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Isolation and Characterization of a Gene Specific to Lager Brewing Yeast That Encodes a Branched-Chain Amino Acid Permease

YUKIKO KODAMA,* FUMIHIKO OMURA, AND TOSHIHIKO ASHIKARI

Institute for Fundamental Research, Suntory Research Center, Mishima-gun, Osaka 618-8503, Japan

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We found two types of branched-chain amino acid permease gene (*BAP2*) in the lager brewing yeast *Saccharomyces pastorianus* BH-225 and cloned one type of *BAP2* gene (Lg-*BAP2*), which is identical to that of *Saccharomyces bayanus* (by-*BAP2*-1). The other *BAP2* gene of the lager brewing yeast (cer-*BAP2*) is very similar to the *Saccharomyces cerevisiae BAP2* gene. This result substantiates the notion that lager brewing yeast is a hybrid of *S. cerevisiae* and *S. bayanus*. The amino acid sequence homology between *S. cerevisiae* Bap2p and Lg-Bap2p was 88%. The transcription of Lg-*BAP2* was not induced by the addition of leucine to the growth medium, while that of cer-*BAP2* was induced. The transcription of Lg-*BAP2* was repressed by the presence of ethanol and weak organic acid, while that of cer-*BAP2* was not affected by these compounds. Furthermore, Northern analysis during beer fermentation revealed that the transcription of Lg-*BAP2* was repressed at the beginning of the fermentation, while cer-*BAP2* was highly expressed throughout the fermentation. These results suggest that the transcription of Lg-*BAP2* is regulated differently from that of cer-*BAP2* in lager brewing yeasts.

Lager brewing yeasts (bottom-fermenting yeasts) were originally classified as *Saccharomyces carlsbergensis* (8) but have been recently reclassified as *Saccharomyces pastorianus*, which is thought to be a natural hybrid between *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (25). Tamai et al. (22) and Yamagishi and Ogata (27) reported that the genome of lager brewing yeasts consists of both types of chromosomes, those originating from *S. cerevisiae* and those from *S. bayanus*, indicating that the lager brewing yeasts have two types of allele, i.e., one that has its origin from *S. cerevisiae* and the other from *S. bayanus*. Fujii et al. (5) and Tamai et al. (23) reported that lager brewing yeasts contain two types of *ATF1* gene and *HO* gene, respectively, one similar to that of *S. cerevisiae* and the other identical to that of *S. bayanus*. On the other hand, it has been reported that lager brewing yeasts contain an *S. cerevisiae* type gene and a *Saccharomyces monacensis* type gene (1, 9, 10). The amino acid sequence homology between the *S. cerevisiae* type gene and the non-*S. cerevisiae* type gene in lager brewing yeasts is high (75 to 94%) (1, 5, 9, 10, 23). It is as yet unclear whether all the non-*S. cerevisiae* type genes found in lager brewing yeasts originated from the same species (e.g., *S. bayanus* or *S. monacensis*).

In brewing, transport of branched-chain amino acids (i.e., leucine, valine, and isoleucine) is very important, specifically because the metabolites of these compounds are converted to higher alcohols, which are some of the most important flavors in alcoholic beverages. The branched-chain amino acids are transported by at least four permeases, which are the general amino acid permease (Gap1p) (7), the branched-chain amino acid permeases (Bap2p and Bap3p) (2, 6), and the high-affinity tyrosine permease (Tat1p) (4). Gap1p can transport all naturally occurring amino acids, including citrulline and D-amino acids (7, 12), and is active during growth on poor nitrogen sources, such as proline. Gap1p activity is downregulated transcriptionally and posttranslationally in response to preferred nitrogen sources, such as glutamine, aspargine, and ammonia (21). Under these conditions, most of the branched-chain amino acids are transported by Bap2p, Bap3p, and Tat1p. The transcription of the branched-chain amino acid permease genes (*BAP2* and *BAP3*) is induced by some amino acids, such as leucine and phenylalanine, in the medium (2, 3), and this induction requires Ssy1p as a sensor for external amino acids (4).

We found that the constitutive expression of *BAP2* in a brewing yeast strain accelerated the rates of assimilation for branched-chain amino acids, while the disruption of *BAP2* did not affect assimilation rates for these amino acids during the brewing process (13). This suggests that there are possibly other functional permeases present during the brewing process. These could be Bap3p, Tat1p, and/or other branched-chain amino acid permease homologues, which exist in lager brewing yeasts.

In this paper, we report on the isolation and characterization of the non-*S. cerevisiae* type *BAP2* gene found in lager brewing yeast.

MATERIALS AND METHODS

Strains and media. Yeast strains used in this work are listed in Table 1. The *Escherichia coli* strain JM109 (*recA1* Δ[*lac-proAB*] *endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F′*traD36 proAB lacI*^{q}*Z* ΔM15) (28) (TOYOBO Co., Ltd.) served as the plasmid host. Growth and handling of *E. coli* bacteria, plasmids, and yeast strains followed standard procedures (18, 19). Yeast cells were grown at 30°C in yeast extract-peptone-dextrose (YPD) medium (18), YPM medium (1% yeast extract, 2% bacto-peptone, 2% maltose), or SD medium (2% glucose, 0.67% Yeast Nitrogen Base without amino acids; Difco). Yeast transformation was performed using the lithium acetate method (11). The selections for positive clones were carried out on either YPD plates supplemented with 300 μg of G418/ml, YPD plates supplemented with 10 mM formaldehyde, or YPD plates supplemented with 1 μg of aureobasidin A (Takara Shuzo Co., Ltd.)/ml. For the

* Corresponding author. Mailing address: Institute for Fundamental Research, Suntory Research Center, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-6503, Japan. Phone: 81-75-962-8802. Fax: 81-75-962-8262. E-mail: Yukiko_Kodama@suntory.co.jp.

TABLE 1. Yeast strains used in this study

Yeast species and strain	Source and remarks
S. pastrianus BH-225	Lager brewing yeast from our own stock
S. bayanus IFO1127	*S. bayanus* type strain from IFO[a]
S. cerevisiae X2180-1A	*MAT***a** *SUC2 mal mel gal2 CUP1* from YGSC[b]
S. cerevisiae YK006	*MAT***a** *SUC2 mal mel gal2 CUP1 gap1*::*AUR1-C*
S. cerevisiae YK007	*MAT***a** *SUC2 mal mel gal2 CUP1 gap1*::*AUR1-C ssy1*::*SFA1*
S. cerevisiae YK008	*MAT***a** *SUC2 mal mel gal2 CUP1 gap1*::*AUR1-C ssy1*::*SFA1* with a vector (pYCGPY)
S. cerevisiae YK009	*MAT***a** *SUC2 mal mel gal2 CUP1 gap1*::*AUR1-C ssy1*::*SFA1* with pYCGPYBP2 [*PYK1p-BAP2*]
S. cerevisiae YK010	*MAT***a** *SUC2 mal mel gal2 CUP1 gap1*::*AUR1-C ssy1*::*SFA1* with pYCGPYLgBP [*PYK1p*-Lg-*BAP2*]

[a] IFO, The Institute for Fermentation, Osaka, Japan.
[b] YGSC, Yeast Genetics Stock Center, University of California, Berkeley.

suppression of the growth defect of the Δ*gap1* Δ*ssy1* strain, SLD agar plates (0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate [Difco], 2% glucose, 0.1% leucine, 2% agar) were used. For the transcription analysis in poor nitrogen source, SPM medium (0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate [Difco], 2% maltose, 0.1% proline) was employed.

Southern analysis. Yeast genomic DNA was prepared according to the standard method (18). The genomic DNA was digested with appropriate restriction enzymes, fractionationed in a 1% agarose gel, and transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia, Buckinghamshire, United Kingdom). Labeling probe DNA, hybridization, membrane washing, and detection of hybridizing probe DNA were carried out with a Gene Images random-prime labeling and detection system (Amersham Pharmacia). The hybridization and washing temperature was 60°C, except in the case of low-stringency conditions (50°C).

Cloning and DNA sequencing of Lg-*BAP2*. Lg-*BAP2* was cloned by colony hybridization from *Spe*I libraries of the lager brewing yeast *S. pastorianus* BH-225 and *S. bayanus* IFO1127 constructed in pBlueScript SK(−) (20). An about 2.7-kb *Spe*I fragment was cloned from each strain and sequenced using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Construction of plasmids. The construction of plasmids for the disruption of the general amino acid permease gene (*GAP1*) (12) and amino acid sensor gene (*SSY1*) (4) was carried out using PCR techniques. The plasmids and oligonucle-

TABLE 2. Plasmids and oligonucleotides used in this study[a]

Name	Description or sequence
Plasmids	
pGAP1	1.9-kb fragment containing *GAP1* in pUC19
pTDH3-AUR1C	3.1-kb fragment containing *TDH3-AUR1-C* in pUC19
pΔGAP1	*gap1*::*TDH3-AUR1-C* in pUC19
pUC-SFA1	2.5-kb fragment containing *TDH3-SFA1* in pUC119
pUC-SSY1	1.4-kb fragment containing SSY1 (nucleotide positions −715 to −4 and 1817 to 2550) in pUC18
pΔSSY1	*ssy1*::*TDH3-SFA1* in pUC18
pYCGPY	Centromere-based vector with *TDH3-G418r*
pBAP2Sph	4.6-kb *Sph*I fragment containing *BAP2* in pUC18
pBAP2ES	4.6-kb *Sph*I/*Eco*47III fragment containing *BAP2* in pUC18
pBAP2ORF	2.0-kb *Sac*I/*Kpn*I fragment containing *BAP2* in pUC18
pYCGPYBP2	*BAP2* in pYCGPY
pYCGPYLgBP	Lg-*BAP2* in pYCGPY
Oligonucleotides	
001	5′CTCGAGCTCATGAGTAATACTTCTTCGT-3′
002	5′-CTCGGATCCCTTTAGATTAATGACGAGA-3′
003	5′-TCAGAGCTCGGTACCGGAGCTTACCAGTTCTCA-3′
004	5′-TCAAAGCTTCTGTTTATGTGTGTTTATTCG-3′
005	5′-AAGCTTAATGTCCGCCGCTACTGTTGG-3′
006	5′-GTCGACGTTGGTAGTTAGGAACAGGC-3′
007	5′-GGTACCGGAGCTTACCAGTTCTCACA-3′
008	5′-TAAAAGCTTCTGTTTATGTGTGTTT-3′
009	5′-GAATTCAGGAAAAACTCGAGAGTTACTAG-3′
010	5′-GGTACCTCAAGGAACTTCCCTATTTTTAAGC-3′
011	5′-GGTACCCAGTGTCGACAGGTTATTCTAGTCCTTGGGTTG-3′
012	5′-GGATCCAGGTAACCAACTTCTTCGCTCTT-3′
013	5′-GATCACCCGGGT-3′
014	5′-AAGCTTAGATCTTCAGAACTAAAAAAATAATAAGGAA-3′
015	5′-TCTAGAGCTCTGTGATGATGTTTTATTTGTTTTGATT-3′
016	5′-TCTAGAACAGGATCCTTGGTTGAACACGTTGCCAAG-3′
017	5′-GAATTCACTAGTGATCCGGAGCTTTCAATCAAT-3′
018	5′-GAGCTCTTTGAATGCCATTATCAGAAGAC-3′
019	5′-GCGGCCGCTAACGACTAGTGTCCGAACTTTA-3′

[a] The underlined nucleotides were added to generate restriction sites.

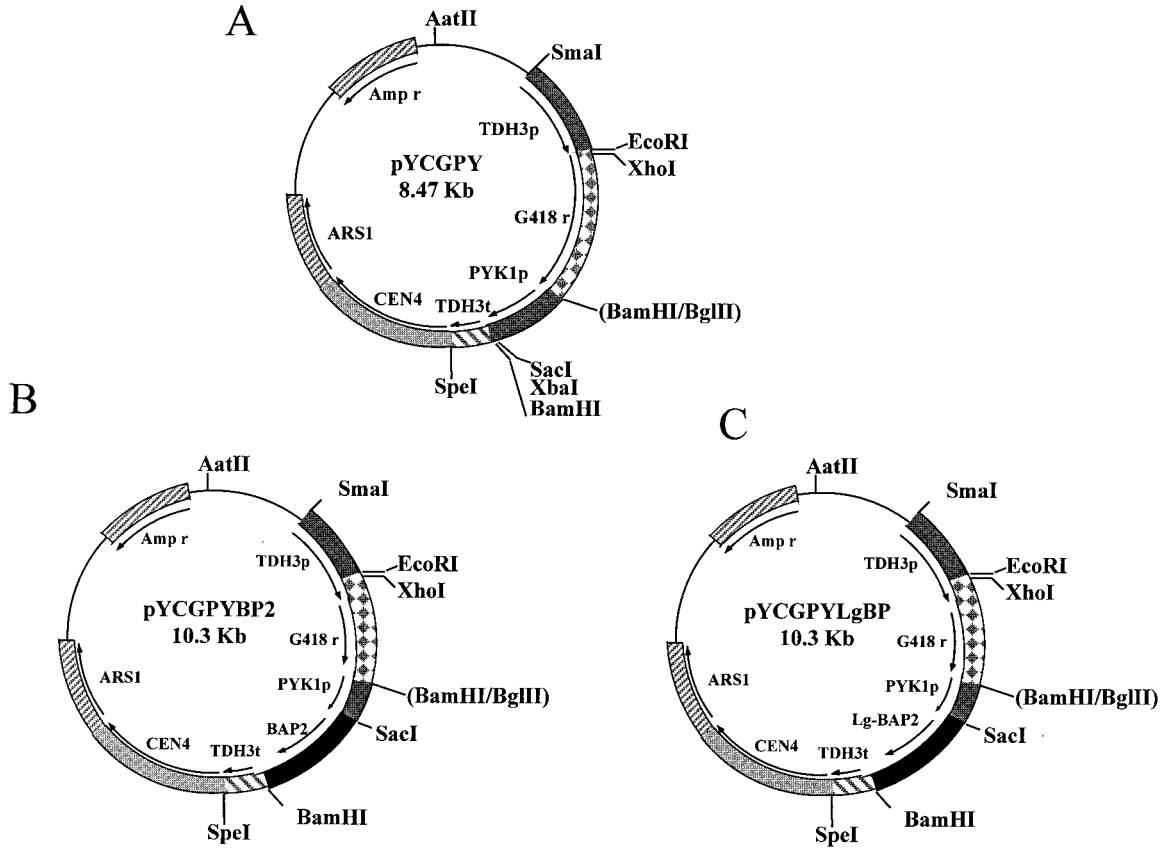

FIG. 1. The structures of the plasmids. (A) PYCGPY is a centromeric vector containing yeast centromere sequence (*CEN4*), yeast autonomously replicating sequence (*ARS1*), yeast glyceraldehyde-3-phosphate dehydrogenase promoter (*TDH3*p), the G418 resistance gene (G418r), the yeast pyruvate kinase promoter (*PYK1*p), the yeast glyceraldehyde-3-phosphate dehydrogenase terminator (*TDH3*t), and the ampicillin resistance gene (Amp^r). (B and C) *BAP2* and Lg-*BAP2* were inserted in the *Sac*I-*Bam*HI site of PYCGPY and named PYCGPYBP2 and PYCGPYLgBP, respectively.

otides used are listed in Table 2. Amplified PCR products were subcloned using the TOPO TA Cloning kit (Invitrogen) according to the supplier's instructions. The integrity of PCR fragments was verified by sequencing. The *GAP1* coding region was prepared by PCR with genomic DNA of X2180-1A as template using the oligonucleotides 001 and 002 as primers. After digestion with *Sac*I and *Bam*HI, the 1.9-kb *GAP1* open reading frame (ORF) was inserted into the *Sac*I-*Bam*HI gap of pUC19 (28) to yield pGAP1. The yeast glyceraldehyde-3-phosphate dehydrogenase (*TDH3*) promoter was amplified from pIGZ2 (14) by PCR using the oligonucleotides 003 and 004 as primers. After digestion with *Sac*I and *Hin*dIII, the *TDH3* promoter was ligated with the 2.0-kb *Hin*dIII-*Sal*I fragment encoding *AUR1-C* (aureobasidin A resistance gene) prepared from pAUR112 (Takara Shuzo Co., Ltd.), and inserted into the *Sac*I-*Sal*I gap of pUC19 to yield pTDH3-AUR1C. The plasmid pTDH3-AUR1C was digested with *Xba*I, repaired, and resealed with phosphorylated *Bgl*II linkers. The 3.1-kb *Kpn*I-*Bgl*II *TDH3-AUR1-C* fragment was excised and inserted into the *Kpn*I-*Bgl*II gap of pGAP1 to give pΔGAP1. Plasmid pΔGAP1 was linearized at *Sac*I and *Xba*I sites before transformation of yeast cells.

A formaldehyde resistance gene, *SFA1* (24) of *S. cerevisiae*, was used as a dominant selective marker in the construction of the Δ*ssy1* strain. The *SFA1* gene fragment (nucleotide positions −1 to 1401) was prepared by PCR with genomic DNA of X2180-1A as a template using the oligonucleotides 005 and 006 as primers. The oligonucleotide primers 007 and 008 were used for amplification of the *TDH3* promoter as described above. The resultant 1.4-kb *Hin*dIII-*Sal*I fragment for *SFA1* and 1.1-kb *Kpn*I-*Hin*dIII fragment for the promoter sequence were ligated together into the *Kpn*I and *Sal*I sites of the pUC119 vector (26) to give pUC-SFA1. The 0.7-kb *SSY1* 5′-flanking region (nucleotide positions −715 to −4) was obtained by PCR with genomic DNA of X2180-1A as a template by using the oligonucleotide primers 009 and 010. The 0.7-kb *SSY1* 3′-flanking region (nucleotide positions 1817 to 2550) was obtained in the same way, using the oligonucleotide primers 011 and 012. The 5′-flanking fragment was digested with *Eco*RI and *Kpn*I, and the 3′-flanking fragment was digested with *Kpn*I and *Bam*HI. The resultant fragments were ligated together into the *Bam*HI and *Eco*RI sites of the pUC18 vector (28), in which the original *Sal*I site had been eliminated. The resulting plasmid was designated pUC-SSY1. To construct pΔSSY1, the 2.5-kb *Kpn*I-*Sal*I fragment was excised from pUC-SFA1 and inserted into the *Kpn*I-*Sal*I gap of pUC-SSY1. pΔSSY1 was digested with *Eco*RI and *Bam*HI prior to the transformation of YK006 (Δ*gap1*) to obtain a Δ*gap1* Δ*ssy1* double disruptant (YK007).

The plasmid pYCGPY (Fig. 1A) is a centromeric vector that allows expression of genes placed downstream of the yeast pyruvate kinase (*PYK1*) promoter and contains the kanamycin resistance (G418^r) gene (15) for use as a selective marker. The G418^r gene embraced by the *TDH3* promoter and terminator sequences was prepared as a 2.5-kb *Bam*HI fragment from pIGZ2 (14) and cloned into the *Bam*HI site of YCp50 (17) to give YCpG418^r. YCpG418^r was partially digested with *Bam*HI and self-ligated with a phosphorylated oligonucleotide, 013, to eliminate one of the two *Bam*HI sites present in YCpG418^r. The resulting plasmid (YCpG418^{r}Sma) was then digested with *Aat*II and *Bam*HI to release a 2.9-kb *Aat*II-*Bam*HI fragment. To obtain the *PYK1* promoter region (nucleotide positions −798 to −1), PCR was carried out with the oligonucleotides 014 and 015 as primers and genomic DNA of X2180-1A as the PCR template. The *TDH3* terminator region was amplified from plasmid pIGZ2 by PCR, with the oligonucleotides 016 and 017 as primers. The 0.8-kb *Hin*dIII-*Xba*I *PYK1* promoter fragment and the 170-bp *Xba*I-*Eco*RI *TDH3* terminator fragment were coligated into the *Hin*dIII-*Eco*RI gap of pUC19 to create pPTPYK1. The 1.0-kb *Bgl*II-*Spe*I fragment was then excised from pPTPYK1 and ligated with

the 2.9-kb *Aat*II-*Bam*HI fragment from YCpG418^{s}Sma and the 4.6-kb *Spe*I-*Aat*II fragment from YCp50 to obtain pYCGPY.

A 9.0-kb DNA fragment containing the *BAP2* locus was isolated from a genomic library derived from *S. cerevisiae* (strain X2180-1A). The 4.6-kb *Sph*I fragment containing the *BAP2* ORF was excised and subcloned into the *Sph*I gap of pUC18 to give pBAP2Sph. pBAP2Sph was digested with *Sma*I and *Eco*47III and resealed to give pBAP2ES. The 5′-flanking region was trimmed with exonuclease III, and a 500-bp *Sac*I-*Pst*I fragment encompassing the 60-bp 5′-flanking region and part of the *BAP2* ORF was prepared and ligated together with a 1.5-kb *Pst*I-*Kpn*I fragment from pBAP2ES into the *Sac*I-*Kpn*I gap of pUC18 to give pBAP2ORF. Eventually the *Sac*I-*Bam*HI fragment from pBAP2ORF, containing the *BAP2* ORF, was inserted into the *Sac*I-*Bam*HI gap of pYCGPY, thus creating pYCGPYBP2 (Fig. 1B), in which the *BAP2* gene is controlled by the constitutive *PYK1* promoter. The Lg-*BAP2* ORF was prepared as a *Sac*I-*Bam*HI fragment by PCR with genomic DNA of the brewing yeast BH-225 as a template using the oligonucleotides 018 and 019 as primers. The fragment of Lg-*BAP2* ORF was inserted into the *Sac*I and *Bam*HI sites of pYCGPY and placed under control of the constitutive *PYK1* promoter, to yield pYCGPYLgBP (Fig. 1C).

Northern analysis. Total RNA was prepared according to the standard method (18). Agarose gel electrophoresis was carried out with 45 μg of RNA per lane, and subsequent Northern analysis was performed with ^{32}P-labeled DNA fragments as probes. After hybridization, the blot was washed twice with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as described previously (18). This condition is sufficiently stringent to distinguish the transcripts of cer-*BAP2* and *Lg-BAP2* in lager brewing yeast BH-225 using a *BAP2* fragment (nucleotide positions +180 to +891) isolated from *S. cerevisiae* X2180-1A and an Lg-*BAP2* fragment (+1 to +1300) from BH-225 as probes. For the detection of other genes, approximately 700-bp PCR products from genomic DNA of X2180-1A were used as probes.

Fermentation conditions. The fermentation was performed in a 2-liter fermentation tube. The initial wort gravity was 12% (wt/vol) prepared with 100% malt. The fermentation was carried out at 12°C with a pitching rate of 15×10^6 cells/ml of wort and a dissolved oxygen content of 9 ppm.

Nucleotide sequence accession numbers. The nucleotide sequence data of Lg-*BAP2* from lager brewing yeast BH-225 and by-*BAP2*-1 from *S. bayanus* IFO1127 have been deposited with DDBJ under the accession numbers AB049008 and AB049009, respectively.

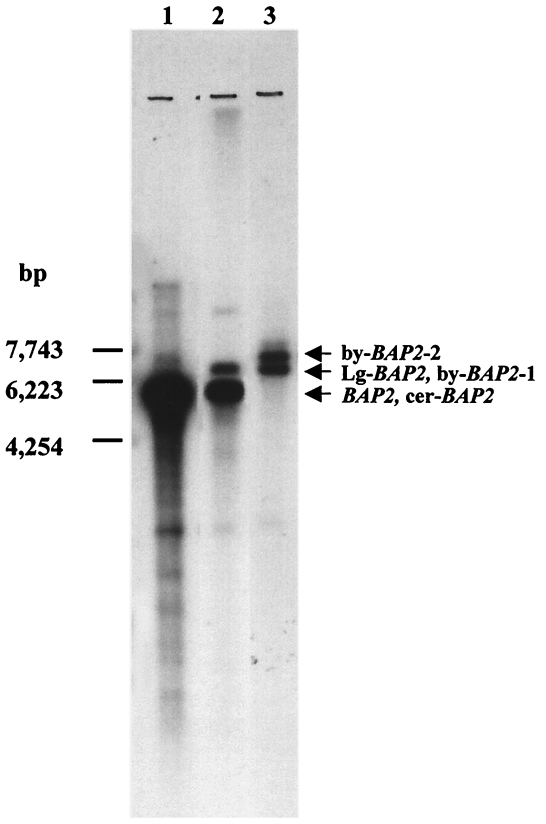

FIG. 2. Genomic Southern hybridization with *S. cerevisiae BAP2* probe (nucleotides 180 to 891). Genomic DNA was digested with *Xba*I. Lane 1, *S. cerevisiae* X2180-1A; lane 2, lager brewing yeast BH-225; lane 3, *S. bayanus* IFO1127.

RESULTS

Southern analysis of *BAP2* in lager brewing yeast, *S. cerevisiae*, and *S. bayanus*. To investigate the existence of the non-*S. cerevisiae*-type of *BAP2* in a lager brewing yeast, low-stringency (50°C) Southern analysis of yeast genomic DNAs from *S. cerevisiae* X2180-1A, lager brewing yeast strain BH-225, and *S. bayanus* IFO1127 was carried out using a fragment of the *BAP2* gene (nucleotide positions +180 to +891) from *S. cerevisiae* X2180-1A as a probe. A 5.5-kb DNA fragment from *S. cerevisiae* X2180-1A hybridized to this probe (Fig. 2, lane 1), while two fragments from *S. bayanus* IFO1127 hybridized to this probe (Fig. 2, lane 3). Lager brewing yeast strain BH-225 showed two fragments, one identical in size to that of *S. cerevisiae* and the other identical to one of the two bands of *S. bayanus* IFO1127 (Fig. 2, lane 2). This result suggests that the lager brewing yeast BH-225 possesses two *BAP2* genes, one similar to that of *S. cerevisiae* and the other similar to that of *S. bayanus*. As shown in Fig. 2, we named the *S. cerevisiae* type *BAP2* gene and the non-*S. cerevisiae* type *BAP2* homologue in BH-225 cer-*BAP2* and Lg-*BAP2*, respectively, and we named the two *BAP2* homologues in *S. bayanus* IFO1127 by-*BAP2*-1 and by-*BAP2*-2, respectively. A DNA fragment encompassing the 5′-flanking region and the open reading frame of cer-*BAP2* (nucleotide positions −886 to +1827) in BH-225 was isolated by PCR using chromosomal DNA of BH-225 as a template. The sequence similarity between the isolated fragment and the *BAP2* sequence in the *Saccharomyces* Genome Database (SGD) (http://genome-www.stanford.edu/Saccharomyces) was proven to be 99.3% (data not shown).

Cloning of the *BAP2* homologue from lager brewing yeast and *S. bayanus*. For the cloning of the non-*S. cerevisiae* type *BAP2* homologue (Lg-*BAP2*) from lager brewing yeast, we obtained a 1.3-kb PCR fragment of Lg-*BAP2* from lager brewing yeast BH-225 by using primers designed on the *BAP2* sequence. The DNA homology between the *BAP2* sequence in SGD and Lg-*BAP2* in this region was about 80% (data not shown). Southern analysis was carried out using this PCR fragment of Lg-*BAP2* as a probe. As shown in Fig. 3A, this fragment did not hybridize to X2180-1A (lane 1 and lane 4). Lager brewing yeast BH-225 showed one fragment that hybridized with this probe, which was similar in size to one of the two fragments of *S. bayanus* IFO1127 (lanes 2 and lane 5). *S. bayanus* IFO1127 showed two fragments that hybridized with this probe, one identical in size to that of Lg-*BAP2* of BH-225 (by-*BAP2*-1) and another one (by-*BAP2*-2) (lane 3 and lane 6). The hybridization intensity of by-*BAP2*-1 with the probe (Lg-*BAP2*) was higher than that of by-*BAP2*-2, suggesting that the DNA homology between by-*BAP2*-1 and Lg-*BAP2* is higher than that between by-*BAP2*-2 and Lg-*BAP2*.

Further Southern blot analysis indicated that Lg-*BAP2* from BH-225 and by-*BAP2*-1 from IFO1127 could be cloned as approximately 2.7-kb *Spe*I fragments (Fig. 3B). Thus, *Spe*I-libraries of genomic DNA of BH-225 and IFO1127 were con-

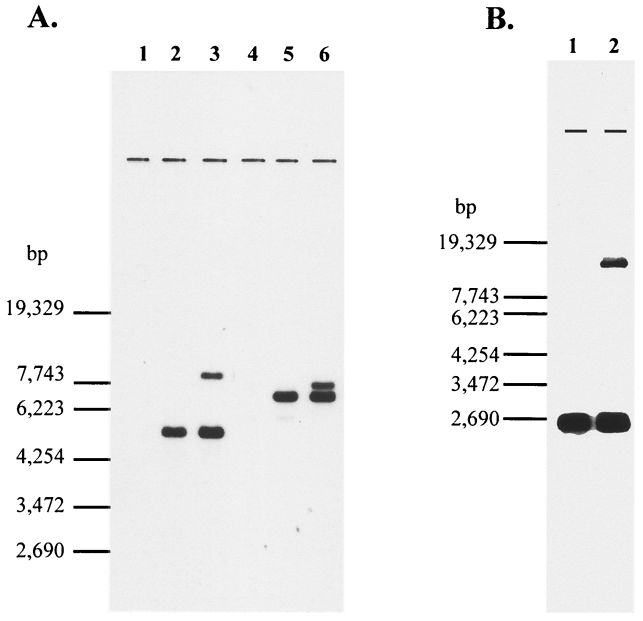

FIG. 3. Genomic Southern hybridization with the Lg-*BAP2* probe. (A) Genomic DNA was digested with *Eco*RV (lanes 1 to 3) and with *Xba*I (lanes 4 to 6). Lane 1 and lane 4, *S. cerevisiae* X2180-1A; lane 2 and lane 5, lager brewing yeast BH-225; lane 3 and lane 6, *S. bayanus* IFO1127. (B) Genomic DNA was digested with *Spe*I. Lane 1, lager brewing yeast BH-225; lane 2, *S. bayanus* IFO1127.

structed in pBlueScript SK(−) and the clones containing the Lg-*BAP2* or by-*BAP2*-1 were isolated by colony hybridization, using the PCR fragment of Lg-*BAP2* as a probe. The *Spe*I fragment of approximately 2.7 kb in size which contains the whole ORF and 5′-flanking region (about 800 bp) was obtained from each strain. From the results of DNA sequencing, it was revealed that the sequence of Lg-*BAP2* from BH-225 was 100% identical to that of by-*BAP2*-1 from *S. bayanus* IFO1127. DNA homology of the 5′-flanking region (about 800 bp) and the open reading frame between the *BAP2* sequence in SGD and Lg-*BAP2* was 60 and 80%, respectively. The amino acid sequence comparison between Bap2p from SGD and Lg-Bap2p from BH-225 is shown in Fig. 4. Deduced amino acid sequence homology between Bap2p and Lg-Bap2p was 88%. This homology seems to be comparable with previously reported homology between lager brewing yeast-specific proteins and their *S. cerevisiae* counterparts: Met2p (94%), Ilv1p (95.7%), Ilv2p (92.3%), partial Ura3p (93%) (9) and Atf1p (75.7%) (5).

There are some reports regarding the chromosomal structure of lager brewing yeasts (22, 27). In these reports, it is shown that lager brewing yeasts have chromosomes that are a mix of those from *S. cerevisiae* and from *S. bayanus*. From the results of pulsed-field electrophoresis and subsequent Southern analysis, the sizes of chromosomes carrying Lg-*BAP2* and by-*BAP2*-1 in the lager brewing yeast BH-225 and *S. bayanus* IFO1127, respectively, were identical (*S. bayanus* chromosome 12). Additionally, the sizes of chromosomes harboring cer-*BAP2* and *BAP2* in BH-225 and *S. cerevisiae* X2180-1A, respectively, were identical (*S. cerevisiae* chromosome II) (data not shown).

Function of Lg-Bap2p as a branched-chain amino acid permease. Branched-chain amino acids are transported by at least four transporters, which are the general amino acid permease (Gap1p) and high- and low-affinity transporters specific for branched-chain amino acids (Bap2p, Bap3p, and Tat1p). Didion et al. (4) showed that deletion of the gene for amino acid sensor *SSY1* in a Δ*gap1* strain abolishes branched-chain amino acid uptake to an extent similar to that of the Δ*gap1* Δ*bap2* Δ*bap3* Δ*tat1* strain. To confirm the function of Lg-Bap2p as a branched-chain amino acid permease, we attempted the constitutive expression of Lg-*BAP2* in YK007 (Δ*gap1* Δ*ssy1*) and investigated the complementation of the growth defect of YK007 on SLD agar plates, which contained leucine as the sole nitrogen source. Lg-*BAP2* could complement the growth defect of YK007 on SLD agar plates as well as *BAP2* (Fig. 5). These results indicate that Lg-Bap2p, as well as Bap2p, is functional as a leucine transporter.

The expression profile of branched-chain amino acid permease genes in lager brewing yeast BH-225. Since DNA homology of the promoter region between *BAP2* in SGD and Lg-*BAP2* from lager brewing yeast BH-225 was rather low, we anticipated that cer-*BAP2* and Lg-*BAP2* in BH-225 are differently regulated. As it is reported that transcription of the branched-chain amino acid permease genes (*BAP2* and *BAP3*) is induced by several amino acids, especially by leucine (2, 3), we investigated the transcriptional induction of cer-*BAP2* and Lg-*BAP2* in BH-225 in response to leucine addition. The transcription of cer-*BAP2* (detected with a *BAP2* probe) was induced by leucine 30 min after addition, while the transcription of Lg-*BAP2* was not induced (Fig. 6).

Furthermore, we investigated the change in the transcription levels of cer-*BAP2* and Lg-*BAP2* in response to nitrogen starvation, because brewing yeast cells undergo the nitrogen starvation during the latter period of fermentation. The cells were transferred from amino acid-rich medium (YPM) to nitrogen-poor medium (SPM), and the mRNA level was analyzed (Fig. 7A). The transcription levels of both cer-*BAP2* (detected with the *BAP2* probe) and Lg-*BAP2* did not change after transfer to nitrogen-poor medium, while the transcription of *DUR1* (urea amidolyase gene, inducible in response to nitrogen starvation) was induced after transfer to nitrogen-poor medium.

We also investigated the transcription of cer-*BAP2* and Lg-*BAP2* in response to various stresses, because brewing yeast cells are put under such stresses as a high concentration of alcohol, low pH, and osmotic stress due to a high concentration of sugars during beer fermentation. Some stress-inducible genes, such as a heat-shock-protein-encoding gene, *HSP30*, are induced in the latter period of fermentation (data not shown). As shown in Fig. 7B, the transcription of Lg-*BAP2* was repressed when cells were treated with ethanol and weak organic acid (1 mM sorbate), while other treatment, such as osmotic stress (27% maltose) and heat shock (37°C), did not affect its transcription. Conversely, the transcription of cer-*BAP2* (detected with the *BAP2* probe) was not affected by any of these treatments. The control gene for stress induction, *HSP30*, was induced when cells were treated with ethanol, weak organic acid, and heat shock.

Finally, we investigated the transcription of cer-*BAP2* and Lg-*BAP2* during beer fermentation by Northern analysis. The transcription level of Lg-*BAP2* was rather low at the beginning

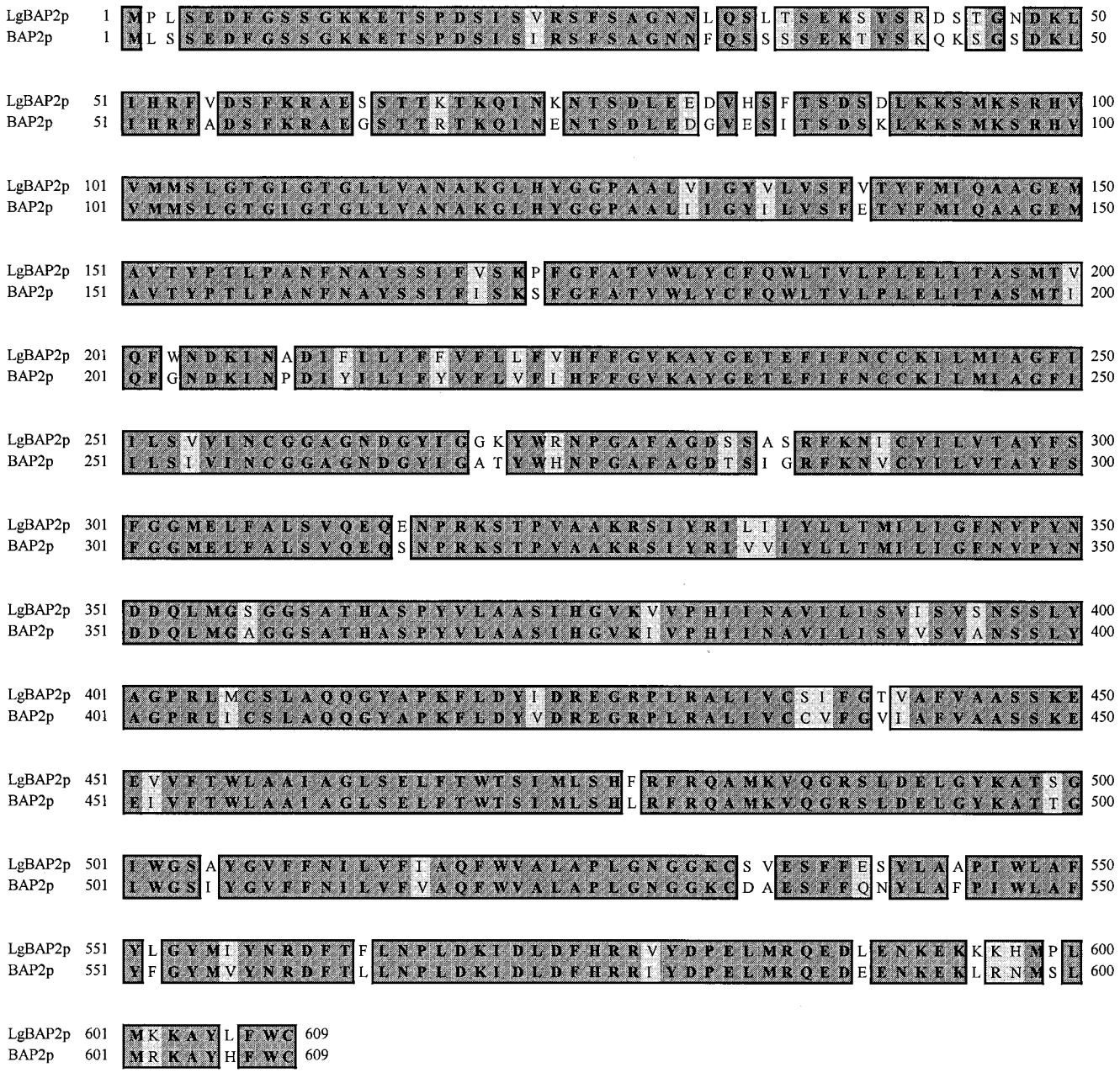

FIG. 4. Amino acid sequence homology between the Lg-Bap2 protein of the lager brewing yeast BH-225 and the Bap2 protein of *S. cerevisiae*. The numbers indicate the amino acid positions. Amino acid sequence identities between two proteins are shaded.

of the fermentation period, while cer-*BAP2* (detected with the *BAP2* probe) was highly expressed throughout the fermentation (Fig. 8).

DISCUSSION

We have been investigating the transport of branched-chain amino acids during the brewing process, specifically because metabolic regulation of these compounds is important in the flavor control of alcohol beverages.

In this report, we investigated the branched-chain amino acid permease genes in a lager brewing yeast and found that it has two divergent *BAP2* genes, one similar to that of *S. cerevisiae* and the other similar to that of *S. bayanus*. We have cloned the non-*S. cerevisiae* type *BAP2* homologue from a brewing yeast, BH-225 (Lg-*BAP2*), and another *BAP2* homologue from *S. bayanus* IFO1127 (by-*BAP2*-1), and found that they were 100% identical to each other. This result substantiates the notion that lager brewing yeast is a hybrid between *S. cerevisiae* and *S. bayanus*.

The results of Southern blot analysis revealed that *S. bayanus* IFO1127 has another *BAP2* homologue (by-*BAP2*-2), which is different from Lg-*BAP2* and by-*BAP2*-1 (Fig. 2 and 3). We have found that *Saccharomyces uvarum* IFO 0615 (type

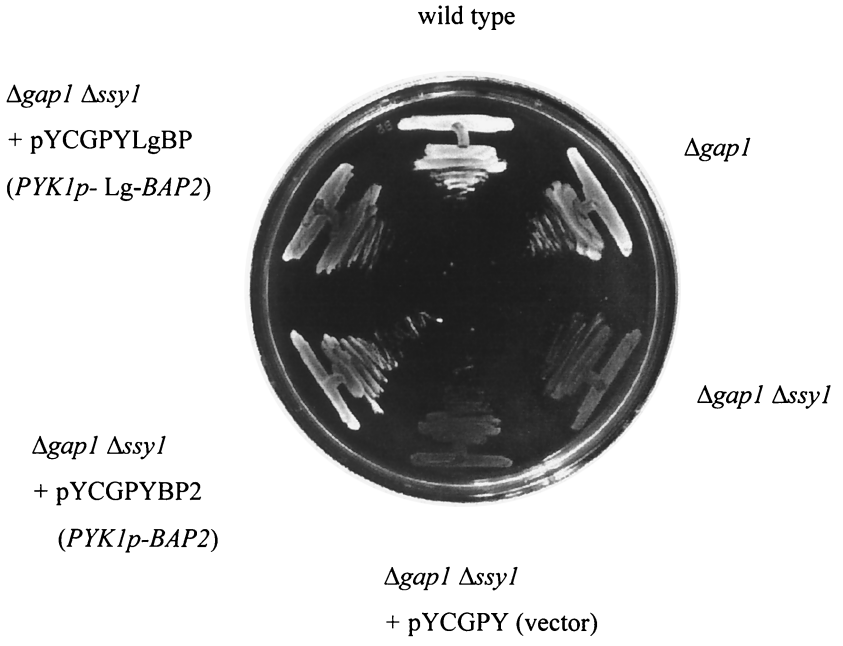

FIG. 5. Growth phenotypes of strains X2180-1A (wild type), YK006 (Δ*gap1*), YK007 (Δ*gap1* Δ*ssy1*), YK008 (Δ*gap1* Δ*ssy1*, pYCGPY [vector]), YK009 (Δ*gap1* Δ*ssy1*, pYCGPYBP2 [*PYK1p-BAP2*]), and YK010 (Δ*gap1* Δ*ssy1*, pYCGPYLgBP [BP*PYK1p*-Lg-*BAP2*]) after 3 days of growth on an SLD agar plate which contained leucine as the sole nitrogen source.

strain) showed a fragment that hybridized with the Lg-*BAP2* probe, which is similar in size to by-*BAP2*-2 (data not shown). It suggests that IFO1127 could be a hybrid between *S. bayanus* and *S. uvarum*. Rainieri et al. (16) have reported that the type strain of *S. bayanus* and other strains that have been classified as *S. bayanus* lack homogeneity and have hypothesized that they are natural hybrids. Our results may support this hypothesis.

The transcription analysis revealed that Lg-*BAP2* is regulated differently from cer-*BAP2* in the brewing yeast. The former is not induced by the addition of leucine, whereas the latter is. The *BAP2* promoter harbors putative binding sites for Gen4p and Leu3p (6), which also exist in the Lg-*BAP2* promoter, but it has been shown that these sites are not involved in the transcriptional induction of *BAP2* by leucine (3). De Boer et al. (2) showed that a portion of the *BAP3* promoter (from −418 to −392 relative to the ATG start codon [UAS_{aa}]) is necessary and sufficient for the induction of *BAP3* transcription by amino acids. The element found in the *BAP2* promoter (nucleotide positions −417 to −400) is very similar to the UAS_{aa} of the *BAP3* promoter, while the corresponding region

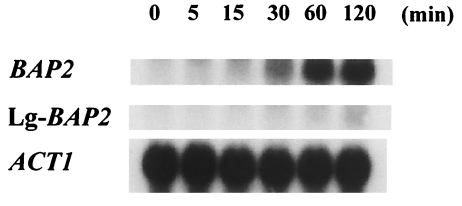

FIG. 6. The transcription of *BAP2* homologues in the lager brewing yeast BH-225 after addition of leucine was analyzed by Northern blotting. Cells were pregrown overnight on SD medium at 30°C. From these precultures, main cultures were inoculated at an optical density at 600 nm of 0.5 in fresh SD medium and grown subsequently to an optical density at 600 nm of 0.65 (for 4 h) at 30°C. Then, leucine was added to a final concentration of 2 mM. Total RNA was isolated at different time points after leucine addition and hybridized with *BAP2*, Lg-*BAP2*, and *ACT1* as probes. *ACT1* was used as a loading control.

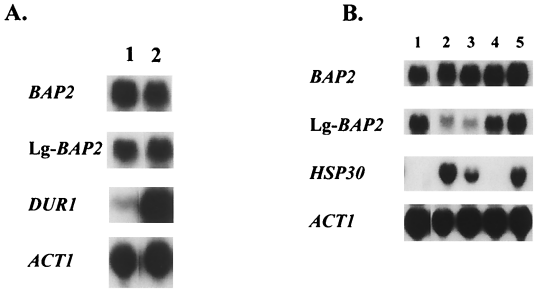

FIG. 7. (A) The transcription of *BAP2* homologues in the lager brewing yeast BH-225 in response to nitrogen starvation was analyzed by Northern blotting. Total RNA was isolated after cultivation for 4 h in YPM medium (lane 1) and after transfer to SPM medium and was cultivated for 2 h (lane 2) and hybridized with *BAP2*, Lg-*BAP2*, *DUR1*, and *ACT1* as probes. The blot that was hybridized with Lg-*BAP2* probe was overexposed for comparison of the transcriptional level in these conditions. *ACT1* was used as a loading control, and *DUR1* was used as a control nitrogen starvation-induced gene. (B) The transcription of *BAP2* homologues in the lager brewing yeast BH-225 in response to various stresses was analyzed by Northern blotting. Total RNA was isolated after incubation in YPM medium at 30°C (lane 1); in YPM medium containing 8% ethanol at 30°C (lane 2); in YPM medium containing 1 mM sorbate (pH 4.5) at 30°C (lane 3); in YPM medium containing 27% maltose at 30°C (lane 4); and in YPM medium at 37°C (lane 5) and hybridized with *BAP2*, Lg-*BAP2*, *HSP30*, and *ACT1* as probes. *ACT1* was used as a loading control, and *HSP30* was used as a control stress-induced gene.

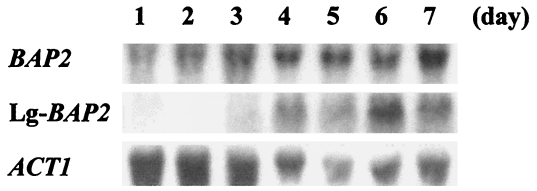

FIG. 8. The transcription of *BAP2* homologues in the lager brewing yeast BH-225 during wort fermentation was analyzed by Northern blotting. Total RNA was isolated after 1, 2, 3, 4, 5, 6, and 7 days of the fermentation period and hybridized with *BAP2*, Lg-*BAP2*, and *ACT1* as probes. *ACT1* was used as a loading control.

of the Lg-*BAP2* promoter is rather different (data not shown). This result supports the hypothesis that the UAS_{aa} is necessary for the induction of transcription by leucine. Since the wort prepared with 100% malt contains about 1 to 2 mM leucine, the difference of the transcription level of cer-*BAP2* and that of Lg-*BAP2* in the beginning of the fermentation period may be due to the difference of these genes in responsiveness to leucine.

The transcription of Lg-*BAP2* seemed to be induced in the latter period of fermentation. In this period, most amino acids are exhausted and yeast cells are exposed to nitrogen starvation. However, the transcription of Lg-*BAP2* was not induced in a poor nitrogen source, suggesting that the induction of Lg-*BAP2* transcription in the latter period of fermentation is not due to nitrogen starvation. Yeast cells are also put under a lot of stress during fermentation. The concentration of alcohol increases and wort pH decreases in the latter period of fermentation. However, since the transcription of Lg-*BAP2* was repressed in the presence of alcohol and weak acid (Fig. 7B), the induction of Lg-*BAP2* transcription in the latter period of fermentation is not due to these stresses.

The mechanisms that differentiate the transcription profiles of cer-*BAP2* and Lg-*BAP2* during fermentation are expected to be very complicated because there are likely a lot of factors which could affect the transcription of these two genes. One of them could be the leucine concentration in wort. Further investigations are under way to clarify the factors involved in the distinct regulation of cer-*BAP2* and Lg-*BAP2*. It is our aim to determine which phenotypes of lager brewing yeast are mainly attributed to the genes derived from non-*S. cerevisiae* type chromosomes. We hope that the identification and analysis of lager brewing yeast-specific genes that are essential for beer fermentation will help us improve the quality of beer production.

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