RESEARCH ARTICLE

Adaptive evolution of the lager brewing yeast *Saccharomyces pastorianus* for improved growth under hyperosmotic conditions and its influence on fermentation performance

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Abstract

An adaptive evolution method to obtain stable *Saccharomyces pastorianus* brewing yeast variants with improved fermentation capacity is described. The procedure involved selection for rapid growth resumption at high osmotic strength. It was applied to a lager strain and to a previously isolated ethanol-tolerant strain. Fermentation performance of strains was compared at 15 °P wort strength. A selected osmotolerant variant of the ethanol-tolerant strain showed significantly shorter fermentation time than the parent strain, producing 6.45% alcohol by volume beer in 4–5 days with mostly similar organoleptic properties to the original strain. Diacetyl and pentanedione contents were 50–75% and 3-methylbutyl acetate and 2-phenylethyl acetate 50% higher than with the original strain, leading to a small flavour change. The variant contained significantly less intracellular trehalose and glycogen than the parent. Transcriptional analysis of selected genes at 24 h revealed reduced transcription of hexose transport genes and increased transcription of the *MALx1* and *MALx2* genes, responsible for α-glucoside uptake and metabolism. It is suggested that an attenuated stress response contributes to the improved fermentation performance. Results show that sequential selection for both ethanol tolerance and rapid growth at high osmotic strength can provide strains with enhanced fermentation speed with acceptable product quality.

Introduction

Brewing yeast strains presently in use have been isolated at a time when fermentation conditions were far removed from those which the strains are expected to cope within today's industrial breweries. Isolated yeast strains are stored frozen as pure cultures, often for decades, and are re-activated to create fresh inocula for fermentation. A single batch of lager brewing yeast is recycled in the brewery a number of times (typically 5–20 fermentations) before being discarded and the process repeated with a fresh culture from storage. As a result, brewing yeast cells are afforded no opportunity to evolve and adapt to the changes in brewing practices that have occurred since the middle of the 20th century, when frozen storage of production strains became common practice. The brewing yeast cell's fitness to ferment is dependent on its ability to adapt to the dynamic and often stressful conditions encountered throughout the brewing process. Challenges encountered during brewery fermentation include changes in oxygen availability, osmotic stress, CO_2 accumulation, nutrient limitation and ethanol toxicity (Gibson *et al.*, 2007). Intensification of the brewing process, particularly through the widespread use of high-gravity worts with strengths significantly exceeding the 12 °Plato (12 g extract per 100 g wort) used in traditional brewing, has placed further demands on brewing yeast, with the result that fermentation rates can be significantly reduced and residual sugars can be present in finished beers (Stewart & Russell, 1993). Improvement in yeast fermentation performance with faster and more complete conversion of sugars to alcohol as well as shorter production time is desirable, not only for profitability, but also for improved efficiency and sustainability of the brewing system.

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FEMS YEAST RESEARCH

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Genetic modification of brewing yeast cells has shown great potential for improvement in yeast fermentation, with numerous phenotypes modified to optimize performance (Dequin, 2001; Saerens *et al.*, 2010). Examples include improved exploitation of wort through increased maltose and maltotriose transport and utilization (Vidgren *et al.*, 2009), increased uptake of proline (Omura *et al.*, 2005) and reduced hydrogen sulphide release (Iijima & Ogata, 2010) as well as changes in the intensity and timing of flocculation (Verstrepen *et al.*, 2001; van Mulders *et al.*, 2009). In other cases, new phenotypes have been introduced to yeast through the expression of exogenous genes. These have included expression of acetolactate decarboxylase to prevent α-acetolactate and diacetyl formation, thereby obviating the need for beer maturation (Kronlöf & Linko, 1992), expression of β-glucanase to improve the filterability of beer (Penttilä *et al.*, 1987) and the expression of amyloglucosidases to utilize wort dextrins, increasing the utilization of wort carbohydrates and reducing the calorific content of the beers produced (Cole *et al.*, 1988). However, low consumer tolerance has, so far, precluded the commercial use of genetically modified brewing yeast. A more 'natural' approach to enhancing brewing yeast attributes is adaptive evolution, which has previously been used to improve fermentation performance in higher gravity worts. Blieck *et al.* (2007), for example, isolated two variants of a lager yeast strain with improved fermentation performance after successive fermentations with UV-treated yeast in very high-gravity wort (> 22 °P). Huuskonen *et al.* (2010) treated brewing yeast cells with ethyl methanesulfonate (EMS) and exposed the mildly mutagenized cells to conditions typical for the final stages of very high-gravity fermentations, that is, anaerobic beer with high concentrations of ethanol and with maltose and maltotriose as the sole sources of fermentable sugar. Yeast variants (here called 'ethanol-tolerant' variants) with an ability to survive these conditions for extended periods were isolated and several exhibited improved fermentation performance in very high-gravity (24 °P) wort. This improvement was particularly evident towards the end of the fermentation, when conditions resembled those under which the variants were selected. A somewhat similar approach was used by Yu *et al.* (2012) but involving EMS and UV mutagenesis.

In the present work, a procedure for the generation and selection of genetically stable yeast variants able to grow rapidly at high osmotic strength was designed. Isolated variants were then tested for their ability to ferment 15 °P wort. It was hypothesized that the variants would perform faster in early fermentation, when high concentrations of sugars impose hyperosmotic stress. The procedure was applied to the same mutagenized lager yeast population described by Huuskonen *et al.* (2010) and to an ethanol-tolerant strain derived from this population. The organoleptic qualities of beers produced were evaluated. Some of the results have been previously presented (Ekberg, 2011).

Materials and methods

Worts

Brewer's all-malt wort was made at VTT Technical Research Centre of Finland. Worts (15 °P) were prepared with Espoo city water (all other water was deionized and filtered through active carbon using the MilliQ Water System; Millipore Corporation, MA). Worts were collected hot (> 90 °C) and stored at 0 °C until use. Stored worts were mixed before use to re-suspend the settled solids evenly. The composition of these worts is shown in Table 1.

Yeast strains

Strains used in this investigation are deposited in the VTT Culture Collection and include the *Saccharomyces pastorianus* lager brewing yeast strain VTT-A63015, herein referred to as A15, and an ethanol-tolerant variant VTT-A05197 derived from A15, herein referred to as A197. Huuskonen *et al.* (2010) earlier compared the fermentation performances of A15 and A197, therein called T24.1. Osmotolerant variants generated in this investigation are deposited as VTT-A11219 and VTT-A11218 and are herein referred to as A219 and A218, respectively. In Table 2, the descriptions and abbreviations of the strains used in the experiments are listed. Validation of oligonucleotide probes for gene expression analysis involved the use of the *Saccharomyces cerevisiae* ale strain VTT-A75060 (A60) and the type strain of *Saccharomyces eubayanus* VTT-C12902 (C902; deposited as CBS 12357 at CBS-KNAW Fungal Biodiversity Centre).

Table 1. Wort composition

	15 °P
Glucose concentration (g L^{-1})	19.6
Fructose concentration (g L^{-1})	3.4
Maltose concentration (g L^{-1})	53.4
Maltotriose concentration (g L^{-1})	17.1
Attenuation limit (%)	86
pH	5.3
Free amino nitrogen (FAN) (mg L^{-1})	304.3
European bitterness units (EBU)	37.9
Calcium (mg L^{-1})	69
Magnesium (mg L^{-1})	190
Zinc (mg L^{-1})	0.14

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Table 2. Yeast strains used in the experiments

Strain	Description	Abbreviation
VTT-A63015	Industrial lager brewing yeast (*S. pastorianus*)	A15
VTT-A05197*	Ethanol-tolerant variant of A15	A197
VTT-A11219	Osmotolerant variant of A15	A219
VTT-A11218	Osmotolerant variant of A197	A218
VTT-A75060	Industrial ale brewing yeast (*S. cerevisiae*)	A60
VTT-C12902	*S. eubayanus* type strain	C902

*Previously referred to as T24.1 (Huuskonen *et al.*, 2010).

Mutagenesis

The yeast strain A15 was grown to stationary phase in yeast peptone medium (YP; 10 g of yeast extract and 20 g of peptone per litre) containing 20 g of maltose per litre. Yeast was harvested by centrifugation, washed with sterile water, suspended in 0.1 M sodium phosphate (pH 7.0) to 25 mg fresh yeast per mL and mutagenized with EMS essentially as described previously (Huuskonen *et al.*, 2010). EMS (60 μL) was added to 3.0 mL of the yeast suspension, and the mixture was shaken at room temperature (*c.* 20 °C) for 60 min. The EMS reaction was quenched by adding 20 mL of sodium thiosulfate (50 g L^{-1}). Mutagenized yeast cells were collected by centrifugation, washed twice with sodium thiosulfate (50 g L^{-1}) and suspended in sterile saline (9 g NaCl per litre). Dilutions were spread onto agar plates containing YP plus 20 g maltose per litre to determine the proportion of dead cells, which was close to zero (from 0% up to 15%). The remaining yeast, a pool of about 3×10^8 mutagenized cells, was inoculated into 1 L of YP containing 40 g maltose per litre and grown to stationary phase, giving about 25 g fresh yeast. Stock suspensions of this mutagenized yeast were prepared by transferring the yeast to 30% glycerol and storing at −80 °C as 1-mL aliquots containing 200 mg fresh yeast.

Enrichment of variants growing rapidly at high osmotic strength

Two hundred microlitre of a glycerol stock suspension containing 40 mg fresh mass of mutagenized A15 or non-mutagenized A197 yeast was used to inoculate 100 mL of YP medium containing maltose (40 g L^{-1}) and sorbitol (210 g L^{-1}) in a 250-mL Erlenmeyer flask. Cultures were incubated at 25 °C with shaking (120 r.p.m.) until an OD_{600} of *c.* 10 was achieved. At this point, a sufficient volume of the culture was transferred to a fresh 100 mL of the same sorbitol-supplemented growth medium to give a starting OD_{600} of 0.15, and the culture was incubated as before. This process was repeated until the approximate number of cell generations, based on change in OD_{600}, reached 200 (at least 27 successive cultures). No precautions were taken to exclude air during this enrichment process, so the metabolism is expected to have been respirofermentative. Samples of the yeast populations were taken periodically during this exposure period, washed and stored as glycerol stocks at −80 °C.

Isolation of variants showing rapid growth

Frozen glycerol stocks of A15, its ethanol-tolerant variant (A197) and the sorbitol-exposed yeast populations collected previously were thawed on ice and the cell number estimated using a NucleoCounter (YC-100; ChemoMetec A/S, Denmark). Populations were diluted and transferred to three YP plus maltose (40 g L^{-1}) agar plates containing 210 g sorbitol per litre and to three YP plus maltose (40 g L^{-1}) agar plates without sorbitol, at viable cell densities of *c.* 200 per plate. Plates were incubated at 24 °C for 9 days, and the number of visible colonies emerging on plates was recorded daily. The number of new colonies emerging each day was expressed as a percentage of the total number of colonies visible after 9 days (no further colonies appeared after this time). Some of the colonies that appeared relatively early on sorbitol-supplemented plates (potential variants) were transferred individually to 50 mL YP containing 40 g maltose per litre and shaken overnight at 24 °C. The yeasts were then harvested and stored in 30% glycerol at −80 °C.

Genetic stability

Two potential variants (A218 and A219) and the original strains (A15 and A197) were grown repeatedly in YP containing 40 g maltose per litre, but no sorbitol. Each successive culture was inoculated with the preceding culture as described above until each strain had grown through at least 100 generations without hyperosmotic selective pressure.

Minifermentations

To rapidly screen the fermentation properties of isolated variants, minifermentations (20 mL of wort in 50-mL Corning tubes) were performed as previously described (Guimarães & Londesborough, 2008), except that air locks containing glycerol were used to prevent evaporative loss of mass instead of overlayering the wort with mineral oil. Yeast samples for these minifermentations were grown in YP containing 40 g maltose per litre.

Tall tube (static) fermentations

To imitate industrial brewery fermentations, 15 °P wort was fermented with selected strains in stainless steel

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cylindroconical vessels (so-called tall tubes) with dimensions of 6 cm diameter × 100 cm height (2 L) or 12 cm diameter × 115 cm height (10 L), essentially as previously described (Rautio & Londesborough, 2003; Vidgren *et al.*, 2009). Because most brewery fermentations are pitched (inoculated) with yeast cropped (harvested) from a preceding fermentation, we pitched each 'generation 1' tall tube fermentation with yeast cropped from an otherwise identical 'generation 0' fermentation. Data presented are from the 'generation 1' fermentations. The overall procedure was as follows:

Generation 0 fermentations: Frozen yeast suspensions in 30% glycerol were thawed and used to inoculate 500 mL autoclaved YP containing 40 g maltose per litre in 1-L Erlenmeyer flasks. Cultures were incubated overnight at 25 °C with shaking (120 r.p.m.) and then transferred to 1.5 L of 15 °P wort to achieve an OD_{600} of 0.15. These cultures were incubated at 16 °C with shaking for 48 h and then moved to 0 °C. After 16 h, most of the supernatant was decanted from each flask and the settled yeast was mixed into a smooth slurry. Samples of slurry (about 5 mL each) were weighed and then centrifuged (10 min at 9000 ***g***). The pellets were weighed, and the slurry was diluted with decanted supernatant to 20 g centrifuged yeast mass per 100 g of slurry. Tall tubes containing 10 L of oxygenated (10 mg dissolved oxygen per litre measured with an Oxygen indicator 26073; Orbisphere Laboratories, Switzerland) 15 °P wort were pitched with this slurry to a concentration of 5 g fresh centrifuged yeast per litre. This generation 0 fermentation was allowed to proceed in a room at 15 °C until 80% apparent attenuation was reached [apparent attenuation is a measure of how completely wort carbohydrates have been converted into ethanol; this and other brewing terms are explained by Vidgren *et al.* (2009)]. The partially settled yeast was then cropped from the bottom of the tall tubes as a slurry mixed with beer (*c.* 2 L), transferred to 0 °C and allowed to sediment for 16 h. Most of the supernatant was then decanted, and a slurry containing 200 g centrifuged yeast mass per litre was prepared as described above and used within 2 h to pitch the generation 1 fermentation.

Generation 1 fermentations: Tall tubes containing 2 or 10 L of 15 °P wort freshly oxygenated to *c.* 10 mg dissolved oxygen per litre were pitched with a slurry of cropped yeast prepared as described above. The fermentations were allowed to proceed in a room at 15 °C. Samples (30 mL from 2 L fermentations; 100–200 mL from 10 L fermentations) were withdrawn daily. Samples were centrifuged (9000 ***g*** for 10 min at 1–5 °C) and supernatants used for wort/beer analyses. The pellets were washed with water, weighed, re-suspended in water to 200 mg centrifuged yeast mass per mL and used for yeast analyses.

Beer conditioning

When the 10 L 'generation 1' fermentations were completed, sedimented yeast slurry (about 2 L) was removed from the bottom of the tall tube and left to sediment overnight at 0 °C (the settled yeast was used for analyses of cropped yeast). The rest of the tube contents (about 8 L) was collected as unconditioned (green) beer and was matured for 7 days at 15 °C and then 2 days at 0 °C. After maturation, the beer was sterile filtered, diluted to 4.5% alcohol by volume (ABV) and bottled aseptically in 0.33 L brown glass bottles under CO_2 to give 4.0 g L^{-1} dissolved CO_2. Bottled beer was stored at 0 °C until use.

Yeast analyses

Dry yeast masses were determined by drying portions of washed yeast slurry overnight at 105 °C. Intracellular glycogen was extracted and assayed according to Schulze *et al.* (1995). Intracellular trehalose was extracted with boiling water and assayed enzymatically as described (Guimarães *et al.*, 2008). Intracellular glycerol was extracted with boiling water and assayed enzymatically with glycerol kit (R-Biopharm AG, Germany). Yeast cell viability was measured with the NucleoCounter.

Sampling for gene expression analysis

After 24-h fermentation, yeast was harvested from tall tubes for transcriptional analysis by anaerobically withdrawing wort containing 50–200 mg fresh mass of yeast. Yeast was immediately separated from wort by filtration with glass-fibre filter discs (Whatman GF/B 47 mm Ø, Kent, UK). The biomass was immediately washed with RNAse-free (dimethyl pyrocarbonite-treated) water, transferred to tared screw-cap tubes and stored in liquid nitrogen at −80 °C. This sampling procedure took < 5 min.

Transcriptional analysis

Transcriptional analysis was performed with the TRAC assay essentially as described earlier (Rautio *et al.*, 2007). Sample tubes were weighed to give the fresh yeast mass. Frozen yeast samples were suspended (50–200 mg fresh weight per mL) in lysis buffer (PlexPress, Finland). Yeast was disrupted with 500 μL of acid-washed glass beads (Sigma) twice in a MagNA Lyzer cell homogenizer (Roche) for 45 s at full speed. Hybridization reaction mixtures contained in 125 μL, yeast lysate (100 μg biomass; 100–150 ng polyA RNA), 4 pmol biotinylated oligo(dT) capture probe (Ella Biotech, Munich, Germany), 0.5 pmol each of labelled detection probe

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(PlexPress), 75 μL HybMix (PlexPress) and 1.5 fmol of ssDNA as internal standard (PlexPress). The hybridizations were carried out in 96-well PCR plates (ABgene, Epsom, UK) at 60 °C for 60 min. The steps following hybridization, including affinity capture, washing and elution, were automated with a magnetic bead particle processor KingFisher 96 (Thermo Electron, Vantaa, Finland) in 96-well plates as follows: (1) affinity capture of hybridized RNA targets to 50 μg of streptavidin-coated Sera-Mag SpeedBeads (ThermoFisher Scientific) for 15 min at room temperature, (2) washing of the beads five times for 1.5 min in 100 μL of Wash Buffer (PlexPress) at room temperature, (3) elution of probes with 10 μL of formamide (Applied Biosystems, Foster City, CA) for 10 min at 37 °C. The eluates were analysed by capillary electrophoresis with an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). To calibrate the separation of the detection probes by size, GeneScan-120LIZ size standard (Applied Biosystems) was added to each sample. The identity of the probes was determined by the migration speed and the quantity by the peak area. To minimize nonbiological variation in the TRAC assay, the signal intensities measured for the target genes were normalized between samples using the signal measured for the ssDNA internal standard.

Probe design and validation

Oligonucleotide probes were designed using algorithms presented earlier (Kivioja *et al.*, 2002). Criteria used were the following: melting temperature, T_m, limits 59–75 °C, GC% limits 38–62, maximum free energy change in hybridization%G_H of −15 kcal mol^{-1} (Le Novére, 2001) and minimum target energy change Ac of −10 kcal mol^{-1} (Luebke *et al.*, 2003). A maximum repeat size of 15 nt and a maximum similarity of 80% were used as probe specificity criteria. T_m values were calculated with the nearest neighbouring method according to Le Novére (2001) using 10 nM nucleic acid and 750 mM salt concentrations. The data for *in silico S. pastorianus* transcriptome were generated from the NCBI assembly (SM18211v1) of *S. pastorianus* Weihenstephan 34/70 (project PRJNA29791). The assembly was re-annotated to hybrids between *S. cerevisiae* and *S. eubayanus* using supplemental data provided by Nakao *et al.* (2009). The target-specific detection probe oligonucleotides, labelled at the 5′-ends with 6-FAM, HEX, ATTO550 or ATTO565, were synthesized by Ella Biotech. The HPLC-purified oligonucleotide detection probes were organized into pools according to their migration in capillary electrophoresis. The probes were designed to be specific to either *S. cerevisiae* or *S. eubayanus* parts of the *S. pastorianus* genome. The specificity testing of the probes was performed using yeast collected from shake flask cultures with an ale strain of *S. cerevisiae* (A60) or the type strain of *S. eubayanus* (C902). Yeast was cultured in YP medium containing maltose (4% w/v) at a temperature of 25 °C and on a shaking table at 120 r.p.m. Yeast samples were taken at intervals throughout the growing period and were prepared for TRAC analysis as described above. Each designed *S. cerevisiae* and *S. eubayanus* probe was tested for potential cross-hybridization in the TRAC assay using 100 μg of lysed yeast samples from both *S. cerevisiae* and *S. eubayanus* cultures.

Wort and beer analyses

Wort densities were determined using an Anton Paar DMA58 density metre. The pH was monitored according to Analytica-EBC method 9.35 (European Brewery Convention, 2004). Ethanol was determined by quantitative distillation according to Analytica-EBC method 9.2.1 (European Brewery Convention, 2004). Samples (4 mL) of green beers for aroma analysis were clarified by filtration (0.45 μm) and alcohols, esters and acetaldehyde then determined by headspace-GC/MS. 1-Butanol was used as internal standard. Samples were first incubated at 60 °C for 30 min. One millilitre of sample was then injected in the splitless injector (260 °C; flow 14.9 mL min^{-1}) of the gas chromatograph (Agilent 6890 Series; Palo Alto, CA) combined with an MS detector (Agilent 5973 Network MSD) and SPME autosampler (Combipal; Varian Inc.). Analytes were separated on a BPX5 capillary column of 60 m × 0.25 mm with phase thickness 1.0 μm (SGE Analytical Science Pty Ltd, Australia). Helium was used as carrier gas at a constant flow of 1.7 mL min^{-1}. The temperature program was 50 °C for 3 min, then 10 °C min^{-1} to 100, 5 °C min^{-1} to 140 and 15 °C min^{-1} to 260 °C, where the temperature was held for 1 min. MSD was operated in electron-impact mode at 70 eV, in the full scan *m/z* 40–550. The ion source temperature was 230 °C and the interface was 280 °C. Compounds were identified by the retention times of authentic standards and by their mass spectra on Palisade Complete 600 K Mass Spectral Library (Palisade Mass Spectrometry) and quantitated with a standard curve. Total vicinal diketones (diacetyl and pentanedione; VDKs) and free amino nitrogen were measured according to Analytica-EBC methods 9.10 and 9.24.2 (European Brewery Convention, 2004), respectively. Beer foam stability was measured after carbonation and bottling according to Analytica-EBC method 9.42 (European Brewery Convention, 2004). Sensory analysis of the bottled beers was performed by Analytica-EBC triangular test method 13.7 (European Brewery Convention, 2004), using 10 trained testers.

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Results

Screening of variants yielded clear differences in sorbitol resistance

Mildly mutagenized A15 yeast was exposed repeatedly to hyperosmotic conditions before being transferred to agar plates with or without 210 g sorbitol per litre. The number of newly visible colonies emerging each day from these populations was counted over a period of 9 days (Fig. 1a and c). On control plates without sorbitol, the majority of colonies (> 90%) were observed after 2-day incubation. On plates containing sorbitol, the emergence of colonies was population dependent. With untreated A15 strain, colonies mostly (58%) appeared 4 days after inoculation, while treated populations produced visible colonies earlier. The time of emergence was inversely related to the duration of the populations' prior exposure to hyperosmotic conditions. The population that had passed through 27 consecutive cultivations (*c.* 200 cell generations) in the presence of sorbitol produced over half of its visible colonies after only 2 days of incubation. Some of these rapidly growing colonies were isolated, cultivated and stored. A feature of colony emergence on the sorbitol-supplemented plates was a second peak in colony emergence about 6 days after inoculation. These late-appearing colonies consisted of respiratory-deficient 'petite' cells unable to grow with glycerol as the sole carbon source (data not shown). Like A15, the A197 strain produced most visible colonies 2 days after inoculation of control plates (Fig. 1b) but 4 days after inoculation of plates containing sorbitol (Fig. 1b and d). After exposure to hyperosmotic conditions for 33 successive cultivations, an A197-derived population generated most visible colonies on plates containing sorbitol already on the third day following inoculation (Fig. 1d).

Enhanced performance confirmed in 2-L-scale fermentations

Mini-fermentations with 25 mL of wort were used to screen five picked colonies that grew rapidly in the presence of sorbitol. Four of these single cell colonies produced yeast that fermented faster than the original A15 or A197 strains. Two of these potential variants, A219 (derived from A15) and A218 (derived from A197), were selected and compared with their parent strains in 2-L-scale fermentations with 15 °P wort pitched with 'generation 1' yeast batches cropped from previous fermentations. Fermentations by the two parent strains, A15 and A197, proceeded at similar rates (Fig. 2a). In earlier work (Huuskonen *et al.*, 2010), A197 fermented faster than A15 near the end of fermentations, and the advantage was smaller in 15 °P than in 25 °P fermentations that reached much higher ethanol concentrations. A219 (osmotolerant variant of A15) fermented faster than

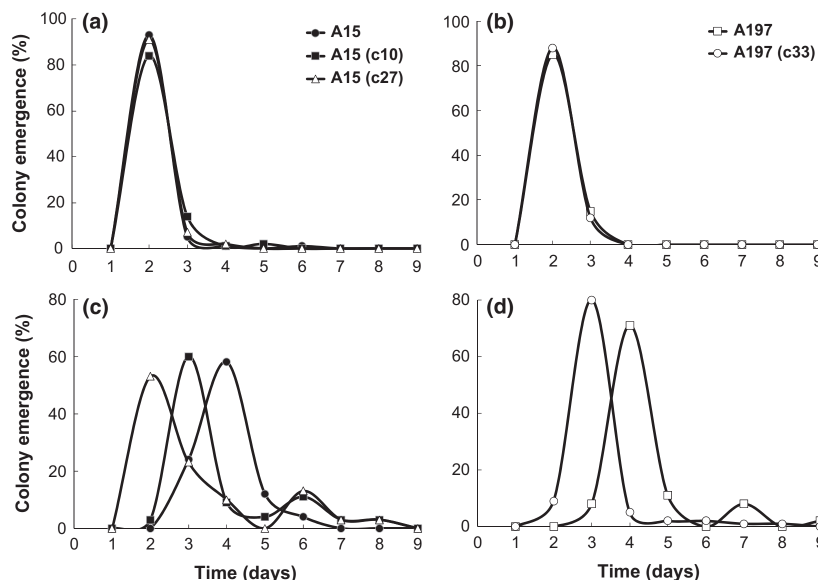

Fig. 1. Emergence of visible colonies from control and hyperosmotically exposed populations of A15 (a, c) and A197 (b, d) lager yeasts. Samples were spread on agar plates containing YP and 40 g maltose per litre without (a, b) or with (c, d) 210 g sorbitol per litre. The number of new colonies emerging each day is expressed as a percentage of the total number of visible colonies on day 9. The exposed populations had been grown repetitively in YP containing 40 g maltose and 210 g sorbitol per litre for 10 (c10), 27 (c27) or 33 cultivations (c33). Results are all averages from three replicate agar plates each producing at least 160 visible colonies on day 9.

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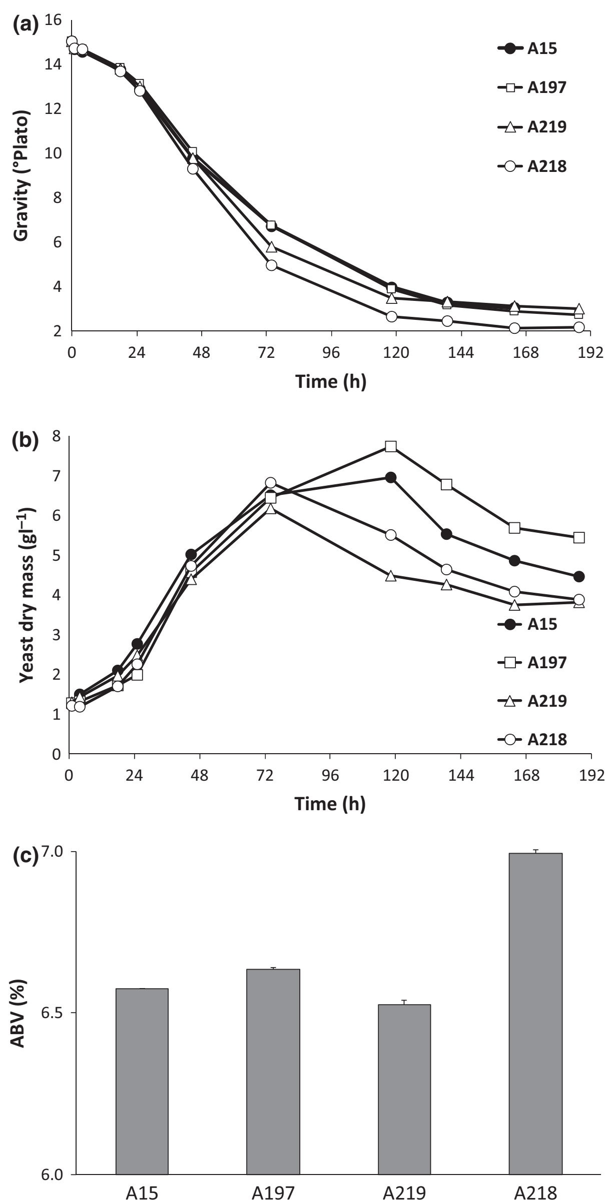

Fig. 2. Two-litre-scale fermentations with the production lager strain (A15), a previously isolated ethanol-tolerant variant (A197) and osmotolerant variants of each produced in this study. Yeasts were pitched at 5 g L^{-1} into 15 °P all-malt wort, and fermentations were carried out at 15 °C. Wort gravity (a), cell dry mass in suspension (b) and final ABV (% v/v) are shown. Data are from single fermentations. Error bars show the range of results from duplicate measurements.

the parent strains in mid-fermentation (reaching a wort gravity of 5.8 °P on day 3, when both parents had reached only 6.7) but slowed in the final stages. A218 (osmotolerant variant of the ethanol-tolerant A197) also fermented faster in mid-fermentation (reaching 4.9 °P on day 3) and maintained its advantage to the end of the fermentation. The final gravity reached was 2.2 °P for A218 compared with values between 2.9 and 3.1 for the other strains (Fig. 2a). These values corresponded to final apparent attenuations of 85% for A218 and between 79% and 81.5% for the other strains (the fermentation limit of the wort was 86%). Final ABV was 7.0% for the beers made with A218 and 6.5–6.6% for the other beers (Fig. 2c). In these 15 °P wort fermentations, growths of the osmotolerant variants and original strains were very similar up to 72 h, after which the mass of variant yeasts in suspension began to decrease, whereas the original strain's amount increased further until 120 h after pitching (Fig. 2b). Simultaneous fermentations carried out with variant yeast that had been exposed to stress-free conditions (YP containing 4% maltose and no sorbitol) for 100 cell generations prior to fermentation gave identical results showing that the variants were genetically stable (data not shown).

Detailed analysis of fermentation behaviour of A218 strain confirmed enhanced performance at the expense of elevated VDK levels

The above results showed that strain A218, derived from A15 by two successive selection procedures (first for ethanol tolerance and then for osmotolerance), was a genetically stable strain exhibiting fast fermentation of 15 °P wort. It was selected for more detailed analyses of its behaviour during fermentation and of the organoleptic quality of the produced beer. The progress of 10-L fermentations with 'generation 1' (cropped) yeast is shown in Fig. 3 (results with the generation 0 yeast were essentially similar; data not shown). The reason for the overall faster fermentation on 10-L scale compared with 2-L scale is not clear, but the fermentation temperature may have been 1–2 °C higher. The relatively narrow 2-L tall tubes remained at ambient temperature, whereas the wider 10-L tubes warmed up during the rapid fermentation stage because of greater metabolic heat generation per unit surface area. From 24 h onwards, fermentations by A218 were faster than those by A15 and reached an apparent attenuation of 80% in 72 h. In comparison, it took 193 h for A15 strain to reach only 78% attenuation. Final alcohol contents were correspondingly higher for A218 than for A15 (Table 3). Sedimentation occurred earlier with the variant compared with the original strain. Viability remained above 95% in both strains throughout the fermentation (data not shown).

Some key features of the fermentations are listed in Table 3. Greater alcohol content and lower present gravity were reached during the fermentation with A218 (the target attenuation value of 80% was not reached with A15). Final glucose and maltose levels were similar in the two beers, but maltotriose was much lower after fermen-

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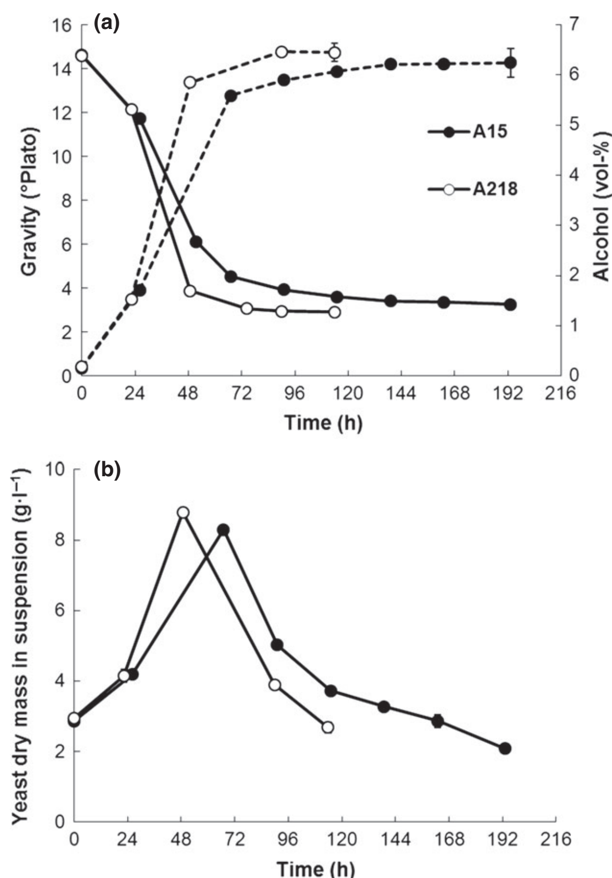

Fig. 3. Ten-litre-scale fermentations with the original (A15) and variant (A218) lager brewing yeast strains showing change in wort gravity and alcohol level (a) and yeast dry mass in suspension (b). Solid lines represent gravity and dashed lines alcohol content in the top panel. Solid markers represent A15 and open markers A218 in all panels. Strains were pitched at 5.0 g fresh yeast per litre into 15 °P wort. Fermentations were carried out at 15 °C. Values are averages of duplicate fermentations. Error bars show the range of results from duplicate measurements.

tation by A218. Free amino nitrogen content was lower in beer made with A218, suggesting greater use, presumably for yeast growth. Final pH values were similar for the two yeasts. Diacetyl and pentanedione contents were both about threefold higher in the A218 green beer than the A15 green beer. Foam stability was slightly reduced in the variant beer.

Intracellular storage carbohydrates and glycerol profiles were altered between strains

Yeast samples for assays of storage carbohydrates and glycerol were taken at the end of the generation 0 fermentation, 5–10 min after pitching the generation 1 fermentations and then daily (Fig. 4). At the end of the generation 0 fermentation, trehalose content of A15 was 33.8 mg g dry yeast^{-1} and of A218 19.3 mg g dry yeast^{-1}. Immediately after pitching, trehalose content had dropped to 18.4 mg g dry yeast^{-1} in A15 and to 5.6 mg g dry yeast^{-1} in A218. Trehalose content was therefore over three times higher in A15 at the beginning of generation 1 fermentation. Glycogen (4.4 in A15 and 3.3 mg g dry yeast^{-1} in A218) and glycerol levels (7.7 both in A15 and A218) were, however, the same at the beginning with both yeasts. These values at the end of generation 0 fermentation were 13.2 and 4.3 mg g dry yeast^{-1}, respectively, in the case of glycogen, and 3.3 and 4.3 mg g dry yeast^{-1}, respectively, in the case of glycerol. During the first 70 h of fermentation, A15 accumulated almost four times more trehalose than A218, reaching a maximum value of 39.0 mg g dry yeast^{-1}, whereas the maximum content found in A218 was only 10.5 mg g dry yeast^{-1}. Also in the case of glycogen, A15 accumulated almost two times more (28.0 mg g dry yeast^{-1}) than A218 (16.8 mg g dry yeast^{-1}). After the

Table 3. Key data from the 10-L-scale fermentations. Error values show the range of values from duplicate or quadruplicate measurements

	A15	A218
Duration of the fermentation (day)	8.0	4.5
Final gravity (°P)	3.26 ± 0.01	2.92 ± 0.00
Beer glucose concentration (mg L^{-1})	112 ± 5.2	113 ± 0.6
Beer fructose concentration (mg L^{-1})	8.1 ± 0.3	14.1 ± 0.3
Beer maltose concentration (mg L^{-1})	367 ± 1.0	364 ± 3.5
Beer maltotriose concentration (mg L^{-1})	4654 ± 326	1207 ± 57
Beer alcohol content (vol-%)	6.24 ± 0.29	6.45 ± 0.18
Beer free amino nitrogen (mg L^{-1})	197.0 ± 5.2	169.0 ± 2.7
Free amino nitrogen utilized (%)	35	44
Beer pH	4.56 ± 0.01	4.33 ± 0.01
Diacetyl (mg L^{-1}), adjusted to 4.5 vol-%		
Green beer	0.07 ± 0.004	0.22 ± 0.012
Mature beer	0.04 ± 0.003	0.07 ± 0.003
Pentanedione (mg L^{-1}), adjusted to 4.5 vol-%		
Green beer	0.05 ± 0.002	0.13 ± 0.007
Mature beer	0.02 ± 0.001	0.03 ± 0.001
Beer foam stability (s), NIBEM*	61-120-185	56-113-170

*NIBEM device measures beer foam collapse time over a distance of 30 mm after transfer of beer to a glass with CO_2 gas flushing. First value is the time taken for foam head to collapse 10 mm after the initial collapse of 10 mm, second 20 mm and third 30 mm.

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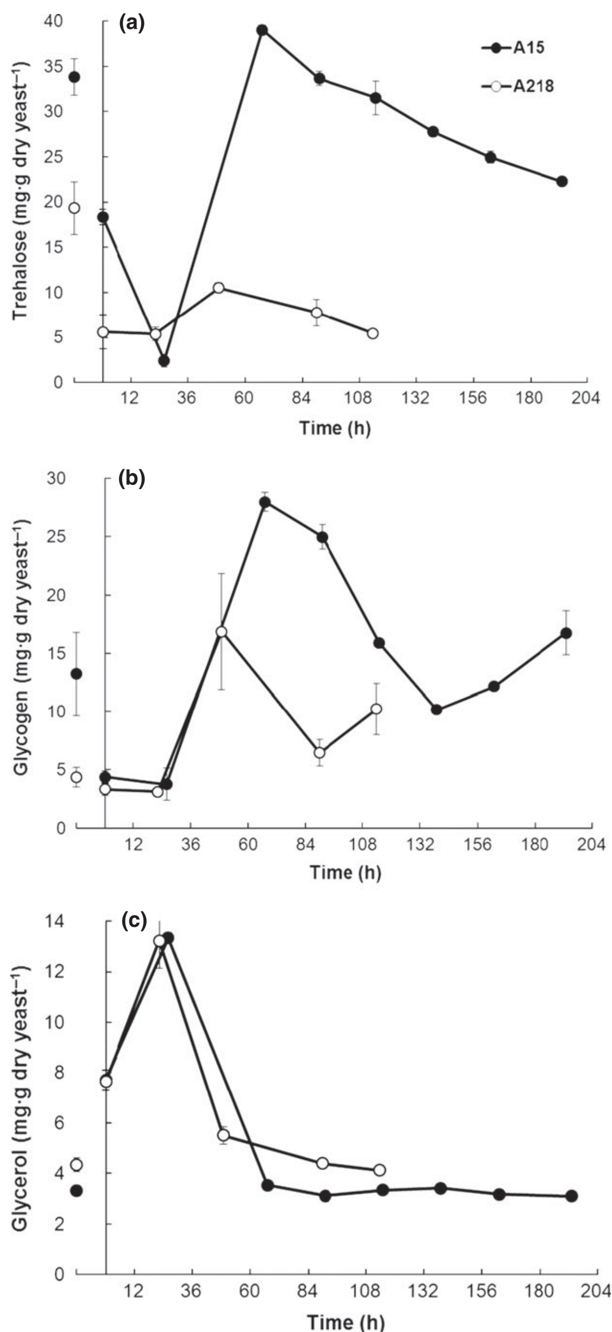

Fig. 4. Trehalose (a), glycogen (b) and glycerol (c) contents during the 10-L fermentations. Solid markers represent the original strain (A15) and open markers the variant (A218) in all panels. Values before 0 h mark represent the values for cropped yeast at the end of generation 0 fermentations. Strains were pitched at 5.0 g fresh yeast per litre into 15 °P wort, and fermentations were carried out at 15 °C. Values are averages of quadruplicate measurements, and error bars show the ranges when these are greater than the size of the markers.

initial accumulation, the changes in glycogen contents followed a similar pattern until the end of fermentations. Accumulation and dissimilation of glycerol was not significantly altered in A218 with respect to A15. Extracellular glycerol was not measured.

Concentrations of some flavour compounds were altered

The VDKs diacetyl and 2,3-pentanedione were analysed both in the green beer before maturation and in the conditioned, bottled beer (Table 3). Other flavour compounds were analysed only in the green beer (Table 4). Amounts of acetaldehyde, alcohols and esters that contribute to beer flavour were similar in beers made by A15 and A218 except that 3-methylbutyl acetate (giving a pear/banana flavour) and 2-phenylethyl acetate (rose flavour) were about 45% higher in the A218 beer, whereas ethyl decanoate (fruity, apple flavour) was about 40% lower. Ethyl hexanoate (apple like) and ethyl octanoate (fruity, wine like) and 3-methylbutyl acetate (banana) were above their taste thresholds in undiluted green beers, but below after dilution to sales strength (4.5% ABV; Table 4). VDK levels (almost threefold higher in the variant green beer) decreased during maturation (Table 3), but diacetyl in bottled A218 beer was still sufficiently high to be noticed by some tasters. Maybe because of this, a statistically significant (*P*-value 0.013) sensorial difference between the beers was noticed in the triangle test. However, when panellists were asked to indicate which beer had more off-flavour or aroma, the difference between beers was not statistically significant.

Gene expression analysis revealed differences between strains during early fermentation

Yeasts were sampled for gene expression analysis immediately before pitching and 24 h after pitching. Differences

Table 4. Concentrations of flavour-active compounds (mg L^{-1}) and their reported taste thresholds (Meilgaard, 1975) in green beers made with A15 and A218 strains. Values are adjusted to a sales strength of 4.5% alcohol by volume

	A15	A218	Threshold
Acetaldehyde	1.1	0.68	25
1-Propanol	2.5	3.3	800
2-Methylpropanol	2.6	2.4	200
3-Methylbutanol	12	14	70
2-Methylbutanol	5.4	5.3	65
2-Phenylethylalcohol	0.25	0.27	125
3-Methylbutylacetate	0.62	0.93	1.2
Ethyl acetate	14	15	30
Ethyl hexanoate	0.12	0.15	0.21
Ethyl octanoate	0.90	0.83	0.9
2-Phenylethylacetate	0.07	0.10	3.8
Ethyldecanoate	0.14	0.08	1.5

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in fermentation were not observed until after 24 h, so that at 24-h the yeast strains still had similar environments and differences between their gene expression patterns are expected primarily to reflect genetic differences between the strains. TRAC analyses were made using probes that recognize 21 genes chosen because they are involved in sugar uptake (believed to limit fermentation rate), accumulation of trehalose, glycogen and glycerol or certain stress responses. Expression of hexose transport genes was generally over twofold greater in A15 than in A218 24 h after pitching (Table 5), despite the presence of residual glucose in both worts at this time (data not shown). Lower transcript levels in the A218 samples were also found for the α-glucoside transporter genes Seub*AGT1* and *MPH2/3* (negligible transcription of the nonfunctional Sc*AGT1* gene was observed but is not reported here). The α-glucoside transporter genes *MALx1* (both orthologues) also showed greater transcript levels in A218. The probe for Sc*MALx1* recognizes mRNA of a number of genes including *ScMAL11, ScMAL21, ScMAL31, ScMAL41, ScMAL61*, while the Seub*MALx1* probe binds to the single *S. eubayanus*-derived *MALx1* ORF present in the *S. pastorianus* genome. The A218 strain also showed greater transcript levels for the α-glucosidase genes *MALx2*, particularly with the *S. cerevisiae* form of the gene (Sc*MALX2*). Both *MALx1* orthologues and both *MALx2* orthologues were strongly induced (12- to 55-fold) between 0 and 24 h, always more strongly in A218.

Nonspecific *NTH1* probe signals were similar in the two strains and showed little change between 0 and 24 h. Transcription levels of the trehalose synthase genes Sc*TPS1* and Seub*TPS1* at 0 h were lower in A15 than in the variant, but then more strongly induced so that by 24 h the levels were similar in the two strains.

Differential activity of the genes responsible for the production of glycerol (NAD-dependent glycerol 3-phosphate dehydrogenase) was observed (Table 5). Activity was found to be both homologue- and orthologue-specific. Both *GPD1* orthologues (essential for osmoadaptation; Ansell *et al.*, 1997) were strongly induced between 0 and 24 h, whereas the *GPD2* orthologues (involved in redox regulation; Ansell *et al.*, 1997) were not. The *S. eubayanus* form of *GPD1* showed relatively greater transcription in the A218 strain than in A15. Transcription of the *S. cerevisiae* form of this gene was not different in the two strains. Conversely, the *S. cerevisiae* form of the *GPD2* gene showed fourfold greater transcript levels in A15 than in the variant while the *S. eubayanus* form of the gene showed a twofold decrease in transcription. These very different orthologue-specific responses were not evident when a nonspecific *GPD2* probe was used to measure transcription.

While absolute signal levels for both orthologues of *GSY2* (the gene responsible for most glycogen synthesis) were similar at 24 h, the induction following pitching was greater for A15 (2.4–2.8 fold) than for the variant strain (no change), which also accumulated less glycogen (Fig. 4). Transcription of two genes (Sc*GPH1* and Sc*GDB1*) involved in dissimilation of glycogen was, however, greater in A15.

With respect to stress response genes, where differences were observed between the two strains, the variant strain had the lower transcript levels, with the exceptions of Sc*MSN2* and Seub*PDR5*. Higher levels in A15 were found for disparate groups of genes including *HSP*, *MSN* and *PDR* genes (Table 5).

Discussion

Our aim was to test whether brewer's yeast variants exhibiting faster growth under hyperosmotic stress would also ferment brewer's wort faster and more completely. Repetitive culturing of lager yeast in the presence of 210 g sorbitol per litre proved to be an efficient method to enrich the yeast population with cells (potential variants) that were quickly able to start growing at high osmotic strength. For untreated A15, most cells formed visible colonies on sorbitol-supplemented agar after 4 days, and this time was decreased to 2 days for the population after 27 enrichment cycles. For A197, a smaller change was observed, from 4 to 3 days after 33 enrichment cycles. This enrichment method was successful when applied to a mutagenized population (EMS-treated A15) or a nonmutagenized population (A197).

Most (80%) of the tested variants that showed rapid growth on 210 g sorbitol per litre also fermented 15 °P wort faster than the original strains. Thus, enrichment selection for respirofermentative growth at high osmotic strength led efficiently to variants showing faster fermentation of brewer's wort. Variant A219 derived from A15 was faster than A15 in mid-fermentation, but showed little or no overall improvement. However, variant A218 derived from A197 (the ethanol-tolerant derivative of A15) was faster in mid-fermentation than both A197 and A15 and also showed a large overall improvement, reaching a greater final attenuation than the original strains and reaching it 2 days (30%) earlier (Fig. 3). It is notable that although the ethanol-tolerant variant (A197) showed little or no improvement in 15 °P wort compared with its parent strain, A15 (Huuskonen *et al.*, 2010), marked improvements in overall fermentation speed and extent were obtained when the (unknown) mutation(s) in A197 were combined with those leading to improved growth in the presence of high sorbitol. The dominant stresses change during fermentation of high-gravity wort. At the

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Table 5. Gene transcription as determined by TRAC. Yeast samples were taken just prior to pitching and 24 h after pitching. Columns 2 and 3 show TRAC signals at 24 h in fluorescence (FLU) units. Values are means of duplicate assays ± half the range of the mean. Columns 4 and 5 show the ratio of 24-h signals to 0-h signals. Oligonucleotide probes were designed for specific gene orthologues within the hybrid *Saccharomyces pastorianus* (*Saccharomyces cerevisiae* vs. *Saccharomyces eubayanus*) genome. Specificity was tested using an ale strain of *S. cerevisiae* (Sc) and the type strain of *S. eubayanus* (Seub). Specificity is denoted in parentheses following the gene names. Nonspecific probes (that recognize both Sc and Seub orthologues) are not followed by parentheses. ND denotes not determined and occurred when 0 h values were too low to detect using the TRAC method

	FLU units		24 h : 0 h ratio	
Gene	A15	A218	A15	A218
Hexose transport				
HXT2 (Sc)	1331 ± 197	615 ± 248	ND	10.2
HXT2	6299 ± 63	2206 ± 158	6.3	2.6
HXT3 (Sc)	13 187 ± 582	4759 ± 75	4.8	1.5
HXT4 (Sc)	2016 ± 94	129 ± 26	ND	ND
HXT4 (Seub)	813 ± 0	823 ± 85	ND	ND
HXT5	1163 ± 212	755 ± 247	0.3	0.4
α-Glucoside metabolism				
AGT1 (Seub)	15 972 ± 19	7606 ± 776	1.5	0.7
MPH2/3 (Sc)	31 750 ± 293	11 346 ± 782	4.4	1.0
MALx1 (Sc)	89 103 ± 582	138 439 ± 5535	12.0	19.6
MALx1 (Seub)	11 206 ± 303	21 409 ± 468	7.3	17.5
MALx2 (Sc)	145 856 ± 212	251 127 ± 20 466	40.2	54.9
MALx2 (Seub)	24 997 ± 237	30 315 ± 246	46.6	49.2
Trehalose metabolism				
TPS1 (Sc)	12 282 ± 1208	13 395 ± 292	2.0	1.6
TPS1 (Seub)	11 151 ± 346	9856 ± 530	2.9	1.6
ATH1	4996 ± 139	5211 ± 88	0.8	1.1
ATH1 (Seub)	1272 ± 271	1059 ± 57	0.3	0.3
NTH1 (Sc)	75 156 ± 15 723	25 633 ± 440	2.7	1.3
NTH1 (Seub)	2269 ± 329	6850 ± 1372	0.6	2.7
NTH1	6005 ± 187	5180 ± 168	1.1	1.0
Glycerol synthesis				
GPD1 (Sc)	22 675 ± 1295	21 002 ± 1116	5.8	5.3
GPD1 (Seub)	15 831 ± 1059	23 540 ± 888	4.2	11.1
GPD2 (Sc)	1927 ± 351	504 ± 61	2.0	1.0
GPD2 (Seub)	796 ± 0	1316 ± 89	1.2	ND
GPD2	17 866 ± 246	17 973 ± 2433	1.2	1.5
Glycogen metabolism				
GSY2 (Sc)	6491 ± 448	6424 ± 262	2.8	1.1
GSY2 (Seub)	14 375 ± 403	12 310 ± 571	2.4	1.0
GPH1 (Sc)	17 336 ± 409	11 369 ± 353	11.2	4.0
GDB1 (Sc)	2810 ± 407	1381 ± 108	0.8	0.3
Stress response genes				
HSP104 (Sc)	5123 ± 680	4674 ± 137	2.4	1.5
HSP104 (Seub)	6971 ± 59	5593 ± 432	3.3	1.6
MSN2 (Sc)	914 ± 0	1667 ± 26	1.6	ND
MSN2 (Seub)	1295 ± 86	1027 ± 3	0.9	0.9
MSN2	5211 ± 320	4126 ± 83	2.2	2.3
MSN4 (Sc)	4890 ± 284	3197 ± 86	4.1	4.0
MSN4 (Seub)	4418 ± 1130	3923 ± 164	3.6	2.7
PDR5 (Sc)	14 440 ± 1048	14 689 ± 387	9.0	12.7
PDR5 (Seub)	1814 ± 68	2924 ± 281	ND	ND
PDR5	6603 ± 182	5960 ± 13	3.5	3.8
PDR10 (Sc)	3947 ± 62	3573 ± 92	1.0	1.4
PDR10 (Seub)	3184 ± 355	1519 ± 159	2.2	1.4

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start, sugar concentrations (> 90 g L^{-1}) and osmotic strength (> 0.3 M) are high, whereas at the end sugar concentrations and osmotic strength are low, but ethanol and CO_2 concentrations are high and oxygen and other nutrients are depleted. Thus, effective strain development to improve the performance of a brewer's yeast needs to select for more than one new character.

The stronger flocculation observed in the faster-fermenting strains A219 and A218 can be an additional industrial advantage, because it facilitates cropping of the yeast and downstream processing of the green beer. Variant A218 was genetically stable through more than 100 generations without selection pressure. It was recovered from wort fermentations with a viability of > 95%. It appears to be suitable for industrial applications although the effect of repeated repitching (beyond two cycles) on viability has not yet been studied.

We do not know the nature of the mutation(s) in variants A218 and A219. Glycerol accumulation is a characteristic response of yeast to hyperosmotic conditions (Attfield & Kletsas, 2000) and its prevention leads to osmosensitive strains (Albertyn *et al.*, 1994; Siderius *et al.*, 2000). However, the parent strain A15 and variant A218 exhibited very similar glycerol accumulations, with sharp peaks immediately after pitching. Thus, the faster fermentation by variant A218 was not caused by a change in glycerol content. In contrast, A218 did not exhibit the large accumulation of intracellular trehalose shown by A15 during the second day of fermentation (Fig. 4). This early accumulation of trehalose is typical during brewing fermentations (Pratt *et al.*, 2007) and is more pronounced in higher gravity worts (Majara *et al.*, 1996; Reinman & Londesborough, 2000), suggesting it may be related to hyperosmotic stress. The variant A218 also accumulated less glycogen than did A15. It is tempting to speculate that the low intracellular levels of especially trehalose (and perhaps also glycogen) between 24 h and the end of the fermentation facilitated the higher fermentation rate of the variant compared with A15.

Trehalose has a well-documented role in protecting yeast cells against many stresses (Gadd *et al.*, 1987; Nwaka *et al.*, 1994; Sharma, 1997; Silljé *et al.*, 1999; Benaroudj *et al.*, 2001; Kandror *et al.*, 2004) including high salinity (Siderius *et al.*, 2000) and hyperosmotic conditions that prevent growth (Hounsa *et al.*, 1998). Its protective role is attributed, at least in part, to its ability to stabilize cell membranes and proteins by substituting for water molecules on the surface of macromolecules (Mansure *et al.*, 1994; Singer & Lindquist, 1998; Sola-Penna & Meyer-Fernandes, 1998). However, it is also known that intracellular trehalose is immediately degraded when starved yeast encounters fermentable sugar (van der Plaat, 1974), including when yeast is pitched into brewery wort (van Dijck *et al.*, 1995; Reinman & Londesborough, 2000; Blieck *et al.*, 2007). Conceivably, high trehalose levels retard metabolic activity, especially when adaptation to a dynamic environment requires rapid changes in proteins and membranes. There is evidence suggesting that a strong stress response in general and trehalose accumulation in particular may inhibit fermentation. Lager and wine yeasts exhibited weak stress responses during fermentation (Brosnan *et al.*, 2000; Carrasco *et al.*, 2001). Lager yeast variants with faster fermentation of very high-gravity worts accumulated less trehalose and glycogen than their parent (Blieck *et al.*, 2007). Strongly fermenting sake yeast strains exhibited a muted stress response and disruption of the *MSN2*/*MSN4* genes that trigger the general stress response (including trehalose accumulation; Martinez-Pastor *et al.*, 1996) accelerated fermentation (Watanabe *et al.*, 2011).

TRAC analyses showed some differences between A15 and the variant A218 in the expression of selected genes at 24 h after pitching. This was before environmental conditions began to diverge, so that the observed differences may be strain dependent rather than consequences of the different fermentation speeds. Strain-dependent differences in gene expression might be causative of the observed differences in fermentation speed. The transport of α-glucosides into yeast is believed to limit the speed of wort fermentations (Kodama *et al.*, 1995; Rautio & Londesborough, 2003; Alves *et al.*, 2007). Probes for Sc*MALx1* (which recognizes the almost identical genes, Sc*MAL11*, Sc*MAL21*, Sc*MAL31* and Sc*MAL41*) and Seub*MALx1* showed increased transcription of these α-glucoside transporter genes in the variant compared with A15. ScMalx1 transporters cannot carry maltotriose efficiently (Han *et al.*, 1995; Salema-Oom *et al.*, 2005; Alves *et al.*, 2008). The Seub*MALx1* probe recognizes mRNA of the Seub*MALx1* gene, which is present as a single copy in the lager genome (Nakao *et al.*, 2009). The properties of the SeubMalx1 transporter have not been reported. If it can carry maltotriose, its increased expression in the A218 variant might contribute directly to the faster utilization of maltotriose by the variant. Faster utilization of maltose may also lead, indirectly, to enhanced consumption of maltotriose in later fermentation, by decreasing competition between maltose and maltotriose for transporters such as SeubAgt1 and Mtt1 that can carry both (We did not yet design a probe for *MTT1* that met our specificity criteria). The lower expression of HXT genes in the variant might also indirectly promote α-glucoside transport, because smaller amounts of Hxt transporters (carrying glucose and fructose) in the plasma membrane would mean that a greater area of the plasma membrane is available for other membrane proteins, including α-glucoside transporters. The capacity of yeast to express

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functional transporters in the plasma membrane is limited (Opekarová *et al.*, 1993; van der Rest *et al.*, 1995).

The present gene expression data do not explain the lower levels of trehalose and glycogen in variant A218 compared with the parent A15 strain. Trehalose and glycogen metabolisms are largely controlled at post-translational levels, for example, by protein kinase A (PKA)-catalysed phosphorylation of enzymes in these pathways (François & Parrou, 2001). There was no difference in the apparent PKA activity (as measured by the SignaTECT® cAMP-dependent protein kinase assay system) of A218 and A15 at 24 h after pitching (B. Gibson, unpublished data). Measured stress response genes were expressed at similar levels in the two yeasts, except that Sc*MSN4* and Seub*PDR10* were 40–100% higher in A15, whereas Sc*MSN2* and Seub*PDR5* were 60–80% higher in A218. A negative correlation between *MSN* gene function and fermentation performance was observed by Watanabe *et al.* (2011), but the probable effect of simultaneous decrease in Sc*MSN4* and increase in Sc*MSN2* is not clear.

Previous work has also shown that some orthologous genes in *S. pastorianus* are differentially expressed during fermentation (Rautio *et al.*, 2007; Horinouchi *et al.*, 2010). Different orthologues may have distinct functions. The *S. eubayanus* form of the *KEX2* gene (encoding kexin protease), but not the *S. cerevisiae* form, has a role in supporting growth of *S. pastorianus* at low temperature (Yamagishi *et al.*, 2010). The *S. cerevisiae* form specifically of *ILV6* is associated with diacetyl production by *S. pastorianus* (Duong *et al.* (2011). In the current study, from 15 studied pairs of orthologues, 7 (*HXT4*, *NTH1*, *GPD1*, *GPD2*, *MSN2*, *PDR5* and *PDR10*) showed different relative expression levels of the *S. cerevisiae* and *S. eubayanus* forms in the A15 and A218 strains (Table 5). A more complete understanding of the specific functions of the different orthologues is needed, for example, to facilitate targeted development of brewing yeast strains (Yamagishi *et al.*, 2010; Duong *et al.*, 2011).

The levels of most yeast-derived flavour compounds were not changed to such an extent that organoleptic properties of beer would be influenced. However, VDKs were 40% higher in bottled beer from A218. In particular, final diacetyl was above the taste threshold (0.05 mg L^{-1}; Mawer & Martin, 1978). This is disadvantageous because diacetyl imparts a butterscotch flavour not desired in lager beers. The higher levels probably result from the shortened fermentation time, which gives less opportunity for the yeast to reduce VDKs (Verbelen *et al.*, 2008, 2009). VDK levels might be reduced by appropriate process changes, for example maturation at higher temperature than in the present work. Foam stability, which is also important to consumers (Bamforth, 2000), was slightly decreased in the beer made with strain A218.

Our selection procedure generated large numbers of cells that grew rapidly at high sorbitol levels. So far we have examined only two pure isolates (A218 and A219). Other variants may have different mutations, and their fermentation characteristics should be examined. Blieck *et al.* (2007) and Huuskonen *et al.* (2010) showed the potential of adaptive evolution to improve fermentations of very high-gravity worts. The dual osmotolerant–ethanol-tolerant variant A218 described here may also be advantageous with very high-gravity worts, which offer substantial cost-savings to the industry (Casey *et al.*, 1984; Blieck *et al.*, 2007; Huuskonen *et al.*, 2010).

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