Chromosome Bisection in the Yeast *Saccharomyces cerevisiae* Facilitated by Yeast Artificial Chromosomes Bearing a Site-specific Recombination System

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A chromosome bisection method was constructed using yeast artificial chromosomes (YAC) and a GALI-promoted site-specific recombination system. This method was applied to bisect chromosome IV into the left and right parts at the centromere region. The bisection occurred at frequencies of about 10% when the recombination site DNAs were integrated onto YAC and chromosome IV in the same direction, but were less than 10⁻³ when they were in opposite directions. Reconstruction of the original chromosome IV from the bisected chromosomes was also induced by galactose at high frequencies. Loss of the left part chromosome was found at the frequencies of 0.9 × 10⁻³ after hybrid cells between the chromosome-bisected strain and a normal haploid were subcultivated in a complete medium. The bisection and reconstruction of chromosome IV and deletion of the left part chromosome were demonstrated by electrophoretical karyotyping.

We have reported an efficient system for specific chromosome loss in *Saccharomyces cerevisiae*. With this system any chromosome in diploid cells can be lost at extremely high rates (more than 40%) to yield 2n-1 aneuploid cells. The aneuploid cells originally form smaller colonies, but subcultivation of the aneuploid cells frequently gives rise to normal size colonies whose cells have restored the chromosome balance toward the 2n euploidy during mitotic cell growth. Through this process we can easily convert any heterozygous pair of chromosomes into homozygous (or uniparental) ones in hybrid cells. We applied this technique to assess how much each chromosome contributed to ethanol productivity in molasses. Conversion of a heterozygous pair into a homozygous one of chromosome V or chromosome IX, for example, in a hybrid between YOY655 and X2180-1A showed changes in the ethanol productivity. These results suggest that the chromosome V of YOY655, a haploid strain isolated from an alcohol yeast, has some recessive gene(s) to increase ethanol productivity and, in contrast, the chromosome IX of a laboratory strain, X2180-1A, has some recessive gene(s) to decrease it. It is important to identify the loci and functions of those potential genes for better understanding of high ethanol productivity. For this purpose, we tried tetrad analysis on the hybrid strain mentioned above, but such classical genetics gave little information about the number and loci of those potential genes (Y. Oda, unpublished data).

In this study, we constructed a chromosome bisection system as a way to identify the chromosomal part in which some unknown gene(s) should be located. Using this system, chromosome IV, the largest chromosome in *S. cerevisiae*, was efficiently bisected into the left and right parts. Loss of one part of the bisected chromosome leaving the other part alone in diploid cells and reconstruction from the bisected chromosomes into chromosome IV were also possible in this system.

Materials and Methods

**Plasmids.** Plasmids pKO301 and pKO302 (Fig. 1), which had a centromere (CEN4), a pair of telomeres (TEL), an autonomous replication sequence (ARS), the site-specific recombinase gene (R) placed downstream of the GALI promoter, the recombination site (RS) sequence, URA3, and TRP1 were constructed using DNAs from the YAC5, the GALI-R gene of the pHM153, and a 2.1-kb *Sal*--*Sac* I fragment containing the RS sequence. pKO301 and pKO302 were different from one another only in the direction of the RS sequence. These pKO plasmids were digested with the BamHI restriction enzyme to remove the 2-kb *His3* DNA on transformation. The 14-kb linear yeast artificial chromosomes thus yielded were designated as YAC301 and YAC302. They carry ARS, TRP1, and one TEL at one end, and R, RS, URA3, and another TEL at the other end (Fig. 1). pHK10-20 was constructed by inserting a 3.8-kb BamHI-BamHI RS-*His3* DNA into the Hpal site, splitting the CEN4 region of YCp19.3,5 pHK11-2 was a derivative of pHK10-20 in which only the direction of the RS sequence was different from pHK10-20 (Fig. 1). The RS-*His3* DNA fragment was derived from either pHK7-1 or pHK7-2, each of which was constructed by ligation of a 2.1-kb *Sal*--*Sac* I RS DNA at the *XhoI* site in *His3* of YLP1. A 5.3-kb *XhoI*-*XhoI* DNA of either pHK10-20 or pHK11-2, which contained a partial CEN4 region split by the RS-*His3* DNA, was used for the integration of the RS sequence into the CEN4 region of chromosome IV by homologous recombination.

**Yeast strains.** NBW5 (*MATa ade2-1 thr2-3,112 leu2-3,112 his3-123 trp1-308 pho80-1*) was supplied by Professor Y. Oshima, Osaka University. X2180-1A (*MATa suc2 mal2 mel2 gal2 CUP1*) was purchased from the Yeast Genetic Stock Center (Berkeley, California) and X2180-1Au (*MATa ura3 suc2 mal2 mel2 gal2 CUP1*) was its ura3 mutant isolated for this study.

**Media and cultivation.** YPAD and YPAGal media contained 10 g of yeast extract, 20 g of polypeptone, 400 mg of adenine sulfate, and 20 g of either glucose or galactose, respectively, per liter. S90u was a synthetic medium containing 6.7 g of Yeast Nitrogen Base without amino acids (Difco Laboratories, Detroit, MI, U.S.A.) and 20 g of glucose per liter, and when necessary appropriate nutrients were added. S91g medium was a synthetic medium containing 20 g of galactose as a sole carbon source, 6.7 g of Yeast Nitrogen Base without amino acids, and all the needed nutrients except for uracil and tryptophan. FOA medium contained 1 g of 5-fluoroorotic acid, 50 mg of uracil, 6.7 g of Yeast Nitrogen Base without amino acids, and 20 g of glucose. Solid media contained 2% agar. Yeast cells were cultivated at 30°C in liquid media with shaking or on solid media.

**Genetic methods.** Transformation of yeast was done by the LiCl method. For the contour-clamped homogeneous electric field
Results and Discussion

Construction of strains for chromosome bisection

For the integration of the RS sequence, NBW5 cells were transformed into histidine prototrophy (His\(^+\)) with the 5.3-kb \(XhoI\)-\(XhoI\) DNA fragment of either pHK10-20 or pHK11-2 (Fig. 1), and the His\(^+\) strain YHK101 or YHK102, respectively, was isolated. Hybridization analysis with a 1.8-kb \(BamHI\)-\(BamHI\) \(HIS3\) DNA as the probe indicated that the \(XhoI\)-\(XhoI\) DNA fragments were integrated on chromosome IV by homologous recombination in these strains (data not shown).

Introduction of YAC into yeast cells

YHK101 cells were transformed with either YAC301 or YAC302, and the Ura\(^+\) (uracil prototrophy) transformant YHK101/YAC301 or YHK101/YAC302, respectively, was isolated on SGlu medium. Similarly, the Ura\(^+\) transformant YHK102/YAC301 or YHK102/YAC302 was isolated using YHK102 cells as the host. Since the YACs had \(URA3\) and \(TRP1\) as the genetic markers, these Ura\(^+\) transformants showed the Trp\(^+\) phenotype as well. These YACs were unstable in the transformants, and Ura\(^-\) and Trp\(^-\) colonies frequently arose after they were grown in YPAD medium. For example, when those cells were cultured overnight in YPAD medium and then spread on YPAD plates, 98 to 100% of the grown colonies were Ura\(^-\) and Trp\(^-\). This mitotic instability is probably because these artificial chromosomes are too small to be maintained normally under no selective pressure.

Isolation of chromosome-bisected strains

When site-specific recombination between chromosome IV and the YAC occurs at each RS site as shown in Fig. 2, a 530-kb chromosome (Chr. IV-S) with the indigenous centromere and a 1100-kb chromosome (Chr. IV-L) with the YAC-origin centromere will be newly yielded. These new chromosomes might be large enough to be maintained stably during mitotic cell division even in the nutrition medium.\(^{8-10}\) Moreover, if either of the new chromosomes was lost, such haploid cells might be unable to live. Therefore, chromosome-bisected strains could be obtained as stable Ura\(^+\) and Trp\(^+\) colonies.

Cells of YHK101/YAC301, YHK101/YAC302, YHK102/ YAC301, or YHK102/YAC302, which had been grown in SGlu medium lacking uracil and tryptophan, were cultivated overnight in SGlu medium to induce the site-specific recombination between chromosome IV and the YAC. These cultures were transferred to YPAD medium at the inoculation ratio of 1/300 and cultivated for 24 h. The cultures were spread on YPAD plates to grow colonies after appropriate dilution. The grown colonies were replicated onto SGlu medium lacking uracil and tryptophan, and the number of Ura\(^+\) and Trp\(^+\) colonies was counted. The results (Table) showed that 9 to 11% of the replicated colonies were Ura\(^+\) and Trp\(^+\) in YHK101/YAC301 and YHK102/YAC302, but no Ura\(^+\) and Trp\(^+\) colonies were found in YHK101/YAC302 or YHK102/YAC301. In other experiments, the final cultures were directly spread on the minimal SGlu medium at a density of \(10^5\) cells/plate, but neither YHK101/YAC302 nor YHK102/YAC301 formed colonies in 4 days, while around 100 colonies were grown in YHK101/YAC301 and YHK102/YAC302. These differences could be explained from the combination of direction in which RS DNAs were inserted onto chromosome IV and YAC. When the two RS DNAs are inserted in the same direction to each centromere position, the site-specific recombination between chromosome IV and the YAC yields two new unicentric chromosomes. However, when the two RS DNAs are in opposite directions, it yields one dicentric chromosome and one acentric chromosome, and would result in the loss of such abnormal chromosomes.\(^4\) This was the case for YHK101/YAC302 and YHK102/YAC301.

Confirmation of chromosome bisection

Chromosomal DNAs of the chromosome-bisected strains
were analyzed by CHEF electrophoresis. Since there were no differences in the electrophoregrams among the chromosome-bisected strains (data not shown), only YHK101/YAC301-CB1 and YHK102/YAC302-CB1, which were representative strains derived from YHK101/YAC301 and YHK102/YAC302, respectively, were compared with the reference strain YHK101 (Fig. 3). The electrophoretical karyotypes indicate that bands of chromosome IV DNA disappeared and in place two new bands appeared as pointed by arrows (IV-L and IV-S) in both YHK101-YAC301-CB1 and YHK102/YAC302-CB1. Southern blot analyses of chromosomal DNAs were done using labeled probes of 0.8-kb EcoRI-PstI TRP1, 3.6-kb HindIII—HindIII PHO2, and 0.8-kb BglII—EcoRI GAL1—R DNAs (Fig. 3). Since the chromosome IV and YACs have CEN4, TRP1, and their flanking sequences, there is a possibility that homologous recombination would occur at a position in these homologous sequences, yielding bisected chromosomes. If the homologous recombination occurred in the CEN4 or TRP1 regions, it should yield two chromosomes, both of which have TRP1/trp1 sequences. On the other hand, if site-specific recombination occurred at the RS sites as expected, only the larger one of the new chromosomes should have both TRP1 and trp1 sequences. The blot patterns shown in Fig. 3 support the latter case. This also coincides with the findings that the chromosome-bisected strains were isolated from YHK101/YAC301 and YHK102/YAC302 at such high frequencies as nearly 10% only when the transformed cells were cultivated in SGal medium. In contrast, no chromosome-bisected strains were found in SGlu cultures, just as in the case of YHK101/YAC302 and YHK102/YAC301 (data not shown). Such a high frequency as 10% has never been reported in chromosome bisection through homologous recombination.9,10

In this work, we chose chromosome IV for the target of bisection because the YACs themselves had a portion of chromosome IV DNA as described above, and consequently there were some possibilities that the homologous recombination between these sequences might disturb the bisection of chromosome IV in an unexpected way. However, our data suggest that the bisection was induced by galactose rightly as expected through the site-specific recombination. This system might be applicable to any chromosome only if the RS DNA is targeted to the desired position of a specific chromosome to be bisected.

**Reversibility of chromosome bisection**

Since the site-specific recombination is reciprocal in nature,9 cultivation of chromosome-bisected cells in galactose medium is expected to induce the reconstruction from bisected chromosomes of the original chromosome IV and YAC. Such strains would easily lose the reconstructed YAC and become Ura− and Trp−. This was confirmed in the following experiments. Cells of YHK101/YAC301-CB1 and YHK102/YAC302-CB1 were cultivated overnight in YPAGal medium and the cultures were spread on YPAD plates. Then, the grown colonies were replica-plated onto SGlu medium lacking uracil and tryptophan to score the ratio of Ura− or Trp− colonies. The results indicate that 156 of 213 (73.2%) and 192 of 236 colonies (81.4%) were Ura− and Trp− in YHK101/YAC301-CB1 and YHK102/
YHC302-CB1, respectively. On the contrary, no Ura\(^{-}\) or Trp\(^{-}\) colonies were found in 136 to 193 of colonies tested after the YHC101/YAC301-CB1 and YHC102/YAC302-CB1 had been grown in YPAD medium. The Ura\(^{-}\) and Trp\(^{-}\) strains showed the same electrophoretic karyotypes as did YHC101 and YHC102 (data not shown) and this indicates the completion of reconstruction of chromosome IV. The reversible recombination may be useful to construct a chimera chromosome, a chromosome consisted of one part of intrinsic and the other part of foreign ones.

**Deletion of one part of bisected chromosomes**

If either part of the bisectioned chromosomes was lost in a haploid strain, no cells might survive. However, even if such a loss happened in diploid cells constructed by a cross between the chromosome-bisectioned strain and a normal haploid, the resultant cells could still live. The diploid cells that lose chromosome IV-S (530-kb) with \( URA3 \) marker will become Ura\(^{-}\) (see Fig. 2). Therefore, YHC101/YAC301-CB1 was crossed with X2180-1Au and the prototrophic diploid, YX301-CB1, was obtained. The diploid cells were subcultivated in YPAD medium with 50 mg/liter of uracil, and then plated on FOA medium in which only Ura\(^{-}\) cells of \( ura3 \) genotype could selectively grow.\(^{11} \) The results indicate that 5-fluoro-orotate-resistant colonies (designated YX301-CB1-FOA\(^{+}\)) arose at the frequencies of \( 0.9 \times 10^{-3} \) after two times of subcultivation (more than 10 generations), and they were all Ura\(^{-}\). Electrophoretical analyses of four YX301-CB1-FOA\(^{+}\) strains showed that they had all lost the chromosomes IV-S (Fig. 4).

In this work, we used laboratory strains for the test of chromosome bisection. The chromosome bisection by this system was very efficient and reversible. However, this system should be revised when we apply it to industrial strains, because most of them are prototrophic and hence we cannot directly use this system as it needs at least two genetic markers for the completion of chromosome bisection. It is easier to obtain \( ura3 \) mutants of industrial strains by isolating 5-fluoro-orotate resistant colonies on FOA medium.\(^{11} \) Therefore, we are programming a chromosome bisection system in which only the \( ura3 \) marker is needed. Previously, we constructed a chromosome conversion system that was feasible even in industrial yeast strains.\(^{11} \)
Our long-range goal is to use these chromosome engineering skills for the search for some potential genes or practically useful genes that have never been identified on yeast chromosomes.

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