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De Novo Hybridization Approach Expands Diversity of Industrially Applicable Lager Yeast Strains with Unique Genome Composition

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ABSTRACT

Lager beer is produced by *Saccharomyces pastorianus*, which is a natural allopolyploid hybrid between *S. cerevisiae* and *S. eubayanus*. Lager strains are classified into two major groups based largely on genomic composition: group I and group II. Group I strains are allotriploid, whereas group II are allotetraploid. A lack of phenotypic diversity in commercial lager strains has led to substantial interest in the reconstitution of *de novo* allotetraploid lager strains by hybridization of *S. cerevisiae* and *S. eubayanus* strains. Such strategies rely on the hybridization of wild *S. eubayanus* isolates, which carry unacceptable traits for commercial lager beer such as phenolic off-flavors and incomplete utilization of carbohydrates. Using an alternative breeding strategy, we have created *de novo* lager hybrids containing the domesticated *S. eubayanus* subgenome from an industrial *S. pastorianus* strain by hybridizing this strain to a variety of *S. cerevisiae* ale strains. Five *de novo* hybrids were isolated that had fermentation characteristics similar to those of prototypical commercial lager strains but with unique phenotypic variation due to the contributions of the *S. cerevisiae* parents. Genomic analysis of these *de novo* lager hybrids identified novel allotetraploid genomes carrying three copies of the *S. cerevisiae* genome and one copy of the *S. eubayanus* genome. Most importantly, these hybrids do not possess the negative traits that result from breeding wild *S. eubayanus*. The *de novo* lager strains produced using industrial *S. pastorianus* in this study are immediately suitable for industrial lager beer production.

Keywords: *Saccharomyces*, *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, lager, hybrid, genetics

Introduction

The lager beer yeast *Saccharomyces pastorianus* is an allopolyploid (a strain that contains more than two homologous sets of chromosomes, which are derived from two different species of yeast) hybrid between *S. cerevisiae* and the cryotolerant species *S. eubayanus* (1,2). Individual lager strains are classified into two major groups: group I (Saaz type) or group II (Frohberg type). Group I and group II strains have some differences in their genomic composition and in fermentation characteristics. Phenotypically, group I strains are generally more cryotolerant when compared with group II strains, whereas group II strains display more robust fermentation rates and yields (3,4). Importantly, group I strains display some phenotypic variation in maltotriose utilization. Some group I strains have an increased maltotriose consumption rate, whereas others have a non-maltotriose-utilizing phenotype (5). Genetically, group I lager strains are characterized by an allotriploid (three sets of chromosomes derived from two different species of yeast) genome composed of a diploid complement of *S. eubayanus* chromosomes and a haploid set of *S. cerevisiae* chromosomes (4). Group II lager strains are allotetraploids (four sets of chromosomes, derived from two different species of yeast), with diploid sets of both *S. eubayanus* and *S. cerevisiae* chromosomes (4,6), with a few exceptions (7). The ploidy of these two groups of strains is also illustrated in Figure 1. One of the more widely accepted hybridization models for the origins of both lager lineages suggests that an initial hybridization event occurred between a haploid *S. cerevisiae* ale strain and a diploid *S. eubayanus* strain (8,9). This progenitor lager strain then underwent multiple chromosomal recombination events to found the group I lineage (10). Subsequently, a second hybridization event occurred between this progenitor and a second *S. cerevisiae* ale strain, to found the group II lineage (8,9). On the other hand, recent sequencing data using chromosome level assemblies suggests that, in fact, both group I and group II lager strains originate from a single hybridization event (7), and that the differences between all known modern group I and II strains are primarily the result of differing evolutionary trajectories following a population bottleneck created by the isolation and propagation of pure cultures during the industrialization of brewer's yeast (7).

This strong phenotypic and, by extension, genotypic selection of lager yeast has resulted in a modern reliance on only a handful of closely related lager yeast strains (from each of the group I and group II lineages) for the entirety of lager beer production globally (11). Not surprisingly, this lack of diversity in available lager strains has created substantial interest in the creation of

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new lager strains. To date, efforts have been focused on trying to create *de novo* "lager-like" alloploid hybrids, by selective mating of *S. eubayanus* to *S. cerevisiae* ale strains using either non-genetically modified (GM) mating strategies (12–16) or plasmid-assisted mating (17). As the cryotolerance of *S. eubayanus* is a critical component of the lager yeast phenotype, other cryotolerant species, including *S. mikitae*, S. *kudriavzevii*, *S. arbicola*, and *S. uvarum*, have also been hybridized to *S. cerevisiae* in an effort to create novel cryotolerant hybrid strains for both beer and wine fermentations (18). More recently, the inheritance of *S. eubayanus* mitochondria has been shown to be a major factor influencing cryotolerance of *S. cerevisiae* × *S. eubayanus* hybrids (19).

Attempts to create *de novo* lager hybrids using *S. eubayanus* isolates and *S. cerevisiae* ale strains have successfully created hybrid strains capable of fermenting beer at lager temperatures (10–13°C). However, because of their use of wild, undomesticated *S. eubayanus*, these strains also possess a number of other undesirable attributes that generally preclude resulting strains from the production of prototypical lager beers. One such issue is the inheritance of a phenolic off-flavor (POF)-positive phenotype from wild *S. eubayanus*, a trait that was lost during the domestication of *S. pastorianus* and is not present in the *S. eubayanus* subgenome of *S. pastorianus* (6,20,21). Indeed, all known wild isolates of *S. eubayanus* are POF-positive (15,16,22). Another complication of using wild *S. eubayanus* isolates in *de novo* lager hybridization is the inability of known *S. eubayanus* isolates to consume maltotriose (3,14,23–25), one of the major sugars in brewer's wort. Recent studies have demonstrated that the domesticated *S. eubayanus* subgenome of *S. pastorianus* spontaneously evolved maltotriose transporters—and thereby the ability to ferment maltotriose (21,26).

A number of strategies have been, or could be, employed to overcome the limitations of using wild *S. eubayanus* in *de novo* lager hybridization. Adaptive laboratory evolution of either wild *S. eubayanus* or resulting *de novo* hybrids is a commonly used approach to mitigate undesirable phenotypic traits (22,27,28). Finally, molecular biology tools, such as recombinant DNA and/or CRISPR-Cas9 gene editing, could be used for strain bioengineering. Indeed, the POF phenotype has been removed from the *S. eubayanus* genome by deleting the *PAD1* and *FDC1* genes using CRISPR-Cas9 prior to *de novo* hybridization (29); however, the use of genetic engineering tools results in genetically modified organisms (GMOs) that may not be accepted by consumers and/or industry.

An alternative strategy to create industrially suitable *de novo* lager yeast strains is to directly access the already-domesticated *S. eubayanus* subgenome by isolating mating-competent spores derived from an allotetraploid *S. pastorianus* strain (30,31). Furthermore, it has also been shown that selectable auxotrophies (the inability of an organism to synthesize a particular metabolite) can be induced using non-GMO methodologies in

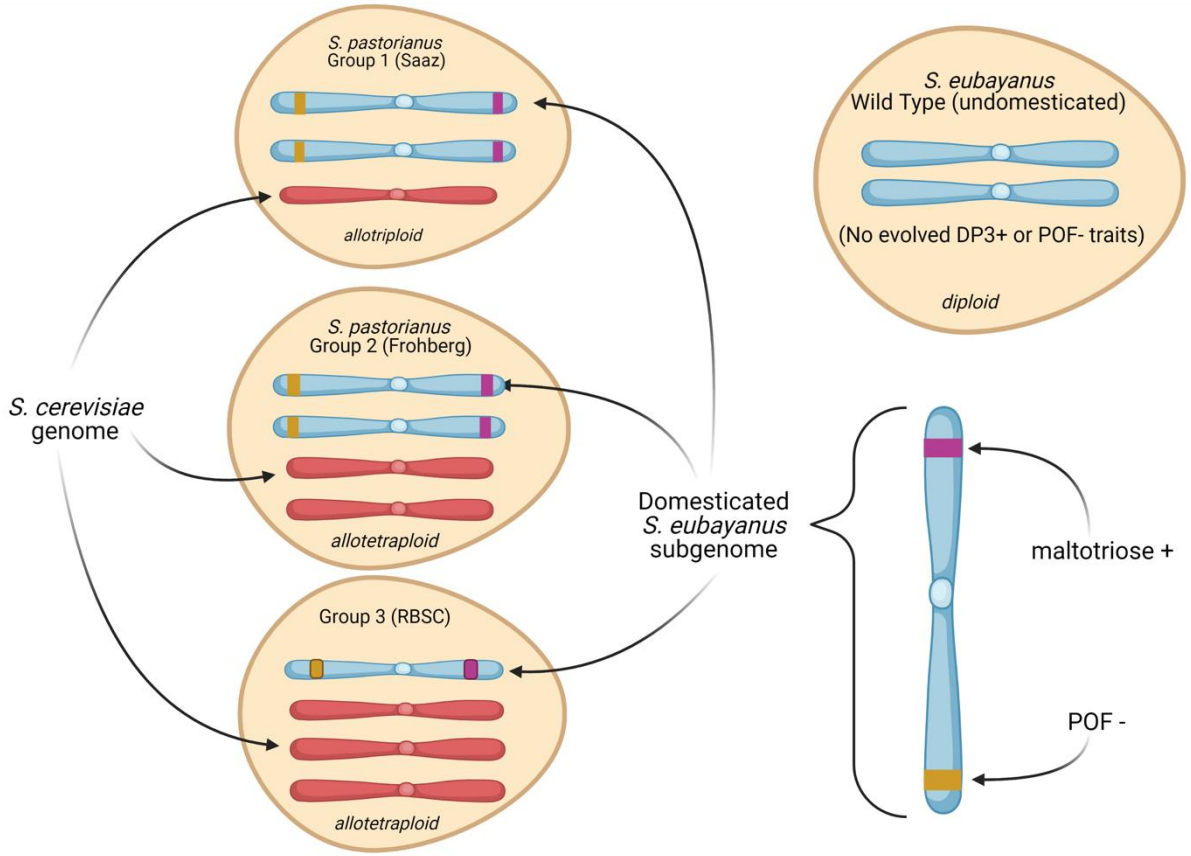

Figure 1. Illustration depicting the karyotypes of group I and group II lager strains compared with the novel lager hybrids forming the new group III category. The diploid genome of wild *S. eubayanus* is also shown in comparison to the domesticated *S. eubayanus* subgenome originating from lager strains. The POF-negative trait and ability to efficiently consume maltotriose are hallmarks of the *S. eubayanus* subgenome domestication and are depicted in the figure to differentiate it from wild *S. eubayanus*. *S. cerevisiae* subgenomes are shown in red with the *S. eubayanus* subgenomes shown in blue. Created using BioRender.com.

these *S. pastorianus* spore-isolates (32). Taken together, these techniques, referred to as "rare mating", provide a feasible, non-GM option for rare mating of *S. pastorianus* spore-isolates (containing the domesticated *S. eubayanus* subgenome and mitochondria) with *S. cerevisiae* strains to create "domesticated" *de novo* lager hybrids. Interestingly, a similar approach was used to create lager-ale hybrids that had improved stress resistance and fermentation performance at warmer ale temperatures (31). However, to the best of our knowledge, this strategy has not been used previously to develop *de novo* lager yeast strains immediately suitable for industrial lager beer production—that is, with the ability to ferment at lager temperatures (10–13°C) and with suitable sugar utilization, stress tolerance, and aroma production, including a POF-negative phenotype.

In this study, we create and characterize *de novo* lager hybrids, which incorporate the domesticated *S. eubayanus* subgenome from *S. pastorianus*, using the aforementioned non-GM approach. We show that such *de novo* hybrid strains have fermentation performance and phenotypic attributes that make them suitable to be applied as industrial lager strains. Importantly, by using different parental strains, we show that we can expand the genetic and phenotypic diversity of the novel lager strains while retaining the lager-like qualities. Lastly, by using whole genome sequencing of selected *de novo* hybrids, we demonstrate that these strains possess a unique allotetraploid genomic composition of three *S. cerevisiae* genome complements to one *S. eubayanus*. Taken together, this study provides a novel, non-GM tool for the creation *de novo* lager hybrids that does not necessitate the use of wild or modified *S. eubayanus* strains. The development and characterization of a set of such hybrids—created by combining the domesticated *S. eubayanus* subgenome with diverse *S. cerevisiae* parents—facilitates the expansion of the lager strain clade by introducing a novel group of strains that are suitable for industrial lager beer production.

Materials and Methods

Strains, Media, and Culture Conditions

All strains used in this study are listed in Table 1. Strains were cultured in YEG (1% yeast extract, 2% glucose) or YMM (1% yeast extract, 2% malt extract, 2% maltose) at 21°C, with agitation. For auxotrophic selection, three different media were used: SD+5FOA (1.9 g/L of yeast nitrogen base [YNB] without ammonium sulfate or amino acids, 2% glucose, 0.5 g/L of 5-fluoroorotic acid, and 2% agar), SD+FAA (1.7 g/L of YNB without ammonium sulfate or amino acids, 5% glucose, 0.74 g/L of complete supplement mixture lacking tryptophan, 10 mg/L of L-tryptophan, 0.5 g/L of 5-fluoroanthranilic acid, and 2% agar), and SD+αAA (1.7 g/L of YNB without ammonium sulfate or amino acids, 2% glucose, 30 mg/L of L-lysine, and 20 g/L of DL-2-α-aminoadipate). All plates were incubated at 25°C. Strains undergoing sporulation were grown in YEG to late exponential phase and subcultured into SPO medium (0.05% glucose, 0.1% yeast extract, and 1% potassium acetate) and incubated at 21°C for 7 days.

Table 1. List of strains used in this study

Strain name	Classification	Species	Source
CBS1513	Group I lager	*S. pastorianus*	NCYC
W3470	Group II lager	*S. pastorianus*	Commercial
US-05	Ale yeast	*S. cerevisiae*	Commercial
CBS12357	Wild yeast	*S. eubayanus*	NCYC
RB-1141	Novel hybrid	*S. pastorianus*	This work
RB-1186	Novel hybrid	*S. pastorianus*	This work
RB-2215	Novel hybrid	*S. pastorianus*	This work
RB-2251	Novel hybrid	*S. pastorianus*	This work
RB-2403	Novel hybrid	*S. pastorianus*	This work
RB-10	Lager parent	*S. pastorianus*	Isolate from W3470[a]
RB-24	Lager parent	*S. pastorianus*	Isolate from W3470[a]
RB-40	Lager parent	*S. pastorianus*	Isolate from W3470[a]
RB-253	Ale parent	*S. cerevisiae*	RBSC[b]
RB-7	Ale parent	*S. cerevisiae*	Isolate from NCYC-1113[a]
RB-8	Ale parent	*S. cerevisiae*	Isolate from NCYC-1113[a]
RB-48	Ale parent	*S. cerevisiae*	RBSC[b]

[a] Diploid meiotic segregant isolated from spores of specified strain.
[b] Diploid strain from RBSC's culture collection, originally isolated from nature.

Diploid Isolation, Auxotroph Generation, and Rare Mating

Tetraploid parental strains (Table 1) were sporulated, and spores were released by digesting the ascus wall in a Zymolyase solution consisting of 5 mg/mL of Zymolyase (Nacalia Tesque, Japan) and 1 M sorbitol. Digestion was carried out at 30°C for 4 h. Spores were then resuspended in water and vortexed to facilitate lysis of any remaining vegetative cells. Spore suspensions were then plated out in serial dilutions and incubated at 25°C for 7 days to allow viable spores to form colonies. Cells obtained from smaller colonies were observed microscopically for reduced cell size compared with the 4 N parental strains (33), at which point their ploidy was determined by flow cytometry. Variants confirmed to possess roughly half of the parental genome content (~2 N) were screened for fermentation characteristics, and those found to inherit similar features as their parental strain were chosen for rare mating. Auxotrophs were obtained as previously described (34–36). Hybridization of auxotrophs was performed by rare mating as previously described with minor modification (37,38). Successful hybridization was confirmed by measuring DNA content by flow cytometry and cross-checking for loss of resistance to 5-FOA, FAA, or αAA.

Wort Preparation and Fermentation Sampling and Analysis

Wort was prepared by dissolving 120 g/L of dry malt extract (Briess USA, CBW Golden Light) and 60 g/L of Brewers crystals (BrewCraft USA, 56% maltose corn syrup solids) in the required amount of water at 90°C, kettle (Blichmann BrewEasy, Lafayette, IN) was brought to a boil for 5 min, 1.47 g/L of Magnum hops were then added and boiled for 30 min to achieve an IBU of 30, 0.12 g/L of nutrients (Wyeast Beer Nutrient Blend, Odell, OR) was added, and the boil was continued for 10 min.

Laboratory-scale fermentations were carried out under a standardized system of assessment for the purposes of benchmarking each yeast's fermentation performance.

Yeast from agar plates was aerobically propagated in 5 mL of YMM broth (1% yeast extract, 2% maltose, 2% malt extract) in culture tubes at 21°C for 24 h with agitation. Cultures were then scaled up to 50 mL of YMM broth in baffled culture flasks and incubated at 21°C for 48 h with agitation. Cells were counted via hemocytometer and pitched into either 80 or 3,000 mL of 15°P wort at a rate of 1.5×10^6 cells/mL/°Plato and incubated isothermally at 13°C. Wort was oxygenated by manual agitation, per a standard protocol that has consistently generated 8 ppm of dissolved oxygen in the past. The particular dissolved oxygen levels were not measured for this study. Fermentations were carried out statically in 3.79 L glass bottles. Fermentation kinetics at the 3,000 mL scale were tracked by daily monitoring for sugar con-

sumption via high-performance liquid chromatography (HPLC) analysis and at the 80 mL scale were tracked by weight loss.

Initially, each strain was grown to an OD_{600nm} of 1.0 and its cell density determined using a hemocytometer and normalized to its OD_{600nm} reading. Throughout the course of the fermentation, a 1 mL aliquot was carefully taken from the center of the fermentation vessel so as not to disturb the settled yeast, diluted, and an OD_{600nm} reading obtained. The cells in suspension value was calculated by multiplying the OD_{600nm} by the normalized OD_{600nm} for each strain. At the end of the primary fermentation, the fermentation vessel contents were stirred in order to suspend all yeast cells uniformly. Samples were taken and cell viability determined using methylene blue staining and quantified by counting using a hemocytometer.

Sugar, Organic Acid, Glycerol, and Ethanol Analysis by HPLC-RID

The sugars, organic acids, glycerol, and ethanol of wort and beer samples was quantitated by HPLC with a refractive index detector (HPLC-RID) (1260 Infinity II, Agilent, Santa Clara, CA) equipped with a Rezex ROA-Organic Acid H+ (8%) column (300 × 7.8 mm, Phenomenex, Torrance, CA).

Sample preparation and analysis were carried out as previously described (39) with a few modifications. Fermentation samples were centrifuged at 15,000 rpm for 5 min, followed by filtration of supernatant through a 0.45 µm nylon membrane filter. The filtrate obtained was loaded for sugar, organic acid, glycerol, and ethanol analysis using a mobile phase of 0.005 N H_2SO_4 at 60°C with a flow rate of 0.5 mL/min for 35 min. Serial dilutions of standard solutions of compounds of interest were prepared and analyzed to establish standard calibration curves.

Volatile Aromatic Compound Analysis by Headspace GC-MS

Beer samples were centrifuged at 4,000 rpm for 10 min, and 5 mL of the obtained supernatant was pipetted into a 20 mL headspace vial sealed by a metal screw cap with a PTFE/silicone septum for quantitation of acetaldehyde, dimethyl sulfide (DMS), acetone, higher alcohols, and esters by an Agilent GC 7890B coupled with a PAL autosampler (RSI 85, CTC Analytics, Zwingen, Switzerland) and an MS detector (5977B, Agilent).

Beer samples were incubated at 40°C for 20 min to reach equilibrium, followed by injection of 1 mL headspace with syringe temperature set at 70°C, inlet temperature at 200°C, and a split ratio of 10. Separation of volatile compounds was carried out with a DB-WAX UI capillary column (60 m × 0.25 mm, 0.25 um film thickness, Agilent) and helium as the carrier gas with a constant flow of 1.2 mL/min. The oven program was as follows: held initial temperature at 35°C for 1 min; increased to 120°C by 15°C/min; increased to 180°C by 5°C/min; further increased to 250°C by 20°C/min; and held at 250°C for 5 min. The temperature of the transfer line to MS was set at 250°C. The detection was performed at TIC mode (*m/z* 25–250) with electron-impact ionization at 70 eV, ion source temperature at 230°C, and quadrupole temperature at 150°C.

Acetone, DMS, ethyl valerate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, phenethyl acetate (MilliporeSigma, St. Louis, MO), and pre-made standard mixture containing acetaldehyde, *N*-propanol, ethyl acetate, isobutanol, isopropyl acetate, ethyl propanoate, active amyl alcohol, isoamyl alcohol, isobutyl acetate, ethyl butanoate, *N*-butyl acetate, and isoamyl acetate (SPEX CertiPrep, Metuchen, NJ) were used to establish standard calibration curves. Ethyl methyl sulfide and 3-octanol were used as the internal standards for quantitation of DMS and the rest of the compounds, respectively.

Analysis of Vicinal Diketones (VDKs) by UPLC-QDa

VDK analysis of beer samples was carried out according to the method of Blanchette et al. (40) with a few modifications. Beer samples were centrifuged at 15,000 rpm for 4 min. The supernatant (1.5 mL) was transferred to a 2 mL screw-cap tube with addition of 27.8 µL of 0.2 N H_2SO_4 to adjust pH to 3.5, followed by 90 min heating at 60°C to convert all VDK precursors to VDKs. Subsequently, 2,3-butandione (diacetyl) and 2,3-pentanedione in the heated beer samples were derivatized by *o*-phenylenediamine dihydrochloride. The derivatization products (2,3-dimethylquinoxaline and 2-ethyl-3-methyl-quinoxaline) were separated and detected by ultra-performance liquid chromatography equipped with a quadrupole dalton detector (UPLC-QDa) (Acquity H-Class, Waters, Milford, MA) equipped with a Waters UPLC BEH C18 Column (2.1 × 50 mm, 1.7 µm). Probe temperature, capillary voltage, and cone voltage of the QDa were set at 600°C, 800 V, and 12 V, respectively. Positive ions of *m/z* 159.1 and *m/z* 173.1 were monitored for quantitation of diacetyl and 2,3-pentanedione, respectively.

A series of standard solutions of diacetyl and 2,3-pentanedione were prepared, derivatized, and analyzed to establish standard calibration curves.

Intracellular Glycogen and Trehalose Analysis in Yeast Cells

The intracellular glycogen and trehalose content in yeast cells was analyzed based on the methods used by Becker (41) and Parrou and François (42) with a few modifications.

Glycogen. Homogenous cell suspension (3 mL) was sampled and washed with 0.9% NaCl solution at 4°C. Intracellular glycogen in the washed cell pellet was released by adding 1 mL of 0.25 M Na_2CO_3 solution followed by boiling for 2 h. After cooling to room temperature, 0.6 mL of 1 M acetic acid and 2.4 mL of 0.2 M sodium acetate were added to bring the pH to 5.2 for the subsequent glycogen hydrolysis by adding 50 µL of 26 U/mL amyloglucosidase solution at 57°C for 40 h. The supernatant of the hydrolysate was loaded for glucose quantitation by HPLC-RID with the same method settings as described above.

Trehalose. Homogenous cell suspension (10 mL) was sampled and washed with 0.9% NaCl solution at 4°C. After discarding the supernatant, 10 mL of Milli-Q water was added to resuspend yeast cells. Once yeast was fully suspended, 10 min boiling in a water bath was carried out immediately to extract trehalose. The supernatant of the extract was collected and analyzed by HPLC-RID for trehalose (see detailed HPLC-RID method). Trehalose standard solutions were prepared and analyzed for calibration.

Yeast content in cell suspension (mg/mL) was determined by recovering yeast cells from 10 mL of cell suspension and drying the yeast pellet at 80°C for 3 h. Glycogen content of yeast was calculated as equivalent glucose amount (mg) released from glycogen per 100 mg of yeast (dry basis, %, w/w). Trehalose content was presented as trehalose amount (mg) extracted from 100 mg of yeast (dry basis, %, w/w).

Reference Genomes

Reference genomes of *S. cerevisiae* S288c R64-1-1 (43) and *S. eubayanus* (44) were merged into a chimeric reference genome.

Sequencing and Raw Reads Alignment

Genomic DNA was purified using a standard phenol-chloroform based extraction. DNA was then treated with RNaseA for 1 h at 37°C and was repurified with an additional phenol-chloroform extraction. Sequencing libraries were prepared using the

Nextera DNA Flex library preparation kit, according to the manufacturer's instructions (Illumina). Libraries were pooled and loaded onto a NextSeq High Output flow cell, generating 150 bp paired-end reads. Raw base call data were converted into FastQ format using the bcl2fastq conversion software from Illumina (version 2.19). Library preparation and sequencing were performed at the Sequencing and Bioinformatics Consortium at the University of British Columbia (Vancouver, BC, Canada). Raw reads quality control was performed in FASTQC (45). Read alignment to our chimeric reference genome was performed using BWA (46). Duplicated reads were marked using Picard tools (47). Base recalibration was performed using GATK (48,49) according to the GATK and NGS data processing workflow (50).

Calling and Annotation of SNPs/INDELs

SNP/INDEL variants were called using the GATK software suite and recommendations (48–50). Variant sites were filtered using the following parameters: depth > 40, quality by depth > 2, Fisher strand < 60, root mean square of mapping quality > 40. Variants annotation was computed using SnpEff (51) and its associated *S. cerevisiae* r64-1-1v86 and *S. eubayanus* annotations databases.

Allele Frequency and Parental Origin

SNP filtering was performed using bcftools (52), and allele frequency computing and plotting was performed using the R environment, with vcfR (53) and karyoploteR (52) as additional packages.

Assembly

For each strain, reads were assembled into scaffolds using ABySS (54) with a median N50 of 19,666 across studied strains. The length of assemblies is reported here as the sum of length of the distinct scaffolds obtained with ABySS. To compute the length of each subgenome (S288C or *eubayanus*) we assembled the reads mapping to the corresponding regions independently and, similarly, reported the cumulative length of distinct scaffolds.

Results

Generation of *De Novo* Lager Hybrids by Rare Mating of *S. cerevisiae* and *S. pastorianus* Meiotic Segregants

To develop *de novo* lager yeast hybrids using the domesticated *S. eubayanus* subgenome of *S. pastorianus*, we isolated allodiploid meiotic segregants (or spores) from a group II lager strain and rare mated them to a variety of diploid *S. cerevisiae* ale strains. In order to do so without the use of antibiotic resistance or other foreign markers, auxotrophic variants of the diploid spores from a group II lager strain unable to synthesize tryptophan (trp-), and different diploid ale strains unable to synthesize lysine or uracil (lys- or ura-) were generated using non-GMO methods. These auxotrophs were then rare mated, and hybrids were selected for on minimal medium to isolate prototrophic hybrids. These prototrophs would be daughter cells that have inherited the ability to synthesize tryptophan from the lys- or ura- parent, and the ability to synthesize lysine or uracil from the trp- parent. Colonies from each set of rare matings were subcultured onto minimal medium for two generations to ensure a pure culture and then confirmed as tetraploid by measuring DNA content using flow cytometry. *Bona fide* tetraploid hybrids were then screened for lager-appropriate phenotypic traits in laboratory-scale fermentations of standard wort (15°P) at 13°C. The top five performing hybrids based on initial criteria of suitable fermentation kinetics, high maltotriose utilization, complete consumption of maltose, and low production of off-aroma compounds (i.e., DMS, acetaldehyde, diacetyl, and ethyl acetate) were carried forward for further characterization. Hybrids originated from the following crosses of diploid parental strains: **RB-1141** (RB-10 × RB-253), **RB-1186** (RB-10 × RB-8), **RB-2215** (RB-24 × RB-7), **RB-2251** (RB-24 × RB-48), and **RB-2403** (RB-40 × RB-48).

De Novo Lager Hybrids Exhibit Broadened Temperature Tolerance

Temperature tolerance is a key differentiator of *S. cerevisiae* ale strains and *S. pastorianus* lager strains: the former grow well at 37°C but poorly at 7°C, and the later are incapable of growth at 37°C but grow robustly at 7°C (55). To assess the temperature tolerance phenotype of the novel lager hybrids, we compared the temperature growth profile of the novel lager hybrids to prototypical group I (CBS1513) and group II (W3470) lager strains, *S. eubayanus* (CBS12357), and a common industrial *S. cerevisiae* ale strain (US-05). Dilution plating on YEG medium at 7, 25, and 37°C was used to establish the strains' relative temperature tolerance. As expected based on their established temperature profiles as cryotolerant and cryosensitive strains, respectively (19), *S. eubayanus* exhibited robust growth at 7°C, whereas US-05 showed relatively poor growth at 7°C (Fig. 2). Of the novel hybrid lager strains, RB-1141, RB-1186, and RB-2251 had similar growth to the *S. pastorianus* controls (CBS1513 and W3470) at 7°C, with RB-1141 being the most cryotolerant, whereas RB-2215 and RB-2403 had slightly weaker growth at 7°C (Fig. 2). When comparing relative growth at 37°C, neither of the *S. pastorianus* control strains nor the *S. eubayanus* strain was able to grow, consistent with previous observations (18,19,31); however, all of the novel hybrids were capable of growth at 37°C, similar to the US-05 *S. cerevisiae* control (Fig. 2), and all strains had relatively similar growth at the control temperature of 25°C. Taken together, these data indicate that our novel hybrids display heterosis with respect to growth temperature, gaining the ability to grow from 7°C to 37°C, effectively extending their temperature tolerance range.

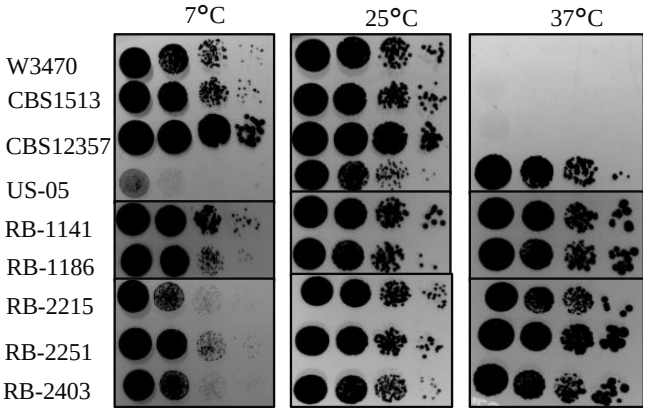

Figure 2. Temperature tolerance of novel hybrids compared with group I (CBS1513), group II (W3470), *S. eubayanus* (CBS12357), and *S. cerevisiae* (US-05) controls at 7, 25, and 37°C. Each spot represents a 10-fold serial dilution (10^4 to 10^1 cells/spot) on YEG medium.

De Novo Hybrid Lager Strains Display Phenotypic Similarities to Traditional Lager Strains in Lager Beer Fermentations

In order for any novel lager strain to be suitable for industrial beer production, it must possess suitable traits for lager beer fermentation. Among others, these include complete consumption of maltose and efficient maltotriose utilization at cooler lager fermentation temperatures (10–13 °C), all the while producing low levels of acetic acid and other off-aroma compounds. We therefore assessed each hybrid yeast's performance in laboratory-scale lager beer brewing, as compared to the group I and group II strains, W3470 and CBS1513, respectively. Briefly, 80 mL fermentations of 15°P wort were inoculated in quadruplicate and incubated isothermally at 13°C. Fermentations were harvested after 14 days and their sugar profiles determined by HPLC analysis. As shown in Table 2, glucose was completely consumed by every strain. Maltose utilization was greater than 98% for all strains with the exception of CBS1513, which only consumed 94.9% of the maltose (3.9 g/L residual maltose). With respect to maltotriose, all strains exhibited high consumption of maltotriose, consuming between 86.4 and 90.2%. RB-1141 had the overall highest consumption of fermentable sugars (i.e., glucose, maltose, and maltotriose combined), resulting in the highest ethanol concentration (7.4% v/v), compared with between 7.1 and 7.3% for the other strains. As expected, none of the strains consumed any dextrins (DP4+ sugars), as they all lack the *STA1* gene required for dextrin utilization (56–59). Acetic acid concentration was also measured, and we observed that two of the hybrids, RB-1141 and RB-2403 had elevated levels (0.14 g/L) compared with W3470 (0.10 g/L) and CBS1513 (0.01 g/L), whereas all other hybrids were in between these two lager reference strains.

To analyze fermentation kinetics, diacetyl production, and flocculation in the context of larger scale brewing, we next performed 3 L laboratory-scale fermentations. Of note, it has been previously observed that both diacetyl (60) and flocculation (61) are better represented in larger volume fermentations, with the increased volume allowing for sampling with minimal disruption to the flocculated yeast in the vessel bottom. Fermentations were carried out isothermally at 13°C in 15°P wort, and samples were taken every 24 h.

Comparing maltose consumption, the best performing strain was RB-2215 (finished on day 5) followed by RB-1141, RB-2251, W3470, RB-2403, and CBS1513, with RB-1186 being the slowest, finishing on day 11 (Fig. 3A). For maltotriose consumption, the fastest strain was CBS1513 (finishing on day 3), followed by strains RB-2215, W3470, RB-2251, RB-1141, and RB-2403, with the slowest being RB-1186, which was still not finished consuming maltotriose after day 14 (Fig. 3B). Of note, the ability of the group I control strain CBS1513 to rapidly consume maltotriose has been previously observed (5), a finding supported by our results. With respect to diacetyl, we observed that all strains differed in the peak concentration achieved on day 2, as well as the ability to reduce diacetyl concentrations toward the end of the fermentations. The strain with the highest peak diacetyl production was CBS1513 at almost 2,400 mg/L, followed by RB-2215, RB-2403, RB-1186, RB-2251, and W3470, and RB-1141 had the lowest peak at only 1,190 mg/L. When considering diacetyl reduction, strain RB-2215 had the greatest reduction capacity, followed by RB-2251, RB-2403, CBS1513, W3470, and RB-1141, and RB-1186 had the lowest reduction capacity. Lastly, flocculation throughout the course of the fermentation was monitored by counting total cells in suspension (CIS). All strains started at the same level due to identical inoculation rates. During the initial growth phase (2–3 days), a sharp increase in CIS was observed for all strains followed by a drop in CIS (indicative of flocculation), with the exception of RB-1186, which had a more gradual increase in CIS, peaking after day 6. Following initial peak CIS, the strain with the fastest flocculation rate was RB-2403, followed by RB-1141, RB-2251, W3470, RB-2215, and RB-1186, with CBS1513 being the slowest to flocculate (Fig. 3D). Only strains CBS1513 and RB-1186 remained in suspension by day 14 of the fermentation, indicating non-flocculant phenotypes.

Upon completion of fermentation (day 14), both yeast viability and yeast vitality were assessed, by methylene blue staining and by measuring intracellular glycogen/trehalose levels, respectively. Of note, glycogen is a critical intracellular storage carbohydrate used as a major energy reserve during the initial stages of fermentation and is important for long-term survival during yeast storage (62,63), and trehalose is an important component of yeast stress tolerance (62,64), both of which play critical roles in the ability to repitch a yeast strain into subsequent fermentations (65). As CBS1513 and W3470 are both industrial workhorse strains capable of being repitched multiple times, we compared each of the *de novo* hybrids to these strains as indicators repitching potential. Strain RB-1186 had the highest glycogen content level, followed by strains CBS1513, RB-2403, RB-2251, W3470, RB-1141, and RB-2215 (Table 3). For trehalose, we observed strain CBS1513 with the highest levels followed by W3470, RB-2403, RB-2251, RB-1186, RB-2215, and RB-1141 with the lowest (Table 3). Viability was determined for each strain on the final day of fermentation (day 14). Strains W3470, CBS1513, RB-2251, and RB-2403 all had very high viability levels (>95%), whereas strains RB-2215, RB-1186, and RB-1141 had lower viability levels between 78.4 and 91.7%.

Lastly, the POF-phenotype of the *de novo* hybrids was tested by measuring the ability of each strain to decarboxylate ferulic acid *in vitro*, as described previously (66). As expected, W3470 and CBS1513 tested POF-negative, whereas *S. eubayanus* (CBS12357) tested POF-positive, consistent with previous

Table 2. Sugar utilization of novel hybrids and control group I and group II strains[a]

Strain	Glucose (g/L)	Maltose (g/L)	Maltotriose (g/L)	Dextrins (g/L)	Acetic acid (g/L)	Ethanol (v/v)
Wort	16.1	77.1	26.4	34.7	0.01	0.0
W3470	0 ± 0 (100%)	1.3 ± 0.03 (98.3%)	3.6 ± 0.04 (86.4%)	34.7 ± 0.14	0.10 ± 0.010	7.2 ± 0.03
CBS1513	0 ± 0 (100%)	3.9 ± 0.34 (94.9%)	3.1 ± 0.07 (88.3%)	35.0 ± 0.13	0.01 ± 0.002	7.1 ± 0.01
RB-1141	0 ± 0 (100%)	0.8 ± 0.02 (99.0%)	2.6 ± 0.10 (90.2%)	34.9 ± 0.25	0.14 ± 0.010	7.4 ± 0.05
RB-1186	0 ± 0 (100%)	0.9 ± 0.02 (98.8%)	3.0 ± 0.05 (88.6%)	35.1 ± 0.17	0.06 ± 0.004	7.3 ± 0.02
RB-2215	0 ± 0 (100%)	1.2 ± 0.03 (98.5%)	3.6 ± 0.01 (86.5%)	34.8 ± 0.19	0.09 ± 0.009	7.3 ± 0.03
RB-2251	0 ± 0 (100%)	0.8 ± 0.01 (99.0%)	3.1 ± 0.06 (88.2%)	35.0 ± 0.13	0.09 ± 0.007	7.3 ± 0.03
RB-2403	0 ± 0 (100%)	0.9 ± 0.05 (98.8%)	3.2 ± 0.06 (87.8%)	34.8 ± 0.18	0.14 ± 0.009	7.3 ± 0.05

[a] Values for unfermented wort are shown, and % utilization for glucose, maltose, and maltotriose shown in brackets next to terminal concentrations.

studies on the POF-positive phenotype of *S. eubayanus* (16). All of the novel lager hybrids were POF-negative, consistent with the POF status of their constituent parent strains.

Volatile Profiling of Novel Hybrid Strains Reveals Diverse Aroma Production Profiles

The ability of a lager yeast strain to ferment wort sugars to ethanol at low temperatures, while producing acceptable levels of acetic acid and diacetyl, and flocculating out by the end of the fermentation are critically important performance characteristics for industrial lager beer production. Equally important, especially for product identity, is the ability of strains to produce a lager-specific volatile profile, with characteristic on-aromas, and free from off-aromas. To assess the fermentation volatile profile of the *de novo* lager hybrids, we measured the volatiles present in finished beer fermentations, compared with those present in fermentations conducted with group I and group II strains, CBS1513 and W3470. We used principal component analysis (PCA) of measured volatile compounds and focused primarily on beer volatile compounds from three major groups of compounds: acetate esters, ethyl esters, and fusel alcohols. Some off-aromas were also included, as they also make up the main sensory components of beer (e.g., DMS, acetaldehyde, diacetyl, and acetone). As represented in Figure 4, principal component (PC) 1 and PC2 carry 33 and 22% of the dataset's total variance, and each of them significantly segregates strains from each other (analysis of variance for strain vs. PC1: f = 28.8, p = 4.3e-09; strain vs. PC2: f = 108.6, p = 1.1e-14). Strain RB-2403 clustered most closely to the group I CBS1513 strain, whereas strains RB-1141 and RB-1186 clustered closer to the group II strain W3470. This patterning was also observed in the hierarchical clustering of strains as shown in Figure 5. Strains RB-2251 and RB-2215 clustered separately (mostly according to PC2), indicating they carry the most different, and therefore unique, volatile profiles compared with the rest of the hybrids. Only hybrids RB-1141 and RB-1186 were somewhat similar to each other. All other hybrids demonstrated divergent volatile profiles when compared with each other.

To further analyze the volatile data and validate the previous PCA, a comparison of specific volatile compounds measured from each strain is shown as a heatmap in Figure 5. Each compound was normalized across all strains on a scale from 0 (lowest measurement) to 1 (highest measurement). Strain RB-2215 had the highest relative production of acetate esters, whereas strain RB-1141 had the highest relative production of ethyl esters. Strain RB-2215 also had the highest production of higher alcohols. With respect to off-aromas, strain W3470 had the highest production of acetone and acetaldehyde, and strain RB-2403 had the highest production of DMS and diacetyl. In contrast, strain RB-1186 showed the lowest production of

Table 3. Measurement of the key vitality indicators, as determined by glycogen and trehalose concentration (expressed as % cell weight), and cell viability, as measured by methylene blue staining[a]

Strain	Glycogen (w/w, %)	Trehalose (w/w, %)	Viability (%)
W3470	15.3 ± 0.19	5.68 ± 0.18	96.4 ± 1.04
CBS1513	33.7 ± 0.52	6.57 ± 0.12	98.8 ± 0.30
RB-1141	12.8 ± 0.41	2.27 ± 0.02	78.4 ± 1.64
RB-1186	35.0 ± 0.46	4.35 ± 0.04	87.5 ± 0.22
RB-2215	12.6 ± 0.04	2.83 ± 0.08	91.7 ± 0.98
RB-2251	23.3 ± 0.28	5.29 ± 0.14	97.3 ± 0.35
RB-2403	28.1 ± 0.25	5.48 ± 0.03	98.9 ± 0.29

[a] All samples were taken on the final day of fermentation.

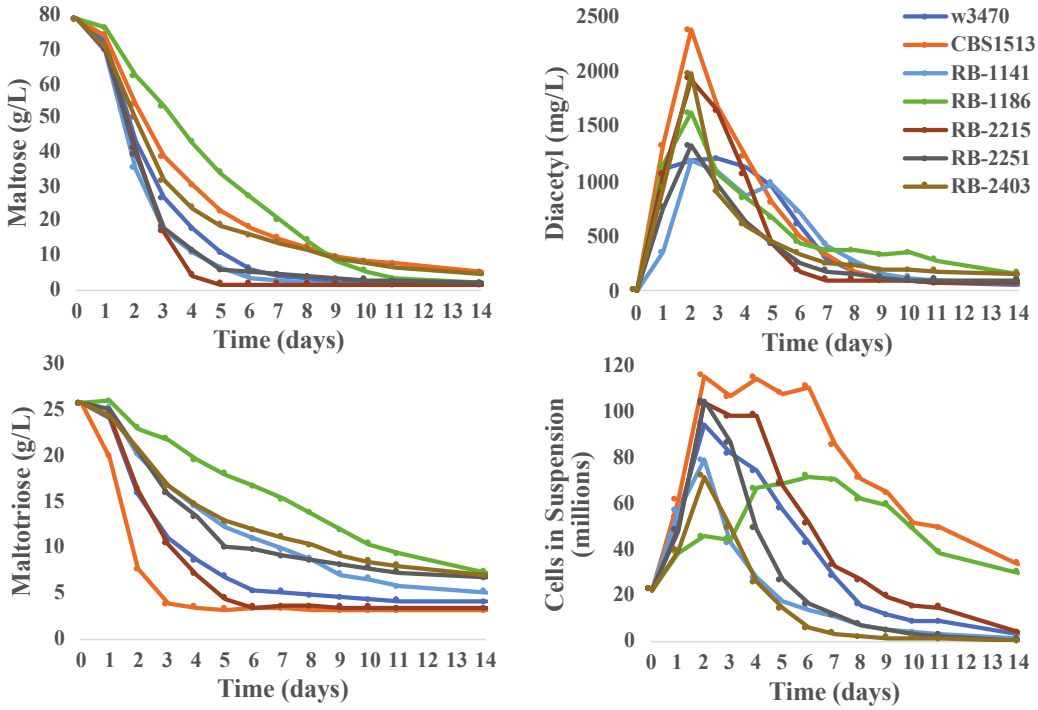

Figure 3. Phenotypic characterization of lager hybrids during beer fermentations performed isothermally at 13°C in 15°P wort in single replicates. **A,** Maltose consumption; **B,** maltotriose consumption; **C,** diacetyl production; and **D,** cells in suspension (flocculation), shown over a 14-day time course. Control strains are shown with solid square markers and novel lager hybrids with solid circle markers.

acetone, acetaldehyde, DMS, and diacetyl. Taken together, the data shown in Figures 4 and 5 demonstrate a diversity in volatile compound production between strains across all three major beer volatile compound groups. When compared with control lager strains, the novel lager strains generally produce fewer off-aromas overall and exhibit volatile profiles suitable for lager beer production.

Novel Lager Hybrids Contain Both *S. cerevisiae* and *S. eubayanus* Subgenomes

Having found that the novel lager hybrids exhibited favorable lager beer-producing phenotypes in a brewing application, we turned our focus to the genomic composition of these hybrids. To characterize the genomic features of these strains and demonstrate the validity of our hybridization method, we used whole genome sequencing to determine the constitution and origin of each strain's nuclear and mitochondrial genome. Our methods allowed for the proportions of each subgenome and the mitotype to be characterized, as well as confirmation of ploidy values obtained by flow cytometry, identification of possible copy number variations/segmental duplications, and determination of the parental origin of the genetic material from each individual novel hybrid (see Turgeon et al. [2021] [67] for in-depth analysis).

Assessment of the proportion of *S. cerevisiae* and *S. eubayanus* DNA in each strain, conducted using ratios calculated from the proportion of sequenced nucleotides mapping to the *S. cerevisiae* and the *S. pastorianus* subgenomes, indicated that they all contain both *S. cerevisiae* and *S. eubayanus* subgenomes (see Turgeon et al. [2021] [67] for in-depth analysis).

In addition to the nuclear genome, we determined the mitotype of each strain. Unlike the nuclear genome, mitochondria are inherited from only one parent (68); accordingly, the sequencing analysis showed that the control strain US-05 had *S. cerevisiae* mitochondria, whereas the control lager strains CBS1513 and W3470 both had *S. eubayanus* mitochondria. Interestingly, we observed both mitotypes among the collection of novel hybrids, with two hybrids (RB-1141 and RB-1186) carrying *S. eubayanus* mitochondria and three hybrids (RB-2215, RB-2251, and RB-2403) carrying *S. cerevisiae* mitochondria.

Whole Genome Sequencing Unveils Unique Karyotype for Novel Lager Hybrids

To more precisely determine the distribution of *S. cerevisiae* and *S. eubayanus* DNA in the novel hybrids, as well as confirm their parental origins, we characterized the inherited allele frequency across the genome. Overall, the novel hybrids displayed a karyotype showing a majority of triploid regions across the *S. cerevisiae* subgenome and a single copy of the *S. eubayanus* subgenome. Importantly, our results were corroborated by the measured ploidy state as determined by flow cytometry, similar to previous studies (see Turgeon et al. [2021] [67] for in-depth analysis).

To study the parental origins of each hybrid's genome, and specifically to determine the source of each hybrid's *S. cerevisiae* subgenome, the chromosome-wide median allele frequency of each selected parental allele was calculated for each novel hybrid strain (67). Importantly, the novel hybrids generally inherited two copies of their *S. cerevisiae* subgenome (median allele frequency of 0.6) from their *S. cerevisiae* parent and one copy of the *S. cerevisiae* subgenome from their *S. pastorianus* parent (median allele frequency of 0.3). This is true for RB-1141, RB-1186, RB-2251, and RB-2403; however, RB-2215 exhibited the converse pattern, with one copy of the *S. cerevisiae* subgenome coming from the *S. cerevisiae* parent and two coming from its *S. pastorianus* parent. Overall, the novel hybrids displayed a unique 3:1 chromosome ratio between the *S. cerevisiae* and *S. eubayanus* subgenomes (Fig. 1). Of course, in all hybrids, the single copy of the *S. eubayanus* subgenome was inherited from the *S. pastorianus* parent.

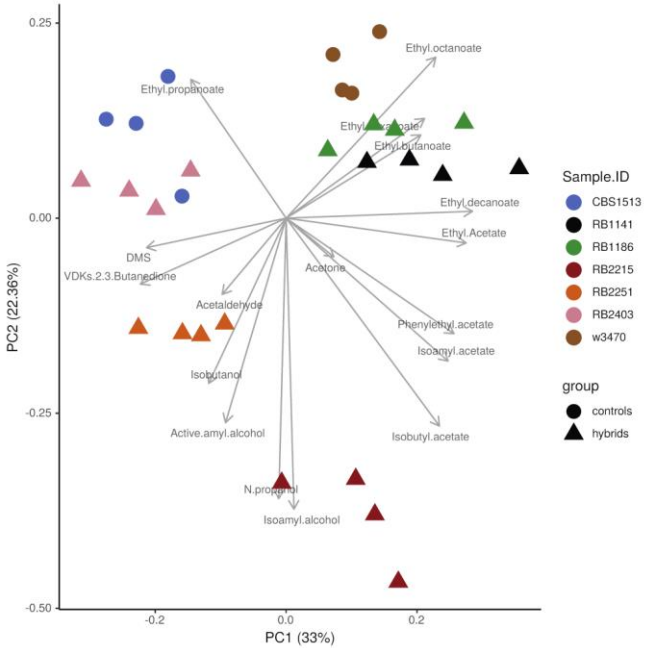

Figure 4. Two-dimensional principal component (PC) analysis of volatile compound production by novel lager hybrids and control group I (CBS1513) and group II (W3470) *Saccharomyces pastorianus* strains.

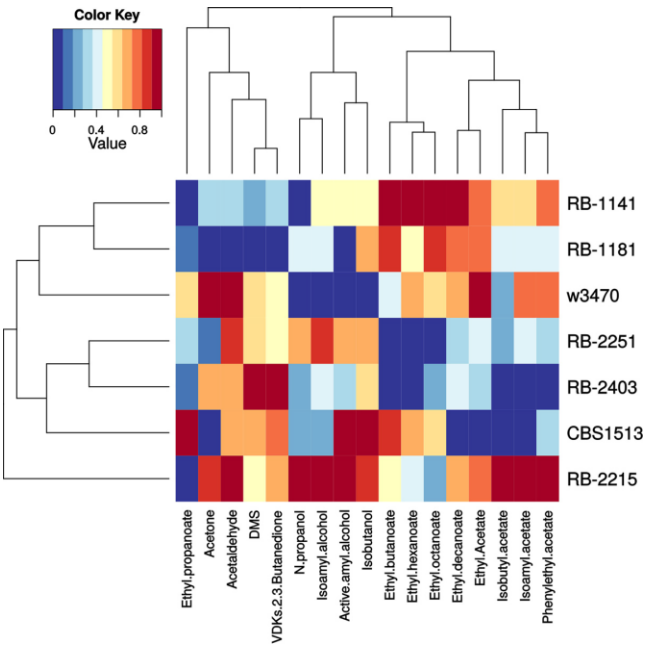

Figure 5. Visual representation of the volatile compound production of novel lager hybrids and control group I (CBS1513) and group II (W3470) lager strains. Colors represent the range of compound concentrations normalized for each strain from 0 = the lowest concentration (blue) to 1 = the highest concentration (red).

Discussion

Previous research on reconstituting *de novo* lager hybrids has aimed at better understanding the evolutionary history of modern lager strains, as well as attempts to increase the phenotypic diversity of lager strains to drive product innovation through novel aromas and flavors in lager beer. In this study, we developed an alternative *de novo* hybridization technique that overcomes previous challenges in strain development by directly breeding the domesticated *S. eubayanus* subgenome from *S. pastorianus* lager strains with different *S. cerevisiae* ale strains. We characterized five de novo lager hybrids with favorable lager fermenting phenotypes, and their specific attributes were characterized compared with prototypical group I (CBS1513) and group II (W3470) lager strains, as it relates to lager beer production.

The ability of our novel hybrid strains to grow robustly at both 7 and 37°C indicates a broadened temperature range compared with traditional ale or lager strains. This trait may allow our novel hybrids not only to perform at cool lager temperatures but also to be suitable in applications as ale strains at warmer temperatures for both fermentation and propagation, although this was outside the scope of the present study. When evaluated in beer fermentations at lager temperatures (13°C), our results indicate that our approach can generate *de novo* lager hybrid strains that are suitable for lager beer production based on their lack the POF trait and ability to efficiently ferment maltose and maltotriose, reduce diacetyl, flocculate during fermentation, and produce volatile profiles that are low in off-aromas and have comparable but unique profiles compared with prototypical industrial lager strains.

Lastly, genomic characterization of the novel lager hybrid strains revealed a unique genomic composition, in which each hybrid had approximately three genomic copies of the *S. cerevisiae* genome to one copy of the *S. eubayanus* subgenome. This novel genomic structure has, to the best of our knowledge, never been observed in either natural lager strains or laboratory-bred *de novo* lager strains. All currently characterized lager strains fall into either the allotriploid group I category, with the genomic composition of 1:2 *S. cerevisiae* to *S. eubayanus*, or into the allotetraploid group II category, with the genomic composition being 2:2, which includes *de novo S. pastorianus* strains created from the hybridization of wild *S. eubayanus* and *S. cerevisiae*. Our novel lager hybrids fall into what we propose as a group III category, containing a 3:1 *S. cerevisiae* to *S. eubayanus* genomic structure (Fig. 5). We also observed hybrids that contained either a *S. cerevisiae* or *S. eubayanus* mitotype, but this did not appear to strongly correlate with increased cryotolerance, nor did it have an effect on growth above 37°C, indicating additional nuclear genes are important in temperature tolerance in our novel hybrids. This finding was unexpected, as it has been shown that the *S. eubayanus* mitotype is a critical factor in cryotolerance (68); however, the role of nuclear genes in cryotolerance has also been shown for *Saccharomyces* species (13,69,70), and the presence of complementary or compensatory nuclear genes in our hybrids may explain our observations. The parental origin of each chromosome is also traced against each parental genome, and we demonstrate that (with some minor exceptions, most notably RB-2215) each hybrid inherited two copies of the *S. cerevisiae* genome from the *S. cerevisiae* parent, one from the *S. pastorianus* parent, and of course the lone *S. eubayanus* copy originating from the *S. pastorianus* parent.

With respect to fermentation phenotypes, our data suggest that lager hybrids created using our approach can be selected for lager beer fermentation performance while displaying phenotypic diversity across different beer making parameters. This was expected because all the novel hybrids share similar *S. eubayanus* subgenomic heritage, partial *S. cerevisiae* heritage from the *S. pastorianus* parental strains, but highly divergent *S. cerevisiae* genomic content from the *S. cerevisiae* ale parent, and the hybrids were selected based on similar performance criteria. It is important to note that our results are based on laboratory-scale fermentations. Furthermore, all hybrids displayed the unique allotetraploid karyotype of 3:1 *S. cerevisiae* to *S. eubayanus* for most chromosomes, and the specific parental origin of each chromosome was identified. The phenotypic and genotypic diversity of such hybrids could be further expanded by incorporating *S. cerevisiae* parents from different geographical or ecological niches with *S. pastorianus* diploids. Different genomic compositions could also be explored by breeding of allodiploid *S. pastorianus* to haploid *S. cerevisiae* (2:1 *S. cerevisiae* to *S. eubayanus* allotriploid) or to diploid *S. eubayanus* (1:3 *S. cerevisiae* to *S. eubayanus* allotetraploid). Thus, our hybrids not only show promise as industrial lager beer cultures but may also serve as tools to study the domesticated *S. eubayanus* subgenome and provide insight into key genomic features critical for lager strain domestication.

Taken together, we have demonstrated that using our non-GMO breeding approach, we can create novel lager strains with unique and diverse genomes yet possess favorable fermentation performance and volatile profiles for lager beer production. It is important to note that in order to validate our novel lager hybrids at industry scale, follow-up studies will be required to determine which of the five hybrids is most suitable for scale-up in a brewery setting. It is also worth noting that it is critically important to undergo a rare mating step to produce higher ploidy strains (tetraploid in this study), because the spore-derived allodiploid *S. pastorianus* parent strains themselves are poor performers compared with the original parents (data not shown), and it has been suggested that higher ploidy is critical for functional attributes of lager hybrids (15).

In conclusion, we have shown that using our approach it is possible to create lager hybrids with a novel genomic composition, which when combined with appropriate selection methods can generate strains that perform like industrial lager strains yet exhibit unique phenotypic diversity and increased growth at higher temperatures. Furthermore, this approach, when incorporated with highly divergent *S. cerevisiae* parental strain, should allow the phenotypic diversity of industrial lager cultures to be vastly expanded and help brewers develop hybrid beer styles, or even completely novel beer styles.

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