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BRIEF REPORT

Adaptive Laboratory Evolution Uncovers Potential Role of a DNA Helicase Mutation in *Torulaspora delbrueckii* Increased Sulphite Resistance

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ABSTRACT

Wine industry has faced pressure to innovate its products. *Saccharomyces cerevisiae* has been the traditional yeast for producing alcoholic beverages, but interest has shifted from the conventional *S. cerevisiae* to non-*Saccharomyces* yeasts for their biotechnological potential. Among these, *Torulaspora delbrueckii* is particularly notable for its ability to enrich wine with novel flavours. During winemaking, sulphites are added to suppress spoilage microorganisms, making sulphite tolerance a valuable characteristic of wine yeasts. Adaptive laboratory evolution in liquid and solid media improved sulphite resistance in two *T. delbrueckii* strains, achieving, in the best case, a fourfold increase from 0.50 to 2.00 mM of sodium metabisulphite, highlighting the potential of these evolve strains for winemaking applications. Genomic analysis revealed SNPs/InDels in all the strains, including a novel unique missense mutation common to the four evolved isolates, but absent from the parental strains, located in chromosome VIII (protein TDEL0H03170, homologue of *S. cerevisiae MPH1*). These genes code for a protein catalogued as an ATP-dependent DNA helicase, known for its role in maintaining genome stability by participating in DNA repair pathways. We propose that this valine-to-serine mutation, common to all the evolved isolates, helps the evolved strains repair sulphite-induced DNA damage more effectively.

1 | Introduction

Winemaking is a highly complex process characterised by the intricate interplay between grapes and various microorganisms, including filamentous fungi, yeast and bacteria. In the initial stages of fermentation, grape must contain a diverse range of yeasts, encompassing both *Saccharomyces* and non-*Saccharomyces* species (Jolly, Augustyn, and Pretorius 2003). *Torulaspora delbrueckii* stands out as one of the most valuable non-*Saccharomyces* yeast species with the potential to enrich the standard wine market (Benito, Calderón, and Benito 2019). This recognition in the winemaking sector is due to several notable traits that enhance the final products when *T. delbrueckii* is used, including the role this species plays in maintaining low levels of acetaldehyde and acetic acid, elevating the overall quality of the wine (Ciani, Beco, and Comitini 2006; Pacheco et al. 2012; Silva-Sousa et al. 2022). However, mainly due to its high sensitivity to sulphites, ethanol and carbon dioxide pressure, this

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yeast is less effective than *Saccharomyces cerevisiae* at finishing wine fermentation (Velázquez et al. 2020). Recently, Silva-Sousa et al. (2022) reported a detailed and extensive phenotypic and metabolic characterisation of a large collection of *T. delbrueckii* strains. In this study, a strong correlation between the phenotypic heterogeneity and the strains' technological groups was obtained, emphasising the convenience of computational approaches in delineating relevant features among the groups. Additionally, our research group has placed a major focus on the study of *T. delbrueckii* genomic particularities, mainly due to the remarkable biochemical, genomic and phenotypic features exhibited by this non-*Saccharomyces* species (Fernandes et al. 2021; Santiago et al. 2021; Silva et al. 2022).

Sulphites, also known as sulphiting agents, have antibacterial, antioxidant and antioxidasic properties, making them highly effective agents for controlling the winemaking microbial environment. Sulphites comprise a group of sulphur-based compounds capable of releasing sulphur dioxide (SO_2). Widely utilised in the winemaking since the late 18th century (Nadai et al. 2016), sulphites are known to be toxic to microorganisms above a certain concentration and their tolerance is known to be species and strain-dependent. For example, *S. cerevisiae* strains are considered to be relatively resistant to sulphites, adding 50 mg/L of SO_2 to spontaneous fermentation often limits the growth of some strains, although the addition of 20 mg/L appears to have a small effect on their growth, in contrast to the spoilage yeast *Brettanomyces bruxellensis* that can grow in the presence of 600 mg/L of SO_2 (Conterno et al. 2006; Curtin, Kennedy, and Henschke 2012; Lage et al. 2019). However, there are currently no other chemical compounds known that can fully substitute sulphites, owing to their multifunctional properties (Ribereau-Gayon et al. 2005; Nadai et al. 2016). Until the moment, there is only one study that assessed the resistance to sulphites of nine *T. delbrueckii* strains, concluding that the concentration of 40 mg/L of free SO_2 affects the growth of some strains, and the maximum concentration tested (60 mg/L) affected most of the strains. In the course of winemaking, SO_2 is added to musts and wines, for example, in the form of potassium metabisulphite, typically at concentrations ranging from 25 to 150 mg/L (Ribereau-Gayon et al. 2005; Vigentini et al. 2013). In winemaking, the equilibrium between the chemical species of sulphites is dependent on the pH of the grape must. The molecular SO_2 is most prevalent from pH 0–2, the bisulphite anion (HSO_3^-) from pH 2–7 and the sulphite (SO_3^{2-}) from pH 7–10. Considering that the wine pH varies between 3 and 4, the dominant species is the bisulphite anion (Divol, du Toit, and Duckitt 2012). Ingram (1948) highlighted the inhibitory effects of free SO_2 species on the general growth of microorganisms, specifying it on yeasts. The antimicrobial potential of SO_2 is believed to stem from its ability to passively diffuse through the microbial plasma membrane (Stratford, Morgan, and Rose 1987; Lage et al. 2019). In *S. cerevisiae* cells, the adverse effects of SO_2, HSO_3^-, or SO_3^{2-} accumulation encompasses ATP depletion resulting from the inhibition of glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenases (Hinze and Holzer 1986). Furthermore, SO_2 disrupts the plasma membrane structure and damages proteins, vitamins or coenzymes through disulphide bond cleavage (Lage et al. 2019). From a technological perspective, increasing yeasts resistance to sulphites is an attractive challenge and remains a significant hurdle in the winemaking process.

There are several genetically modified yeasts available on the market, for the enhancement of wine aroma and the treatment of must/wine. However, none of them enhances the sulphite resistance of the yeasts (Coulon et al. 2006; Husnik et al. 2006; Grossmann et al. 2011), but the accessibility and utilisation of these in the industry depends on strict regulatory legislations in each country and also the consumer acceptance of genetically modified organisms (GMOs), for this reason it is critical to explore alternatives (Dicarlo et al. 2013; Gonzalez et al. 2016; Fernandes et al. 2021; Turnbull, Lillemo, and Hvoslef-Eide 2021). Adaptive laboratory evolution (ALE) offers a way to accelerate evolution in the laboratory with the possibility of favouring specific phenotypes (Dragosits and Mattanovich 2013). Not being considered a genetic manipulation technique, ALE presents a perfect solution to adapt microorganisms for industry utilisation avoiding the concerns associated with the use of GMOs. Among its main advantages, ALE is easy to apply, does not require prior knowledge of the relationships between genotype and phenotype and can induce phenotypes based on complex traits that include several intracellular pathways, like stress tolerance and rapid cell growth (Lee and Kim 2020). This method entails a progressive increase of a certain substance concentration in order to induce long-term adaptability in the microorganisms across several generations under particular selective conditions (Fernandes et al. 2023).

In the present work, we applied ALE to enhance the resistance of *T. delbrueckii* to sulphites and evaluate relevant genomic changes in the evolved isolataes, providing a non-GMO solution to improve fermentation performance. By creating novel *T. delbrueckii* strains with increased sulphite resistance for winemaking applications, we can establish the basis for further implementation of the evolved isolates in biotechnological applications.

2 | Materials and Methods

2.1 | Yeast Strains and Media

A *T. delbrueckii* strain collection, composed of 44 isolates from different geographical and technological origins (Supporting Information S1), and previously characterised phenotypically (Silva-Sousa et al. 2022), and genomically (Silva et al. 2022), was used in the present study. Each strain, preserved at −80°C in cryotubes containing glycerol (30%, v/v), was pre-cultivated overnight in YPD medium (20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract), at 30°C, 200 rpm, before each experiment. Evaluation of the strains' resistance to sulphites was performed using YPD medium supplemented with sodium metabisulphite (SMBS). pH adjustment to 3.5 was done using 2 M L-(+)-tartaric acid. For ALE experiments, two strains were chosen, based on their SMBS basal resistance—strain T04, the *T. delbrueckii* type strain with a very low resistance to sulphites and strain T34, a wine strain already showing a considerable basal resistance.

2.2 | Sulphite Tolerance Assay

Phenotypic characterisation of the sulphite tolerance of the *T. delbrueckii* strain collection was performed in triplicate, according to the spot assay protocol described by Park, Lopez, and

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Bakalinsky (1999). Briefly, yeast pre-cultures were adjusted to an optical density of 1 ($OD_{640nm} = 1$). Five microlitre of serial diluted cell suspensions (four 10^{-1} serial dilutions), were plated on petri dishes containing YPD medium (pH 3.5) supplemented with SMBS at different concentrations—0.00, 0.25, 0.50, 1.00, 1.50, 2.00, 2.50 and 3.50 mM (0.00, 47.53, 95.05, 190.10, 285.15, 380.20, 475.25, 665.35 mg/L). Results were documented through photographs using an image acquisition system. A strain was considered resistant to a certain concentration of SMBS if considerable growth was visually observed in the 10^{-0} dilution, with at least a residual growth detected in the dilution of 10^{-1}.

2.3 | Adaptive Laboratory Evolution

ALE experiments were performed in two different conditions: liquid and solid media (Figure 1). In the first case, yeasts were inoculated at an $OD_{640nm} = 0.1$ in YPD liquid medium (pH 3.5) supplemented with a starting concentration of SMBS of 0.25 mM. The inoculums were incubated at 30°C and 200 rpm, and the yeast growth was regularly monitored through OD_{640nm} measurements. At the mid-exponential phase of growth, approximately at $OD_{640nm} = 3$, the cultures were transferred to a fresh medium with a higher concentration of SMBS. This approach was repeated for all the concentrations considered—0.25, 0.50, 1.00, 1.50, 2.00, 2.50 and 3.50 mM—, with three repetitions at each concentration, before transfer to the subsequent one, to ensure a good adaptation of the cells.

In solid media, YPD plates containing different concentrations of SMBS were prepared, similarly to what was performed in the spot assay, and grown colonies were directly transferred to the subsequent concentration, starting at 0.25 mM, until a maximum of 4.50 mM and using two intermediate transfers of the same concentration, as in the liquid media approach. Three different approaches were used: (1) single colony (SC), where only one isolated colony was directly transferred to another plate with fresh medium and with the corresponding higher concentration of SMBS; (2) yeast biomass (YB), in which a considerable amount of cells were directly transferred after 7 days of the detection of the first colony, to ensure that an entire population of the resistant yeast was transferred and (3) active growth (AG), in which the transfer was directly performed as soon as sufficient biomass emerged. The plates were incubated at 30°C, and the results were registered as photographs on the day of the transfer. The SMBS concentration considered as the growth limit, in terms of tolerance that was attributed to each strain, was the last one in which growth was observed. For both approaches (liquid and solid ALE experiments), resistant colonies were isolated and stored at −80°C in glycerol (30%, v/v).

During liquid ALE experiments, and to establish a correlation between the OD_{640nm} and the number of cells, a Neubauer chamber was used to count the cells. Subsequently, these results were used to calculate the number of generations (n) and the cumulative number of cell divisions (CCD), using the method proposed by Lee et al. (2011). In particular, the following expressions were used, in which N_0 and N correspond to the initial and final number of cells measured in a defined volume, respectively:

$$n = \ln(N/N_0)/\ln(2)$$

$$\mathrm{CCD} = \sum \left(N_{0,i}\left(2^{ni} - 1\right)\right)$$

2.4 | Characterisation of the Evolved Isolates

To characterise the evolved isolates obtained using ALE in liquid media, we performed growth curves in YPD medium (pH 3.5) supplemented with different concentrations of SMBS (0.00, 0.25, 0.50, 1.00, 1.50 and 2.00 mM), measuring OD_{640nm} over time and registering turbidity over 7 days of incubation time. The evolved isolates of ALE in solid media were assessed through the spot assay as described above. Additionally, cell size and morphology were evaluated in evolved isolates, in comparison with the parental strains, using an optical microscope and measuring the

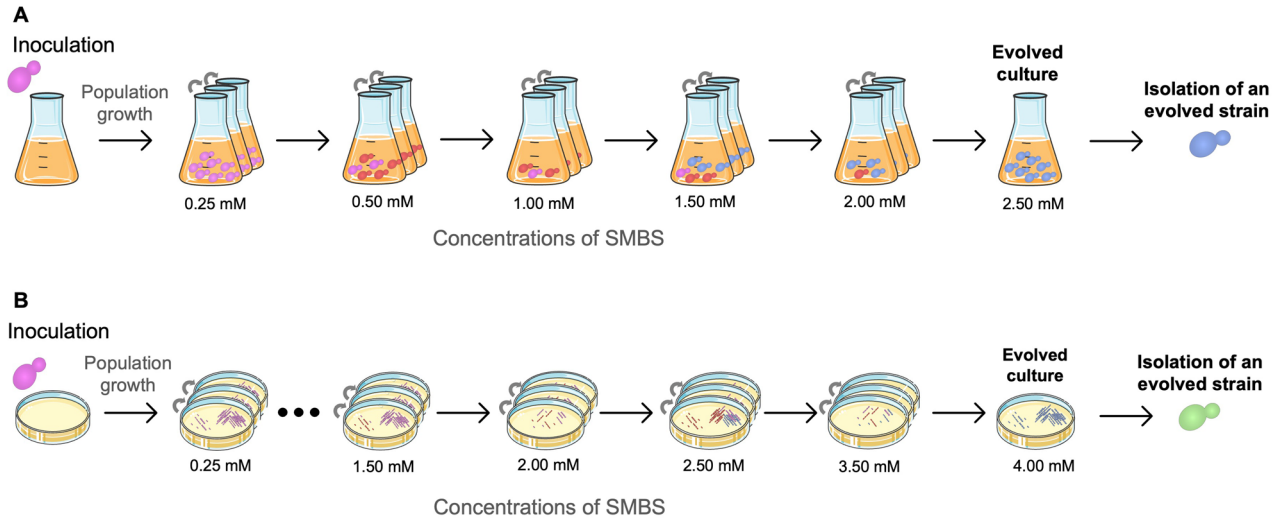


FIGURE 1 | Schematic overview of Adaptive Laboratorial Evolution experiments. Parental strains (T04 and T34) were cultured in YPD (pH 3.5), and aliquots of the cells' population were sequentially transferred into fresh media with increasing concentrations of SMBS, for long-term period, both in liquid (A) and solid (B) media. Intermediate concentrations between 0.25 and 1.50 mM of SMBS were omitted from image (B). This figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported licence.

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cell size of 20 randomly chosen replicates of each strain (T04P, T04L, T04S, T34P, T34L and T34S), using the ImageJ Software. Statistical analysis (two-way ANOVA) was performed using GraphPad Prism Version 9.5.0.

2.5 | DNA Extraction and Genome Sequencing

Genomic DNA samples of the best-performing evolved isolates in liquid (T04L and T34L) and in solid media (T04S, T34S) were isolated using the DNeasy UltraClean Microbial Kit (Qiagen), according to the manufacturer's recommendations. Whole-genome sequencing was performed at Novogene (UK) using Illumina MiSeqII, with over ~100× coverage. Raw data was deposited in NCBI-SRA database with the BioProject accession number PRJNA1135909.

2.6 | Bioinformatic Data Analysis

Raw sequencing reads were checked for quality using FastQC Software v.0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Clean reads were assembled into scaffolds using MEGAHIT v.1.2.9 (Li et al. 2015). Next, assembly quality was assessed using QUAST software v.5.2.0 (Gurevich et al. 2013). Annotation of variants in terms of SNPs (Single Nucleotide Polymorphisms) and InDels (insertion–deletion) was performed using SnpEff (Cingolani et al. 2012) and copy number variations (CNVs) were assessed using Control-FREEC software v.11.6 (Boeva et al. 2012), comparing each evolved strain against the respective parental strain.

To understand specific variations within the set of genes known to be related with sulphite resistance and how/if they correlate with the resistance phenotype exhibited by the strains, a comparative analysis was carried out for the entire collection and the results were represented as a cladogram. Eight genes, widely known to be related with sulphite resistance according to the literature, were selected—*FCY21*, *COS9*, *MET22*, *FZF1*, *MNN4*, *ARG3*, *SSU1* and *BIO3* (Gong et al. 2023; Lage et al. 2019; Park, Lopez, and Bakalinsky 1999). A BLAST analysis was performed to obtain the genetic sequence of these genes in the 41 strains of *T. delbrueckii* whose genome was already sequenced (deposited in NCBI), using the sequence of the *T. delbrueckii* type strain CBS1146 as query, in particular our previous improved annotation (Santiago et al. 2021). Three strains of *T. delbrueckii* were excluded from this analysis due to genome quality issues. After that, sequence alignment and phylogenetic analysis using neighbour-joining algorithm were performed in MEGA software v.11.0.11 (Tamura, Stecher, and Kumar 2021) and in Interactive Tree of Life (iTOL) (Letunic and Bork 2019), respectively.

3 | Results

3.1 | Determination of *T. delbrueckii*'s Maximal Tolerance to Sodium Metabisulphite

The tolerance of 44 *T. delbrueckii* strains to sulphites, specifically to SMBS, was evaluated by measuring the strains' ability to proliferate at various SMBS concentrations using a spot assay. Supporting Information S2 illustrates the complete spot assay for the 44 strains, the results being summarised in Figure 2, overviewing the entire population tolerance levels. At 0.25 and 0.50 mM of SMBS all the 44 strains were able to grow, at all tested dilutions (Figure 2A). However, for concentrations higher than 0.50 mM, we observed a clear heterogeneity between strains in relation to the maximum SMBS level tolerated (Supporting Information S2). As expected, a gradual decline in the number of strains able to grow at each concentration was observed: 41 strains at 1.00 mM, 33 strains at 1.50 mM, 30 strains at 2.00 mM, 27 strains at 2.50 mM, and, at the highest tested concentration, 3.50 mM, growth was detected only in 16 strains, representing 36% of the strains under study.

In Figure 2B, strains were categorised by their technological groups, along with their maximum tolerances to SMBS. Remarkably, strains from arboreal/soil group exhibited a higher tolerance, with most strains thriving even at the highest tested concentration of 3.50 mM, marking this group the most homogeneous. In contrast, strains associated with wine, bread and unknown origins exhibited a wide tolerance range, from 1.00 to 3.50 mM. The group related to other beverages showed a slightly narrower tolerance spectrum within the same range. On the other hand, strains from the food, water and clinical groups were the most sensitive to SMBS, with a maximum tolerance limit of only 0.50 mM.

3.2 | ALE of Two *T. delbrueckii* Strains

To enhance sulphite tolerance in *T. delbrueckii* strains, we specifically targeted two strains with different basal tolerance levels in spot assays, the type strain, T04, able to grow only in low levels of SMBS (1.00 mM) and a strain from a wine environment, T34, able to withstand a moderate SMBS concentration (2.00 mM). These strains underwent ALE through successive transfers in either liquid and solid media, as depicted in Figure 1. Subsequent to these evolutionary adaptations, the evolved strains resulting from both experimental conditions were thoroughly assessed for their enhanced tolerance to sulphites.

During ALE in liquid media, we quantified the number of generations (n) and the CCD, as detailed in Supporting Information S3. The evolutionary process encompassed eight evolutionary lines—five derived from T04 and three from T34, with two T34 lines unable to complete the procedure. Ultimately, lines that achieved the highest SMBS concentration were selected for further analysis and were named T04L and T34L throughout the work. The T04L line began growing at an SMBS concentration of 0.25 mM and reached 2.00 mM over 50 days, resulting in approximately 82 generations and 6.61×10^9 CCD. Conversely, the T34L line, starting at 1.00 mM of SMBS, reached 2.00 mM in 27 days, yielding around 51 generations and 4.57×10^9 CCD. The assessment of the number of generations and CCD in strains obtained after ALE experiments in solid media was not feasible due to the impossibility of measuring the parameters essential for the calculations.

In solid media, the T04 strain was subjected to three distinct transfer strategies: SC, YB and AG. The SC strategy involved a single assay with a duration of 30 days, starting at a SMBS concentration of 0.25 mM and concluding at 1.00 mM. For the YB strategy, a single 109-days assay spanned from 0.25 to 1.50 mM

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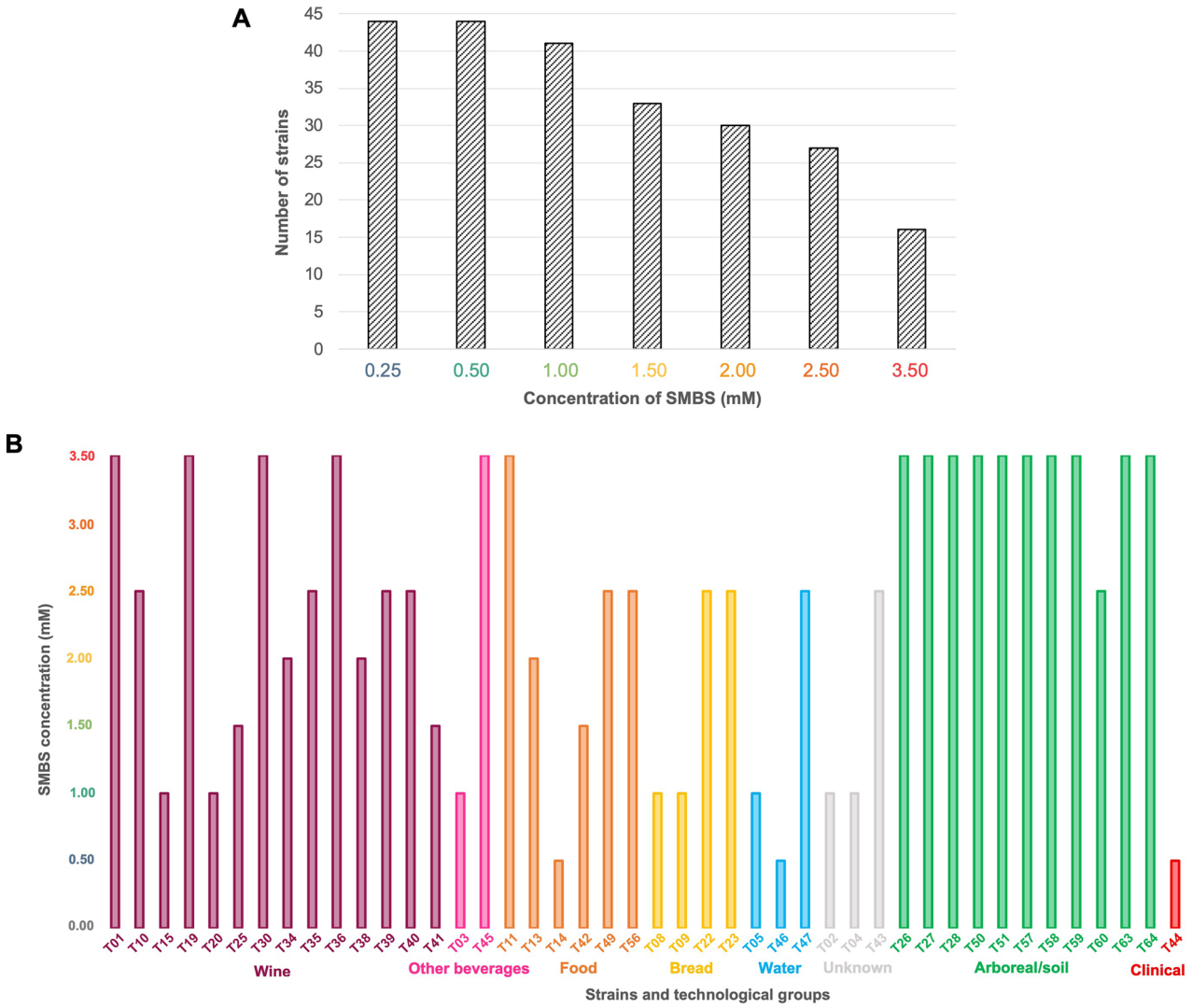


FIGURE 2 | Assessment of the maximum tolerance of 44 *T. delbrueckii* strains to SMBS. (A) Overview of results presented in Supporting Information S2, showing the number of *T. delbrueckii* strains able to grow at each SMBS concentration (0.25–3.50 mM). (B) Maximum tolerance of the strains categorised by their origin and technological group.

of SMBS. The AG strategy, that we conclude to be the most effective, consisted in three independent assays with durations ranging from 70 to 135 days, starting at 0.25 mM and achieving up to 4.00 mM of SMBS. The T34 strain, benefiting from prior optimization of solid media assays, underwent the AG strategy in two evolutionary lines, with experiments lasting 28 and 32 days and reaching final SMBS concentrations of 3.00 and 3.50 mM, respectively. Similarly to liquid media, the best evolved isolates, that is the ones achieving a higher concentration of SMBS, for T04 and T34 strains were selected and were named T04S and T34S, respectively.

3.3 | Phenotypic Evaluation of Evolved Strains

To validate the increased sulphite tolerance of the strains evolved in liquid media (T04L and T34L), their growth was assessed in YPD media containing various concentrations of SMBS (0.25–3.50 mM), alongside their respective parental strains (T04P and T34P), for comparative analysis (Figure 3A). The parental strain T04P showed a basal tolerance up to 0.50 mM of SMBS, in liquid media, whereas its evolved counterpart, T04L, confirmed a significantly enhanced tolerance, capable of growing in concentrations up to 2.00 mM—fourfold higher than the parental strain. For the T34 strain, the parental T34P displayed a basal tolerance of up to 2.00 mM. Post-evolution, T34L's tolerance improved, allowing growth up to 2.50 mM of SMBS.

To validate the enhanced sulphite tolerance phenotype after ALE in solid media, the spot assay (Figure 3B) was employed, testing resistance of evolved strains T04S and T34S across SMBS concentrations ranging from 0.00 to 3.50 mM. The maximum tolerance of a strain was determined based on substantial growth in the agar plates at the first two dilutions (10^0 and 10^{-1}), aligning with the screening method used for the entire *T. delbrueckii* strain collection. According to this criterion, both T04S and T34S showed significant enhancements in resistance, revealed by their apparent growth in the agar plates, displaying the ability to grow up to 3.25 mM of SMBS. Specifically, T04S's resistance increased more than three-fold compared to its parental strain, while T34S's resistance improved over 1.5 times of its original one.

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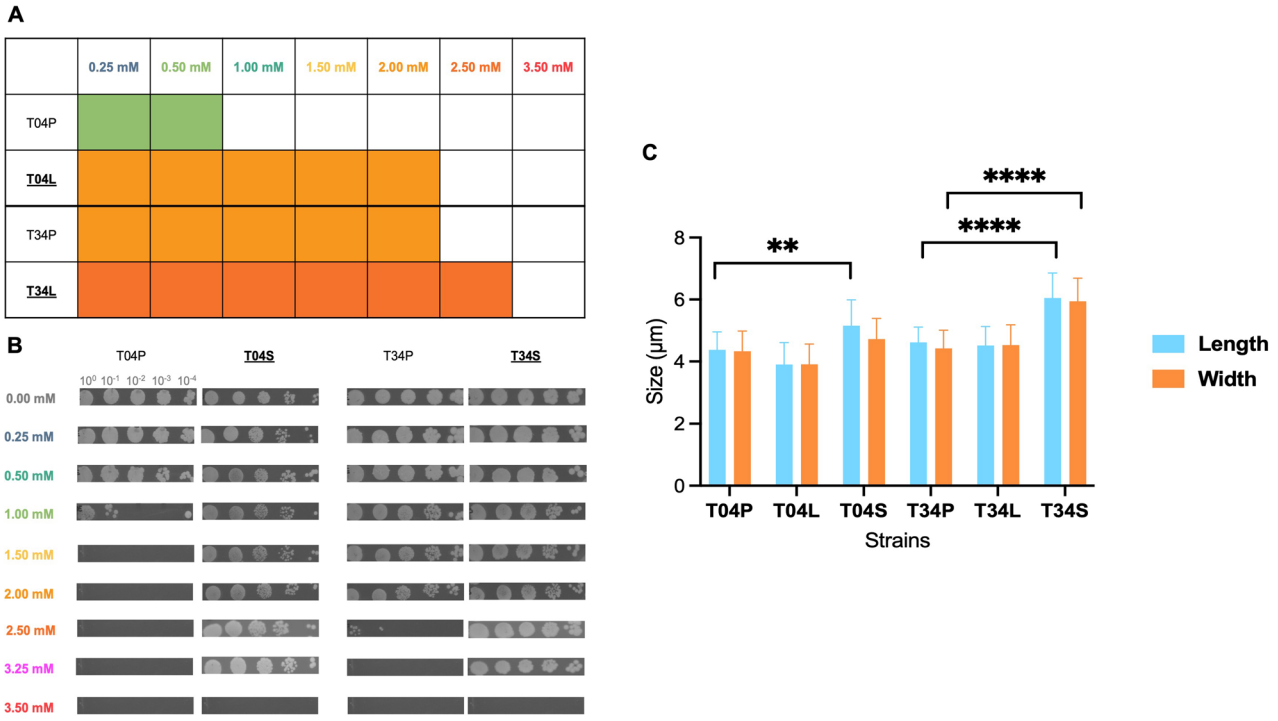


FIGURE 3 | Phenotypic evaluation of the evolved *T. delbrueckii* strains, selected following ALE experiments in liquid and solid media. (A) The tolerance of the evolved strains in liquid media (T04L and T34L) to SBMS was characterised through growth curves and subsequently compared with the parental strains (T04P and T34P). Coloured squares represent the positive growth of each strain in the test concentration. Uncoloured squares represent concentrations that were inhibitory for strains' growth. (B) To assess the resistance of the evolved strains in solid media (T04S and T34S), spot assays were conducted with these strains, using SMBS concentrations ranging from 0 to 3.50 mM, and then the results were compared with the ones of the parental strains (T04P and T34P). Each test was performed in triplicate to ensure accuracy. (C) Size of the parental and evolved strains. The size (µm) of the evolved strains (T04L, T04S, T34L, and T34S) was measured with ImageJ Software and then compared to the parental strains (T04P and T34P). The error bars indicate the standard deviation of 20 biological replicates. Statistical differences were determined through a two-way ANOVA. p value < 0.001 (**); p value < 0.0001 (****).

Additionally, we also assessed changes in cell morphology and size of evolved strains compared with parental strains, by optical microscopy. Morphological examination revealed no alterations in cell shapes (spherical, ovoid, or ellipsoidal) as a result of the ALE experiments (data not shown). However, significant changes were observed in the size of the yeast cells, specifically in terms of length and width (Figure 3C). Cell lengths varied from 2.49 to 7.96 µm, with the shortest average length recorded at 3.90 (±0.71) µm for strain T04L and the longest at 6.05 (±0.81) µm for strain T34S. Notably, ALE in solid media led to significant length variations for both strains T04S ($p < 0.001$) and T34S ($p < 0.0001$). Similarly, cell widths spanned from 2.87 and 7.58 µm, with T04L showing the narrowest average width of 3.91 (±0.66) µm and T34S the widest at 5.94 (±0.75) µm. Significant width differences were particularly evident between strains T34P and T34S ($p < 0.0001$).

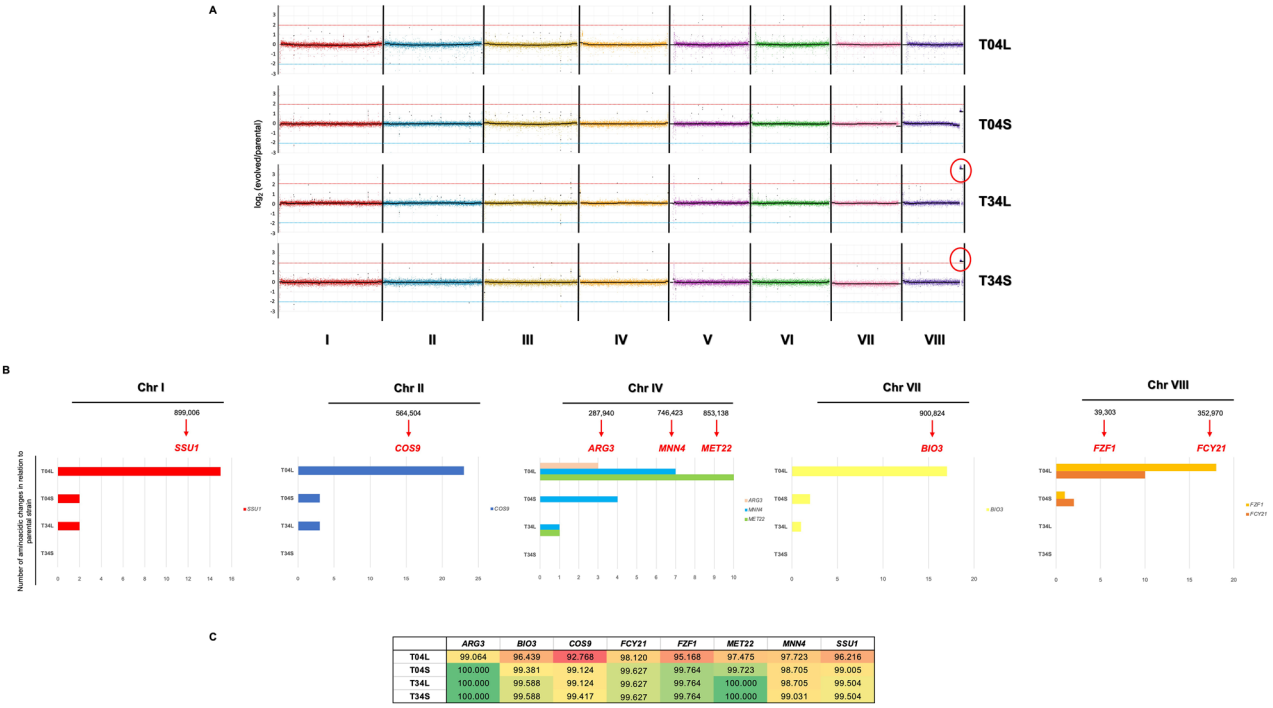
3.4 | Genomic Analysis

Following ALE experiments, in order to identify genomic differences associated with the higher tolerance obtained, the genome of the evolved strains T04L, T04S, T34L and T34S were sequenced. After quality control and genome assembly, we conducted a comparative analysis of scaffolds from the evolved yeast strains against their parental genomes previously sequenced (Silva et al. 2022) to identify SNPs, InDels and variation in gene copy numbers.

To first understand the genetic underpinnings that drove the evolution of the yeast strains in a general way, we assessed CNVs across the entire chromosomes of the evolved strains, comparing with the respective parental genomes (Figure 4). This aspect of genomic analysis is crucial for identifying regions of the genome that have undergone duplication or deletion, events that can significantly impact gene expression and phenotypic traits. Figure 4A presents a striking pattern of CNVs increase at the terminal regions of chromosome VIII in evolved isolates of strain T34 (highlighted using a red ellipsis). Strain T04S showed also this tendency, although at a smaller extent. Interestingly, this CNV pattern did not extend to other chromosomes, suggesting a unique selective pressure or genetic stability in those regions.

To expand this analysis and understand if the regions with expanded CNVs harbour genes with known importance to explain sulphites resistance, we analysed the amino acid changes in eight key genes selected from literature as relevant—*ARG3*, *BIO3*, *COS9*, *FCY21*, *FZF1*, *MET22*, *MNN4* and *SSU1* (Gong et al. 2023; Lage et al. 2019; Park, Lopez, and Bakalinsky 1999). In Figure 4B, we annotated these variations in the genomes of the evolved strains, in comparison with the parental strains,

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	ARG3	BIO3	COS9	FCY21	FZF1	MET22	MNN4	SSU1
T04L	99.064	96.439	92.768	98.120	95.168	97.475	97.723	96.216
T04S	100.000	99.381	99.124	99.627	99.764	99.723	98.705	99.005
T34L	100.000	99.588	99.124	99.627	99.764	100.000	98.705	99.504
T34S	100.000	99.588	99.417	99.627	99.764	100.000	99.031	99.504

FIGURE 4 | Copy number variations in the genome of evolved *T. delbrueckii* strains. (A) Genome view of yeast chromosomes, illustrating CNV patterns in the four evolved strains. The y-axis represents log2 CNV evolved/parental, with each dot representing a genomic window of 200 bp. The red ellipses highlight the most significant increases in CNVs. (B) Number of variations (number of amino acid changes in genes related to sulphite resistance or susceptibility, in comparison with the parental strain. The location of the genes (chromosome and base pair) in relation to the type strain is also indicated. (C) Heatmap illustrating the percentage of similarity of the selected genes, comparing the evolved strains and the *T. delbrueckii* type strain (T04).

focusing on their chromosomal locations and potential implications for functional adaptations in the evolved strains. This analysis sheds light on the specific genetic modifications that contributed to the strains' evolved phenotypes. Notably, T04L stood out for its widespread amino acid changes across multiple genes, indicating a broad scope of genetic adaptation, whereas T34S displayed no such alterations, suggesting a different evolutionary path or mechanisms of adaptation. Chromosome II emerged as a hotbed for genetic changes, with *COS9*, coding for an endosomal protein involved in turnover of plasma membrane proteins, in T04L, experiencing a substantial shift of 23 amino acids, alongside notable modifications in *ARG3*, coding for ornithine carbamoyltransferase which catalyses the biosynthesis of the arginine precursor citrulline. Interestingly, the three key genes located on chromosome IV—*ARG3*, *MNN4* (involved in mannosylphosphorylation of N-linked oligosaccharides) and *MET22* (involved in sulphate assimilation pathway in methionine biogenesis and in salt tolerance)—showed increased number of SNPs in strain T04L. Aiming to further elucidate these genetic divergences, we employed a heatmap to compare the gene similarity percentages between the type strain and the evolved variants (Figure 4C). The analysis highlights the extent of genetic divergence in T04L, with all the analysed genes showing alterations. *COS9* demonstrated the most significant deviation—7.2% changes—, while *ARG3* exhibited the smallest differences—0.9%. In contrast, strain T04S revealed more modest changes when compared with the respective parental genome, with no variations in *ARG3* and a slight 1.3% shift in *MNN4*. When examining the strains that evolved from T34P (T34L and T34S), both showed analogous patterns of gene alterations, revealing no alterations in genes *ARG3* and *MET22* and displaying minor alterations of 1.3%–2.0% in gene *MNN4*, respectively. These findings not only underscore the genetic flexibility and adaptive capacity of yeasts but also highlight the specific chromosomal regions and genes that are pivotal in the evolutionary response to different environmental pressures, paving the way for targeted genetic engineering and strain improvement strategies for industrial applications.

To understand if these genes are also changed in natural strains and if their sequence variation can have implications on strains' basal resistance to sulphites, we analysed also their variability in 41 strains of our *T. delbrueckii* collection, comparing with the *T. delbrueckii* type strain (T04) and crossing these results with the resistance capacity detailed in Figure 2. Results in the form of cladograms are presented in Supporting Information S4 and show that for some genes, for example *SSU1*, one of the most important genes related to sulphite metabolism, some differentiated clades are observed, being correlated with strains' resistance levels. Even when considering the heatmap of panel B, representing the percentage of similarity in the key genes between the type strain and the remaining isolates, it is possible to observe that gene *COS9* stands out as the gene with the highest differences in gene sequences, with strains T03, T05, T14, T44, T57, T58 and T59 exhibiting the most significant differences in the majority of the genes.

With the purpose of finding new relevant gene mutations that help explain the increase of sulphite resistance in the evolved isolates, we did a thorough analysis of all the SNPs, insertions and

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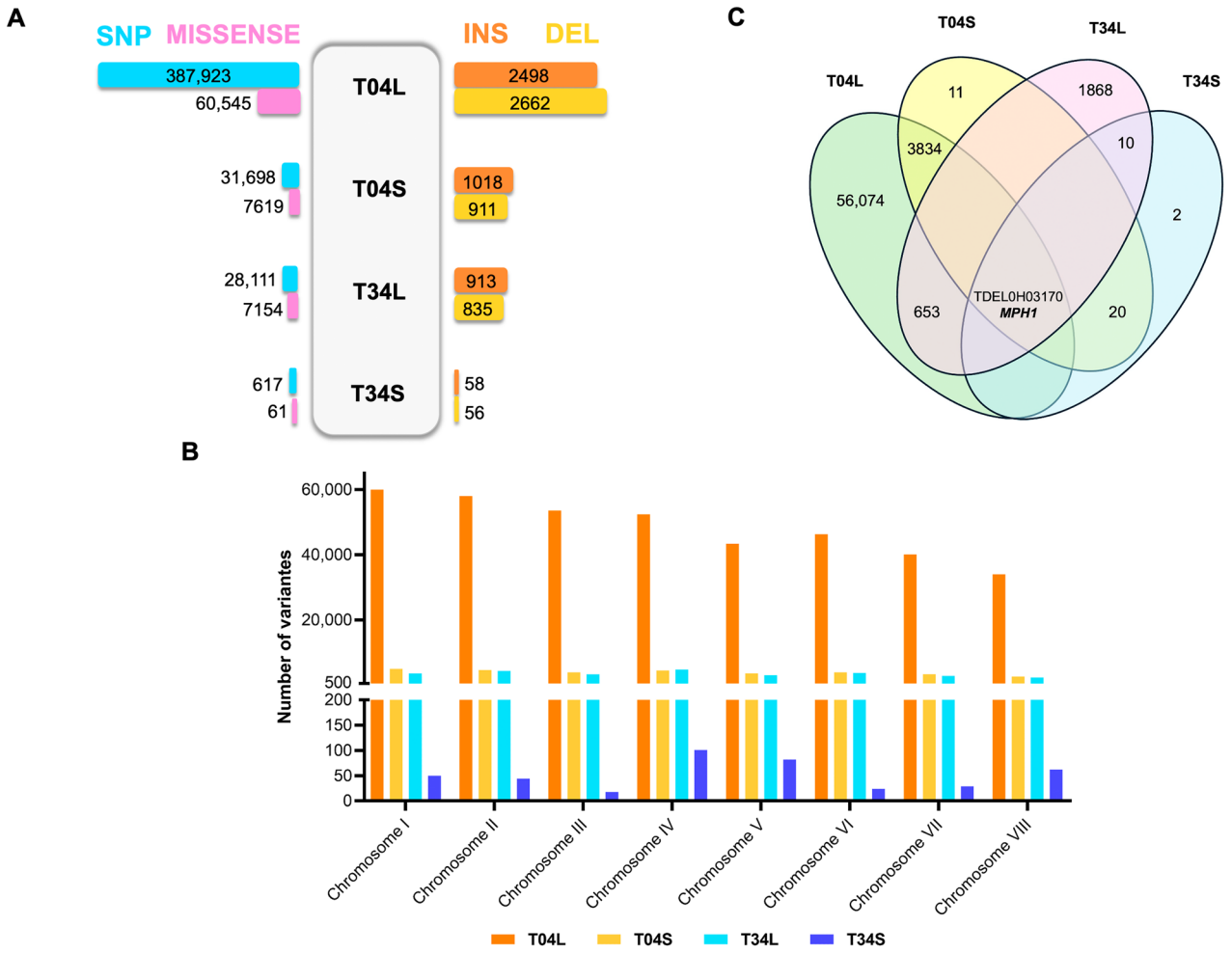


FIGURE 5 | Genetic variation of the evolved strains, in comparison with the respective parental genomes. (A) Number of single nucleotide polymorphisms (SNPs; total and missense only), insertions (Ins) and deletions (Del) in the genomes of each evolved strain. (B) Number of variants per chromosome in the genomes of each evolved strain. (C) Venn diagram representing the number of shared missense mutations between the four evolved genomes.

deletions across the four evolved strains, in comparison with the respective parental genome. Figure 5A summarises the total distribution of SNPs, revealing a predominance of SNPs over insertions and deletions in all cases. Remarkably, strain T04L exhibited the highest total variant count (390,074), contrasting sharply with T34S, which had the fewest (713). When considering only variants leading to missense mutations (substitution of one amino acid for another in the protein product), strain T04L showed also a surprisingly high number (60,565). In Figure 5B, the variant distribution across chromosomes for each evolved isolate was detailed, in comparison with their parental genomes. T04L showed a superior variant count across all eight chromosomes compared to T04S. Conversely, T34S, which evolved on solid media, displayed the most significant variation between chromosomes, especially on chromosomes II and III. The variants in T04S and T34L were similarly distributed among chromosomes, underscoring the unique evolutionary trajectories and genomic plasticity of these strains under different selective pressures.

Narrowing the analysis on the missense alterations, hypothesized as the most important to explain the evolved phenotype, we compared the ones that were shared between the strains with increased resistance (Figure 5C). Surprisingly, only one variant was found to be shared between the four evolved strains, independently of the adaptation experiments being performed in liquid or solid media, being absent from both T04 and T34 parental strains. This nonsynonymous mutation was located in chromosome VIII and represented an aminoacidic change from valine to serine in the position 934 of protein *TDEL0H03170*. Even though being a hypothetical protein according to the most updated annotations on *T. delbrueckii* genome, it is a homologue of the *S. cerevisiae* Mph1, which encodes an known to encode an ATP-dependent DNA helicase known for its role in maintaining genome stability by participating in DNA repair pathways, homologue of the *S. cerevisiae* Mph1. To confirm the association of the *MPH1* gene in the increased resistance to sulphites, we performed a validation experiment using a *S. cerevisiae* Δ*mph1* strain (Supporting Information S5). A spot assay was conducted with both wild-type and Δ*mph1* strains under increasing concentrations of SMBS. Both strains showed comparable growth up to 0.50 mM, but the Δ*mph1* strain grew better than the wild type for higher concentrations and was the only one to exhibit growth at 1.00 mM. These findings confirm that de *MPH1* gene is associated with enhanced sulphite resistance, supporting our genomic analysis results.

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4 | Discussion

The potential advantages of utilising *T. delbrueckii* over conventional *S. cerevisiae* strains in different fermentation processes have garnered significant attention in recent years. Particularly regarding wine fermentation, understanding the physiological characteristics of *T. delbrueckii* under stress conditions has emerged as a key focus in addressing the challenges associated with its application in commercial wine production (Ramírez and Velázquez 2018). Ongoing investigations that have explored diverse strategies aimed at overcoming these obstacles, with the goal of fully exploring the benefits of using *T. delbrueckii* strains.

One of the obstacles that *T. delbrueckii* can face in grape must fermentation is the presence of sulphites. In the present work, we characterised the sulphite tolerance of a collection of *T. delbrueckii* strains to understand the species variability regarding the response to this antimicrobial agent. Our results (Figure 2) demonstrated the variability of maximum tolerances observed within *T. delbrueckii* species, showing that the maximum concentration tested of 3.50 mM is required to inhibit the growth of 64% of the *T. delbrueckii* collection. Considering that *S. cerevisiae* has been reported to exhibit resistance up to 4.0 mM of potassium metabisulphite (García-Ríos et al. 2019), it is plausible to hypothesize that some *T. delbrueckii* isolates may possess similar tolerance as *S. cerevisiae*. Regarding a possible association between the maximum tolerance of each strain to SMBS and its technological group, results showed that isolates from arboreal/soil environments appear to exhibit the highest tolerance (3.50 mM), with only one strain (T60) demonstrating a slightly lower tolerance (2.50 mM). The robust tolerance displayed by these strains may be attributed to the harsh environmental conditions from which they were isolated, such as tree bark, fallen leaves and soil, where yeasts may be exposed to various sulphate compounds, including copper sulphate, commonly used in agriculture (Rusjan 2012). This result was also found before for yeasts from other origins (De Guidi et al. 2024), in which in the presence of copper in the must, wine strains produce more H_2S than other isolates that were not previously exposed to it. The authors have found that exposure to copper triggers a higher expression of genes encoding the two subunits of the sulphite reductase—*MET5*, *MET10*—leading to a higher production of H2S. No clear relationship was observed in our work for the remaining technological groups. Our findings regarding the heterogeneity of *T. delbrueckii* strains are consistent with previous results from the literature (Comitini et al. 2011).

The cladogram analysis shown in Supporting Information S4 provides insights into the genetic diversity among *T. delbrueckii* strains, for genes coding for proteins described to be related to sulphite resistance or susceptibility: *ARG3*—ornithine carbolyltransferase; *BIO3*—transaminase involved in biotin biosynthesis; *COS9*—endosomal protein; *FCY21*—putative purine-cytosine permease; *FZF1*—transcription factor involved in sulphite metabolism; *MET22*—bisphosphate-3′-nucleotidase; *MNN4*—putative positive regulator of the mannosylphosphate transferase *Mnn6p*; *SSU1*—transporter of the major facilitator superfamily involved in sulphite efflux (Lage et al. 2019; Gong et al. 2023). Notably, a discernible relationship between strain resistance and clades is observed in the *SSU1* gene, likely due to its role as the principal membrane transporter involved in sulphite metabolism. However, the differences observed in panel B of the same image, particularly in the *COS9* gene, do not exhibit a clear correlation with sulphite resistance, as strains with varying tolerances to SMBS (ranging from 0.50 to 3.50 mM) display similar genetic variations.

Successful ALE strategies were implemented to evolve strains to acquire higher resistance to sulphites, making them more suited for wine production. The selection of T04 and T34 was made under the assumption that strains with low to moderate tolerance levels would undergo more prominent genetic alterations compared to highly resistant strains. Based on our previous discussion about the advantages of employing both liquid and solid media to perform ALE (Fernandes et al. 2023), strains were evolved towards improved sulphite resistance using both solid and liquid media approaches. Notably, the solid media approach employed in this study was innovative, with no prior publications utilising this specific method, to the extent of our knowledge. To determine the optimal methodology for evolving strains in solid media, three distinct approaches were explored. In all approaches, it was assumed that the evolved culture would exhibit enhanced adaptation to the medium under the same selective pressure. However, the SC approach proved unsuccessful as the inoculum failed to develop at 1.00 mM SMBS even after 30 days of experimentation, indicating a lack of adaptation. The YB approach, lasting 109 days, only reached a concentration of 1.50 mM SMBS, falling short of significant improvement. In contrast, the AG approach yielded the most promising results, achieving a concentration of 4.50 mM in solid media over 135 days. This method's efficacy was due to the transfer occurring during the cells' AG phase, ensuring heightened metabolic activity. Analyses of the evolved isolates from the AG approach (T04S and T34S) confirmed the optimization of the ALE protocol in solid media.

In recent years, ALE in liquid media has gained prominence in scientific research. In optimization processes, strain behaviour was assessed under stress conditions imposed by the experiment, including pH 3.5 and varying SMBS concentrations. Throughout the ALE experiment, strains were expected to demonstrate increased tolerance and proliferation in media containing SMBS. To evaluate the performance of the evolved strains in both liquid and solid media, we followed the same methodology used before in the growth curves and spot assay, respectively. Results (Figure 3) show that in the growth curve assays (Figure 3A), a notable enhancement in resistance was observed across all evolved strains. Specifically, T04L demonstrated a significant improvement of 1.50 mM (approximately four times its original resistance), while T34L exhibited a smaller improvement of 0.50 mM (roughly 1.25 times more resistant). These results surpass those achieved by Bartel et al. (Bartel et al. 2021) in ALE experiments with the native sulphite-resistant strain *B. bruxellensis*, where an enhanced sulphite tolerance ranging from 1.6 to 2.5 times higher was reported. This discrepancy was expected given the inherent difficulty in inducing resistance in microorganisms with already optimised mechanisms. Additionally, Supporting Information S3 illustrates that most batch cultures exhibited a reduction in the time (days) between the first and last batch (third), indicating evident adaptation progress and facilitating experiment monitoring. Parameters typically scaled for quantifying ALE experiments include the number of generations and CCD. While the number of generations is commonly used, it overlooks DNA polymerase errors during cell growth and chromosomal

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replication. Therefore, CCD, which accounts for mutations proportional to the number of cell divisions during ALE, offers a more meaningful measure for time scaling experiments. Taking this into account, Lee et al. (2011), postulated that the CCD is a more meaningful measure to timescale this experiment where the CCD enhances with the number of generations and with the time of the experiment (Camps et al. 2003; Lee et al. 2011). In the spot assay (Figure 3B), T04S displayed a threefold enhancement in resistance (from 1.00 to 3.25 mM), while T34S showed a 1.5 times enhancement (from 2.00 to 3.25 mM). As this method has not been described in the literature previously, our successful implementation thereof marks a novel contribution to the field. Contrast to Alves et al. (2021), who observed elongated bacterial cells post-ALE, our morphological analysis did not reveal such changes. However, we noted an increase in cell size in the strains evolved in solid media (T04S and T34S) compared to the parental strains, consistent with observations by Mavrommati, Papanikolaou, and Aggelis (2023) in evolved strains of *S. cerevisiae* which had their sized changed.

Regarding whole-genome sequencing of the evolved strains, analysis of the number of variations in the genome of each evolved strain (Figure 5), in comparison with the genomes of the respective parental isolates, revealed, firstly, a lack of proportionality between the number of variations and the experiment duration—T04L (387,923 SNPs; 50 days), T04S (31,698 SNPs; 135 days), T34L (28,111 SNPs; 27 days) and T34S (617 SNPs; 32 days). Furthermore, ALE in liquid media appeared to have a greater impact on the number of mutations arising on each strains' genomes than ALE in solid media. Notably, the number of generations and CCD described for T04L and T34L did not correlate with the number of variations, despite similar values for the two strains. Figure 5B demonstrates a relatively equal distribution of variations across the eight chromosomes, except for strain T04L which shows a higher number of variants in the first chromosomes, and a remarkably higher number of total variants in comparison with the other evolved isolates (Figure 5A). Considering CNVs (Figure 4), a common feature among most evolved strains was an increase in CNVs in the terminal region of chromosome VIII. Other studies have observed similar differences in CNVs during the adaptation to stress, in particular regarding heat, nutrient limitation and ethanol tolerance (Lynch et al. 2008; Yona et al. 2012; Payen et al. 2014; Voordeckers et al. 2015). Even though these telomeric regions of chromosomes are known to have a general absence of coding genes, our previous genome annotation of the *T. delbrueckii* type strain Santiago et al. (2021) revealed the existence of 11 potential coding genes in this particular location, within chromosome VII terminal region. However, a BLAST analysis (data not shown) revealed that all those proteins matched with also hypothetical proteins of other yeast species (including *S. cerevisiae*), and, in this way, no identification of known proteins in that region was possible. Additionally, our previous work assessing *T. delbrueckii* adaptation to different environments and how it reflects in the strains' genomes (Silva et al. 2022) revealed that chromosome VIII is important to explain the adaptation of some strains to bread fermentation environments since a particular group of seven strains possess syntenic inactivated remnants of *GAL2* and *GAL10* in this chromosomic region.

In addition to the genomic variations previously discussed, the most remarkable finding in our study is the identification of a common missense mutation in gene *TDEL0H03170*, codifying a hypothetical protein homologous to the *S. cerevisiae* gene *MPH1* and functionally annotated as an ATP-dependent DNA helicase, a protein known to play a critical role in DNA repair and homologous recombination (Prakash et al. 2005; Panico et al. 2010). The mutation, causing a valine to serine alteration, consistently observed across all evolved isolates and absent in the parental strains, suggests a significant adaptive advantage under the selective pressures of sulphite resistance. This mutation is located in the C-terminal of Mph1, a region described to be important for its interaction with different partner proteins in the *S. cerevisiae* homologue (Banerjee et al. 2008; Chavez, Agrawal, and Johnson 2011; Xue et al. 2014, 2015).

The presence of this mutation may imply an enhanced ability for DNA repair in the evolved isolates, potentially contributing to a greater genomic stability in environments with high levels of sulphites. Sulphites are known to cause oxidative stress and DNA damage, and functional alteration of a DNA helicase involved in repair mechanisms could provide a protective effect, enabling the cells to better cope with the stress induced by sulphite exposure. This aligns with existing knowledge that mutations in DNA repair genes can confer resistance to various stressors, including chemical agents and radiation, by improving the efficiency of damage repair and reducing the accumulation of deleterious mutations (Branzei and Foiani 2008; Hanawalt and Spivak 2008). Moreover, *S. cerevisiae*'s *MPH1* is involved in the avoidance of crossover during homologous recombination, a process that can prevent the loss of genetic information and maintain chromosomal integrity. This could be particularly advantageous in stressful environments, where the preservation of essential genomic regions is critical for survival. The observed mutation might affect the helicase activity or its interaction with other proteins involved in DNA repair pathways, thereby modifying the cellular response to DNA damage in a way that enhances sulphite tolerance. This finding is significant because it highlights a specific genetic adaptation mechanism in *T. delbrueckii* that has not been previously described in the context of sulphite resistance. Further functional characterisation of this mutation could provide deeper insights into its role and potential applications in industrial processes where sulphite resistance is desirable, such as wine fermentation. Further experiments are required to elucidate the mechanistic basis behind variations observed in our strains and validate their adaptive significance, in particular to evaluate also how an increase in copy number of genes located in the telomeric region of chromosome VIII can help this particular strain thrive in growing concentrations of sulphites.

The enhanced sulphite resistance observed in the evolved *T. delbrueckii* strains indicates their potential to withstand standard winemaking conditions. However, further studies are required to confirm their fermentative performance and evaluate any changes in the organoleptic properties of the resulting wine, such as flavour, aroma and mouthfeel, to ensure that these improvements do not compromise the sensory qualities of the final product.

5 | Conclusion

This study successfully enhanced sulphite resistance in *T. delbrueckii* using ALE in both solid and liquid media, marking significant progress in yeast strain improvement. The innovative

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solid media ALE protocol demonstrated a strong potential for industrial applications, achieving higher resistance levels compared to traditional methods. Genomic analysis of the evolved strains revealed diverse mutation profiles, underscoring the distinct evolutionary pathways induced by each ALE method. Notably, a common missense mutation in the gene *TDEL0H03170*, encoding a DNA helicase, emerged as a key adaptation conferring sulphite resistance. These findings highlight the effectiveness of tailored ALE strategies in generating desired phenotypic traits and lay the groundwork for further research into the genetic mechanisms of stress resistance in yeast. Future work should focus on the functional validation of these genetic changes and expanding ALE protocols to other media and stress conditions to enhance the utility of *T. delbrueckii* in various industrial applications.

Author Contributions

Carolina Osório: methodology, investigation, software, writing – original draft, writing – review and editing. **Ticiana Fernandes:** methodology, investigation, writing – original draft, writing – review and editing. **Teresa Rito:** funding acquisition, writing – original draft, writing – review and editing. **Pedro Soares:** funding acquisition, writing – original draft, writing – review and editing. **Ricardo Franco-Duarte:** conceptualization, methodology, investigation, software, supervision, writing – review and editing, writing – original draft. **Maria João Sousa:** conceptualization, investigation, funding acquisition, supervision, writing – original draft, writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Genomic data are available at the NCBI-SRA database with the BioProject accession number PRJNA1135909. The remaining data is available in Table S1. Here is the DOI for the published Supporting Information: 10.6084/m9.figshare.28129961.

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