and mutant strains. (a) Read coverage fold change between mutant and WT strains by chromosome and subgenome. Dotted lines represent the start of each chromosome. Chromosome copy number gain (gold), chromosome loss (grey), and chromosomal translocations (blue). (b) Estimated copy number of *Saccharomyces cerevisiae* (red), *Saccharomyces eubayanus* (blue), hybrid (red/blue), and translocated (green) chromosomes in CBS1538 and W34/70 and their respective mutant strains; mutant 11.1 and mutant 9.7.

(Figure 5a). Just 15 genes are upregulated, and 87 genes downregulated in the mutant in all three conditions. The gene set upregulated in all three conditions includes the genes *Sc ARO9* and *Sc ARO10* (Supporting Information: Tables S5 and S6) encoding for aromatic aminotransferase II and phenylpyruvate decarboxylase, respectively, the enzymes responsible for the first two steps of the Ehrlich pathway. Also of note is the upregulation of *MET32*, the global transcriptional regulator of methionine biosynthetic genes (Supporting Information: Table S6).

Genes involved in sulphur metabolism are upregulated in mutant 9.7 in minimal medium and on Day 2 (Supporting Information: Tables S5 and S8) along with the associated sulphur metabolism gene ontology (Figure 5b). Gene ontologies associated with homologous recombination and mismatch repair were also enriched in mutant 9.7 (Figure 5b). Downregulated gene ontologies in WS 9.7 in minimal medium include sugar metabolism and amino acid metabolism including β-alanine metabolism, tryptophan metabolism and valine, leucine, and isoleucine degradation. Day 4 also had downregulated ontologies associated with amino acid metabolism, affecting tyrosine, glycine, serine, and threonine metabolism as well as genes associated with membrane biosynthesis (Figure 5b).

Consistent with the reduced genomic changes in mutant 11.1, we observed fewer DEGs between the mutant and the parental strain CBS1538. The majority of DEGs were condition-specific with just 5 genes upregulated and 11 genes downregulated in all three conditions (Figure 5a). Interestingly amongst the genes upregulated in all three conditions are genes encoding *HSP82*, *TEF2*, and *MAL32* (Supporting Information: Table S7). The heat shock genes *HSP104*, *HSP30*, *HSP26* and *SSA4* are also upregulated in mutant 11.1 on Day 2 in wort and genes encoding hexose transporters are upregulated on Days 2 and 4 in wort. The genes *BAP2* and *BAT2* encoding for a branched chain amino acid permease and branched chain amino acid transaminase respectively are upregulated in minimal medium and the ammonium permease *MEP2* is upregulated on Day 2 in wort (Supporting Information: Table S7). Amongst the commonly downregulated gene pool are several genes encoding ribosomal proteins. There were no gene ontologies enriched in the up- or downregulated gene pools in mutant 11.1.

To determine if the gene expression changes in the mutants were due to the observed chromosomal copy number differences in the mutants, the ratio of gene copy number in the mutant and WT strains for up- and downregulated genes was examined (Figure 5c). For the 9.7 mutant, there is no change in the gene copy number between the mutant and the parental strain (ratio of copy number = 1.0) for most upregulated genes (Figure 5c). A small group of genes located in *Se* Chr I have a higher expression rate due to an increase in copy number of this chromosome. However, for the downregulated gene pool,

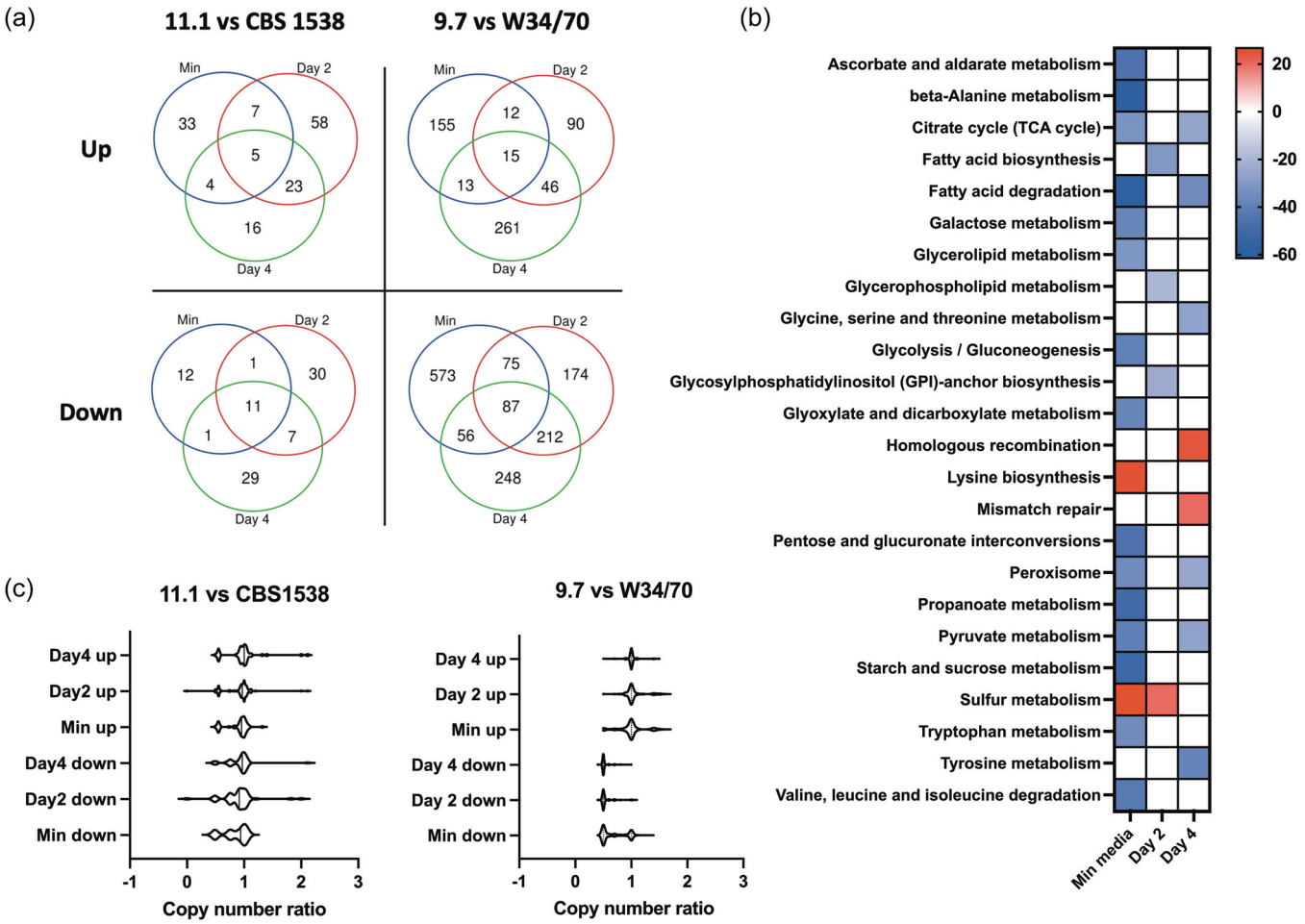

FIGURE 5 (a) Venn diagrams showing numbers significantly ($p < 0.05$) up and downregulated genes in the three experimental conditions: minimal media, wort Day 2, and wort Day 4. (b) Heatmap representing the percentage of genes associated with the listed gene ontologies which are differentially expressed between mutant and wildtype under the different experimental conditions. Upregulated GOs (red), downregulated (blue). Down regulated genes are shown in negative values. White boxes: no significant number of differentially expressed genes under that condition. (c) Distribution of copy number ratios (mutant:wildtype) in the different experimental conditions. Up and Down refers to upregulated and downregulated genes respectively. GO, Gene Ontology.

most genes displayed a reduction in the gene copy number (ratio of copy number = 0.5), except for a subset of genes in the minimal medium condition. For the mutant 11.1, here again, there is no change in the gene copy number ratio for the majority of up- or downregulated genes but a subset of up- and downregulated genes show a reduction in copy number (Figure 5c).

Taken together, the mutants displayed unique gene expression profiles with mutant 9.7 showing differential gene expression of genes associated with amino acid catabolism while mutant 11.1 upregulates several genes associated with amino acid and sugar transport as well as for heat shock proteins.

4 | DISCUSSION

S. pastorianus comprises a set of strains with interesting fermentative traits, including the ability to utilise complex sugars such as maltotriose, to produce aromatic compounds above the sensory threshold, and to ferment at cold temperatures (Dietvorst et al., 2005, 2010; Gibson et al., 2013; Lin et al., 2021; Monerawela & Bond, 2017; Vidgren et al., 2005). Their complex aneuploid genomes underpin these unique physiological traits. The hybrids, formed just 500–600 years ago, appear to still be in genomic flux as evidenced from copy number variations in published sequences of strains (De la Cerda Garcia-Caro et al., 2022; Dunn et al., 2012; Okuno et al., 2016; Salazar et al., 2019). Additionally, CNVs have been observed during a single round of fermentation conducted in high gravity wort at 20°C (James et al., 2008). Indeed, we observed homologous recombination between two known recombination sites on chromosome VII in 2 out of 36 cultures in this study.

The sterile nature of the hybrids creates an evolutionary bottleneck for genetic variation. Approaches such as hybridisation, adaptive evolution, and allele replacement, have been used to generate strains with altered phenotypes such as temperature tolerance, sugar utilisation and aroma production (Krogerus et al., 2017). While successful, such approaches are time-consuming, hybrids can

be unstable and require lengthy successive propagations to stabilise while adaptive evolution can require propagation for 300–400 generations to obtain successful phenotypes. Allele replacement is avoided due to legislation on the use of GMO products and customer rejection of GMO products (Pérez-Torrado et al., 2015).

Here we have established an accelerated evolution approach to obtain lager yeasts with altered flavour profiles through the temporary inhibition of the cells stress response by applying a severe short heat stress or by treatment of cells with radicicol, a Hsp90p, inhibitor. Both treatments have previously been shown to induce aneuploidy and/or chromosomal recombination (Chen et al., 2012; James et al., 2008). Resistance to amino acid analogues of phenylalanine was used to select strains with an increased flux towards the production of higher alcohols and esters. Our results show that radicicol treatment, followed by selection on PFPA produced the greatest number of mutants. Mutants resistant to either amino acid analogue displayed cross resistance, but PFPA was more toxic to the cells than B2TA (Cordente et al., 2018).

4.1 | Chromosomal changes

Hsp90p assists in the folding of an extensive list of clientele proteins (Pennisi et al., 2015; Trepel et al., 2010). Amongst this list are proteins involved in DNA repair, kinetochore assembly and cell cycle checkpoint monitoring. The chromosomal changes identified in the mutants; aneuploidy, homeologous chromosome recombination and translocations can be accounted for by the temporary inhibition of chaperone function of Hsp90p coupled with the selection of clones resistant to amino acid analogues. There appears to be a correlation between the degree of resistance to PFPA and the extent of induced aneuploidy: mutant 9.7 from W34/70 is resistant to 400 μg ml^{-1} PFPA and showed extensive aneuploidy while mutant 11.1 from CBS1538 was only resistant to 100 μg ml^{-1} of PFPA and showed less induced aneuploidy. The difference in induced aneuploidy may also be a consequence to the tolerance of the strains to aneuploidy as CBS1538 is triploid with 48 chromosomes while W34/70 is almost pentaploid at 76 chromosomes.

There appears to be a preferential loss of *Se* over *Sc* chromosomes in the mutants. The recovery period after HSTS (25°C) or the temperature used for radicicol treatment (25°C) may have acted as a selective pressure towards strains with more *Sc* content, such as mutant 9.7.

The recovery period after both HSTS and radicicol treatments may have influenced the selection process of the strains. The temperature of 25°C or the media used may have acted as a selective pressure towards strains with more *Sc* content, such as mutant 9.7.

Previous analysis of chromosome composition following *de novo* generated hybrids of *S. cerevisiae* × *S. eubayanus* observed a similar preferential loss of *Se* chromosomes (Krogerus et al., 2021). The reason for this preferential loss is not currently understood but may reflect a greater fitness of strains with higher *Sc* content, or the essential requirement for some *Sc* alleles. We previously reported that *Sc* alleles are overrepresented in genes upregulated during fermentation in CBS1538 despite the extensive loss of *Sc* chromosomes in this strain (De la Cerda Garcia-Caro et al., 2022). Interestingly, mutant 9.7 has reverted to a near tetraploid ($n = 65$) and there is some reciprocity in subgenome loss to create the preferred stable ploidy for lager yeasts.

Previous studies showed that diploid *S. cerevisiae* strains treated with radicicol acquired an extra copy of chromosome XV, increasing the copy number of *STI1*, a Hsp90 co-chaperone and the multidrug resistance gene, *PDR5*, which are located on that chromosome. Here, we did not see increased copies of chromosome XV, most likely due to differences in selection pressures. Interestingly, we did observe a constitutive overexpression of *Se HSP82*, which encodes for Hsp90p, in mutant 11.1 in all three conditions examined here. The heat shock genes *HSP104*, *HSP30*, *HSP26* and *SSA4*, encoded from the *Se* subgenome, are also upregulated in mutant 11.1 on Day 2 in wort but these heat shock genes are downregulated in the mutant 9.7. Conversely, genes encoding Hsp78p and its co-chaperone Hsp42p, are upregulated in mutant 9.7 in wort on days 2 and 4. Taken together, it appears that the mutants display altered stress responses perhaps as a compensatory response to the imposed stress on the cells.

4.2 | SNPs in the mutants

Surprisingly, we detected few SNPs that were unique to the mutant strains, however crucially amongst those found were SNPs in *ARO4* in both mutants. Aro4p catalyses the first step of the aromatic amino acid biosynthetic pathway, the Shikimate pathway. The activity of Aro4p is inhibited by negative feedback of phenylalanine, tyrosine, and tryptophan. The mutations identified in mutants 11.1 and 9.7 are located in amino acid positions known to be involved in the negative feedback inhibition (Hartmann et al., 2003). Gene expression analysis in the mutants corroborates the in situ increase in aromatic amino acid biosynthesis as we observed the upregulation of *ARO9* and *ARO10*, two genes that are positively regulated by *ARO80* in the presence of increased concentrations of phenylalanine. Increased production of aromatic amino acids in the cell also drives the flux through the Ehrlich pathway towards the production of higher alcohols and esters such as 2-phenylethanol, 2-phenylethyl acetate, tryptophol, and tyrosol, observed in the mutants. Allele variation in *ARO4* may also contribute to the overproduction and the variation of aromatic compounds between the mutant strains. In mutant 9.7, it is the *Sc* copy of *ARO4* that is affected with a SNP present in two of the three copies of the gene while in mutant 11.1, the mutation occurs in the *Se* copy of *ARO4* with one of the three copies of the allele affected. The overproduction of 2-phenylethanol and 2-phenylethyl acetate by the yeast strains have a positive impact in the final beer as these two molecules imparts notes of honey and rose-like flavours. Furthermore, tyrosol and tryptophol contribute to in-mouth sensory properties of beverages (Di Benedetto et al., 2007; Rebollo-Romero et al., 2020; Soejima et al., 2012).

The SNP analysis also uncovered allele frequency changes and LOH in the two variant *S. cerevisiae* subgenomes, confirming the

presence of two types of *Sc* chromosomes in the Group II strains (Okuno et al., 2016). Additionally, we uncovered recombination between the *Sc1* and *Sc 2* chromosomes that has not previously been reported. Such recombination events may contribute to the genetic variation within *S. pastorianus* strains.

4.3 | Gene expression changes in the mutants

In addition to the notable upregulation of *ARO9* and *ARO10* in mutant 9.7 and heat shock genes in mutant 11.1, several additional transcriptome changes were observed in the mutant strains. Of note was the upregulation of *BAP2* and *BAT2*, encoding for a branched chain amino acid permease and branched chain amino acid transaminase and the ammonium permease *Se MEP2* in mutant 11.1. Both *Sc* and *Se MEP1* are upregulated in mutant 9.7. Such upregulation increases the uptake of nitrogen and amino acids into the cell. Genes involved in lysine and methionine metabolism are also upregulated in mutant 9.7. Taken together these changes improve the fluxes toward the production of higher alcohols and esters in the mutants.

The significant loss of chromosome copies in mutant 9.7 accounts for most downregulated genes. Inversely, genes located in chromosomes with a higher copy number show significant higher expression. This confirms our previous finding that gene expression is directly correlated with gene copy number in *S. pastorianus*. Such alterations in the ratios of *Sc* and *Se* alleles may increase proteome diversity through generating chimeric multi subunit complexes with altered composition of subunits thus affecting interactions with substrates.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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