Recombination between Homoeologous Chromosomes of Lager Yeasts Leads to Loss of Function of the Hybrid GPH1 Gene

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Received 12 February 2009/Accepted 5 May 2009

Yeasts used in the production of lagers contain complex allopolyploid genomes, resulting from the fusion of two different yeast species closely related to Saccharomyces cerevisiae and Saccharomyces bayanus. Recombination between the homoeologous chromosomes has generated a number of hybrid chromosomes. These recombination events provide potential for adaptive evolution through the loss or gain of gene function. We have examined the genotypic and phenotypic effects of one of the conserved recombination events that occurred on chromosome XVI in the region of YPR159W and YPR160W. Our analysis shows that the recombination event occurred within the YPR160W gene, which encodes the enzyme glycogen phosphorylase and generates a hybrid gene that does not produce mature mRNA and is nonfunctional due to frameshifts in the coding region. The loss of function of the hybrid gene leads to glycogen levels similar to those found in haploid yeast strains. The implications for the control of glycogen levels in fermentative yeasts are discussed.

One of the recombination events identified by CGH analysis is located on chromosome XVI in the region of YPR159W and YPR160W. DNA to the left of the region hybridizes to S. cerevisiae microarrays, while genes between YPR160W and YPR190C and encompassing approximately 58 kb of DNA displayed a lack of hybridization to these microarrays, suggestive of a hybrid chromosome (3). Whole-genome sequence analysis of the Weihenstephan strain confirmed the existence of hybrid chromosome XVI and indicated the presence a second type of chromosome XVI containing S. bayanus-like sequences to the left of YPR159W (15, 16).

To examine the genotypic and phenotypic outcomes of this recombination event, the right arm of chromosome XVI has been cloned from the yeast strain CMBS-33. Our analysis reveals that the recombination event occurred within the open reading frame (ORF) of YPR160W (GPH1) encoding the enzyme glycogen phosphorylase, which is required for the mobilization of stored glycogen through its conversion into glucose-1-P. The recombination event generates a hybrid gene that does not produce a mature mRNA and is nonfunctional due to frameshifts in the coding region.

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† Supplemental material for this article may be found at http://aem.asm.org/.
‡ Published ahead of print on 8 May 2009.
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 K. Verstrepen). Strain 6701 was from the Guinness yeast collection. The haploid Saccharomyces cerevisiae strain S-150 (MATa leu2-3 leu2-112 his3D1 trp1-289 ura3-52) was obtained from J. Beggs (Edinburgh University).

The haploid S. bayanus strain was obtained from the Collection de Levures d’Intérêt Biotechnologique, Paris, France.

Electrophoretic karyotyping and Southern blotting of lager yeast DNA. The lager yeast strains CMBS-33 and 6701 and the haploid S. cerevisiae yeast strain S-150 were grown overnight in yeast extract (1%)–peptone (2%) supplemented with maltose (2%) (YEPM) to give a final yield of 1.5 × 10^9 cells. The total genomic DNA was isolated, and DNA-agar plugs were prepared as previously described (7).

Electrophoresis was carried out in 1.2% agarose containing 0.5× Tris-acetate (40 mM), EDTA (1 mM) (TAE) buffer (pH 8.5) at a temperature of 14°C using an initial switching time of 60 s for 15 h and a final switching time of 90 s for 9 h at a 120° pulse angle. After the electrophoresis, the gel was stained with ethidium bromide (10 mg/ml). The gel was then transferred to a nylon membrane (Pall) for hybridization as previously described (8). The membranes were prehybridized for 1 h at 68°C, and the hybridizations were carried out in the same solution with the addition of 10 ng of digoxigenin (DIG)-labeled DNA probes corresponding to either YPR159W or YPR160, which were prepared as previously described (8).

Generation of the yeast genomic DNA library. High-molecular-weight DNA from the lager strain CMBS-33 was isolated, using the standard phenol-chloroform extraction method as previously described (3). A total of 100 ng of the genomic DNA, partially digested with Sau3A, was incubated with 25 ng of BamHI-digested CopyControl pCC1BAC cloning ready vector (Epicentre) in sterile water and incubated at 55°C for 10 min. The solution was cooled to room temperature, and 1× Fast-Link ligation buffer (Epicentre), 10 mM ATP, and 2 μl Fast-Link DNA ligase (Epicentre) were added to the cooled solution. The ligation mixture was incubated at 16°C for 4 h and then heated to 65°C for 15 min. The ligation reaction mixture was desalted using an agarose cone on ice for 1 h and then mixed with TransforMax EPI3000 electrocompetent Escherichia coli cells (Epicentre), and the cells were electroporated. Following the recovery of the cells, the transformants were plated in Luria broth containing chloramphenicol (12.5 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside; 0.4 mM) and incubated overnight at 37°C. In total, 7,000 chloramphenicol-resistant colonies were obtained.

Characterization of the bacterial artificial chromosome (BAC) library. Individual clones were transferred to agar plates containing chloramphenicol–X-Gal–IPTG. The plates were incubated overnight at 37°C and the colonies replicated onto a nylon membrane. The membranes were consecutively treated with the following solutions: 10% sodium dodecyl sulfate for 3 min, 0.5 N NaOH, 1.5 M NaCl for 5 min, 0.5 M Tris-Cl, 1.5 M NaCl (pH 7.4) for 5 min, and finally 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 5 min. The membranes were air-dried and UV-cross-linked. DIG-dUTP-labeled DNA probes were generated by the PCR amplification of CMBS-33 genomic DNA using oligonucleotides based on the S. cerevisiae genome sequence for ORF YPR159 and ORFs to the left of this ORF and the S. bayanus genome sequence for YPR160 and ORFs to the right of this ORF (see Table S1 in the supplemental material). Hybridizations were carried out using the pooled probes as previously described (5). Following hybridization, positive clones were identified, isolated, and grown in 2× YT medium (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 liter) supplemented with chloramphenicol (12.5 μg/ml) at 37°C overnight. To verify the selection of positive clones, DNA isolated from the picked colonies was hybridized to individual DIG-labeled probes in the region of interest.

Sequencing. The sequencing of the PCR products and direct sequencing from the BAC clones was performed at GATC, Constance, Germany. The specific primers were designed using the Saccharomyces Genome Database and the Saccharomyces bayanus database (www.yeastgenome.org and www.broad.mit.edu/annotation/fungi/comp_yeasts/), respectively). A list of primers and their genome locations are provided in Table S2 in the supplemental material. Three independent passes in a forward and reverse direction were performed on each fragment.

RNA extraction and Northern blot analysis. RNA extractions and Northern blot analysis were carried out as previously described (5). The primers used for the preparation of the DIG-labeled hybridization probes are provided in Table 1.

Reverse transcriptase reactions. The total RNA (30 μg) was incubated with DNase I (Promega, Inc.) at 30°C for 1 h to remove any contaminating DNA.

Following digestion, reverse transcription reactions were carried out using 2 μg of DNA-treated RNA as the template, a transcript-specific reverse primer (50 nM), and reverse transcriptase (Applied Biosystems) as specified by the manufacturer in a final volume of 20 μl at 25°C for 10 min and 37°C for 120 min, followed by 85°C for 5 s. PCR amplification was carried out using 2 μl of the cDNA as previously described (3). The primers used for cDNA synthesis are shown in Table 1.

Detection and measurement of the glycogen content of yeast cells. Yeast cells were pelleted onto YEP-dextrose or YEPM plates and grown at 30°C overnight. The plates were inverted over iodine crystals. The levels of glycogen were detected by the appearance of a purple coloring of the colony. Additionally, the cell pellets (1 × 10^7 cells) were washed twice with 5 ml ice-cold sterile distilled water and resuspended in 1 ml of 40 mM sodium acetate (pH 4.8). The cells were boiled for 5 min and cooled on ice. Following vortexing (approximately 12 times with 1-min intervals between on ice), the samples were pelleted by centrifugation at 10,000 rpm. The supernatant was transferred to a fresh tube, and 335 μl of amyloglucosidase (230 U/ml, A1602; Sigma) was added to 125 μl of each sample. The samples were incubated at 57°C for 16 h. The amount of glucose-1-P, generated from the hydrolysis of the glycogen, was determined using a glucose assay kit (GAGO-20; Sigma) according to the supplier’s instructions.

Nucleotide sequence accession numbers. Sequences have been deposited with GenBank (accession numbers bankit198460 and FJ834880).

Results

Lager yeasts contain hybrid chromosome XVI. A real-time PCR analysis of genomic DNA from both 6701 and CMBS-33, and correlation with CGH data, predicts the presence of four copies of chromosome XVI in a ratio of three hybrids to one S. bayanus-like chromosome (2, 3, 15, 16). Additionally, in almost all the lager yeast strains tested so far, the telomeric regions of both types of chromosome XVI contain S. cerevisiae-like genes (Fig. 1A), the exceptions being BH-314 and ATCC 24966 (15).

To examine the hybrid nature of chromosome XVI, chromosomal DNA from the lager yeasts CMBS-33 and 6701 and the haploid S. cerevisiae strain S-150 was separated by pulsed-field gel electrophoresis and hybridized to DIG-labeled DNA complementary to either S. cerevisiae YPR159W (ScYPR159W) or ScYPR160. Ethidium bromide staining revealed similar chromosome profiles in all three strains in the high-molecular-weight region of the gel (Fig. 1B). Hybridization to a single chromosome band was observed in the lager DNA samples as well as to the S. cerevisiae DNA with the ScYPR159W probe (Fig. 1C). The intensities of the hybridizing bands in 6701 and CMBS-33 are greater than that observed in the S. cerevisiae sample despite the underloading of the DNA in these samples (Fig. 1C, compare lanes 2 and 3 to lane 1), suggesting a higher copy number for the S. cerevisiae-type YPR159W genes in the two lager strains. A DNA probe in the ScYPR160C ORF also hybridized to a single chromosome band in all three strains; however, the signal was much less

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Sequences have been deposited with GenBank (accession numbers bankit198460 and FJ834880).
The ends of all four types of chromosome XVI contain \textit{S. bayanus}-like genes. The common white region encompasses genes YPR160W to YPR190C. Genomic DNA from \textit{S. cerevisiae} (lane 1), lager strain 6701 (lane 2), and lager strain CMBS-33 (lane 3) were separated by pulsed-field electrophoresis. The gels were either stained with ethidium bromide (B) or probed with DIG-labeled DNA to ScYPR159W (C) or ScYPR160W (D). The arrow points to the hybridizing band.

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FIG. 2. Bacterial artificial chromosome clones (1 to 5) containing regions of chromosome XVI were hybridized to DIG-labeled DNA probes amplified from either \textit{S. cerevisiae} (Sc) or \textit{S. bayanus} (Sb) genomic DNA using the ORF-specific primers.

importance of genome size and complexity in \textit{S. cerevisiae} and \textit{S. bayanus} strains. The reduction in the degree of hybridization with \textit{S. cerevisiae} DNA for genes between YPR160W and YPR190C was confirmed by Southern blotting using other \textit{S. cerevisiae} probes corresponding to ORFs in the region (data not shown).

A sequence analysis of the YPR160W gene reveals that the first 360 nucleotides are 97% identical to \textit{S. cerevisiae} and 83% identical to \textit{S. bayanus}. After this region, there is a significant reversal in the homologies (Fig. 3B). The DNA sequence from nucleotide 330 to the end of the ORF is 94% identical to the \textit{S. bayanus} sequence and shares 85% identity with the corresponding \textit{S. cerevisiae} sequence (see Fig. S1 in the supplemental material). Thus, the recombination event between the parental homoeologous chromosomes appears to have occurred in the 5' region of YPR160. The DNA sequence encompassing the recombination event is shown in Fig. 3C. An alignment of the lager DNA sequences in this region to the equivalent regions in \textit{S. cerevisiae} and \textit{S. bayanus} reveals the presence of an extra three codons in the YPR160W gene sequence (see Fig. S1 in the supplemental material). The translation of the lager YPR160W gene sequence indicates that due to the presence of base insertions, the reading frame is interrupted and that only a truncated form of YPR160W of 15,455 Da could possibly be generated.

The hybrid YPR160 gene is not expressed in lager yeast strains. Since DNA sequence analysis suggested that the hybrid YPR160W gene, hereafter referred to as Lg (lager-type) YPR160W, cannot encode a functional protein but may produce a truncated form, we set out to determine if this gene is expressed in the lager strain. RNA was extracted from the lager strain CMBS-33 and the parental strains \textit{S. cerevisiae} and \textit{S. bayanus} and analyzed by Northern blotting and reverse transcriptase PCR (RT-PCR) using a combination of primers to amplify regions either before, after, or encompassing the recombination site in the Lg YPR160W ORF (Fig. 4A). A

To identify clones encompassing the YPR159W to YPR160W region, the library was screened with a pool of DIG-labeled PCR DNA fragments complementary to ORFs in the region (see Table S1 in the supplemental material). Positive clones were identified, and genomic DNA was isolated from each BAC clone and hybridized to individual \textit{S. cerevisiae} or \textit{S. bayanus} probes corresponding to ORFs in the region (Fig. 2). Of the clones screened, only clone 4 hybridized to both the ScYPR159W and \textit{S. bayanus} YPR160W (SbYPR160W) probes. Interestingly, clone 1 hybridized to the ScYPR159W, SbYPR175C, and SbYPR189W probes and clone 5 to YPR159W and YPR189W, but neither hybridized to the SbYPR160W probe. The differential hybridization of probes to the individual BACs suggests the presence of structurally different forms of chromosome XVI.

DNA was isolated from clone 4 and sequenced as described in Materials and Methods. The complete sequence of the region is available in the supplemental material (see Fig. S1). An analysis of the sequence data reveals that the recombination event occurred within the YPR160W ORF (Fig. 3A). The ORF for YPR159W in the lager yeast strain CMBS-33 is 99% identical to the \textit{S. cerevisiae} YPR159W sequence. The intergenic region between YPR159 and YPR160 is 98% identical to the \textit{S. cerevisiae} sequence and 77% identical to the \textit{S. bayanus} sequence and is of the same length as the similar intergenic region in \textit{S. cerevisiae}, indicating that this region originated from the \textit{S. cerevisiae}-like chromosome in the lager strain (data not shown).

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DNA probe corresponding to the prerecombination site region (region A) detected a transcript in all three strains, although the level of the transcript in the CMBS-33 strain was much reduced (Fig. 4B). Likewise, a probe corresponding to the postrecombination site region (region C) detected a reduced level of transcript in the CMBS-33 strain compared to the level detected in either the S. cerevisiae or S. bayanus strain. When a probe encompassing the recombination site region (region B) was used, no detectable transcript was observed in the CMBS-33 strain. This lack of hybridization cannot be accounted for by the reduced homology of the recombination site DNA probe to the YPR160 gene in CMBS-33 as indicated by

FIG. 3. (A) Gene organization of YPR159W and YPR160W in S. cerevisiae (top panels) and the lager strain CMBS-33 (bottom panels). The gray regions are S. cerevisiae-like and the white regions S. bayanus-like. The coordinates for the S. cerevisiae genes on chromosome XVI are shown, and arrows indicate the direction of transcription. (B) Percentage sequence identity of the first 800 nucleotides of the CMBS-33 YPR160W gene to S. cerevisiae (gray) and S. bayanus (black) homologues. (C) Comparison of YPR160W DNA sequences of CMBS-33 (Lager), S. cerevisiae (S.c), and S. bayanus (S.b.) in the region where recombination has occurred. Nucleotides shown in black are common to all three species, those in red are identical in S. cerevisiae and CMBS-33, those in blue are identical in S. bayanus and CMBS-33, and those in pink are identical in S. cerevisiae and S. bayanus. The nine extra nucleotides at positions 450 to 459 present in CMBS and S. bayanus but absent in S. cerevisiae are shown. The in-frame stop codon in the CMBS sequence is underlined. Numbers on the right indicate the nucleotide position relative to the start codon (not shown) for the lager YPR160 gene. The full DNA sequence is shown in Fig. S1 in the supplemental material.
the percentage of homology of the various probes to the DNA sequences in all three strains (Fig. 4C). The lack of a YPR160W transcript containing the recombination site region (region B) was confirmed by RT-PCR analysis (Fig. 4D, lane 10); however, the transcripts corresponding to the pre- and postrecombination sites (regions A and C, respectively) can be detected (Fig. 4D, lanes 2 and 6, respectively). All three regions can be amplified from the CMBS-33 genomic DNA (Fig. 4D, lanes 1, 5, and 9). The quantification of the transcript levels by real-time RT-PCR confirms that the levels of transcripts from all three regions of the gene are much reduced compared to the levels detected in the S. cerevisiae and S. bayanus strains (data not shown).

To rule out the possibility that sequences within the recombinant site region were absent from the final transcript, perhaps due to the introduction of a splice site in the hybrid gene, cDNA synthesis was carried out using reverse primers 4 and 6 in the postrecombination region (Fig. 4A). cDNAs were amplified using the same primers and forward primer 1 (Fig. 4A) in the prerecombination region. The PCR products of the expected sizes (366 and 708 bp, respectively) can be amplified from the CMBS-33 genomic DNA (Fig. 4E, lanes 1 and 5), but no corresponding cDNA transcripts were detected (Fig. 4E, lanes 2 and 6). Transcripts of the expected sizes were detected in both the S. cerevisiae and S. bayanus strains (data not shown). We interpret these data as reflecting the absence of transcription from the hybrid YPR160W gene but the presence of a YPR160W-like RNA, transcribed possibly from the YPR160W gene on the S. bayanus-like chromosome in these strains.

Haploid glycogen levels are observed in the lager yeast strains. The YPR160W ORF encodes for glycogen phosphorylase (GPH1), which is required for the mobilization of stored glycogen through its conversion into glucose-1-P (12). To determine if the lack of gene expression from the hybrid YPR160W gene affects the glycogen levels in the cells, cellular glycogen levels were determined both qualitatively and quantitatively. First, the S. cerevisiae and S. bayanus parental strains and the lager yeast strain CMBS-33 were grown on glucose- or maltose-supplemented medium and exposed to iodine vapors to visualize the cellular glycogen levels. The glycogen levels were similar for all three strains when grown on either glucose (Fig. 5A)- or maltose (data not shown)-containing medium. The glycogen levels were quantified by measuring the glucose levels following the conversion of glycogen to glucose. Similar levels were detected in all three strains (Fig. 5A). Since the lager yeast strains are considered to be aneuploid but contain a general tetraploid DNA content (J. Usher and U. Bond, unpublished data), we asked if the glycogen content varied with the DNA content by examining the glycogen levels in a series of isogenic S. cerevisiae strains increasing in ploidy from haploid to tetraploid (11). As shown in Fig. 5B, the glycogen levels increased as the DNA content increased, displaying a correlation coefficient of 0.965. In all, the glycogen content of CMBS-33 most closely resembles that observed in the 1n iso-
The translocation generated a fusion between the 5′ region of chromosome IX to the right telomere region of chromosome XIII approximately 20 kbp from the left telomere region of chromosome. This recombination event has resulted in a loss of function of the gene. Flocculation in brewing yeasts is an important phenotypic trait that results in the sedimentation of yeasts to the bottom of the fermentation vat at the end of fermentation.

In this paper, we set out to examine the genotypic and phenotypic outcomes of one of the conserved recombination events between homoeologous chromosomes that occurred between YPR159W and YPR160W on chromosome XVI. A sequence analysis of the region indicates that the recombination site is located in the 5′ region of YPR160W, resulting in the generation of a hybrid gene.

A sequence analysis of the YPR159W gene in CMBS-33, which encodes a protein with glucosidase activity involved in cell wall organization, indicates that the gene differs by just two nucleotides from the annotated sequence of S. cerevisiae YPR159W. The nucleotide changes do not affect the protein coding sequence. The intergenic region between YPR159W and YPR160W is also highly conserved and like in origin.

DNA sequence analysis of the hybrid Lg YPR160W gene indicates that the recombination event has resulted in a loss of function in the CMBS-33 lager strain due to specific base insertions that have led to a frameshift in the ORF. Real-time RT-PCR and Northern blot analysis confirm the lack of expression of the gene; however, using specific DNA probes both upstream and downstream of the recombinant site, a related transcript that has significantly diverged from the sequences of both S. cerevisiae and S. bayanus is detected in CBMS-33. This divergent transcript most likely emanates from the single S. bayanus-like chromosome XVI that has been identified in lager yeasts. This result may also go some way to explain the clones identified in the screening of the BAC library. While clone 4, which was used in this study, was shown to contain a contiguous copy of the region of interest, both clones 1 and 5 (Fig. 2) showed hybridization to other ORFs in the region but not to an SbYPR160W probe. These clones may contain a divergent copy of YPR160W and may represent S. bayanus-like chromosome XVI or an as-yet-unidentified version of chromosome XVI in which sections between YPR159W and YPR175W have been deleted.

The YPR160W gene (GPH1) encodes the enzyme glycogen phosphorylase (12, 21). Together with Gdb1p, a glycogen-debranching enzyme, GPH1p catalyzes the sequential phosphorol-
yss of α-1,4-linked glucose units in glycogen to generate glucose-1-phosphate and glucose, thereby mobilizing stored glycogen for energy utilization. In *S. cerevisiae*, GPH1 expression is regulated by stress-response elements and by the high-osmolarity glycerol mitogen-activated protein kinase pathway and is induced during late exponential growth phase (10, 21). Recent data indicate that the deletion of *GPH1* leads to a shortened life span, stress intolerance, and decreased survival in stationary phase (9). Thus, reduced levels of Gph1p in lager yeast may not necessarily be advantageous. However, the lager yeasts still retain one functional GPH1 gene emanating from the *S. bayanus*-like chromosome. Since cellular glycogen content is determined by the regulation of multiple metabolic pathways and many enzymes, including glycogen synthase, further research will be required to examine the gene dosage effects caused by the loss of three copies of *GPH1* in the lager yeasts.

Finally, it is interesting to note that both of the chromosome rearrangements characterized to date in lager yeasts, namely, the YPR160W recombination event described here and the FLO5-YIL169C fusion observed in the nonflocculent type 1 strain (20), result in loss-of-function rather than gain-of-function phenotypes. It will be interesting to examine the other recombination sites in detail to determine if this is a common trend.

ACKNOWLEDGMENTS

We thank T. C. James for continued expertise and assistance during this project. This research was supported by a grant to U.B. as part of the IITAC consortium from the Higher Education Authority through the Programme for Research in Third Level Institutions (PRTLI-3).

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