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Reduction of off-flavour formation and improved α-oligoglucoside utilization enable use of *Saccharomyces eubayanus* as a novel lager brewing yeast

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Abstract

Lager beer, made by fermentation of barley malt using the hybrid yeast *Saccharomyces pastorianus*, is extremely popular worldwide. The low genetic variation of *S. pastorianus* strains limits the scope of development for novel lager beer types. The wild ancestor *Saccharomyces eubayanus* has potential for use in lager brewing, but its application is constrained by formation of phenolic off-flavours (POF$^+$ phenotype) and limited uptake of the wort α-oligoglucosides maltose and maltotriose. Here, we addressed both limitations, using screening and evolutionary engineering of the Himalayan *S. eubayanus* strain ABFM5L.1, one of the closest known relatives of *S. eubayanus* subgenome of modern hybrid lager yeasts. Prior to improving α-oligoglucoside utilisation, 4-vinylguaiacol formation was abolished by mutagenesis and screening for POF$^-$ mutants. Subsequently, evolution for improved sugar utilisation and reverse engineering identified Se*MALR1* (the *S. eubayanus* ortholog of *S. cerevisiae MAL63*) as a key regulatory control point. Single-nucleotide substitutions in this maltose regulon activator gene enabled rapid maltose uptake and partial maltotriose utilisation in a previously maltose-negative background. The evolved non-genetically modified POF$^-$ strains showed near-complete maltose consumption (>90%–95%) and partial maltotriose consumption (∼25%–32%) in industrial wort. These results reveal substantial regulatory flexibility in Holarctic *S. eubayanus* and support its further development as a novel lager brewing yeast.

Keywords *Saccharomyces eubayanus*, lager brewing, maltose metabolism, off flavour, adaptive evolution, classical strain improvement

Introduction

Saccharomyces eubayanus is a cryotolerant yeast species that came to prominence when it solved a long-standing mystery about the origin of lager yeasts (Libkind et al. 2011). Since its formal identification, it has attracted intense interest from evolutionary biologists (Peris et al. 2014, 2016, Eizaguirre et al. 2018, Langdon et al. 2020) and brewers alike because of its cold tolerance, genomic relationship to the lager hybrid *Saccharomyces pastorianus*, and its potential as a tool to generate new lager strains and flavour profiles (Hebly et al. 2015, Krogerus et al. 2015, Gibson et al. 2017, Turgeon et al. 2021, Giorgetti et al. 2025). Unlike many other *Saccharomyces* species, which had been described ealier, *Saccharomyces eubayanus*, was identified from niche associated with *Nothofagus* tree- and *Cyttaria hariotii* stromata-associated in Patagonia, Argentina. Its discovery provided the long-sought non-*cerevisiae* parent of lager-brewing yeast that had eluded researchers for decades (Rainieri et al. 2006, Libkind et al. 2011). That discovery resolved a core question about the ancestry of lager (cold-fermented) yeasts and opened a new field of research into the wild relatives of domesticated fermentation microbes. Although *S. eubayanus* was first isolated from Patagonian forests in South America, environmental isolates or genomic signatures have been reported from areas of Asia (including Himalayan plateau, West China and Sichuan), parts of New Zealand (Gayevskiy and Goddard 2016), and sporadically in North America (Peris et al. 2014, 2016, Langdon et al. 2020), while traces of the species are present in the genomic heritage of European lager strains even when wild isolates in Europe are rare or hard to find (Bergin et al. 2022). Comparative genomics has shown that the *S. eubayanus* subgenome of *S. pastorianus* is more closely related to isolates from Far-East Asia than to Patagonian strains (Bing et al. 2014). Subsequent work refined this picture, showing that the *S. pastorianus eubayanus* subgenome is not derived from a single wild strain but is instead mosaic, with different chromosomal regions aligning to different *S. eubayanus* haplotypes within the Ho-

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larctic (Eurasian) clade (Peris et al. 2016, Bergin et al. 2022). This mosaicism suggests that either multiple hybridization events occurred, or that the founding *S. eubayanus* population already harboured considerable standing genetic variation. So, *S. pastorianus* (previously called *S. carlsbergensis* or broadly "lager yeast") is an interspecific hybrid formed from *S. cerevisiae* (typical ale yeasts) and a non-*cerevisiae* species; *S. eubayanus* is now recognized as that missing parent. The hybridization event(s), likely occurring several centuries ago in the context of cold fermentation practices in central Europe, combined the cold tolerance of *S. eubayanus* with fermentation traits of S. *cerevisiae* to produce the domesticated lager lineages used worldwide today (Gorter de Vries et al. 2019, Hutzler et al. 2023).

Intrinsically, *S. eubayanus* exhibits several phenotypic traits compatible with brewing. First, it is cryotolerance; *S. eubayanus* grows and ferments at lower temperatures than typical ale strains, a critical attribute that contributed to the emergence of lager brewing. This property makes it useful for low-temperature fermentations and the development of cold-tolerant brewing yeasts. Wild *S. eubayanus* isolates can also produce aroma profiles that differ from those of conventional lager strains, providing opportunities to diversify beer flavour (Mardones et al. 2022, Giorgetti et al. 2025). However, these advantages are counterbalanced by phenotypes that can limit brewing application. Most notably, many *S. eubayanus* strains are phenolic off-flavour positive (POF^+). While this characteristic can be desirable in certain speciality beers, it is not suited to lager brewing. The POF^+ phenotype depends on functional *PAD1* and *FDC1* genes, whose protein products enable the conversion of hydroxycinnamic acids into volatile phenols such as 4-vinylguaiacol (4-VG) and 4-vinylphenol (Clausen et al. 1994, Mukai et al. 2010). A second limitation is α-oligoglucoside assimilation. Many wild *S. eubayanus* isolates do not ferment maltotriose at all, restricting attenuation (i.e. leaving high residual sugars) and lowering alcohol yield in standard brewer's wort compared to domesticated lager strains. South American strains typically consume maltose, whereas many Asian/Holarctic strains do not (Bing et al. 2014, Baker et al. 2015, Brickwedde et al. 2018, Brouwers et al. 2019a, Gorter de Vries et al. 2019). Consequently, pure *S. eubayanus* strains often display sluggish or incomplete fermentations in typical brewing wort

Several approaches have been developed to overcome these drawbacks and harness the genomic diversity of *S. eubayanus*. Intentional interspecific hybridisation of *S. cerevisiae* (laboratory, ale strains or other industrial strains) with *S. eubayanus* has been explored to combine traits such as efficient sugar use, ethanol tolerance, and flocculation from *S. cerevisiae* with cold tolerance and novel aroma contributions from *S. eubayanus* (Gibson et al. 2017, Krogerus et al. 2018, 2022, Mardones et al. 2022, Molinet et al. 2022). The resulting hybrids display heterosis and can outperform either parent for lager-style fermentations, while also being selectable for desired flavour and fermentation kinetics (Brouwers et al. 2019a). Classical strain improvement and adaptive evolution have also been successfully applied, for example, to eliminate the formation of 4-vinylguaiacol (Diderich et al. 2018), or to improve fermentation kinetics and strain performance (Vega-Macaya et al. 2025). Collectively, these strategies have already yielded hybrid strains and evolved *S. eubayanus* derivatives that are improved and suitable for experimental brewing product development. However, no true lager type *S. eubayanus* strain has been developed, despite strategies aimed at eliminating 4-VG and evolving maltotriose assimilation. Screening of UV mutagenized cells with a light absorption assay for ferulic acid quantification enabled the selection of POF^- isolates of the *S. eubayanus* type strain CBS12357, which carried mutations in Se*PAD1* and *SeFDC1* (Diderich et al. 2018). Similarly, combining mutagenesis and evolution of *S. eubayanus* CBS12357 led to the isolation of maltotriose positive mutants from a maltotriose negative parent. This phenotype arose from repeated gene recombination of three maltose transporters, resulting in a newly neo-functionalized maltotriose transporter (Baker and Hittinger 2019, Brouwers et al. 2019b).

In this study, we aimed to develop an *S. eubayanus* strain that lacks 4-VG formation and displays substantially improved utilization of wort maltose and maltotriose, thereby increasing its suitability for lager type beer production. We applied classical strain improvement and adaptive laboratory evolution to the Himalayan *S. eubayanus* strain ABFM5L.1 (Bing et al. 2014, Brouwers et al. 2019a). Following selection for elimination of 4-VG production and improved wort sugar utilization, genome sequencing was used to identify candidate causative mutations. The role of Se*MALR1* (*SeMAL63*) variants was tested by reverse engineering, and engineered strains were characterized under brewing-relevant conditions. Finally, we discuss the evolutionary significance of the regulatory changes in maltose metabolism, and their potential application in brewing.

Materials and methods

Strains, growth conditions, and media

Escherichia coli XL1-Blue (New England Biolabs, Ipswich, MA) was used for plasmid propagation. *Escherichia coli* was cultivated in lysogenic broth medium (LB), supplemented with 100 mg·l^{-1} ampicillin or 50 mg l^{-1} kanamycin at 37°C, with shaking at 200 rpm in an Innova 4000 Shaker (Eppendorf AG, Hamburg, Germany). For solid media, 20 g·l^{-1} bacto agar was added to LB medium.

The *S. eubayanus* strains, used in this study are described in Table 1. *Saccharomyces eubayanus* strains were cultivated in complex medium containing 10 g·l^{-1} bacto Yeast extract, 20 g·l^{-1} bacto Peptone (YP) and either 20 g·L^{-1} glucose (YPD), 5 g l^{-1} glucose and 20 g l^{-1} maltose (YPMG) or 60 g l^{-1} maltose (YPM) at 20°C, while shaking at 200 rpm in an Innova 43/43R shaker (New Brunswick, Nijmegen, the Netherlands). For tall tube precultures, 6% (w/v) maltose was added (YPM). For transformation, YPD agar was prepared by addition of 20 g·l^{-1} bacto agar to YPD medium and supplemented with 200 mg l^{-1} hygromycin for selection. Agar plates were incubated at room temperature (20°C). For cultivation in brewing-like conditions in septum flasks and tall-tubes, industrial wort (Heineken, Zoeterwoude, The Netherlands) was used as previously described (Brickwedde et al. 2017). For sporulation of *S. eubayanus* strains, the cells were incubated in sporulation liquid medium (SLM) (Dymond 2013).

For screening of 4-VG negative mutants and maltose consumption, YPMG medium containing 20 g l^{-1} maltose and 5 g l^{-1} glucose or 12 °P wort was used. Media were supplemented with 1 mM of ferulic acid for the first screening and 10 mM of ferulic acid for confirmation. For the final colony screening, yeast cells were grown on synthetic wort (SWM) as previously described (Diderich et al. 2018).

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Table 1 Strains used in this study.

Strain	Genotype	Comments	Parental strain	
ABFM5L.1	Wild type	Himalayan *S. eubayanus*, Mal$^-$, Malt$^-$, POF$^+$		(Bing et al. 2014, Brouwers et al. 2019a)
CDFM21L.1	Wild type	Himalayan *S. eubayanus,* Mal$^-$, Malt$^-$, POF$^+$		(Bing et al. 2014, Brouwers et al. 2019a)
HTS219		Evolved Himalayan *S. eubayanus* ABFM5L.1 Mal$^-$, Malt$^-$, POF$^-$ mutant	ABFM5L.1	This study
HTS257		Evolved Himalayan *S. eubayanus* ABFM5L.1, Mal$^+$, Malt$^+$, POF$^+$, evolved on SM	ABFM5L.1	This study
HTS258		Evolved Himalayan *S. eubayanus* ABFM5L.1 Mal$^+$, Malt$^+$, POF$^+$, evolved on wort	ABFM5L.1	This study
HTS259		Evolved *S. eubayanus* HTS219 Mal$^+$, Malt$^+$, POF$^-$; evolved on SM	HTS219	This study
HTS260		Evolved *S. eubayanus* HTS219 Mal$^+$, Malt$^+$, POF$^-$; evolved on wort	HTS219	This study
CBS 12357^T	Wild type	*Patagonian S. eubayanus* type strain, Mal$^+$, Malt$^-$, POF$^+$		(Libkind et al. 2011)
HTSE042		Evolved *Patagonian S. eubayanus* type strain CBS 12357 Mal$^+$, Malt$^-$, POF$^-$	CBS12357	(Diderich et al. 2018)
IMI648	*SemalR1*$_{ChrV}$Δ::synPAM	Deletion of *SeMALR1*$_{ChrV}$ and integration of SynPAM	ABFM5L.1	This study
IMG020	*SemalR1*$_{ChrV}$Δ::*SeMALR1*$^{L310\,R}$	Integration of *SeMALR1* mutant	IMI648	This study
IMG021	*SemalR1*$_{ChrV}$Δ::*SeMALR1*^{H311Q}	Integration of *SeMALR1* mutant	IMI648	This study
IMG022	*SemalR1*$_{ChrV}$Δ::*SeMALR1*^{S374I}	Integration of *SeMALR1* mutant	IMI648	This study

DNA isolation, PCR, and cloning

Plasmids were isolated using GeneJET Plasmid Miniprep Kit (#K0503, Thermo Fisher Scientific, Bleiswijk, The Netherlands), according to the manufacturer's instructions. PCR fragments were purified with GeneJET PCR purification kit (#K0702, Thermo Fisher Scientific). For the construction of the CRISPR-Cas9 plasmids, Golden Gate cloning was performed by mixing: 10 ng pUDP002, 20 fmol gRNA plasmid (pUD1567/pUD1568), 2.5 U BsaI restriction enzyme (#ER0291, Thermo Fisher), 2.5 U T4 ligase (EL0011, Thermo Fisher) in 1xT4 ligase buffer (B69, Thermo Fisher) in a final volume of 5 μl. For the assembly the following program was used in a ThermoCycler (Biometra): 25 cycles of (digestion at 37°C for 2 min, ligation at 22°C for 5 min), followed by a final digestion of 10 min at 37°C and 20 min inactivation at 80°C. For colony PCR, genomic DNA of *S. eubayanus* strains was isolated using the LiAc-SDS method (Looke et al. 2011). For amplification of repair fragments and diagnostic PCR on plasmids or genomic DNA, repliQa HiFi ToughMix (#95 200, Quantabio, Beverly, MA) was used according to the manufacturer's protocol. Primers for PCR were synthesized at Merck Life Science (Amsterdam, The Netherlands).

Sequencing and sequence analysis

Strain ABFM5L.1 was sequenced using ONT long-read sequencing (Oxford, United Kingdom). A one-dimensional (1D) sequencing library (SQK-LSK109) was prepared and loaded on a FLO-MIN111 (R10.3) flow cell connected to a MinION using MinKNOW software version 20.10.3. Raw FAST5 files generated by MinKNOW were basedcalled using Guppy (version 5.0.11). Reads with a minimum length of 1000 bp were extracted in fastq format yielding 3.2 Gigabases across 220,182 reads with an average read length of 14.55 kb and an N50 read length of 25.24 kb. Using the obtained data, *de novo* assembly of the Oxford Nanopore fastq files was performed by Canu (version 2.1.1, with setting genomesize = 12 m) (Koren et al. 2017) which resulted in 16 telomere-to-telomere chromosomes. The mtDNA was resolved by *de novo* assembly of the fastq files with Flye (version 2.7.1-b1673, with settings –nano-raw and -g 12 m) (Kolmogorov et al. 2019). The reconstructed ABFM5L.1 genome (16 Canu chromosomes + mtDNA from Flye) was error corrected by mapping an Illumina paired-end library against this genome with BWA (Li 2013), sorted and converted to BAM by applying Samtools (Danecek et al. 2021) and subsequently polished with pilon (version 1.18, with settings -changes -fix bases) (Walker et al. 2014). The error-corrected genome was annotated using the MAKER2 annotation pipeline (version 2.31.9) (Holt and Yandell 2011) as in (Brouwers et al. 2019a).

The strain HTS219 was sequenced using short-read Illumina technology. Paired-end sequencing was performed on a 350 bp PCR-free insert library with an Illumina HiSeq 2500 (San Diego, CA) by Novogene Company Ltd (Hong Kong, China). Raw Illumina reads were mapped using BWA (Li 2013) against the generated assembly of ABFM5L.1, alignments were processed using Samtools (Danecek et al. 2021) and visualized using the Integrated Genome Viewer (Robinson et al. 2011), variant calling was done using Pi-

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lon. Chromosomal copy number variation between ABFM5L.1 and HTS219 was performed using the Magnolya program (Nijkamp et al. 2012) and by calculating coverage average, for this, mapped reads were aligned to the ABFM5L.1 reference using BWA and subsequently extracted and analysed using IGVtools (Thorvaldsdottir et al. 2013). Coverage was calculated with a 25 bp window. The read count mapping was normalized to 1 for the whole chromosome XIII.

Sequencing reads data of the strain ABFM5L.1 can be found under Bioproject (https://www.ncbi.nlm.nih.gov/bioproject) PRJNA528469 (Brouwers et al. 2019a). The extracted raw sequencing data of the ChrIII from strains derived from HTS219 can be found at NCBI under BioProject PRJNA1359756 (Table S1). The *SeMALR1* amplicon derived from HTS257, HTS259, HTS260, IMG020, IMG021 and IMG022 were sequenced by Plasmidsaurus (Eugene, OR) using long read technology from Oxford nanopore. The corresponding data can be found at NCBI under BioProject PRJNA1359756 (Table S1).

Visualization of genome comparisons of ABFM5L.1 and CDFM21L.1 was done using D-Genies (https://dgenies.toulouse.inra.fr/) (Cabanettes and Klopp 2018).

Construction plasmids

Plasmids and primers used for this study are shown in Tables 2 and 3, respectively. Plasmids for co-expression of Cas9 and gRNA were constructed according to (Gorter de Vries et al. 2017). The gRNA templates were *de novo* synthesised at GeneArt (Thermo Fisher Scientific), consisting of the 20 nt gRNA target, flanked by 5'-HH—and 3'-HDV ribozymes and BsaI sites for Golden Gate assembly (Juergens et al. 2018). The following gRNA target sequences were designed: 1) 5'-CGTCACTTGAAATAGAGCCA-3', targeting the Se*MALR1* gene and 2) 5'- AGTAGAATTTCACCTAGACG-3', targeting a synthetic spacer with PAM site (SynPAM). The synthesized gRNA templates were cloned into pMK-RQ plasmid by GenArt, yielding plasmids pUD1567 ($gRNA_{SynPAM}$) and pUD1568 ($gRNA_{SeMALR1}$). Plasmid pUDP002, containing the Cas9 expression cassette and the Hygromycin B resistance gene was assembled using Golden Gate cloning with gRNA plasmids pUD1567 and pUD1568. The Golden Gate assembly mixtures (2 μl) were transformed to *E.coli* XL1 Blue (New England Biolabs, Ipswich, MA) chemical competent cells, using heat-shock. Transformants were selected on LB agar, supplemented by 50 mg l^{-1} kanamycin. Correct assembly of the resulting plasmids pUDP365 (*Spycas9*/$gRNA_{SynPAM}$) and pUDP366 (*Spycas9*/$gRNA_{SeMALR1}$), was verified using diagnostic primers 11 200 & 11 201 and whole plasmid sequencing (Plasmidsaurus).

Construction yeast strains

Saccharomyces eubayanus strains were transformed by electroporation (Gorter de Vries et al. 2017). For the construction of IMI648, ABFM5L.1 was transformed with 500 ng of plasmid pUDP366 and 1 μg repair fragment, obtained by hybridizing equimolar amount of primers 19 128 & 19 129. Replacement of the Se*MALR1* gene on chromosome V by the SynPAM sequence (Perli et al. 2020) was verified by diagnostic PCR using primers 19 132 & 19 133. Plasmid pUDP366 was removed by three rounds of re-streaking on non-selective YPD-agar and plasmid loss was verified by absence of growth on YPD containing 200 mg·l^{-1} hygromycin.

Strains IMG020, IMG021 and IMG022 were constructed by transforming IMI648 with 500 ng of plasmid pUDP365 and 1 μg repair fragments, consisting of one of the evolved Se*MALR1* alleles with 500 bp homology flanks. Repair fragments were amplified from genomic DNA of HTS257, HTS259 and HTS260, using primers 21 789 & 21 790. Transformants were selected on YPD agar containing 200 mg l^{-1} hygromycin for 4–5 days at room temperature. Correct integration was verified by diagnostic PCR using primers 19 132 & 19 133 (Table 3). The *SeMALR1* locus amplified with primer 21 789 and 21 790 was sequence-verified by Nanopore sequencing (Plasmidsaurus), confirming the mutation and the homozygosity of the engineered locus.

Screening for 4-VG-negative and maltose positive colonies in microtiter plates

UV-mutagenesis and screening of 4-VG negative mutants was essentially performed as described previously (Diderich et al. 2018). Briefly, YPD grown cells of strain ABFM5L.1 were harvested by centrifugation, washed and transferred to SLM. After 72 h of incubation, the presence of asci was confirmed and spores were plated on SWM-agar plates and subjected to 70 s of UV light (UVC-lamp, 36 W, MSC-Advantage Biological Safety Cabinet, Thermo Fisher Scientific) for a survival rate of 1%. Approximately 800 colonies were picked and transferred to 96-well microtiter plates using a TECAN Freedom Evo 200 (Tecan, Männedorf, Switzerland) equipped with a Pickolo colony picker (Sci Robotics, Kfar Saba, Israel) into wells containing 150 μl of SWM. Plates were incubated for 5 days at 20°C and subsequently reinoculated in 96-well microtiter plates filled with 200 μl SWM medium supplemented with 1 mM of ferulic acid and incubated for 3 days at 20°C, after which the supernatant was harvested by centrifugation and absorption spectra were determined between 250 and 400 nm on a Tecan infinite M200 Pro spectrophotometer. Duetz low evaporation sandwich covers were used, and shaking was done at 250 RPM in an Infors Multitron orbital incubator (Infors, Bottmingen, Switzerland). For confirmation, the best performing colonies were reinoculated in 8 ml SWM in 50 ml vented tubes and sampled for HPLC analysis after 3 days of growth at 20°C.

Screening for α-oligoglucosides positive *S. eubayanus*

The strains ABFM5L.1 and HTS219 were subjected to serial cultivation in either YPMG (2% maltose,0.5% glucose) or 12 °P wort in vented Greiner tubes, resulting in four independent evolution conditions. Each experiment was performed in duplicate. Precultures were grown in YPD and subsequently transferred to fresh YPMG and 12 °P wort media at an initial optical density (660 nm) of 0.2. Cultures were serially transferred to fresh medium at the same initial OD_{660nm} after 192, 384, 504, and 648 h. At 720 h, the best performing cultures from each replicate evolution line were transferred to septum flask containing full malt wort (17 °P). Each experiment was performed in duplicate. At the end of the fermentation, the cultures were plated on YPD agar. From each of the four evolution lines, 150 colonies were picked into 150 μl YPD. The resulting cultures were transferred to 140 μl of SWM and incubated for 2 days at 12°C. Samples were subsequently filtered, and residual sugar concentrations and ethanol production

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Table 2 Plasmids used in this study.

Name	Relevant genotype	Reference
pUDP002	panARS$^{(OPT)}$ *bla* colE1 *AgTEF1*p-*hph*-*AgTEF1*t *ScTDH3*p-$^{BsaI\text{-}BsaI}$-*ScCYC1*t *AaTEF1*p-*Spycas9*$^{D147Y\ P411T}$-*ScPHO5*t	(Juergens et al. 2018), Addgene #103872
pUD1567	*nptII* colE1 ^{BsaI}HH-gRNA$_{SynPAM}$-HDV ^{BsaI}	GeneArt
pUD1568	*nptII* colE1 ^{BsaI}HH-gRNA$_{SeMALR1}$-HDV ^{BsaI}	GeneArt
pUDP365	panARS$^{(OPT)}$ *bla* colE1 *AgTEF1*p-*hph*-*AgTEF1*t *ScTDH3*p-HH-gRNA$_{SynPAM}$-HDV-*ScCYC1*t *AaTEF1*p-*Spycas9*$^{D147Y\ P411T}$-*ScPHO5*t	This study
pUDP366	panARS$^{(OPT)}$ *bla* colE1 *AgTEF1*p-*hph*-*AgTEF1*t *ScTDH3*p-HH-gRNA$_{SeMALR1}$-HDV -*ScCYC1*t *AaTEF1*p-*Spycas9*$^{D147Y\ P411T}$-*ScPHO5*t	This study

Table 3 Primers used in this study.

Number	Sequence (5'→3')	Purpose
11 200	ACACGCTTTTTCAGTTCGAG	Diagnostic primer to check integration gRNA into pUDP002
11 201	ATGTTACATGCGTACACGCG	Diagnostic primer to check integration gRNA into pUDP002
21 789	GGATCTCTGCCAGAAATTCAAAAGCACGAGAG	Amplification Se*MALR1* variants incl. flanks of HTS257, HTS259 and HTS260
21 790	CTATCTTTGATCTGCCCGAAACTGCAGGTAGAAC	Amplification Se*MALR1* variants incl. flanks of HTS257, HTS259 and HTS260
19 128	AAAAAAAAATTCTCACTTCTCAAACAATAAAAAAAATCTAGCTT TGAATAGTAGAATTTCACCTAGACGAGGACATATTCACTCTTTG CTCCTTCCAACATTCTAAGAAGCAGGTGTTCT	SynPAM with Se*MALR1* flanks
19 129	AGAACACCTGCTTCTTAGAATGTTGGAAGGAGCAAAGAGTGA ATATGTCCTCGTCTAGGTGAAATTCTACTATTCAAAGCTAGATT TTTTTTATTGTTTGAGAAGTGAGAATTTTTTTTT	SynPAM with Se*MALR1* flanks
19 132	CTGTGATTGCGTATGG	Diagnostic primer in left flank Se*MALR1*
19 133	CCCTAAAGATCCCAAG	Diagnostic primer in right flank Se*MALR1*

were determined by HPLC to identify isolates with improved α-oligoglucoside utilization.

Septum flask fermentations

To test sugar consumption and flavour production of *S. eubayanus* isolates, septum flask fermentations were performed in complete full malt wort (16.8 °P) or 1/3 diluted wort at 12°C as previously described by Diderich et al. (2018). Samples were taken with a syringe through the rubber seal to determine extracellular metabolites.

Tall tube fermentations

For characterization in brewing-like conditions, tall tube fermentations were performed as previously described (Bennis et al. 2024, Spaans et al. 2026).

The septum flask and tall tube cultivations presented in this study were performed in duplicate. Given the limited number of independent biological replicates (n = 2 per strain), formal statistical testing would need to be interpreted with considerable caution. Although a two-sample *t*-test can technically be performed with n = 2 per group, the estimation of biological variance is highly uncertain, resulting in very limited statistical power. We therefore decided not to perform formal statistical significance testing. Instead, we report the results of individual biological replicates, which are publicly available through the 4TU.ResearchData repository (https://doi.org/10.4121/776460a8-e6d4-4e06-9f35-3891ecdc0e72.v1). Throughout the manuscript, observed differences are described quantitatively, including percentage differences between strain means where relevant.

Analytical methods

Wort density was determined using an Anton Paar DMA35 instrument according to the manufacturer's recommendations (Anton Paar Netherlands, Breda, The Netherlands). Optical densities were determined using a Libra S70 spectrophotometer (Biochrom, Cambridge, United Kingdom) at 660 nm. Gas outflow from tall tubes was determined with Bronkhorst EL-FLOW® Prestige mass flow meters (Bronkhorst Nederland, Veenendaal, The Netherlands).

The concentration of sugars (glucose, fructose, maltose, maltotriose) and ethanol were determined using HPLC (Agilent Technologies, 1260 HPLC system), equipped with a Bio-Rad HPX-42A column (300 × 7.8 mm, 25 micron) (Bio-Rad) and a 1260 Refractive Index Detector. Elution was performed with milliQ water at a flow rate of 0.5 ml min^{-1} and a column temperature of 75°C, for 30 min. Compounds were measured using a RID at 35°C. Samples

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were filtered prior to analysis, using a 0.2 μm filter. (Brickwedde et al. 2017)

Aromatic compounds ferulic acid and 4-vinylguaiacol were measured using HPLC on an Agilent 1260 LC equipped with an Agilent Zorbax SB-C18 column (4.6 × 5.0, 3.5 μm) at 30°C and a 1260 Infinity Diode Array detector at 214 nm or a Thermo Fisher Vanquish LC equipped with an Agilent Eclipse C18 column (4.6 × 10, 3.5 μm) and a Vanquish DAD detector measuring at 280 nm. A gradient of 20 mM KH_2PO_4 (pH 2) with 1% V/V acetonitrile and acetonitrile was used as eluent at a flow rate of 0.8 ml·min^{-1} with a gradient up to 10% acetonitrile in 6 min, increasing to 40% acetonitrile at 23 min after which 100% buffer with 1% acetonitrile was used up to 27 min (Hassing et al. 2019). Calibration compounds were obtained from Merck Life Science and dissolved in 100% ethanol; dilutions were made in water.

Volatile flavour components were measured using static headspace gas chromatography on an Agilent 7890A GC system. Esters and alcohols were measured from a 2.5 ml sample which was equilibrated for 5 min at 50°C prior to injection of 1 ml of headspace volume using a Combi PAL headspace auto-injector (CTC Analytics, Zwingen, Switzerland). Compounds were separated on an Agilent DB-Wax capillary column (30 m × 320 μm × 1 μm) and analysed by FID at 250°C with a 40 ml·min^{-1} H_2 flow and 10 ml·min^{-1} make-up flow of nitrogen. A split ratio of 5.8:1 with a split flow of 9.88 ml·min^{-1} nitrogen was used, and injector and inlet temperatures were set to 250°C, oven temperatures were increased from 55 to 160°C by 20°C·min^{-1} and a hold time of 4.75 min. Vicinal diketones were measured on the same GC equipment using 0.5 ml·l^{-1} samples which were equilibrated for 30 min at 65°C. Samples were measured on an Agilent CP-Sil 8 CB column (50 m × 530 μm × 1 μm) and detection was by Electron Capture Detector (ECD) at 150°C with a 30 ml·min^{-1} make-up flow of nitrogen. 1 ml of headspace volume was injected, a 1:1 split ratio with a split flow of 8 ml·min^{-1} was used. Oven temperature increased from 35 to 150°C by 30°C·min^{-1}.

Protein structure prediction

Protein structure prediction was performed using Boltz-1 (Wohlwend et al. 2025) with MSA generation by MMseqs2 using Colabfold. As ligands Zn^{2+}, maltose and a short double stranded section (GGATAGACCCTTCATAGTTGCCTAATAGCTTTTGAAATTTTTTTTGGAAAGTCTTGATTAGATGCATA) of the MALT promotor proximal to the MALT transporter gene were used. Visualization was done using PyMOL 3.03 (Schrödinger LLC).

Results

Selection and characterization of a POF$^-$ mutant derived from Himalayan *S. eubayanus* strain ABFM5L.1

To harness the brewing potential of the Holarctic Asian *S. eubayanus* strain ABFM5L.1, two phenotypic traits were targeted: i) the formation of phenolic off-flavours and ii) the inefficient α-oligoglucoside utilization. When grown in 12 °P wort with 1 mM of ferulic acid at 12°C, ABFM5L.1 did not consume maltotriose and utilized less than 30% of available maltose after 200 h (Fig. 1C). Under these conditions, ABFM5L.1 produced 3.0 ± 0.4 mg·l^{-1} of 4-VG confirming that this strain required improvement to meet the standards for lager-type beer production (Fig. 1E). To enable direct use in beverages, we chose classical strain improvement (CSI) methods. While molecular genetic approaches could introduce the desired improvements, CSI avoids regulatory approval requirements and minimizes risk of consumer rejection. For screening POF$^-$ mutants from the ABFM5L.1 background, we applied a previously published assay that monitors conversion of ferulic acid into 4VG by differential absorbance at 335 nm (Diderich et al. 2018). ABFM5L.1 was subjected to UV mutagenesis and screening. A total of 803 mutants were screened using a microtiter plate assay and seven isolates showing a strongly reduced ferulic acid conversion were selected (Fig. 1A). Among these, mutant number 2 showed no detectable 4-VG production. Its absorbance profile was comparable to that of a ferulic acid solution suggesting that ferulic acid remained largely unconverted. Direct HPLC analysis confirmed the absence of detectable 4-VG production upon addition of excess ferulic acid (Fig. 1B), and this mutant was subsequently renamed HTS219. HTS219 was evaluated in septum flask fermentations in 12 °P wort with 1 mM of ferulic acid at 12°C (Fig. 1D). Consistent with the screening results, HTS219 did not produce detectable 4-VG whereas the parental strain ABFM5L.1 produced an average of 3.0 mg l^{-1} (Fig. 1 E). Importantly, elimination of 4-VG production was not accompanied by major changes in the concentrations of the other measured aroma compounds (Fig. 1F and G). Ethyl acetate, isobutanol and isoamyl alcohol, the most abundant compounds measured, differed by only 3.3%, 2.6%, and 8.0%, respectively, between the strain means. Similarly, only small absolute differences were observed for the less abundant acetate and ethyl esters. Thus, the mutagenesis and selection procedure specifically eliminated detectable 4-VG formation without substantially altering the overall profile of the other measured flavour compounds. HTS219 nevertheless retained the inefficient sugar utilization phenotype (Fig. 1D) of its parent ABFM5L.1 (Fig. 1C). Both strains only partially consumed maltose after more than 200 h of fermentation and showed no maltotriose consumption (Fig. 1D and C). This limited utilization of di- and tri-α-oligoglucosides by ABFM5L.1 and its POF$^-$ derivative HTS219 therefore remained a major limitation for their application in lager brewing. Illumina short read sequencing of HTS219 and mapping to the ABFM5L.1 reference assembly revealed the loss of ∼30-kb of the subtelomeric region on the right arm of CHRXIII (nt 942 000–972 000), encompassing the *SePAD1* and *SeFDC1* genes (Fig. 1H). Loss of either *SePAD1* and *SeFDC1*, or both genes has previously been shown to abolish phenolic off flavour formation consistent with the POF$^-$ phenotype of HTS219 (Clausen et al. 1994, Mukai et al. 2010, Diderich et al. 2018). The deletion extends beyond *SePAD1* and *SeFDC1,* resulting in the loss of additional genes in this region. The 30-kb region also harboured *SeSTL1*, *SeFDH1*, SeYOR389W, *SeIRC7*, and *SePAU20*.

Improving α-oligoglucosides transport by adaptive laboratory evolution of *S. eubayanus* ABFM5L.1

Neither *S. eubayanus* ABFM5L.1 nor its derivative HTS219 efficiently consumed α-oligoglucosides, maltose and maltotriose (Fig. 1). To improve α-oligoglucoside utilization, each strain was subjected to adaptive laboratory evolution under two conditions,

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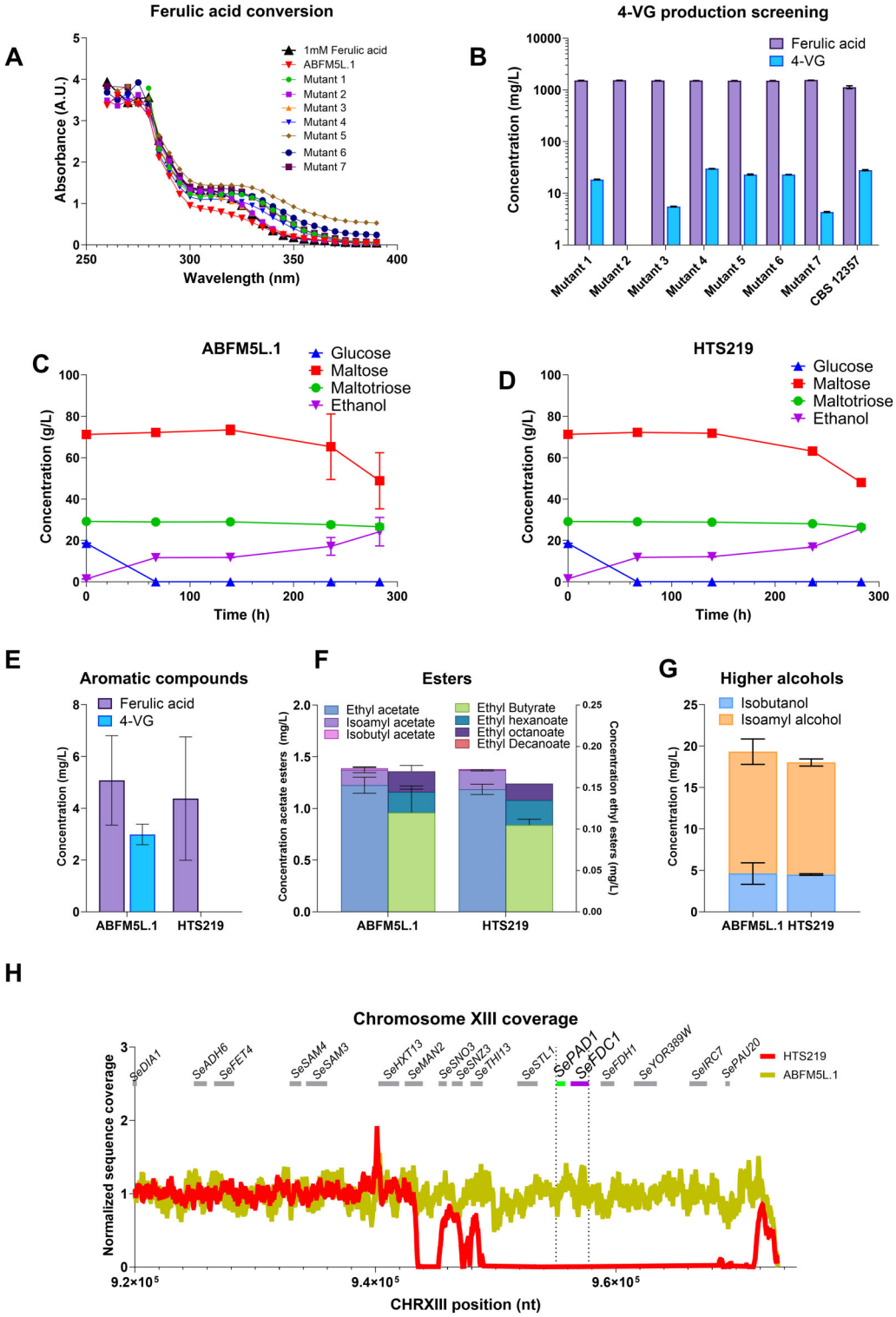

Figure 1 Characterization of the POF$^-$ strain HTS219. (A) Absorbance profiles of ferulic acid conversion of UV-mutagenized mutants. The absorbance scans range from 250 nm to 400 nm. (B) Ferulic acid and 4-VG determination. The selected UV mutagenized mutants as well as the POF$^+$ control, the Patagonian *S. eubayanus* strain CBS 12357, were grown in SWM supplemented with 10 mM of ferulic acid at 20°C. The data represent average and standard deviation of two biological replicates. (C and D) Sugar utilization and ethanol production of *S. eubayanus* strains ABFM5L.1 (C) and its derived POF$^-$ mutant HTS219 (D). The fermentations were performed in septum flasks using 12 °P wort supplemented with 1 mM of ferulic acid at 12°C. The data represent the average and standard deviation (shown as error bar) from two independent biological replicates. (E–G) Production of flavour compounds after 283 h of fermentation in septum flask with 12 °P wort supplemented with 1 mM of ferulic acid. The fermentations were performed at 12 °C. (E) Ferulic acid and 4-VG, (F) acetate and ethyl esters, (G) higher alcohols. The data represent the average and mean deviation from two independent biological replicates. (H) Normalized sequencing coverage of the right arm subtelomeric region of chromosome XIII of the POF$^+$ABFM5L.1 strain and the POF$^-$ HTS219 strain.

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i) in YP with maltose and glucose mix as carbon source and ii) in industrial wort, yielding a total of four evolution lines. Cultures were incubated at 12°C and transferred to fresh medium every five to seven days for six transfers, corresponding to ∼35 generations based on the transfer regime. Maltose utilization became detectable during evolution in all four evolution lines (Fig. S1). Evolved populations were subsequently tested for growth and sugar uptake in septum flask using complete industrial wort (Fig. 2). Control fermentation with the parental strains ABFM5L.1 and HTS219 confirmed earlier results: glucose and fructose were depleted within 50 h, while maltose utilization was marginal (<15% in HTS219) and maltotriose remained unused (Fig. 2A and D). Ethanol concentrations in these controls did not exceed 20 $g{\cdot}l^{-1}$. In contrast, the evolved populations showed substantially improved sugar utilization. The populations evolved in YPMG performed best (Fig. 2E), fully depleting maltose within 150–220 h and consuming over 50% of the maltotriose by 220 h. The HTS219 population evolved in this condition eventually exhausted maltotriose, although fructose depletion was slower than in the parental strains (Fig. 2E). Similar trends were observed in the other populations, albeit with somewhat less efficiency (Fig. 2B, C, and F). Because these data reflected population averages, over 150 single colony isolates were obtained from each evolution lines and screened in SWM containing 25 $g{\cdot}l^{-1}$ maltose and 11 $g{\cdot}l^{-1}$ maltotriose. Colonies that consumed > 90% of the initial maltose in three days were classified as maltose positive. Over 70% of isolates from the ABFM5L.1 and HTS219 populations evolved in SWM were maltose-positive (Fig. 2G). In contrast, the HTS219 population evolved in wort still contained a majority of maltose-negative isolates, suggesting that evolution had not yet reached completion.

Representative single colony isolates were selected, preserved and renamed with the ABFM5L.1 mutant from YPMG medium named HTS257 and from evolution in wort HTS258. For the HTS219 mutants, the mutant evolved in YPMG was named HTS259 and the mutant evolved in wort HTS260.

Characterization of evolved mutants HTS259 and HTS260 with improved α-oligoglucosides utilization in industrial brewing fermentations.4

The two evolved mutants HTS259 and HTS260 derived from the *S. eubayanus* 4-VG negative strain HTS219 were tested in standard E.B.C. tall tube brewing fermentations using 17 °P industrial wort and at 12°C. As controls, we included the parental strains ABFM5L.1 and HTS219 as well as the maltose positive *S. eubayanus* type strain CBS 12357 originating from Patagonia (Libkind et al. 2011) and its 4-VG negative variant HTSE042 (Diderich et al. 2018) to benchmark the performance of the new isolates (Fig. 3). Based on fermentation attenuation, the strains segregated into three groups (Fig. 3A). The first group, comprising the parental strains ABFM5L.1 and HTS219 showed poor fermentation performance with only a marginal reduction of the wort gravity. Both strains remained above 15 °P after 200 h (Fig. 3B and C). The second group, consisting of control strain CBS12357 and its 4-VG negative derivative HTSE042 attenuated the wort to 11 °P, reflecting their ability to consume ∼50% of the available maltose (Fig. 3D). The third group comprised the two evolved mutants HTS259 and HTS260 which attenuated the wort from 18 to 5 °P within 600 h. Among them, HTS259 showed the strongest performance, consuming over 95% of maltose and 32% of the maltotriose, while HTS260 consumed around 90% of the maltose and 25% of the maltotriose (Fig. 3C). HTS219 and its derived strains HTS259 and HTS260 as well as HTSE042 did not produce any measurable 4-vinyl guaiacol, as expected (Fig. 3E). Of all tested strains the evolved HTS259 and HTS260 strains produced the highest concentrations of esters, concomitant with their more complete fermentation, while the concentrations of off-flavour vicinal diketones are reduced further (Fig. 3E).

Sequencing of HTS257-260 mutants revealed mutations in SeMALR1, gene coding for the maltose regulon activator

The four evolved isolates HTS257-260 were sequenced to identify the mutations responsible for their improved α-oligoglucoside utilization. To facilitate identification of mutations, the parental strain ABFM5L.1 was sequenced using both short read Illumina and long read ONT platforms and a novel genome assembly was generated. This new reference revealed that ABFM5L.1 overall was very similar to CDFM21L.1 (Brouwers et al. 2019a), with an overall identity of 99.83% and 19.7 kB and 9.0 kB of unaligned sequence present in CDFM21L.1 and ABFM5L.1 respectively. Structurally, an inter-chromosomal duplication from chromosome V to chromosome XII in the CDFM21L.1 strain relative to ABFM5L.1 could be identified, which disrupted the *MAL* locus on the subtelomeric region of chromosome V. The breakpoint of the recombination event was located in the Se*MALR1* open reading frame, causing a truncated open reading frame (Fig. S2). While the *MAL* loci on ChrXIII were identical between the two strains, as reported previously (Brouwers et al. 2019a), because this inter-chromosomal duplication is lacking in ABFM5L.1, the *MAL* locus on the subtelomeric region of ChrV was more complete in this strain, with Se*MALR1* and the Se*MALT* and Se*MALS* genes, although the Se*MALT* gene carries a frameshift mutation (Fig. 4A).

Short Illumina sequencing reads from the evolved isolates and the parental strain ABFM5L.1 were mapped to this new reference genome. Mutations in *SeMALR1*, located on chromosome V and encoding a transcriptional regulator of the *MAL* regulon, were identified in three of the four evolved strains (Table S2). *SeMALR1* is orthologous to *MAL63* in *S. cerevisiae*. To align the *S. eubayanus* gene nomenclature with the well-established *S. cerevisiae* naming convention, *SeMALR1* was therefore also assigned the alias *SeMAL63*. The names *SeMALR1* and *SeMAL63* refer to the same gene and are used interchangeably throughout this manuscript. Se*MALR1/SeMAL63* shows high similarity (87%) to the Se*MALR1* gene found in the Patagonian CBS 12357 strain on Chr. XVI (Fig. 4B). The remaining isolate carried a mutation in *TUP1* that encodes a transcriptional regulator that functions primarily as a transcriptional co-repressor with Cyc8 (Fig. 4D and E).

Each mutation resulted in an amino acid substitution within the C-terminal maltose response domain (residues 300–end of the protein). Specifically, HTS257 carried a T to G non-synonymous mutation at position 929, resulting in a Leu to Arg substitution at position 310. Similarly HTS259, carried a T to A mutation at posi-

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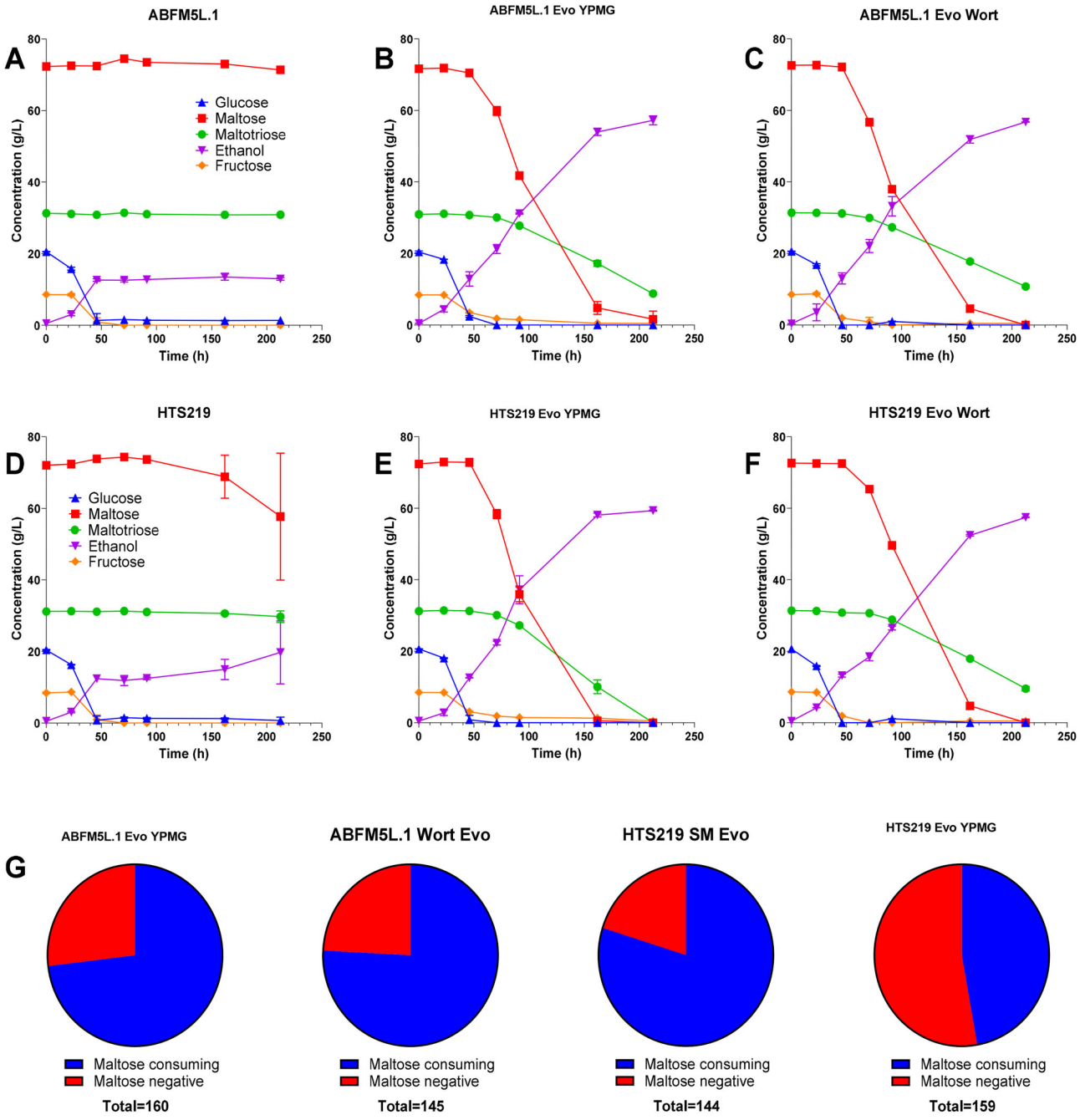

Figure 2 Characterization of evolved populations of *S. eubayanus.* Evolved populations were compared to the parental strains ABFM5L.1 and HTS219 in septum flasks containing complete 17°P industrial wort at 12°C. Sugar consumption and ethanol formation are shown in panels (A–C) for ABFM5L.1 and (D–F) for HTS219 and their derived populations. The data represent average and standard deviation of two independent biological replicates. (G) Fraction of maltose-positive colonies obtained following single colony isolation from each evolved population as determined in microtiter plate assays using. Colonies were considered maltose consuming if more than 90% of available maltose was consumed within three days.

tion 933, resulting in a His to Gln substitution at position 311. Finally, HTS260 carried a G to T non-synonymous mutation at position 1121, resulting in a Ser to Ile substitution at position 374 (Fig. 4). Interestingly, the Se*MALR1/SeMAL63* mutation in HTS257 was homozygous, whereas those in HTS259 and HTS260 were heterozygous, indicating a dominant effect of the mutated allele. To better understand the structural implications of these substitutions and appreciate the position of the mutated residues, a three dimensiaonal structural model of *Se*Mal63 bound to its promoter DNA, a maltose molecule and a zinc ion was generated using Boltz-1 (Wohlwend et al. 2025) (Fig. 4E). The model revealed that the mutated residues were clustered together but were located neither within the main maltose-binding pocket nor within the zinc-finger DNA binding domain. These substitutions might, however, induce subtle structural rearrangements that modulate the protein's function and binding to other proteins. The mutations identified in Se*MALR1/SeMAL63* are consistent with previous findings suggesting that the required maltose and maltotriose transporters

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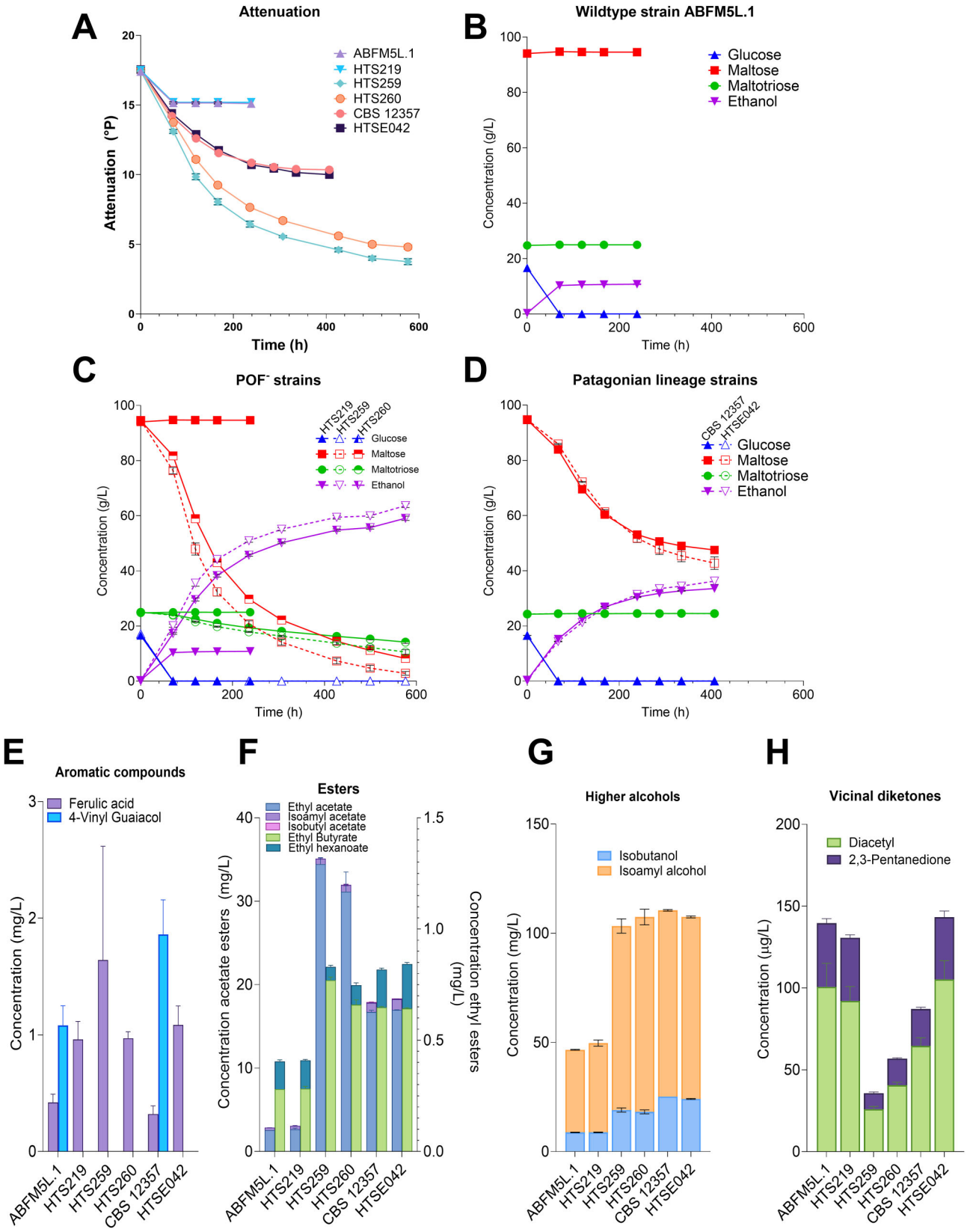

Figure 3 Characterization of evolved *S. eubayanus* strains under brewing conditions. The parental strains ABFM5L.1 and CBS 12357, The POF⁻ strains HTS219 and HTSE042, and the POF⁻ α-oligoglucoside^{+} HTS259 and HTS260 were grown in tall tubes containing full malt wort at an initial gravity of 17 °P and a temperature of 12°C. Each strain was grown in duplicate, and the data represent average and standard deviation of the two independent replicates. (A) Wort attenuation. (B–D) Consumption of sugars and production of ethanol during tall-tube fermentations. (C) Filled symbols: HTS219, open symbols: HTS259, half symbols: HTS260. (D) Filled symbols: CBS 12357, open symbols: HTSE042. (E–H) Flavour compounds produced at the end of fermentation (240 h, 576 h, and 407 h, respectively). (E) Ferulic acid and 4-VG, (F) esters, (G) higher alcohols, (H) vicinal diketones.

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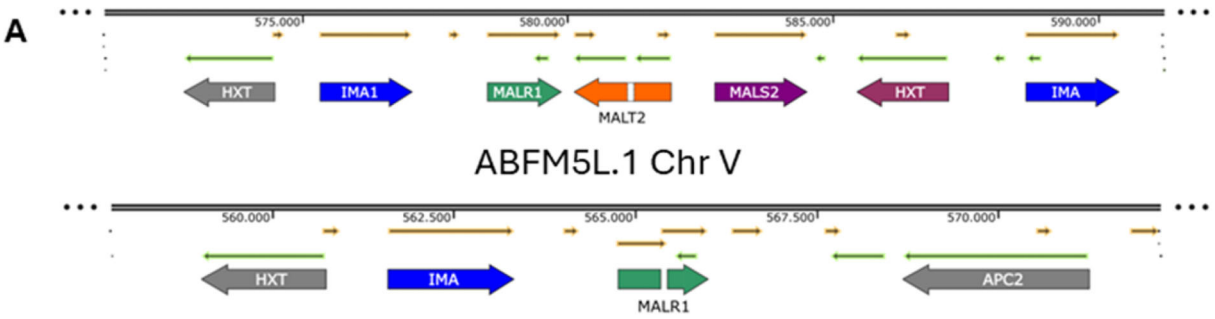
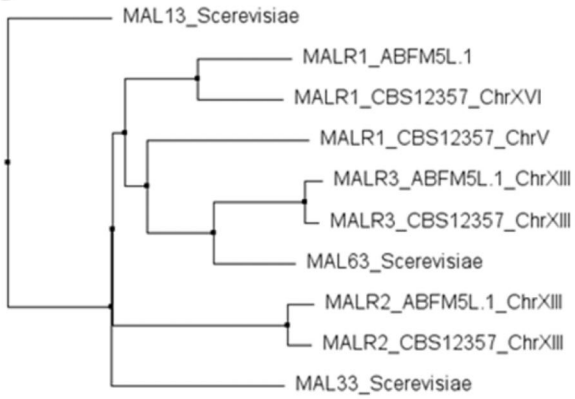


B

	MALR1 ABFM5L.1	MALR2 ABFM5L.1	MALR3 ABFM5L.1	MALR1 CBS12357 CHRV	MALR1 CBS12357 CHRXVI	MALR2 CBS 12357	MALR3 CBS 12357
MALR1 ABFM5L.1	100	73.3	70.1	72.8	86.8	71.9	70.4
MALR2 ABFM5L.1		100	68.3	68.3	76.6	99.4	68.3
MALR3 ABFM5L.1			100	71.4	69.3	68.5	99.4
MALR1 CBS12357 ChrV				100	73.5	68.7	72.1
MALR1 CBS12357 ChrXVI					100	74.1	69.3
MALR2 CBS 12357						100	68.5
MALR3 CBS 12357							100

C



D

Strain origin	Strain isolate name	Mutation
ABFM5L.1 SM evolution	HTS257	*MALR1* Leu-310-Arg
ABFM5L.1 wort evolution	HTS258	*TUP1* Asp-598-Val
HTS219 SM evolution	HTS259	*MALR1* His-311-Gln (heterozygous),
HTS219 wort evolution	HTS260	*MALR1* Ser-374-Ile (heterozygous)

E

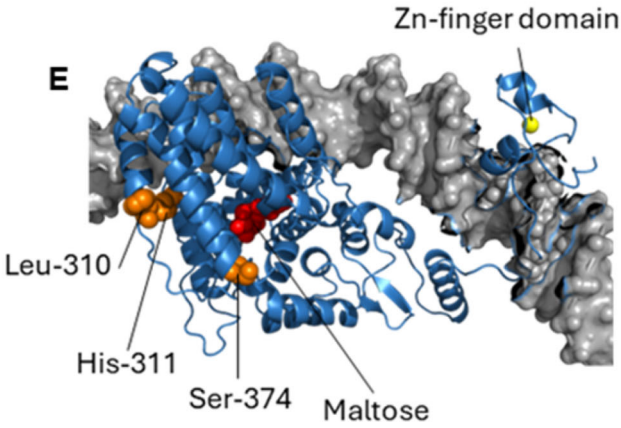


Figure 4 Sequence analysis of *MALR* genes. (A) Comparison of the *MAL* loci on Chr V between the two Himalayan strains ABFM5L.1 and CDFM2L.1. (B) Percentage identity between *MALR* genes from the Himalayan strain ABFM5L.1 and Patagonian strain CBS 12357. (C) Neighbour-joining tree of *SeMALR* genes derived from ABFM5L.1, CBS 12357 and reference *MAL* genes in *S. cerevisiae*. (D) Mutations identified in evolved maltose-positive clones of ABFM5L.1. (E) Three dimensional structural prediction of the *Se*MalR1/*Se*Mal63 protein bound to DNA with the mutated amino acid sites indicated in orange and maltose shown in red.

are present but not actively expressed in these strains (Brouwers et al. 2019a).

In contrast, the mutation identified in HTS258 was a A to T non-synonymous substitution at position 1793 in *TUP1*, resulting in an Asp598Val change. This mutation was not subsequently investigated.

Reverse engineering of SeMALR1/SeMAL63 mutations to enable maltose and maltotriose uptake

To verify the role of the Se*MALR1/SeMAL63* mutations in enabling the maltose and maltotriose uptake observed in the

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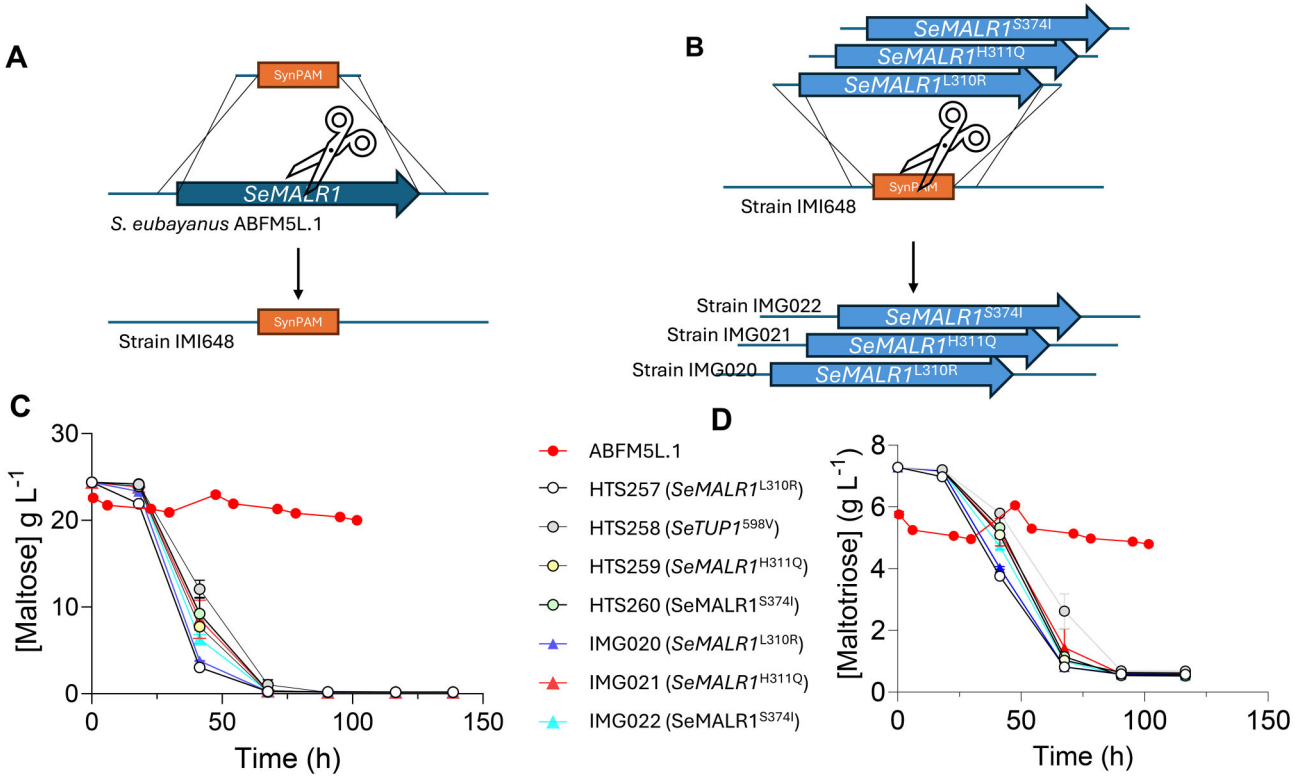

Figure 5 Reverse engineering of *S. eubayanus MALR1* mutations. Schematic representation of the reverse engineering strategy applied. (A) A synthetic gRNA site (SynPAM) was integrated in place of the *SeMALR1* gene on Chr. V in strain ABFM5L.1, yielding strain IMI648. (B) In a second transformation step, the three different *MALR* alleles obtained from the evolved strains were integrated into IMI648, generating strains IMG020-IMG022. (C) Maltose consumption profiles of evolved strains HTS257-HT260 with the reverse engineered strain IMG020-IMG022 and their parental strain ABFM5L.1. (D) Maltotriose consumption profiles of evolved strains HTS257-HTS260 with the reverse engineered strain IMG020-IMG022 and the parent of the engineered strain ABFM5L.1. The strains were grown in septum flask containing 1/3 diluted wort, the cultures were incubated at 20°C. Each strain was cultivated in duplicate. The data represent average and standard deviation of two independent biological replicates.

evolved isolates, the mutated Se*MALR1/SeMAL63* alleles were isolated and integrated into ABFM5L.1. To this end, the native Se*MALR1/SeMAL63* gene on ChrV was first deleted and replaced by a synthetic spacer and PAM site. In a subsequent transformation step, this site was targeted and the mutated alleles identified in the evolved strains were introduced as repair fragments (Fig. 5A and B), resulting in homozygous integration of the mutated allele. The resulting strains IMG020 (*SeMALR1*^{L310R}), IMG021 (*SeMALR1*^{H311Q}) and IMG022 (*SeMALR1*^{S374I}) were first evaluated in septum-flask fermentations using one-third diluted wort, together with the parental strain ABFM5L.1 and the evolved strains HTS257-260 (Fig. 5C and D). The wild-type parent ABFM5L.1 strain did not consume maltose and maltotriose. In contrast, both the evolved isolates and the reverse engineered strains completely consumed maltose and consumed at least 90% of the maltotriose present. Among the evolved isolates, HTS257 (*SeMALR1*^{L310R}/*SeMAL63*^{L310R}) exhibited the best α-oligoglucoside consumption profile. Consistent with this observation, the corresponding reverse engineered strain IMG020, carrying the same *SeMALR1*^{L310R}/*SeMAL63*^{L310R} variant, showed the fastest α-oligoglucoside consumption among the three reverse engineered strains. Although the *SeTUP1*^{D598V} mutation identified in HTS258 was not reverse engineered, HTS258 was evaluated and showed the slowest α-oligoglucoside consumption among the four selected evolved lineages.

The reverse engineered strains IMG020-IMG022 were subsequently evaluated in tall tube fermentations using brewer's malt wort at 17 °P. Under these conditions, the parental strain ABFM5L.1 showed greater attenuation than observed in previous experiments (Figs 1 and 3), decreasing from 17.5 to 10.3 °P. This was accompanied by a decrease in maltose concentration from 95 g l^{-1} at the start of the fermentation to 56 g l^{-1} at the end of the fermentation (Fig. 6), whereas less than 10% of maltotriose was consumed (Fig. 6). In comparison, the reverse engineered strains showed substantially improved wort fermentation. IMG020 (*SeMALR1*^{L310R}/*SeMAL63*^{L310R}) and IMG022 (*SeMALR1*^{S374I}/*SeMAL63*^{S374I}) reached an apparent extract below 5 °P after 600 h, reaching 4.6 $\pm$ 0.0 and 3.7 $\pm$ 0.1 °P, respectively. Maltose consumption reached 94%, 83%, and 98.5% for IMG020, IMG021, and IMG022, respectively (Fig. 6). The corresponding maltotriose consumption was 48%, 30%, and 64%, respectively (Fig. 6). These results demonstrate that the identified *MALR* mutations are sufficient to strongly enhance maltose utilization and confer substantial maltotriose utilization in the parental Himalayan *S. eubayanus* background.

To assess whether introduction of the evolved *SeMALR1/SeMAL63* alleles affected the formation of aroma-active fermentation products, concentrations of esters, higher alcohols and vicinal diketones were measured in fermentations with the parental strain ABFM5L.1 and the reverse-engineered strains

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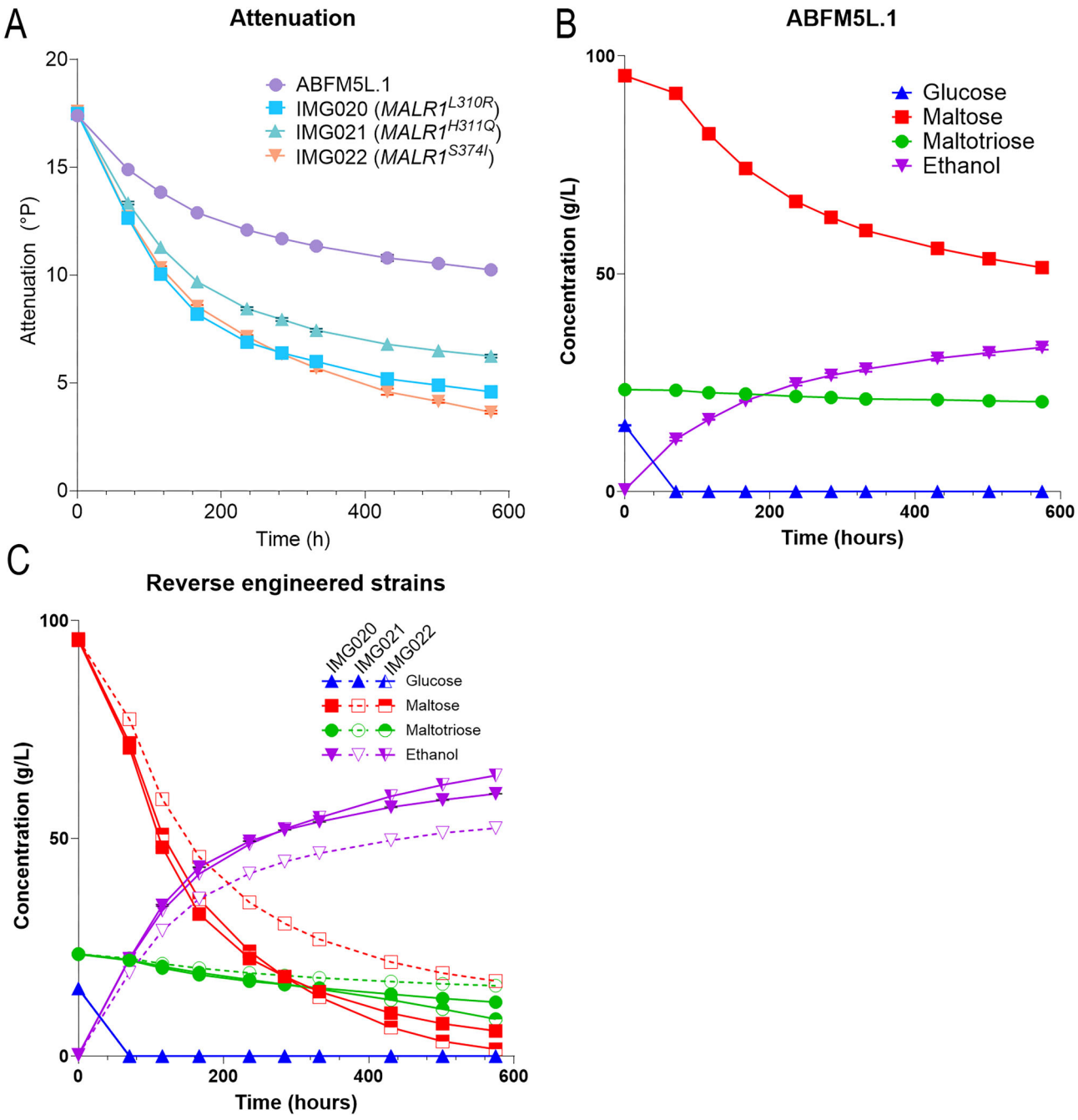

Figure 6 Fermentation performance of reverse engineered IMG020-IMG022 strains in tall tubes. Strains IMG020, IMG021, and IMG022 together with the parental strain ABFM5L.1 were grown in tall tubes containing 17 °P full malt wort at 12°C. The cultures were sampled daily. Each strain was grown in two independent biological replicates. The presented data represent average and standard deviation of two independent replicatee. (A) Wort attenuation during tall tube fermentations with engineered strains expressed in °P. (B) Sugar consumption and ethanol production of wild-type strain ABFM5L.1 in tall tubes. (C) Sugar consumption and ethanol production profiles in tall-tube fermentations of the three *MALR1* engineered strains IMG020, IMG021, and IMG022. .

IMG020, IMG021, and IMG022. Overall, introduction of the *Se-MALR1* alleles substantially altered the metabolite profiles. The most consistent change was observed for ethyl acetate, which increased from an average of 26.3 mg l^{-1} in ABFM5L.1 to 41.1, 39.9, and 44.8 mg l^{-1} in IMG020, IMG021, and IMG022, respectively. This could be explained by the better attenuation observed in these strains. In contrast, isoamyl and isobutyl acetate concentrations remained similar or decreased slightly, while ethyl hexanoate decreased in all three reverse-engineered strains (Fig. S3).

Higher-alcohol formation was also affected. Isobutanol concentrations decreased from 30.1 mg l^{-1} in ABFM5L.1 to 20.4–25.4 mg l^{-1} in the reverse-engineered strains, whereas changes in isoamyl alcohol were comparatively modest and strain dependent (Fig. S3).

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The largest differences were observed in the concentrations of vicinal diketones. Diacetyl was strongly reduced in IMG020 (72.7 μg l-1 compared with 132.5 μg l^{-1} in ABFM5L.1), while IMG021 and IMG022 showed more variable diacetyl concentrations between replicate fermentations. 2,3-Pentanedione was consistently reduced in all reverse-engineered strains, with average concentrations decreasing from 47.3 μg l^{-1} in ABFM5L.1 to 19.0, 28.9 and 35.7 μg l^{-1} in IMG020, IMG021, and IMG022, respectively. Together, these results indicate that the *SeMALR1* alleles affected fermentation metabolite formation beyond their effect on α-oligoglucoside utilization, with particularly pronounced effects on ethyl acetate and vicinal diketone production (Fig. S3).

Discussion

This study demonstrates that a wild Himalayan *S. eubayanus* isolate can be converted into a strain meeting the key technological requirements of lager brewing, absence of phenolic off-flavours (POF^-) and efficient fermentation of wort α-oligoglucosides. In recent years, several Patagonian *S. eubayanus* isolates have been explored in craft and experimental brewing, where they are valued for their cold tolerance and distinctive aroma profiles (Giorgetti et al. 2025). However, their application has largely remained limited to niche or specialty beers due to incomplete attenuation and frequent POF^+ phenotypes (Urbina et al. 2020, Mardones et al. 2022). As a result, *S. eubayanus* has primarily been used either in mixed fermentations or as a genetic donor in interspecific hybrids rather than as a stand-alone lager yeast (Hebly et al. 2015, Mertens et al. 2015, Gyurchev et al. 2022). By combining classical strain improvement with adaptive laboratory evolution (Mans et al. 2018), we show that these limitations are not intrinsic to the species but instead reflect regulatory constraints that can be relieved by a small number of genetic changes, thereby expanding the potential of *S. eubayanus* for broader lager-style brewing applications. Furthermore, this can be achieved without genetic engineering, meaning that the resulting strains can be readily applied in lager brewing.

The genetic determinants for the phenolic off-flavour phenotype are well established, with *SePAD1* and *SeFDC1* genes being required for the decarboxylation of hydroxycinnamic acids, such as ferulic acid, into volatile phenolic compounds (Clausen et al. 1994, Mukai et al. 2010). These genes were targeted using a previously described mutagenesis and screening approach that was used to generate the POF^- strain HTSE042 (Diderich et al. 2018), in which 4-vinylguaiacol production was eliminated by a subtelomeric deletion encompassing both genes. Our finding is consistent with this previous study and with other studies in *S. cerevisiae* in which POF^- phenotypes arise through loss-of-function mutations or chromosomal rearrangements affecting *PAD1* and *FDC1* (Diderich et al. 2018, Mertens et al. 2019). The recurrent occurrence of such deletions may suggest that the subtelomeric region containing these genes is particularly prone to recombination, potentially facilitated by regions of microhomology (Deng et al. 2014). However, this hypothesis is difficult to verify because subtelomeric sequences are often repetitive and incompletely resolved in *Saccharomyces* genome assemblies.

Together with *SePAD1* and *SeFDC1*, the subtelomeric deletion resulted in the loss of several genes that are not expected to be critical under brewing conditions, including *SeSTL1*, *SeFDH1*, *IRC7*, and *PAU20* (Fig. 1H). Most of these genes belong to multigene families, potentially limiting the phenotypic consequences of their loss. Among them, only *IRC7* has been directly associated with brewing-relevant traits. In *S. cerevisiae*, *IRC7* encodes a cysteine-S-conjugate β-lyase that can cleave non-volatile cysteinylated thiol precursors, releasing highly aroma-active volatile thiols during fermentation, including 3-sulfanylhexan-1-ol and 4-methyl-4-sulfanylpentan-2-one (Roncoroni et al. 2011, Svedlund et al. 2022). These compounds have very low sensory thresholds and contribute fruity and tropical aromas, particularly in hop-forward beer styles. The loss of *IRC7* is therefore expected to have a limited impact on conventional lager brewing, in which hops are primarily used to provide bitterness rather than to promote the release of hop-derived volatile thiols and associated fruity aromas.

Importantly, removal of phenolic off-flavour production did not substantially alter the formation of other volatile compounds, supporting the suitability of this approach for brewing applications. Moreover, complete gene deletion may also provide phenotypic stability during industrial propagation, minimizing the risk of reversion to phenolic off-flavour production compared with strains carrying point mutations, as reported in some ale brewing yeasts (Mertens et al. 2019).

A major outcome of this work is the demonstration that inefficient α-oligoglucoside utilization in the Himalayan *S. eubayanus* strain is primarily due to regulatory limitation rather than the absence of functional transporters. Adaptive laboratory evolution consistently selected mutations in *SeMALR1/SeMAL63*, a transcriptional activator of the maltose regulon, rather than in the maltose or maltotriose transporter genes themselves. These results support earlier observations that wild *S. eubayanus* strains often carry intact but transcriptionally silent sugar utilization pathways and strengthen the conclusion that the genetic capacity for α-oligoglucoside uptake was already present but insufficiently expressed under brewing conditions (Brouwers et al. 2019a). This mechanism contrasts with that described for Patagonian *S. eubayanus* strains, where maltotriose utilization has been shown to arise through structural rearrangement of maltose transporters. In those strains, repeated recombination events among Se*MALT* genes resulted in chimeric transporters with neofunctionalized maltotriose uptake capacity (Baker and Hittinger 2019, Brouwers et al. 2019b). Together, these findings indicate that distinct evolutionary solutions underlie improved sugar utilization in different *S. eubayanus* lineages: regulatory derepression in Holarctic strains versus transporter neofunctionalization in Patagonian backgrounds.

Independent evolution lineages repeatedly acquired non-synonymous substitutions in the C-terminal regulatory domain of *Se*MalR1, indicating strong convergent selection acting on this transcriptional regulator. The dominant effect of these mutations, as inferred from their heterozygosity in evolved strains, suggests a gain-of-function mechanism that enhances induction of the maltose regulon or reduces glucose-mediated repression. Structural modelling indicates that the affected residues cluster outside the canonical DNA-binding and maltose-binding domains, consistent with a role in modifying regulatory responsiveness rather than abolishing function. These mutations likely change the interaction with other regulatory proteins, possibly including the Tup1/Cyc8 complex. Indeed, mutations between residue 343 and 375 of the homologous *MALx3* gene in *S. cerevisiae* have been reported to result in constitutive *MAL* gene expression, potentially by disrupting the interaction between *MALx3* and the Tup1/Cyc8

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complex (Higgins et al. 1999). The identification of a mutation in *SeTUP1* in one evolved isolate further suggests the importance of global transcriptional regulation in determining sugar utilization phenotypes. *TUP1* functions as a corepressor involved in carbon catabolite repression, and its mutation likely relaxes repression of maltose-associated genes during mixed-sugar fermentation as previously shown *TUP1* mutations in *S. cerevisiae* (Lin et al. 2017). Although not directly validated here, this result parallels findings in other yeast domestication contexts and reinforces the conclusion that regulatory rewiring, rather than acquisition of new metabolic functions, underlies improved fermentative performance (Voordeckers et al. 2015, Gallone et al. 2016, Gorter de Vries et al. 2019).

Reverse engineering demonstrated that each of the three tested Se*MALR1* alleles was sufficient to strongly enhance maltose and maltotriose utilization in the ABFM5L.1 background, providing direct evidence that *SeMALR1*/*SeMAL63* plays a key role in the evolved phenotype. However, the greater maltose consumption observed for the ABFM5L.1 control during the fermentation evaluation of the reverse engineered strains IMG020-IMG022 (Fig. 5) compared with earlier fermentations (Figs. 1–3) indicates that the parental maltose-negative phenotype is not completely invariant. Screening for the identified *SeMALR1*/*SeMAL63* mutations in both the ABFM5L.1 inoculum and cells collected at the end of the fermentation did not reveal any of these mutations. This observation argues against a strict requirement for *SeMALR1/SeMAL63* mutation for maltose consumption but is consistent with a flexible regulatory limitation and with *SeMALR1/SeMAL63* representing an accessible mutational route towards a strongly maltose-positive phenotype. Moreover, our data suggest that mutations in *SeTUP1* may also enhance α-oligoglucoside utilization, and the contribution of additional, as-yet-unidentified genetic or regulatory changes cannot be excluded.

These findings identify Se*MALR1* as a critical control point in the domestication of Himalayan *S. eubayanus* for brewing. Consistent with this conclusion, work by Quintrel et al. (2026) highlighted that *MALR* (*SeMAL33*) diversity among *S. eubayanus* isolates from *Quercus* and *Nothofagus* trees represents a key evolutionary mechanism influencing maltose adaptation in this species. The generally close association between Holarctic strains and the relatively maltose-poor habitat of *Quercus* trees might explain the absence of maltose uptake in these strains. However, the ease with which this trait was acquired during adaptive evolution shows this might reemerge over relatively short timespans in natural populations when subjected to evolutionary pressure (Quintrel et al. 2026). A similar adaptation was observed in wild Irish *S. eubayanus*, in which evolution selected gain-of-function mutations in the transcriptional regulator *Se*MalR (I243N or W307L) that, together with increased *MAL* locus copy number, improved maltose utilization (Bergin et al. 2026). These findings further highlight the interplay between regulatory mutations and gene copy-number variation in adaptation towards efficient maltose utilization.

The evolved POF$^-$ strains HTS259 and HTS260 showed markedly improved attenuation in industrial wort, outperforming both their parental strain and Patagonian *S. eubayanus* controls. Efficient maltose depletion and partial maltotriose consumption resulted in lower final extract and increased ethanol production, traits essential for lager brewing. Although maltotriose utilization remained incomplete, even partial consumption can significantly improve flavour balance, indicating that further optimization may not be strictly required for practical application. The production of substantial amounts of esters and higher alcohol, combined with low concentrations of the off-flavour diacetyl suggests the potential to generate distinctive beverage flavour profiles. However, side-by-side sensory evaluation against beers produced with commercial lager strains will be required before conclusions can be drawn regarding an expanded flavour space or the absence of undesirable sensory effects. Despite these improvements, the overall fermentation rate remained considerably lower than that of industrially applied hybrid *S. pastorianus* lager strains, resulting in substantially longer process times. In addition, the lack of flocculation remains an important process limitation for efficient biomass separation and yeast repitching.

The rapid emergence of maltose-positive phenotypes through Se*MALR1/SeMAL63* mutation has implications for understanding the evolutionary origins of lager yeasts. It suggests that ancestral *S. eubayanus* populations may have possessed latent fermentative potential that was readily selectable under brewing conditions. Indeed, recent historical and microbiological investigations suggest that *S. eubayanus* may itself have been the dominant yeast species in early lager-type fermentations, rather than merely a contaminant in fermentations dominanted by *S. cerevisiae* (Hutzler et al. 2023). Regardless, regulatory flexibility in the maltose phenotype could have facilitated early hybridization events leading to *S. pastorianus* and may help explain the mosaic nature of the *S. eubayanus* subgenome observed in modern lager strains (Gorter de Vries et al. 2019, Salazar et al. 2019, Hutzler et al. 2023).

In conclusion, this study establishes that a wild *S. eubayanus* strain can be improved for lager relevant traits through classical mutagenesis and adaptive laboratory evolution. The resulting strains combine elimination of 4-VG formation with near complete maltose and partial maltotriose utilization. The identification of *Se*MalR1/*Se*Mal63 as a major regulatory control point highlights the importance of transcriptional control in yeast domestication and provides a mechanistic framework for further strain development. Beyond its practical relevance for brewing, this work advances our understanding of how regulatory evolution enables rapid adaptation of environmental yeasts to anthropogenic fermentations.

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Supplementary material

Supplementary material are available at *FEMSYR Journal* online.

Conflict of interest

The authors declare the following conflict of interest. JD, SZ, TP, and J.-M.D. are inventors on a patent application related to the work described in this study. Owing to the industrial sponsor-

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ship of the project, the strains and materials generated during this study cannot be made available upon request.

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Data availability

The genome assembly of the *Saccharomyces eubayanus* strain ABFM5L.1 and its annotation are publicly available in the 4TU Research Data Repository (https://doi.org/10.4121/776460a8-e6d4-4e06-9f35-3891ecdc0e72.v1). Genome sequencing raw data for the strains ABFM5L.1 are available under accession number Bioproject PRJNA528469 (Brouwers et al. 2019a). Sequencing data of amplicons from strains HTS219, HTS257, HTS258, HTS259, and HTS260 derived from ABFM5L.1 are available at NCBI under BioProject (https://www.ncbi.nlm.nih.gov/bioproject) PRJNA1359756.

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