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RESEARCH ARTICLE

Lager yeasts possess dynamic genomes that undergo rearrangements and gene amplification in response to stress

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Abstract A long-term goal of the brewing industry is to identify yeast strains with increased tolerance to the stresses experienced during the brewing process. We have characterised the genomes of a number of stress-tolerant mutants, derived from the lager yeast strain CMBS-33, that were selected for tolerance to high temperatures and to growth in high specific gravity wort. Our results indicate that the heat-tolerant strains have undergone a number of gross chromosomal rearrangements when compared to the parental strain. To determine if such rearrangements can spontaneously arise in response to exposure to stress conditions experienced during the brewing process, we examined the chromosome integrity of both the stress-tolerant strains and their parent during a single round of fermentation under a variety of environmental stresses. Our results show that the lager yeast genome shows tremendous plasticity during fermentation, especially when fermentations are carried out in high specific gravity wort and at higher than normal temperatures. Many localised regions of gene amplification were observed especially at the telomeres and at the rRNA gene locus on chromosome XII, and general chromosomal instability was evident. However, gross chromosomal rearrangements were not detected, indicating that continued selection in the stress conditions are required to obtain clonal isolates with stable rearrangements. Taken together, the data suggest that lager yeasts display a high degree of genomic plasticity and undergo genomic changes in response to environmental stress.

Keywords Competitive genomic hybridisations · Gross chromosomal rearrangements · Environmental stress · Adaptive evolution

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Introduction

The model of the genome in organisms ranging from bacteria and yeast to human cancer cells, as a stable entity is constantly being challenged and currently evolving to one in which genomes are plastic, capable of responding to environmental stresses (reviewed in Galhardo et al. 2007). Such responses may lead to genomic instability, generating fitter mutants and potentially accelerating adaptive evolution. The yeast strains used in industrial processes such as beer and wine production are under constant environmental stress and therefore must be at the forefront of this adaptive evolution process. Lager yeasts (*Saccharomyces pastorianus*; Rainieri et al. 2006), have complex genomes arising from the fusion of two *Saccharomyces sensu stricto* yeast species, one of which is very closely related to *S. cerevisiae* and the other showing similarity to *S. bayanus* (Grauslund et al. 1995; Rainieri et al. 2006). The resulting hybrid genome appears to have undergone duplication without cell

division leading to an allotetraploid strain, which subsequently underwent selective genomic deletions and rearrangements. The present day lager yeasts are aneuploid possessing unequal numbers of both *S. cerevisiae*-like and *S. bayanus*-like chromosomes (Liti and Louis 2005). Furthermore, recombination has occurred between the homeologous chromosomes generating a number of mosaic or hybrid chromosomes consisting of both *S. cerevisiae* and *S. bayanus* genes (Bond and Blomberg 2006; Bond et al. 2004; Kodama et al. 2005; Nilsson-Tillgren et al. 1981). Previously, we have used competitive genomic hybridisations (CGH) to DNA microarrays to estimate the copy number of *S. cerevisiae*-like genes in two lager yeast strains (Bond et al. 2004) and to examine the mosaic nature of the lager yeast genome. This analysis identified the chromosome locations where genomic rearrangements have occurred in the two strains to generate the mosaic chromosomes, thus identifying potential recombination hot spots between homeologous chromosomes.

Under fermentation conditions, brewery yeasts are exposed to numerous environmental stresses, which affect their vitality and viability. These include, but are not limited to, high osmotic and hydrostatic pressure, high alcohol concentrations, anaerobiosis and temperature fluctuations. When subjected to conditions that are less than physiologically ideal such as those experienced during fermentation, yeasts exhibit a complex array of stress responses (Attfield 1997; Carrasco et al. 2001) such as alterations in transcription and translation, accumulation of stress-protectants and activation of repair functions (Attfield 1997; Brosnan et al. 2000; Zuzuarregui et al. 2005). The efficiency of these processes in a given yeast strain determines its robustness, and whether it is able to perform to the necessary commercial standards in industrial brewing. This is particularly important when part or all of the spent yeast from one fermentation cycle is reused ("re-pitched") in a subsequent fermentation. It has been demonstrated that lager yeasts show reduced viability when they are sequentially re-pitched and especially when using high specific gravity (HSG) wort (Blieck et al. 2007).

The hybrid allotetraploid nature of the lager yeast genome in part appears to confer a degree of tolerance to the stresses experienced during the brewing process as haploid strains of *S. cerevisiae* or *S. bayanus* do not ferment or survive as well as the lager yeasts under brewing conditions. Allopolyploidy is well-known in higher plants and more complex than in the lager yeasts in many cases. For example the wheat hexaploid genome originated from three diploid ancestral species: *Triticum urartu, Aegilops speltoides* and *Aegilops tauschii* (Feldman and Levy 2005). Allopolyploidization leads to the generation of duplicated homoeologous genes (homoeologs), as opposed to paralogous genes (paralogs). The homoeologous genes in polyploids may undergo functional diversification, gene silencing, or retain a similar function (Shitsukawa et al. 2007). Thus it could be argued that the allopolyploid genomes are in an evolutionary flux with a higher rate of viable evolutionary changes than their diploid or haploid counter-parts. Extensive and variable aneuploidy is found in genomes of different lager yeast isolates suggestive of such ongoing changes to their genomes. The brewery yeast strains may have taken advantage of this feature, thereby establishing themselves as a very successful eukaryote group since pre-historic times (Casaregola et al. 2001).

The identification of yeast strains with increased tolerance to brewery stresses is of commercial importance to the brewing industry. We have attempted to select for stress-tolerant lager yeast strains by using a combination of EMS mutagenesis and selection for tolerance to exposure to high temperature and growth in high specific gravity (HGS) wort. In the course of this study, data, both published (Bond et al. 2004; Kodama et al. 2005) and unpublished, had indicated to us that the genomes of different strains of the lager yeasts have distinctly different gene copy numbers of the *S. cerevisiae*-like complement. These findings prompted us to examine the genomes of a selected number of the stress-tolerant strains recovered in our screen. Our studies using CGH show that these stress-tolerant strains have undergone a number of gross chromosomal rearrangements (GCRs). To determine if such GCRs can spontaneously arise when cells are exposed to stress conditions such as fermentation in HSG-wort or at higher than normal temperatures, the genomes of the parental strain and a number of the stress-tolerant strains were compared at the beginning and end of an 8-day fermentation. Our results show that the lager yeast genome shows tremendous plasticity during fermentation, especially when fermentations are carried out in HSG-wort and at slightly higher temperatures. Many localized gene amplifications and general chromosomal instability were evident. However, we did not detect any GCRs in the yeast genome when analysed at the end of a single round of fermentation in standard wort.

Materials and methods

Strains

The bottom fermenting lager yeast strain CMBS-33 was from the Centre for Malting and Brewing Science (Leuven, Belgium) Lager Strain Collection (kindly provided by Dr. K. Verstrepen).

Ethyl methanesulfonate (EMS) mutagenesis of CMBS-33

The EMS mutagenesis scheme was adapted from the Hahn Laboratory (http://www.fhcrc.org/labs/hahn/methods/

genetic_meth/ems_mutagenesis.html). Briefly, a single colony culture of CMBS-33 cells was grown overnight in YEPM (1% yeast extract, 2% Bactopeptone, 2% maltose) at room temperature. Approximately, 1×10^8 cells were harvested and washed once in sterile water and then twice with 0.1 M sodium phosphate buffer (PB) and finally resuspended in 1.7 mL PB. Either 50 or 100 µL of an EMS stock solution (9.4 M; Sigma Chemical Co.) was added to the cell suspension and incubated for 60, 100 or 120 min. Following mutagenesis, the cells were washed extensively in PB and allowed to recover in YEPM for 4 h at room temperature. The percentage survival rates were 60 ± 10% for the 120-min sample. The cells were pooled from the three time points and pelleted. Approximately, 1×10^7 cells were resuspended in 3 mL of prewarmed (either 45, 50 or 55°C) YEPM medium and incubated for 10 min at the corresponding temperature. The cells were diluted to 30 mL in room temperature YEPM medium and allowed to recover at room temperature for 2 h and then seeded onto HSG-wort (specific gravity 1.14 g/mL) agar (2%) plates. Approximately, 200 colonies were recovered from the EMS-treated, 55°C heat-stressed CMBS-33 yeast samples. The yeasts subjected to the lower temperature heat stress showed a lawn of growth under identical plating conditions and were not pursued. Individual yeast colonies from the 55°C heat treatment were picked into 2 × 96 well plates in 300 µL YEPM and grown overnight. Replicates of the plates were made and the master plates were stored frozen at –70°C after adding glycerol to 20%. The 55°C-tolerant colonies were propagated in YEPM at room temperature. Approximately, 1×10^7 cells from each colony were subjected to a second round of heat treatment as before and then seeded onto YEPM agar plates. Twenty-nine of the original 200 selected colonies survived this step. The surviving colonies were re-grown and the process was repeated for a third time. Only 6 of these 29 colonies showed repeated survival through the 55°C treatment. Three of these six clones were used for further analysis.

Fermentations

Wort 40% (w/v) was prepared from dried spray malt (Amber 18EBC; SprayMalt Brewferm; Brouland, Beverlo, Belgium) and kept in a steamer/boiling water bath for 4 h. To estimate the specific gravity of this wort, an aliquot was diluted 1:1 with sterile water, the insoluble materials were removed by centrifugation and the °Brix value for the solution was measured using a pre-calibrated refractometer (Eclipse; Bellingham+Stanley, UK). The wort concentrations are expressed here as °Plato (°P). In general terms, °Brix, °Plato and °Balling scales express the weight percentage of sucrose solutions and relate this weight percentage to specific gravity and are interchangeable. A °Brix value of 1° is approximately 1 g of sucrose dissolved in 100 g of water. Specific gravity is calculated from the °Brix value using the formula SG = 1 + (°Brix/(258.6 – ((°Brix/258.2) × 227.1))). Alternatively, clarified wort (50 µL) is weighed at room temperature in graduated micro-capillaries and the specific gravity is calculated as gram weight/mL. Both methods yielded identical values for specific gravity. Fermentations were carried out in 200 mL cylinders or 2 L cylindroconical vessels at 13 or 22°C as indicated in the figure legends. Wort was supplemented with zinc sulfate (1 mM final concentration). Prior to the addition of yeast (pitching), the wort was aerated with 3 × 15 sec pulses of compressed air (5 psi) bubbling from the bottom of the vessels. The dissolved air content in this wort was approximately 6–8 mg/L. Fermentations were pitched at cell densities of 1–1.5 × 10^7 viable lager yeast cells/mL. The wort was overlaid with mineral oil unless otherwise stated. For all fermentations, the parent and the mutant yeast strains were propagated from a single colony in 500 mL aliquots of YEPM in 2-L flasks with mild aeration at room temperature (20°C) to a cell concentration of $<5 \times 10^7$ mL^{-1}. The required amount of yeast was harvested and resuspended in 20–50 mL wort of the desired concentration and introduced into the vessel through a pre-installed anaerobic plumbing system. Aliquots (10–20 mL) of the wort were removed anaerobically before and at defined times during the 8-day fermentation cycle, to recover the yeast for analysis and to determine the specific gravity of the wort.

DNA labeling and competitive genome hybridisation (CGH)

Genomic DNA was extracted from yeast cell pellets that were resuspended in a buffer containing 4 M guanidinium thiocynate, 1% sorbitol and 100 mM Tris–HCl; pH 8.0; as previously described (Kaiser et al. 1994). Genomic DNA (300 ng) was labeled as previously described (Bond et al. 2004). DNA labeling, microarray fabrication, hybridisation and data collection were performed at the Microarray Facility, Genomics Shared Resources at Fred Hutchinson Cancer Research Institute, Seattle, WA, USA. Genomic DNA from each of the competing DNA pairs was labeled in "random-priming" reactions using either Cy3- or Cy5-tagged nucleotides. Briefly, 10 µL of 30 ng/µL DNA was added to 20 µL of random primer mix (BioPrime Kit; Invitrogen, CA, USA), denatured at 100°C for 10 min and quickly chilled on ice. Labeling reagents (5 µl 10 × dNTP, 11 µl sterile water, 3 µl Cy3-dUTP or Cy5-dUTP, 1 µl Klenow fragment DNA Polymerase I (40–50 U/µl) were added to the denatured DNA-random primer mix. Following overnight incubation at 37°C, the two reactions were pooled and diluted tenfold in 10 mM Tris–HCl, pH 8.0 and 1 mM

EDTA (TE) buffer. The unincorporated nucleotides were removed using spin columns (MicroCon YM30; Millipore Inc., MA, USA) at 10,000*g* for 8 min. The concentrate was washed once in the same device with 400 μL TE buffer containing 100 μg yeast tRNA until the final volume was reduced to approximately 15 μL. The labeled DNA was mixed with 20 × SSC (3 M NaCl, 0.3 M sodium citrate; pH 7.0) to give a final concentration of 1.5× and filter sterilized (0.45 μm) and stored frozen in the dark until use.

Yeast ORF "spotted" microarrays (GPL1914) were constructed employing a set of 6,229 ORF-specific PCR primer pairs (Research Genetics, Huntsville, AL, USA) and over 90 positive and negative control sequences. Individual PCR products were mechanically "spotted" onto glass microscope slides using robotics. The slides were quality checked through low-resolution scanning and blocked with 3% non-fat dry milk powder in 1× SSC for 1 h. Sodium dodecyl sulphate (SDS; 20%) was added to the probe mix to give a final concentration of 0.2%. The sample was heat denatured at 100°C for 2 min, centrifuged for 8 min at 10,000*g* and the supernatant was applied to the hybridization chamber, which already contained 10 μL of 3× SSC. The sealed chamber was hybridized for 16 h at 63°C submerged in a water bath. The slides were washed in the dark sequentially with 1× SSC, 0.1% SDS for 2 min, then multiple washes in 1× SSC without SDS and finally in two washes in 0.2× SSC. The slides were never allowed to dry during the process until just before the scanning when they were dried by centrifugation and scanned on a GenePix 4000B dual-laser scanner (Molecular Devices; http://www.moleculardevices.com/pages/instruments/gn_genepix4000.html). A Microsoft Access database was collated from various GenePix data files and a sub-set of the error-free common data-points across a number of separate experiments. All arrays were normalised online using VARAN software (http://www.bionet.espci.fr) and analysed using either the TIGR-Mev software (http://www.tm4.org/mev.html) or BRB ArrayTools developed by Drs. Richard Simon and Amy Peng Lam (http://www.lincus.nci.nih.gov/BRB-ArrayTools.html).

Results

Isolation of stress-tolerant strains of the lager yeast CMBS-33

To isolate strains with increased stress-tolerance, we used a strategy based on cross-tolerance, whereby cells that have transiently acquired tolerance to one form of stress, such as survival at a higher temperature (heat-shock), can be tolerant to other unrelated stresses, such as growth in higher ethanol concentrations (Attfield 1997; Bond 1988; Sanchez et al. 1992). As a first attempt, we used EMS to mutagenise the lager yeast strain CMBS-33 with the view to introduce random point mutations and selecting mutants that are capable of growth on HSG-wort media. However, the vast number of survivors from this step necessitated the performance of a more stringent selection. Thus, all the survivors from the EMS mutagenesis were subjected en mass to a short heat-shock regime as outlined in the "Materials and methods" section. The survivors were plated on high specific gravity wort, agar (specific gravity 1.14 g/mL; 35°P). To ensure that the selected mutants displayed permanent tolerance to heat stress, the surviving colonies were subjected to successive rounds of exposure to high temperatures. From the initial pool of cells heat-shocked at 55°C, only six clones survived three successive rounds of exposure to 55°C. Three of these clones, designated as C5, C6 and C10, were chosen for subsequent analysis.

Stress-tolerant mutants show gross genomic alterations compared to the parent

The karyotype analysis of a number of individual colonies from each of the selected mutants by clamped homogeneous electrical field (CHEF) electrophoresis showed some electrophoretic differences between the parent and the mutant strains and between the mutants themselves (data not shown). The chromosome banding patterns were identical for each of the colonies originating from a specific mutant, indicating the clonal nature of the three stress-tolerant strains (data not shown). However, due to the complex nature of the allotetraploid lager yeast genome and the insufficient resolution of the technique, it was difficult to assign any noted changes to specific chromosomes. Therefore, we used CGH to analyse the genome composition of the mutant strains by direct comparison to that of the parent. We have previously shown (Bond et al. 2004) that CGH can be a very efficient technique for genome-wide mapping of the recombinations that occurred between the homeologous parental chromosomes of lager yeasts that resulted in the formation of the mosaic chromosomes (Casey 1986; Nilsson-Tillgren et al. 1981). Furthermore, we showed that the ratio of hybridisation (ROH), deduced from hybridisation of the competing fluorescent tags Cy5/Cy3 to the DNA microarrays, directly correlates with the *S. cerevisiae* gene copy number in the lager yeasts as the *S. bayanus* gene complement hybridises weakly, if not at all, under the conditions used (Bond et al. 2004). Thus, changes in the ROH for a given ORF reflect changes in the *S. cerevisiae* complement relative to that of *S. bayanus* in the genome and therefore can be used as the guide to detect specific chromosomal changes.

Using this strategy, we compared the genomes of each of the three mutants C5, C6 and C10 to their parent CMBS-33.

In this case, CGH was performed using genomic DNA from the mutants and the parent, grown up from single colony in YEPM, and differentially labeled with Cy5 and Cy3, respectively. The labeled DNA was hybridised to a *S. cerevisiae* microarrays "Materials and methods". If the two competing DNA samples are identical, a mean ROH of 1.0 is expected while any deviations from this value indicates possible loss or gain of the reactants. To identify changes in chromosome composition, the normalised ROH ($\log_2$) for each ORF was plotted against its location on the chromosome, using the gene order for *S. cerevisiae*. The mutants C5 and C6 displayed distinct changes in ROH on eight different chromosomes (Fig. 1, Table 1), while the ROH values for ORFs in the mutant C10 remained relatively unchanged compared to the parental strain. Interestingly, many of the observed changes in ROH values mapped to previously identified recombination hot spots between the *S. cerevisiae* and *S. bayanus* chromosomes in the lager yeasts (Bond et al. 2004). However, C5 and C6 mutants have acquired two novel recombination sites in the regions of YJL053W on chromosome 10 and YKL165C on chromosome 11 (Fig. 1). The mutants C5 and C6 showed nearly identical ROH patterns although a few notable changes were identified, such as a difference in the ROH values for chromosome V, which distinguishes them as individual mutants (Fig. 1).

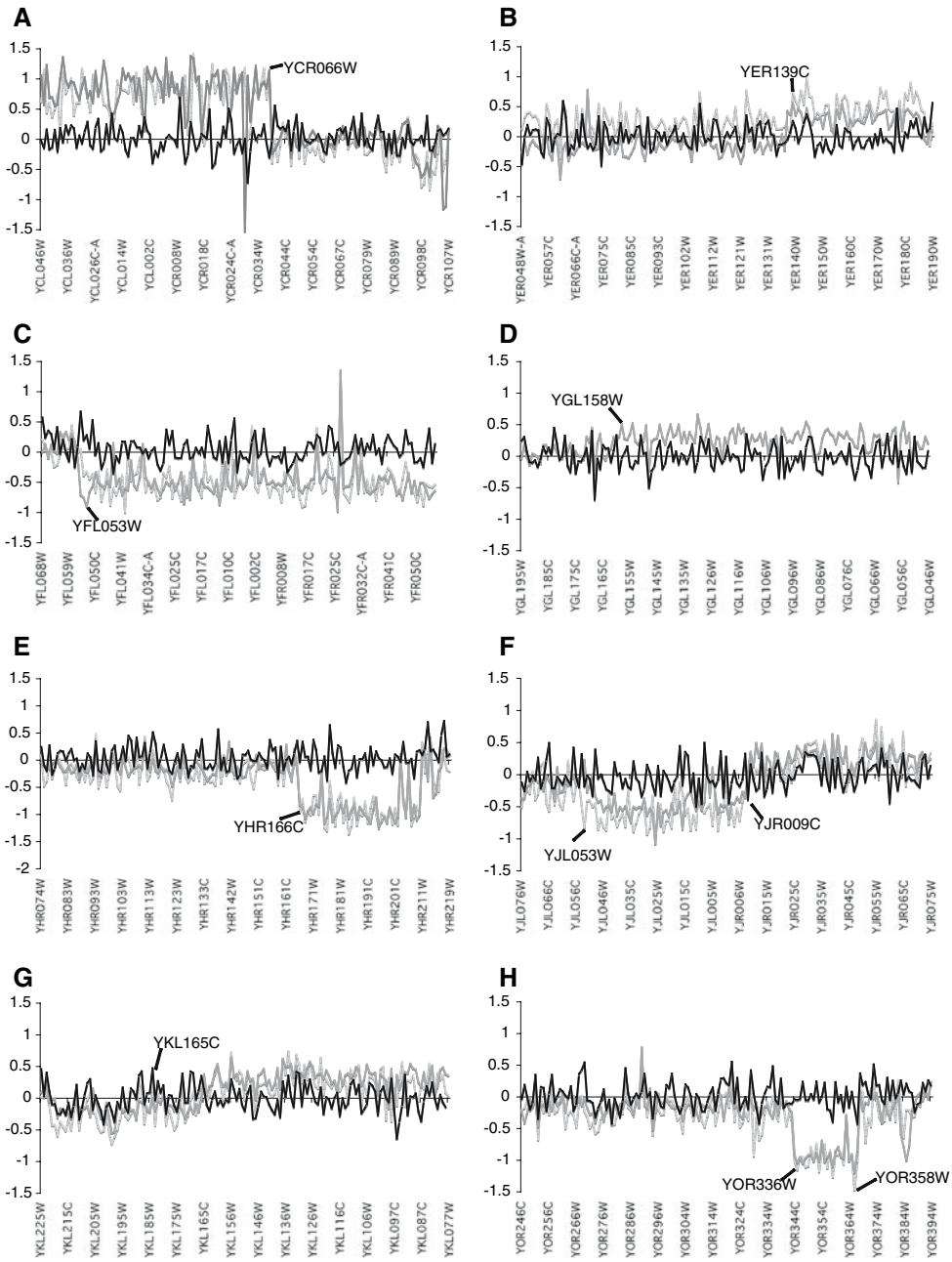

Fig. 1 Chromosomal changes in the mutants C5, C6 and C10. The parent strain CMBS and the mutants C5 *pale grey line*, C6 *grey line* and C10 *black line* were fermented in 19°P wort at 13°C in 2 L cylindroconical vessels. DNA was extracted from cells harvested on day 3 of the fermentation and labeled with Cy5. The parental strain CMBS was labeled with Cy3. The pooled DNA was hybridised to *S. cerevisiae* DNA microarrays. The $\log_2$ value (*y-axis*) of the normalised ratios of hybridisation (ROH) for ORFs (*x-axis*) on regions of chromosomes (**a** III, **b** V, **c** VI, **d** VII, **e** VIII, **f** X, **g** XI, **h** XV) in the mutants C5, C6 and C10 are shown. For complete data set of all chromosomes refer to the supplementary material

Table 1 Locations of chromosomal rearrangements in the mutants C5, C6 and C10-51 as deduced from alterations in ROH values

Chromosome location	C5	C6	C10-51
YCR039C	+	+	+
YER139C	+	+	–
YER162C	–	–	+
YFL052W-054C	+	+	+
YGL172W	+	+	–
YHR166C	+	+	+
YJL053W	+	+	–
YJR009C	+	+	+
YKL165C-167C	+	+	+
YOR344C	+	+	+
YPR160W	–	–	+

The C5, C6 and C10 mutants were originally selected through EMS mutagenesis followed by selection for both heat-tolerance and growth on HSG-wort. EMS mutagenesis generally induces C > T point mutations resulting in C/G to T/A substitutions but rarely can cause strand breaks that lead to inversions or deletions. There have been reports that EMS treatment can lead to a decrease in ploidy in tetraploid strains while some haploid lines registered an increase (Mable and Otto 2001) in ploidy. Another possibility is that the chromosomal changes may have been induced by the combined stress factors of higher temperature and HSG-wort selection regime. To verify this, a second round of mutant selection was carried out in the absence of EMS on the parent CMBS and the mutants C5, C6 and C10. This second generation mutants were heat-tolerant and retained this phenotype for many months over successive generations (data not shown). One such mutant C10-51, which originated from the C10 mutant, also showed chromosomal rearrangements (Table 1 and supplemental data: note that C10 mutant was very much like the parent CMBS and without any chromosome rearrangements; see Fig. 1). Again, the observed rearrangements in C10-51 mostly mapped to the original recombination sites between the *S. cerevisiae* and *S. bayanus* genomes previously identified in both CMBS and a second lager yeast 6701, and are very similar to the rearrangements identified in mutants C5 and C6 (Table 1). Therefore, it is very likely that the chromosomal changes observed in C5, C6 and C10-51 are a consequence of the selection for growth in high specific gravity wort and/or exposure to high temperatures rather than from the chemical mutagen EMS.

Environmental stress can induce chromosome rearrangements in lager yeasts

The initial finding that stress-tolerant mutants contain gross chromosomal changes (GCRs) prompted us to ask if chromosome stability is influenced by the physiological conditions experienced during the brewing process. To examine this, firstly, fermentations using the mutant C10 were carried out over 8 days in HSG-wort (1.08 g/mL; 19°P) and at the low temperature of 13°C used for traditional brewing. DNA was extracted from the mutant C10 at the beginning of the fermentation (day 1) and at the end of the fermentation (day 8). CGH analysis of these DNA samples did not reveal any significant changes in the ROH values between days 1 and 8. However, when similar fermentations were carried out at a slightly higher temperature (20°C; rather than 13°C) and specific gravity wort (1.092 g/mL; 22°P), significant differences in ROH values for specific regions of selected chromosomes were observed. This was true for both C10 and its heat-resistant sub-strain C10-51 in each case comparing the status of day 1 DNA to that of day 8. For example, in both cases, the majority of ORFs showing a greater than a twofold change in ROH between days 1 and 8 were located predominantly on chromosomes I, III and VI (Figs. 2, 3). Additional significant changes in ROH were observed on chromosomes IV (Fig. 2) and XII (Figs. 2, 3) in mutant C10-51. No significant changes were observed when DNA from either C10 or C10-51, extracted on day 1, was differentially labeled with Cy3 and Cy5. This, together with the fact that significant changes between days 1 and 8 in both mutants were restricted to a sub-set of chromosomes rules out the possibility of labelling artifacts (Fig. 3 and supplementary data). The increase in ROH in C10-51 for ORFs on chromosome XII centred around the major ribosomal RNA gene cluster at YLR154C. On chromosome IV, the amplification peak is centred at YDR125C-YDR167W (data not shown). Changes in ROH were also evident at the telomeres of a number of chromosomes but again only when fermentations were carried out at 20°C in 22°P wort (see chromosome III, Fig. 3).

Regional amplification is dependent on the specific gravity of the wort

The results thus far indicate that specific environmental conditions used in fermentation can alter the genetic composition of selected chromosomes of a group of heat-resistant lager yeast strains. To determine if the observed changes were due, in part, to the instability of the genome of these mutants perhaps induced by the selection process or if they reflect a more general response to the stress conditions imposed on the yeast, the genome of the parent strain CMBS-33 was examined under a variety of physiological conditions: quadruplicate fermentations were carried out at 20°C in either high (1.080 g/mL; 20°P) or low (1.065 g/mL; 16°P) specific gravity wort and also under conditions where air was either excluded by overlaying the medium with mineral oil (anaerobic) or not (aerobic). DNA

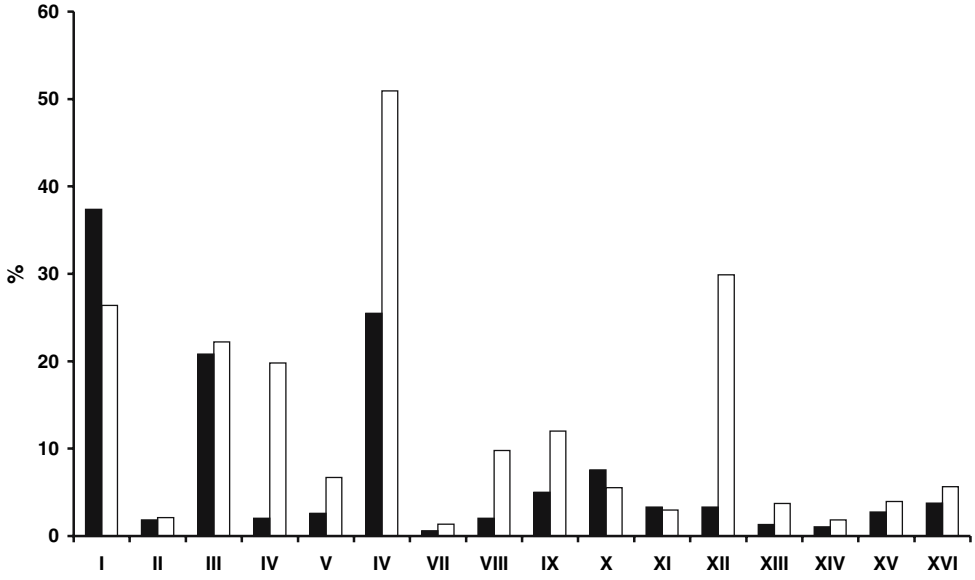

Fig. 2 Differential chromosomal changes in response to high-gravity and high-temperature. Percentage of genes per chromosome showing greater than twofold change in ROH in DNA extracted on days 1 and 8 of fermentations carried out at 20°C and 22°P using the mutants C10 (*black columns*) and C10-51 (*white columns*)

was extracted from yeast samples taken at day 1 or 8 of fermentation and analysed by CGH.

The data show that the higher specific gravity (20°P) wort conditions resulted in a greater degree of chromosomal instability compared to that observed in the lower specific gravity (16°P) wort. Differential changes in ROH between high and low specific gravity were observed for 658 genes (11% of all ORFs). Mapping of these genes to their chromosomal positions, indicated that approximately 50% of these changes occurred in genes located at or near the telomeres (Table 2; Fig. 4a, b). Of the remaining genes, most changes in ROH were confined to small clusters of genes and evenly distributed amongst all chromosomes with the exception of chromosome I where 31% of the genes showed significant changes in ROH (Table 2). The full list of affected genes is available in the supplementary material (Supplementary Table 1).

Again, as in the case of strain C10-51, one of the gene clusters showing higher ROH values in HSG fermentations in the parent strain CMBS-33 spans a region on chromosome XII from YLR130C to YLR180W and flanks a major ribosomal RNA gene cluster containing approximately 100–150 repeated rRNA units (Fig. 5).

While the majority of changes in ROH values between days 1 and 8 were observed in samples fermented in HSG-wort, a number of differences were also evident in samples fermented in low-gravity wort. Specifically, in the latter case, increases in ROH values were observed for large sections of chromosomes IV, IX and XIII (Fig. 6 and supplementary data). Interestingly, these changes noted in low-gravity wort are not confined to the telomeric and sub-telomeric regions and it appears that changes are more likely to occur on chromosomes where telomeric changes are relatively low (see Table 2).

The presence or the absence of oxygen during fermentation did not result in any significant changes of ROH (at 0.001 level of the univariate test), however, a small number of genes showed changes in ROH at a 0.05 level of the univariate test (data not shown). Mapping these changes to individual chromosomes did not reveal any obvious clustering and therefore, we believe that these changes may not be significant and may represent outliers in the data. While the presence of air during fermentation did not in itself induce changes in ROH values under low gravity conditions, the aerated brewing conditions exacerbated the changes in ROH observed in high-gravity fermentations (data not shown). Thus, different stress conditions may generate cumulative effects on chromosome stability.

It should be noted that a number of ORFs, not related to the major clusterings described above, also showed striking differences in ROH values in high versus low specific gravity fermentations. These include the genes YJR115W and YBR134W, which show a differential ratio of 4.7 and 4.6, respectively, in high versus low gravity.

Discussion

In this study, we set out to characterise three mutant lager yeast strains, C5, C6 and C10, generated from the parent CMBS by EMS mutagenesis followed by selecting sequentially for survival at 55°C for 10 min and growth on high specific gravity media. Our analysis of these mutants and their parent strain demonstrates that the genomes are plastic and capable of undergoing gross chromosomal rearrangements and regional amplification in response to stress. Each of the mutant strains retained their fermentative capacity and the mutant C10 consistently showed increased fermentation rates

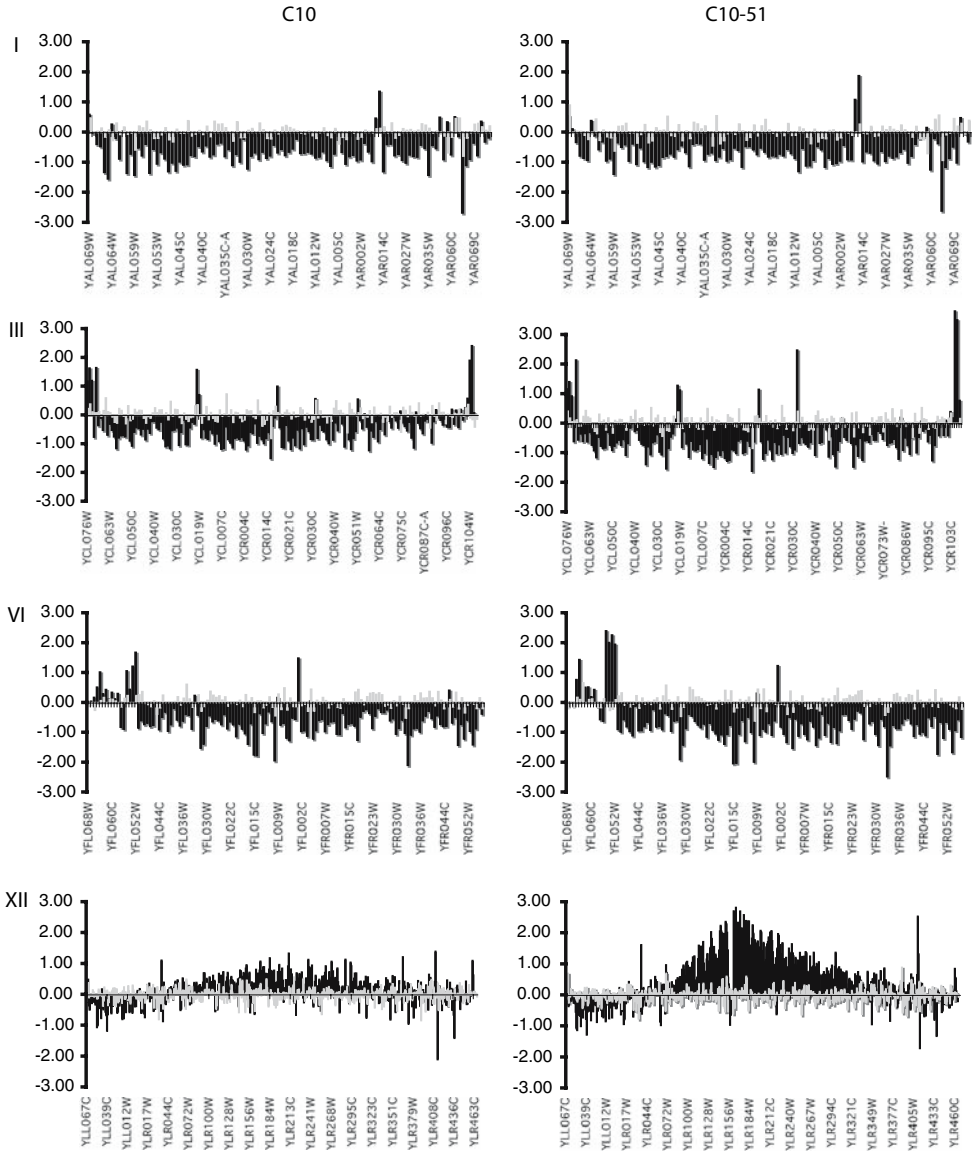

Fig. 3 Chromosome changes in mutants C10 and C10-51 fermented in high-gravity wort. The mutant strains C10 and C10-51 were fermented in high specific gravity wort (22°P) at 20°C in 200 mL cylinders. DNA was extracted at days 1 and 8 of the fermentation and differentially labeled with Cy3 and Cy5. The pooled DNA was hybridised to *S. cerevisiae* DNA microarrays. The $\log_2$ values of the normalised ROH for genes on chromosomes I, III, VI and XII are shown (*black columns*). The ROH values were plotted in relation to their chromosome location in *S. cerevisiae* (*x-axis*). DNA isolated from day 1 of the fermentation for both C10 and C10-51 was differentially labeled with Cy3 and Cy5 (*grey column*)

compared to its parent CMBS-33 (data not shown). The severe stress regime used for the selection of mutants generated clones with fixed genomic rearrangements as evidenced by CGH analysis and by karyotyping. The majority of the rearrangements mapped to previously identified "hot spots" of recombination, which had generated the mosaic chromosomes of the lager yeasts (Bond et al. 2004). However, two new sites in the regions of YJL053C on chromosome X and YKL165C on chromosome XI were identified in mutants C5 and C6. Interestingly, these new sites, as with the previously mapped recombination sites, are also flanked by or in close proximity to Ty elements, tRNA or ARS elements. The conservation of the sites of recombination between different strains of lager yeasts, whether isolated by selection as described here, or amongst established brewery strains, argues that all strains may have evolved through genetic rearrangements at selected recombination hot spots from a common ancestor.

Since it is uncommon for EMS treatment to induce GCRs, we asked the question whether exposure to the stress-selection regime was sufficient to generate GCRs. The selection procedure was repeated using both the parent CMBS and its derived stress-tolerant mutants. Analysis of one clonal mutant (C10-51) derived from C10, revealed a similar set of GCRs in almost the same locations as those found in the C5 and C6. Thus, the GCRs very likely arose as a result of the selection for tolerance to high-temperature and high specific gravity wort. The conservation of the sites where recombination has occurred between the three mutants C5, C6 and C10-51 suggests that these regions represent the major recombination sites in the polyploid *S. pastorianus* strains.

Our results also show that lager yeast genomes can be influenced by the physiological conditions experienced during the brewing process. Although the data presented here

Table 2 Number of genes per chromosome showing changes in ROH values for strain CMBS grown in low- or high-gravity wort

Chromosome	No. of genes with differential ROH values	No. of genes analysed per chromosome	Genes showing differential ROH (%)	Telomeric (%)
I	33	108	31	61
II	35	426	8	43
III	16	176	9	63
IV	74	806	9	34
V	25	286	9	68
VI	20	135	15	100
VII	49	563	9	49
VIII	35	279	13	51
IX	30	225	13	33
X	46	389	12	70
XI	24	336	7	58
XII	62	540	11	42
XIII	69	493	14	23
XIV	44	419	11	57
XV	45	567	8	36
XVI	51	491	10	63

is exclusively from CMBS and its descendants, it is very likely to be a more generalised feature since the rearrangement hot spots are conserved between CMBS and other lager strains (Bond et al. 2004). While GCRs were not identified during a single round of fermentation in HSG and at the traditional low temperatures (10–13°C) used in the brewing industry, we identified significant reproducible changes in ROH values when cells were fermented at higher temperatures (20°C). These changes were not random but were mainly confined to a sub-set of chromosomes. When the mutant strains C10 or C10-51 were fermented for 8 days, very similar changes in the ROH values were observed on chromosomes I, III and VI, where there is a generalized decrease in ROH values on day 8 relative to day 1. Additionally, in the mutant C10-51, ROH values increased on chromosomes IV and XII. The reduction in ROH values observed on chromosomes I, III and VI, most likely reflects an amplification of the *S. bayanus* component of the genome leading to a greater competition for hybridisation to the *S. cerevisiae* microarrays thus resulting in a concomitant reduction in hybridisation by the *S. cerevisiae* component of the genome. Alternatively, there may be a loss of *S. cerevisiae* DNA during the fermentation, however, conceptually it is difficult to reconcile the latter since there is only from two to three rounds of cell divisions between the beginning and the end of a brewing cycle. Likewise, the increase in ROH values observed on chromosomes IV and XII in strain C10-51 most likely reflects an amplification of the *S. cerevisiae* component of the genome. It should also be noted that the strains are described as allotetraploids with some aneuploidy and may behave differently from standard diploid or haploid strains for which most of the data is available. For example, it is known that that aneuploidy in general causes a proliferative disadvantage in yeast, often due to checkpoint delays (Torres et al. 2007) and from a brewers' point of view this is a desirable feature as energy is utilised for fermentation rather than biomass production. The downside may be that some of the replication/repair process may stall and this may depend on the size and ploidy of the chromosomes.

To rule out the possibility that the chromosomal changes, observed during a single round of fermentation with mutants C10 and C10-51, reflected an inherent genome instability in stress-tolerant mutants and to determine if this was a more general process related to fermentation in HSG-wort, we examined the stability of the CMBS parent strain during fermentations in both high and low specific gravity conditions. The data obtained from seven independent experiments indicate that fermentation of CMBS in high-gravity wort resulted in a greater degree of chromosomal instability and/or gene amplification particularly in the telomeric regions. Specific regional amplifications were also evident in low specific gravity fermentations that were not apparent in cells fermenting the high-gravity wort suggesting the possibility that certain chromosomal changes may relate to specific environmental conditions. Furthermore, we also find that combining different stresses (such as exposure of cells to high specific gravity and aerobiosis) exacerbates the effects on chromosome stability. Taken all data together, it appears that chromosomal changes can occur spontaneously during a single round of 8-day fermentation, and while common changes are apparent in different

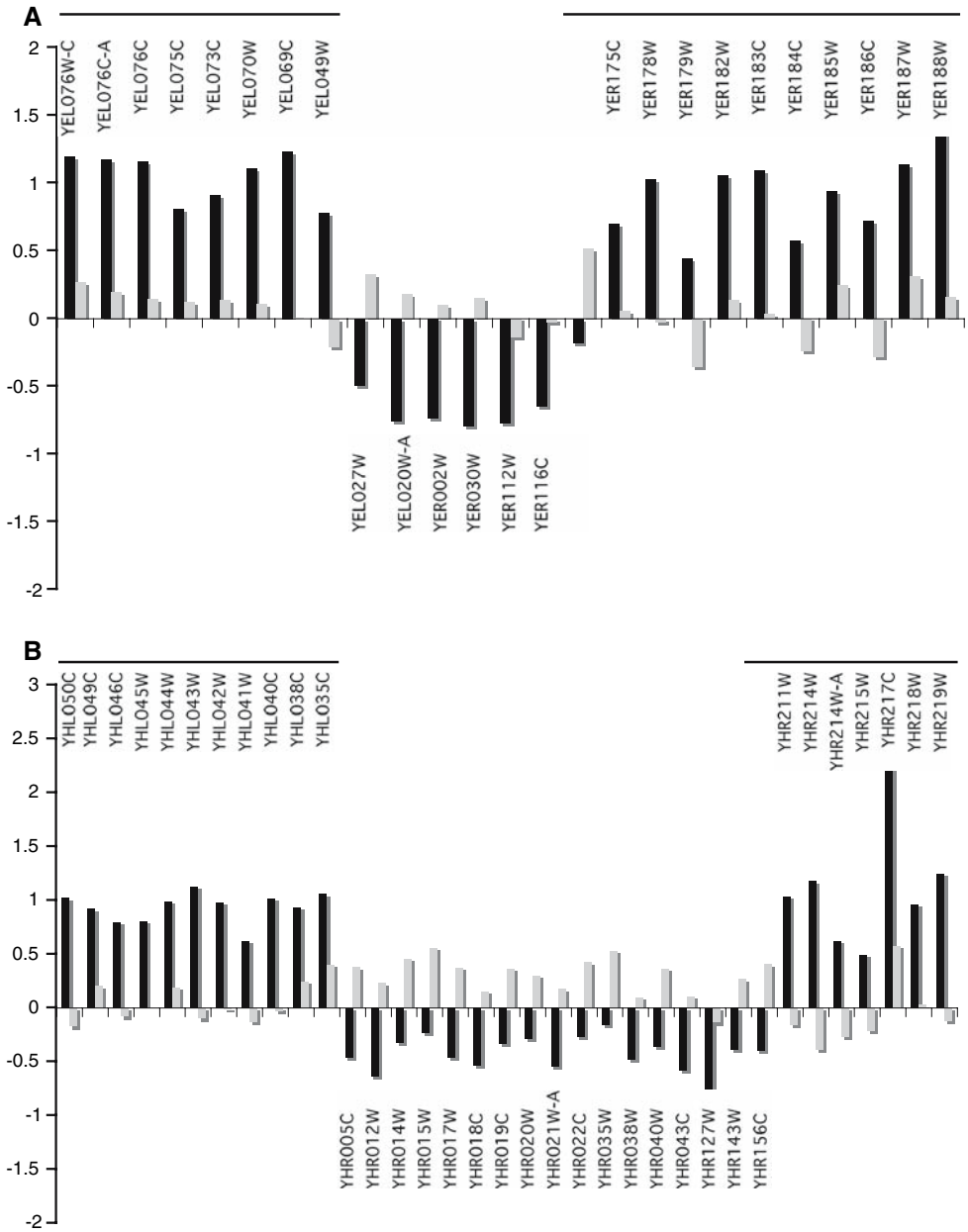

Fig. 4 Fermentation in high-gravity wort generates chromosomal changes in CMBS. The parent strain CMBS was fermented in either high (20°P) or low (16°P) specific gravity wort at 20°C in 200 mL cylinders. DNA was extracted on days 1 and 8 of the fermentation and differentially labeled with Cy3 and Cy5. The pooled DNA was hybridised to *S. cerevisiae* DNA microarrays. The resultant data was analysed using the software BRB Microarray Tools to identify genes showing differential ROH values in high- and low-gravity. ORFs on chromosomes **a** V and **b** VIII showing differential ROH in high- (*black columns*) and low-gravity (*grey columns*) are shown. The names of the ORFs are shown alongside the columns. A *solid line* above the columns indicates telomeric-associated ORFS

strains, each strain shows a unique pattern at the end of an 8-day fermentation. One drawback with CGH analysis is that it only provides a mean ROH value for a population of cells. Fermentations are initiated with yeast grown from a single colony to a concentration of 1–2 $\times$ 10^7 cells/mL and generally cells undergo 2–3 rounds of multiplication during the 8-day brewing period. Thus, any chromosomal changes in a single cell or a small percentage of the total population may remain undetected or may generate some variability in the mean ROH values across ORFs on a given chromosome. Despite this, the statistically significant changes in ROH values stand out from amongst the generalised scatter as observed from the clustering of changes to specific chromosomes. The net result of such changes would be a mixed population of cells at the end of fermentation, some of which may have acquired relevant and/or pertinent traits for survival. Many industrial brewers reuse all or part of the spent yeast recovered at the end of a given fermentation cycle to start a new fermentation ("re-pitching") and such reuse may favour selection and fixing of the favoured trait.

One key finding of this study is the clustered short regions of gene amplification, particularly for genes flanking the major rRNA locus on chromosome XII in both the parental strain CMBS and the mutant C10-51. In *S. cerevisiae*, the ~150–200 rDNA copies are present in a single head-to-tail tandem array. Despite the high demand for rRNA, only about one and a half of the rDNA copies are active, even in rapidly growing cells (French et al. 2003). It

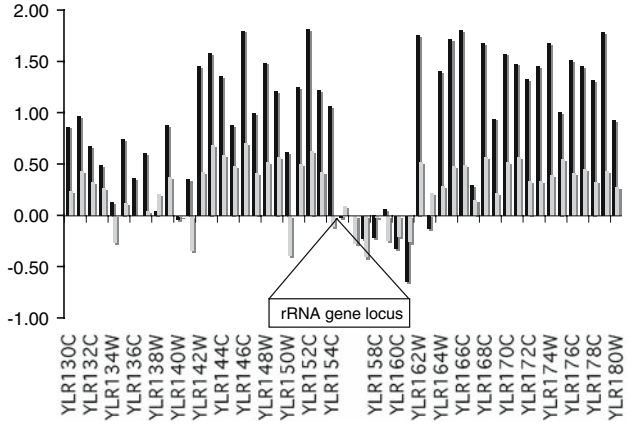

Fig. 5 ORFs flanking the rRNA gene locus are selectively amplified in high-gravity fermentations. The parent strain CMBS was fermented in either high (20°P) or low (16°P) specific gravity wort at 20°C in 200 mL cylinders. DNA was extracted on days 1 and 8 of the fermentation and differentially labeled with Cy3 and Cy5. The pooled DNA was hybridised to *S. cerevisiae* DNA microarrays. The resultant data was analysed using the software BRB Microarray Tools to identify genes showing differential ROH values in high- and low-gravity. Mean ROH of ORFs flanking the rRNA gene cluster (*box*) on chromosome XII in high-gravity (*black columns*; mean 3 independent experiments) and low-gravity (*grey columns*; mean 4 independent experiments) wort. The names of the ORFs are shown below the columns

has been suggested that cells maintain a twofold excess rDNA copy number in attempt to protect the locus from homologous recombination events (often customary in repeat DNA sequences) that might reduce copy number below some threshold required for viability. The Sir2 gene product, a deacetylase enzyme required for gene silencing at both telomeres and silent mating-type (HM) loci, represses both meiotic and mitotic recombination within the rDNA cluster in yeast (Kobayashi et al. 2004). Interestingly, *sir2* mutants have a shortened replicative life span, apparently because mother cells accumulate toxic levels of extra-chromosomal rDNA circles (ERCs; Sinclair and Guarente 1997). Thus the observed increase in rRNA copy number may be indicative of ERCs and may be a good measure of the future viability of these yeast cells. It is also interesting to note that the telomere amplifications observed in this study may soak up significant quantities of Sir2p thus reducing its availability to the maintenance of rDNA clusters. Another such gene cluster that is amplified is found on chromosome I (YAR028W–YAR040W) in the CMBS strain. This cluster is known in *S. cerevisiae* as DUP240, one of the largest gene families in yeast consisting of ten genes with a high level of nucleotide identity (from 50 to 98%), scattered on four chromosomes and arranged either as tandem repeats or as isolated genes (Despons et al. 2006). Five open reading frames (ORFs; YAR027W, YAR028W, YAR029W, YAR031W and YAR033W) are tandemly repeated on chromosome I; the others being YGL051W and YGL053W which are directly repeated on chromosome VII. DUP240 orthologs have been identified only in species of the *Saccharomyces sensu stricto* group including *S. bayanus*. Short repetitive DNA sequences identified in this cluster have been implicated in large chromosomal rearrangements observed at the tandem DUP240 loci on chromosome I through nonallelic recombination events (Leh-Louis et al. 2004). The locus is also flanked by Ty elements. Most DUP240 ORFs encode membrane-associated proteins of ~240 amino acids with two potential transmembrane domains (Despons et al. 2006). Simultaneous deletion of the ten DUP240 ORFs *S. cerevisiae* does not alter cell viability and therefore, it is conceivable that any fluctuations in their gene numbers may not be deleterious.

Similarly, the two other ORFs showing the highest levels of amplification in CMBS are YBR134W and YJR115W, both were once referred to as dubious ORFs in the Saccharomyces Genome Database. It is now known that YBR134W is over-expressed commonly in senescent and cdc48^{S565G} apoptotic cells (Laun et al. 2005), while YJR115W has recently been shown to be a stress-responsive locus that is regulated by the heat-shock protein HSP30 (Ansanay Galeote et al. 2007). It could be argued that the higher copy number of YBR134W may be affected by senescence and apoptosis through some unknown process analogous to that of ERC formation while stress is a known causative factor for amplification albeit in vertebrates, specifically in rodent and human cells. Oxidative stress conditions can influence the interaction of telomeric binding complexes with telomeres (Opresko et al. 2005), which in turn may affect telomeric stability in human cells (Liu et al. 2003; Ziv et al. 2005). Additionally, deletion of Hsp70 genes in mice fibroblasts has been shown to lead to an increased genomic instability that is exacerbated by heat treatment (Hunt et al. 2004). Therefore, chromosome instability resulting from stress may be a universal occurrence and the stress response may play a role in maintaining chromosome stability under stress conditions.

Several recent reviews have highlighted the contribution of repetitive loci in adaptive evolution (Biemont and Vieira 2006; Fablet et al. 2007; Volff 2006) mediated by transposable elements (TEs). It has been suggested that TEs may offer a unique ability for mutation in response to environmental change. Many of the other localised gene amplification events described here are associated with the presence of a TE (Ty), tRNA genes and/or an ARS sequence (which are all known "hot spots" for chromosome rearrangements) in the flanking regions of affected ORFs. It is worth noting that the mobility of the Ty1 is greatly stimulated in response to telomere erosion stress in *est2* mutants, which lack telomerase (Scholes et al. 2003). Thus the observed telomeric changes in our studies may affect the movement of the Ty elements.

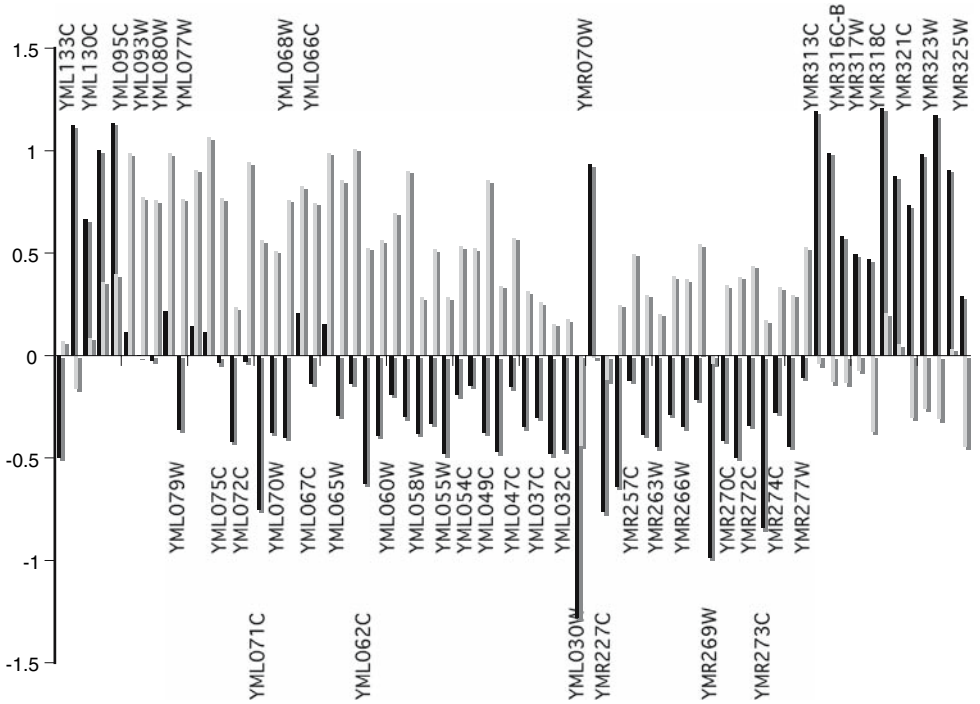

Fig. 6 Localised amplification of ORFS in low-gravity wort. The parent strain CMBS was fermented in either high (20°P) or low (16°P) specific gravity wort at 20°C in 200 mL cylinders. DNA was extracted on days 1 and 8 of the fermentation and differentially labeled with Cy3 and Cy5. The pooled DNA was hybridised to *S. cerevisiae* DNA microarrays. The resultant data were analysed using the software BRB Microarray Tools to identify genes showing differential ROH values in high- and low-gravity. Mean ROH of ORFs on chromosome XIII showing differential ROH in low-gravity (*grey column*; mean 4 independent experiments) and high-gravity (*black column*; mean 3 independent experiments) wort. The names of the ORFS are shown next to the columns. Some ORF names have been removed for clarity. The full data set can be viewed in Supplemental Table 1

Chromosomal rearrangements and DNA amplification most likely initiate from spontaneous and/or replication-induced double strand breaks (DSBs) in the DNA. This may be particularly true for polyploidy strains of *S. cerevisiae*, where high levels of chromosome instability have previously been observed (Mayer and Aguilera 1990). More recently, studies have shown that tetraploid strains of *S. cerevisiae* display an approximate 400-fold increase in chromosome loss compared to isogenic diploid strains (Andalis et al. 2004). Additionally, small but significant increases in chromosomal rearrangements are observed in polyploid strains (Huang and Koshland 2003). Such chromosomal loss and/or rearrangements are not directly related to increases in mitotic frequency, but rather are due in part to defects in microtubule-kinetochore attachment, which can lead to chromosome non-disjunction and thus chromosome loss and increased sensitivity to DSB inducing agents and to microtubule poisons (Storchova et al. 2006). Furthermore, tetraploids show a requirement for homologous recombination for survival suggesting a high degree of spontaneous DNA lesions. The presence of palindromic and/or repetitive sequences (such as Ty elements, tRNA and/or ARS sequences) in the vicinity of such DSBs may invoke intra- or intermolecular recombination events and the formation of dicentric chromosomes. Subsequent rounds of replication can lead to DNA amplification of genes in the vicinity of these sequences (Butler et al. 2002). At anaphase, as the centromeres are pulled to opposite poles, breakage of the dicentric chromosomes can lead to gross chromosomal rearrangements and chromosome non-disjunction (VanHulle et al. 2007).

Taken together, the defects observed in isogenic polyploid *S. cerevisiae* strains and the emerging picture of mechanisms involved in the repair of DSBs in *S. cerevisiae* may aid, in our understanding, the observed dynamic behavior of chromosomes in aneuploid lager yeasts. Such DSBs may either be induced and/or not quickly repaired in response to the stress conditions such as growth in HSG-wort and higher than normal temperatures. In some cases, localised amplification in the region of the DSB can occur leading to chromosome instability thereby affecting the vitality and viability of the strain in question. Continued selection in stress conditions such as those used to screen for the mutants C5, C6, C10 and C10-51 allows the selection of pure clonal strains with stabilised rearrangements with certain advantageous traits. While chromosomal rearrangements may reduce fitness in haploid and isogenic polyploid strains (Andalis et al. 2004), the existence of a

polyploid genome consisting of homeologous chromosome sets with built-in redundancy appears to confer certain selective advantages to the lager yeasts, with respect to their fermentative capacity, adaptability to extreme environmental conditions and ultimately survival.

Our finding is that the exposure to environmental stress can influence chromosome stability may have implications for the brewing industry in particular in the maintenance of pure breed yeast stocks and in the employment of high specific gravity conditions for brewing. Perhaps more importantly, it sheds light on the survival value of polyploidy and allopolyploidy in particular in brewery yeasts.

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