A Structural and Phylogenetic Study of the HO Gene from Saccharomyces bayanus var. uvarum

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A novel HO gene (Uv-HO) was cloned from the Saccharomyces bayanus var. uvarum (abbreviated as S. uvarum in this study) type strain. The coding region of Uv-HO showed relatively high homology (95%) to that of the Sb-HO gene (S. bayanus var. bayanus HO), but not to the HO genes of other Saccharomyces sensu stricto species. However, the 5′ and 3′ non-coding region of Uv-HO showed less similarity (79% and 76% respectively) even to those of the most homologous gene Sb-HO. Motifs of the mating-type control and the cell-cycle control were conserved in the 5′ non-coding region of Uv-HO, but numbers and positions of motifs were different from those of Sb-HO. CHEF-Southern analysis showed that all tested strains of S. bayanus species, including S. uvarum, carried the HO gene on the 1,100-kb chromosome. By HO-typing PCR using mixed primers, which provided a rapid and convenient tool for yeast identification, either the Uv-HO gene or the Sb-HO gene was detected in strains of S. bayanus species, but two strains were found to have both types of HO gene in each genome. These results suggest that S. uvarum has a unique sequence, but might share the same chromosome constitution within S. bayanus species, and that S. bayanus is a heterogeneous species, of which some strains might be natural hybrid.

Key words: Saccharomyces uvarum; Saccharomyces bayanus; HO-endonuclease; HO gene; Saccharomyces sensu stricto

Saccharomyces sensu stricto species are classified into seven sibling species: S. bayanus, S. cariocanus, S. cerevisiae, S. kudriavzevii, S. mikatae, S. pastorianus, and S. paradoxus.1,2 DNA-DNA re-association analysis shows a low relationship between S. cerevisiae and S. bayanus, but S. pastorianus shows an intermediate re-association value with both species. Therefore S. pastorianus is considered to be a natural hybrid of S. bayanus and S. cerevisiae. Recently it was proposed that S. bayanus species can be divided into two sub-group, S. bayanus var. bayanus and S. bayanus var. uvarum (abbreviated as S. uvarum hereafter) by molecular and genetic study.3)

All species belonging to Saccharomyces sensu stricto have characteristic mating-types (a and α) and life cycles (homothallism and heterothallism). In the homothallic strain, mating-type interconversion results in translocation of genetic information from the silent loci HMLα or HMRα to the MAT locus. A site-specific endonuclease encoded by the HO gene generates a double-strand break at the recognition site in the MAT locus to initiate substitution between the silent loci HML or HMR and the MAT locus.5) In the heterothallic strains, a mutation in the HO gene prevents mating-type interconversion between the MAT locus and the silent loci.5) Since the HO gene has the important role in deciding the life cycle, many studies has been reported elucidating the function and regulation of the HO gene in mating-type interconversion.5) We carried out a structural and phylogenetic study of the HO gene because HO is thought to be conserved but to be a characteristic gene among species of Saccharomyces sensu stricto.

Russell et al. cloned the HO gene (Lab-HO) from S. cerevisiae, used in genetic studies, and reported the nucleotide sequence.6) We reported that S. pastorianus had both of the S. cerevisiae-type HO gene (Sc-HO) and the S. pastorianus specific HO gene (Lg-HO). The nucleotide sequences of the Sc-HO gene and the Lg-HO gene were highly homologous to those of the Lab-HO gene and the Sb-HO gene (S. bayanus var. bayanus HO) respectively.7) When chromosomal DNA from type strains and industrial strains were digested with EcoRI, Southern blot analysis showed that a 7-kb band of

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**Materials and Methods**

**Strains and media.** The yeast strains used in this study are listed in Table 1. Media and culture condition were as described previously by Rose et al.8)

**Transformation.** E. coli transformation was carried out using frozen competent cells DH5α (Toyobo, Tokyo, Japan). Yeast transformation was carried out according to the electroporation procedure.9)

**DNA cloning.** S. uvarum type strain NBRC0615 was cultured at 30°C overnight in 100 ml YEPD medium.

DNA was extracted by the method of Rose and Broach.10) Chromosomal DNA was partially digested with Sau3AI and fractionated by the 5–20% sucrose gradient ultracentrifugation. DNA fractions containing from 10 to 15-kb were pooled, precipitated with ethanol, and ligated to the dephosphorylated BamHI site of pUC118. Clones carrying the HO gene were detected by colony hybridization.11)

**DNA manipulations and DNA sequencing.** DNA digestion with restriction enzymes, standard agarose gel electrophoresis, and recovery of DNA from agarose gel were carried out by standard methods.11) DNA sequencing and analysis were described previously.7) Small-scale chromosomal DNA extraction from yeast cells was carried out according to the method described by Hereford et al.12)

**Polymerase chain reaction for amplification of the HO gene.** To detect different types of HO gene from yeast strains, DNA carrying the HO gene fragment was amplified with Taq™ (TakaraBio, Tokyo, Japan) using chromosomal DNA as a template. A 22-mer common primer, P2-1/2 (5’-GCTAA(T/C)TCCTACGTTATGT-GCG-3’; positions 67–88 of ORF), was designed on a highly conserved sequence among the Sc-HO, Lg-HO, and Uv-HO genes. 20-mer primers P3 (5’-CATCAAA-CTGTAAGATTCCG-3’; positions 1704–1685 of ORF) for Sc-HO, 17-mer P4 (5’-GCTTCTCACCATCGAGC-3’; positions 925–909 of ORF) for Lg-HO, and 18-mer P5 (5’-TAAAACACCGTTCCCAGT-3’; positions 582–565 of ORF) for Uv-HO were designed on the specific

**Table 1. Yeast Strains Used in This Study**

<table>
<thead>
<tr>
<th>Species and strain designation</th>
<th>Source and CBS number</th>
<th>Remark or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td>X2180-1A&lt;br&gt;S288C&lt;br&gt;TD4</td>
<td></td>
</tr>
<tr>
<td><strong>S. paradoxus</strong></td>
<td>NBRC 10609/CBS432</td>
<td>Type strain</td>
</tr>
<tr>
<td><strong>S. pastorianus</strong></td>
<td>NBRC 1167/CBS1513</td>
<td>Type strain</td>
</tr>
<tr>
<td><strong>S. bayanus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synonyms of S. bayanus</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. inusitatus</em></td>
<td>NBRC 10563/CBS1546</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. heterogenicus</em></td>
<td>NBRC 1620/CBS425</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. uvarum</em></td>
<td>NBRC 0615/CBS395</td>
<td>Type strain</td>
</tr>
<tr>
<td><em>S. globosus</em></td>
<td>NBRC 10557/CBS424</td>
<td>Type strain</td>
</tr>
<tr>
<td>Industrial strains and a natural isolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wine yeast</td>
<td>NBRC 0613</td>
<td></td>
</tr>
<tr>
<td>Wine yeast</td>
<td>NBRC 0676</td>
<td></td>
</tr>
<tr>
<td>Wine yeast</td>
<td>KY702</td>
<td>our stock culture</td>
</tr>
<tr>
<td>Beer yeast</td>
<td>NBRC 2031</td>
<td></td>
</tr>
<tr>
<td>Brewing contaminant</td>
<td>NBRC 1948</td>
<td></td>
</tr>
</tbody>
</table>

NOBRC, NITE-BRC: National Institute of Technology and Evaluation, Biological Resource Center, Chiba, Japan (http://www.nbrc.nite.go.jp/)

CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (http://www.cbs.knaw.nl)
sequences of the respective genes. PCR amplification was carried out at 25 thermal cycles at 94°C (30 s), 60°C (1 min) and 72°C (1 min) using the four mixed primers. The reaction mixture was electrophoresed on 1.2% agarose gel and visualized with ethidium-bromide.

Contour-clamped homogeneous electric field (CHEF) gel electrophoresis and Southern analysis. Preparation of chromosomal DNA, CHEF electrophoresis, and Southern blot analysis were carried out by the methods described previously. In Southern blot analysis, after at least 12 h of hybridization, the membrane was washed twice in 1 x SSC containing 0.1% SDS at 65°C for 15 min (low stringency condition).

Results

Isolation of the HO gene from S. uvarum type strain

From a gene library constructed from S. uvarum type strain NBRC0615, the Uv-HO gene was cloned by colony hybridization using a labeled 2.4-kb HindIII fragment containing the whole open reading frame (ORF) of Lab-HO. Positive clones contained the overlapping 4-kb HindIII DNA fragment and Southern blot analysis showed that the 4-kb HindIII fragment made strong hybridization with the same probe. We subcloned the 4-kb HindIII fragment into pUC118, and the resulting plasmid, pHOUV1, was used for further analysis.

A 4-kb HindIII DNA fragment was ligated into YEp-type vector pYO326, and the resulting plasmid was introduced into heterothallic haploid laboratory strain TD4 (MATa his4-519 ura3-52 leu2-3, 112 trp1 can). The activity of the Uv-HO gene was confirmed according to the method described previously. As shown in Table 2, 22 of the tested 25 clones were non-maters, which produced asci, and three clones were conserved in the native mating-type. This result indicates that the HindIII DNA fragment contained the Uv-HO gene encoding an active HO-endonuclease.

Sequence comparison of Uv-HO to other species HO genes

One ORF was found in the 4-kb HindIII fragment. The deduced coding region consisted of 586 amino acids, the same amino acid number as the known HO of Saccharomyces sensu stricto species. The HO nucleotide sequence of S. uvarum CBS7001 was found in a data bank. Compared to Uv-HO, six base changes were observed without amino acid change in ORF (data not shown). A partial sequence of wine yeast HO (446-bp) was perfectly matched to that of Uv-HO. This result confirms significant sequence homogeneity within strains classified as S. uvarum.

As shown in Fig. 1, the ORF of Uv-HO showed 95% homology to that of the Sb-HO and Lg-HO genes in nucleotide sequence. However, the ORF of Uv-HO showed only 83–85% homology to that of the HO genes cloned from other species. Two sequences of Saccharomyces-specific-endonuclease were highly conserved in all species (at positions 214–225 and 325–336), except that Gly223 was mutated to Asp223 in Sc-HO. Zn-finger domains at the carboxyl-terminus of the HO protein have been reported to be essential for target sequence recognition. The amino acid sequence deduced from HO ORFs contained Zn-finger domains of one form His-X-X-His (beginning at position 448), five forms Cys-X-X-Cys (beginning at positions 466, 486, 508, 522, and 558), one form Cys-X-X-X-X-His (beginning at position 470), and one form His-X-X-Cys (beginning at position 574). His448-His451 was observed only in Lg-HO, Sh-HO, and Uv-HO. Cys466-Cys469 was replaced by Tyr469 in Lg-HO, Sh-HO, and Uv-HO. This result suggests that the Uv-HO gene forms a group closely related to the Sh-HO and Lg-HO genes.

To evaluate the homology of ORFs and non-coding regions within Saccharomyces sensu stricto species, a bootstrap-tree was constructed by CLUSTAL W. As shown in Fig. 2, a comparison of the ORFs and the 5’ non-coding regions (1450-bp) within eight HO genes showed that Lab-HO and Sh-HO had high homology to Sc-HO and Lg-HO respectively, which were cloned from S. pastorianus. Otherwise, a phylogenetic bootstrap tree shows that Uv-HO is closely related to Sh-HO and Lg-HO, but significantly diverges from other HO genes. Since these results support the conclusion that S. uvarum forms one group of S. bayanus species, further structural analysis of the non-coding region was carried out among HO genes of S. uvarum, S. bayanus and S. pastorianus.

Structure of the non-coding region in S. uvarum, S. bayanus and S. pastorianus HO genes

The 5’ non-coding region of the Uv-HO gene was found to be 79% homologous to that of the Sb-HO gene. Compared to the homology of ORFs, the homology of the 5’ non-coding region was significantly low, between Uv-HO and Sh-HO. It is known that the HO gene is expressed under three restricted conditions: in a or α cells, during the G1 phase, and in the mother cell. Nucleotide sequences involved in these three types of transcriptional control have been reported in the 5’ upstream region of the Lab-HO gene. Although the 5’ upstream region of the Uv-HO gene had less homology to that of Lab-HO and Sh-HO, the nucleotide sequence of the regulatory motifs were conserved.

Table 2. Mating Type Conversion Activity of the Sc-HO, Lg-HO and Uv-HO Genes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of tested clones</th>
<th>Spo+ clones</th>
<th>Mating Type</th>
</tr>
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<tbody>
<tr>
<td>pYO326</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sc-HO/pYO326</td>
<td>25</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Lg-HO/pYO326</td>
<td>25</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Uv-HO/pYO326</td>
<td>25</td>
<td>22</td>
<td>22</td>
</tr>
</tbody>
</table>
As shown in Fig. 3, 12 putative homologous sequences of the α/α repression regulatory motif, 5'-TGATGTANNTN(A/T)NNACA(C/T)GA-3', were found in the 5' upstream region of the Uv-HO gene, while eight α/α repression motifs have been reported to exist in the Lg-HO gene and Sb-HO respectively. Although three α/α motifs on the complementary strand were conserved in these three HO genes, four motifs on the sense strand appeared to be lost from Sb-HO and Lg-HO. The positions of these motifs were conserved from 5' to approximately 500-bp of Sb-HO and Uv-HO, but not in the region from 500-bp to 1,450-bp.

The consensus sequence of the cell cycle control, 5’-NN(T/C)CACGAAAA-3’, was reported in the Lab-HO gene. As shown in Fig. 3, we found 15 cell cycle boxes in the 5' upstream region of the Uv-HO gene. There were six copies with a perfect match, eight copies with a single mismatch to consensus, and one copy with two mismatches. Since 12 cell cycle control motifs have been reported to exist in the Lg-HO and Sb-HO genes, it was thought that the three additional cell cycle boxes were generated in Uv-HO or that three cell cycle boxes were lost from Sb-HO and Lg-HO.

In all Saccharomyces sensu stricto species, the SSB1 gene locates in the 3' downstream region of the HO gene. The non-coding region between HO and SSB1 of Uv-HO was 449-bp, although that of Sb-HO was 394-bp. Alignment analysis indicated that the homology of Uv-HO and Sb-HO was 76% in the 3' non-coding region.

Our results confirm that S. uvarum is more closely related to S. bayanus var. bayanus than to other species, but indicate that S. uvarum diverges from S. bayanus var. bayanus by the accumulation of sequence alterations.
Uv-HO gene distribution among strains classified as *S. bayanus* species

For a rapid method to distinguish three types of HO gene, Sc-HO, Sb-HO=Lg-HO, and Uv-HO, a set of PCR primers was developed. Using mixed primers containing P2-1/2, P3, P4, and P5, 1.6-kb DNA fragment of Sc-HO, 0.85-kb of Lg-HO, and 0.52-kb of Uv-HO could be amplified, but could not be amplified from *S. cariocanus*, *S. kudriavzevi*, *S. mikatae*, or *S. paradoxus* (data not shown). The results of DNA amplification from type strains, NBRC stocked strains, and industrial strains are shown in Fig. 4. Within synonyms of *S. bayanus* species (*S. globosus*, *S. heterogenicus*, *S. inusitatus*, and *S. uvarum*), the type strain of *S. globosus* had only the Sb-HO gene (lane 4), and the type strains of *S. heterogenicus* and *S. uvarum* had only the Uv-HO gene (lanes 7 and 8 respectively). From type strains of *S. bayanus* and *S. inusitatus*, the 0.85-kb and the 0.52-kb DNA fragments were amplified with mixed primer (lanes 5 and 6 respectively). Since two amplified DNA fragments were not detected with any other pairing of primers, we summarized that the type strains of *S. bayanus* and *S. inusitatus* contained both the Sb-HO gene and the Uv-HO gene. Wine yeast KY702 and beer-fermenting yeast NBRC2031 had the Uv-HO gene (lanes 11 and 12 respectively). The 0.52-kb DNA fragment of the Uv-HO gene was amplified from NBRC0676, NBRC1948, and NBRC10558 (lanes 9, 10, and 16 respectively). This result indicates that the Uv-HO gene exists widely in strains belonging to *S. bayanus* species. Almost strains contained either Sb-HO or Uv-HO, but some strains had both Sb-HO and Uv-HO.

**CHEF-Southern blot analysis of chromosome carrying the Uv-HO gene**

A *BglII* DNA fragment (0.4-kb) containing the Uv-HO ORF was used for CHEF-Southern blot analysis as a probe, and a membrane was washed in the low stringent condition. As shown in Fig. 5, *S. cerevisiae* showed weak hybridization, corresponding to the 1,620-kb chromosome IV (lane 1). *S. pastorianus* showed strong hybridization to Lg-HO, located on the 1,100-kb chromosome, and weak hybridization to Sc-HO, located on the 1,500-kb chromosome (lane 2), as reported previously. *S. uvarum* produced strong hybridization on the 1,500-kb chromosome (lane 6). *S. bayanus* type strain and synonyms (*S. inusitatus*, *S. heterogenicus*,

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**Fig. 3.** Comparison of the 5’ Non-Coding Regions of the Lab-HO, Sc-HO, Lg-HO, Sb-HO, and Uv-HO Genes.

Negative numbers on upper bar indicate nucleotide numbers of upstream regions from the initiation codon. The vertical lines indicate the position of the cell cycle control motif, the open boxes indicate the position of the mating-type control motif on the sense strand, and dotted boxes indicate the position of the mating-type control motif on the complementary strand.

**Fig. 4.** Species Identification by PCR-Amplified HO Gene Fragments Using Mixed Primers.

Lane 1, DNA size marker, λ/ScyI; lane 2, *S. cerevisiae* X2180-1A; lane 3, *S. pastorianus* type strain NBRC1167; lane 4, *S. globosus* type strain NBRC10557; lane 5, *S. bayanus* type strain NBRC1127; lane 6, *S. inusitatus* NBRC10563; lane 7, *S. heterogenicus* type strain NBRC1620; lane 8, *S. uvarum* type strain NBRC0615; lane 9, wine yeast NBRC0676; lane 10, brewing contaminant NBRC1948; lane 11, wine yeast KY702; lane 12, beer yeast, NBRC2031; lane 13, *S. bayanus*, NBRC10549; lane 14, *S. bayanus*, NBRC10550; lane 15, *S. bayanus*, NBRC10551; lane 16, *S. bayanus*, NBRC10558.
and *S. globosus*) also produced strong hybridization bands on the 1,100-kb chromosome (lanes 3, 4, 5, 7 respectively). PCR amplification, shown in Fig. 4, revealed that the type strain of *S. bayanus* and *S. inusitatus* contained both Sb-*HO* and Uv-*HO*. CHEF-Southern blot analysis indicated that a hybridization band of Uv-*HO* and a band of Sb-*HO* overlapped on the 1,100-kb band in strains of *S. bayanus* species. Two hybridization bands of *S. inusitatus* might have resulted from a structural alteration, in that an additional approximately 1,200-kb chromosome was rearranged from one of the 1,100-kb chromosomes carrying the *HO* gene. We summarize that *S. bayanus* species strains contained the Uv-*HO* gene or/and the Sb-*HO* gene on the 1,100-kb chromosome.

**Discussion**

Nguyen *et al.* reported that the structure of rDNA spacer region of *S. uvarum* is different from that of *S. bayanus* based on PCR-RFLP analysis, and suggested that *S. uvarum* might form a sub-group of the species *S. bayanus*.22) Rainieri *et al.*23) and our present study support their proposal.

This study indicates that the *S. bayanus* type strain contains both the Uv-*HO* gene and the Sb-*HO* gene, but only one strong hybridization band was observed on the 1,100-kb chromosome by CHEF-Southern. We concluded that each *HO* gene was located on the respective chromosome, which overlapped on one band of CHEF-Southern blotting, because a spore progeny, B19-3C,24) which was obtained from the *S. bayanus* type strain, contained only the Uv-*HO* gene (unpublished data). This information indicates that Uv-*HO* and Sb-*HO* were located on the respective but same-sized chromosomes, and that the *HO*-containing chromosomes were independently segregated to progenies through meiosis.

*S. cerevisiae* carries the *HO* gene on chromosome IV, and *S. paradoxus* has been reported to have the *HO* gene on the high molecular weight chromosome corresponding chromosome IV of *S. cerevisiae*.25) On the other hand, Ryu *et al.* reported that the 1,100-kb chromosome of *S. bayanus* strains, on which the *HO* gene located, was rearranged from chromosome II and chromosome IV of *S. cerevisiae*.21,24) Our result, that the 1,100-kb chromosomes of *S. uvarum* carried the Uv-*HO* gene, suggests that the same chromosome rearrangement occurred in *S. uvarum* as in *S. bayanus* var. *bayanus*, and that the strains might share a similar chromosome constitution, but nucleotide sequence of the Uv-*HO* gene lost homogeneity from that of the Sb-*HO* gene by accumulation of sequence alterations. These results imply that *S. bayanus* var. *bayanus* and *S. uvarum* diverged from a common ancestor, which branched from *S. cerevisiae* by chromosomal rearrangement.

Although the nucleotide sequence of the Uv-*HO* gene showed less homology to that of the Sb-*HO* gene, nucleotide sequences of the ORF as well as the non-coding regions of the Sc-*HO* gene and the Lg-*HO* gene cloned from *S. pastorianus* were substantially identical to those of the Lab-*HO* gene and the Sb-*HO* gene respectively. This result suggests that *S. uvarum* diverged from *S. bayanus* much earlier than *S. cerevisiae* and that *S. bayanus* made a hybrid to form a species *S. pastorianus*.

Our *HO* typing PCR identified the sub-group of *S. bayanus* species rapidly and easily. This method has advantages over the PCR-RFLP22) of the rDNA spacer region, and the previous PCR21) detecting the chromosome rearrangement occurring in *S. bayanus* species.
Our PCR analysis showed the HO gene polymorphism in S. bayanus species; the S. globosus type strain had only the Sb-HO gene, type strains of S. heterogenicus and S. uvarum had only the Uv-HO gene, and type strains of S. bayanus and S. inuistatus had both the Sb-HO gene and the Uv-HO gene. Recently, Rainieri et al. also reported that several S. bayanus strains contained mixed genomes consisted from S. bayanus var. bayanus and S. uvarum by PCR-RFLP analysis of 48 genes and partial sequencing of 16 genes. In our study of the ACT1 gene coding actin protein, Sb-HO and Uv-ACT1 were shuffled independently from the Sb-HO or Uv-HO gene among S. bayanus species strains (manuscript in preparation). Why did such different types of genes co-exist and why were they shuffled in their genome? A simple answer is that S. bayanus type strain NBRC11279 as well as some other strains belonging to S. bayanus species are natural hybrids. Our results imply that ancestors of S. bayanus var. bayanus and S. uvarum once evolved independently from a common ancestor, but mated to contain both genomes, and then strains carrying the shuffled genome, which are now classified as S. bayanus species by DNA-DNA hybridization, might have appeared by chromosome loss or meiosis.

References

22) Nguyen, H.-V., and Gaillardin, C., Two subgroups within the Saccharomyces bayanus species evidenced by PCR amplification and restriction polymorphism of non-transcribed spacer 2 in the ribosomal DNA unit.

