Development of a Highly Efficient Gene Targeting System Induced by Transient Repression of YKU80 Expression in Candida glabrata†

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In the pathogenic yeast Candida glabrata, gene targeting to generate knockouts and “knockins” is a potentially powerful method for the analysis of gene function. Its importance increased after the C. glabrata genome sequence project, but progress in the field is hampered by inefficient mechanisms for gene targeting. With the use of 40-bp homologous flanking DNA, no gene targeting was identified. To address this issue, YKU80 was disrupted, leading to an increase in targeting efficiency of 5.1% using 40-bp flanking homologous DNA. To harness the beneficial effects of YKU80 inactivation on gene targeting frequency without incurring any negative effects, such as synthetic sickness or lethality, we developed a new system whereby the expression of YKU80 was restored following a transient knockdown of expression during transformation. Strains used for this new system carried a SAT1 flipper in the YKU80 promoter region, which was used to repress expression during transformation but was spontaneously excised from the locus after the transformation. By using this strain, DNA damage induced by methyl methane sulfonate, H2O2, UV irradiation, and hydroxyurea before and during gene targeting was evaluated and the mutation rate of URA3 was determined. No significant effects of the SAT1 flipper on these processes have been identified. After the SATI flipper is excised, a 34-bp FLP recombination target sequence is left in the promoter region. However, the levels of mRNA transcription were restored and no difference in the survival ratio in vivo compared to that with the YKU80 wild-type strain was identified.

The incidence of opportunistic fungal infection has been increasing, particularly in patients who are immunocompromised by human immunodeficiency virus infection or leukemia or who are receiving immunosuppressive therapy for organ transplantation or chemotherapy for cancer. Systemic fungal infections in such patients are often life threatening. Candida albicans and Candida glabrata are among the opportunistic pathogenic fungi that cause candidiasis (7). C. albicans remains the most commonly encountered species in clinical practice but other species (non-C. albicans) species, such as C. glabrata, now cause significant levels of disease (35). In one study, C. glabrata was the second most commonly documented species, isolated from up to 20% of all candidiasis patients (31). It is known that C. glabrata has a low susceptibility to fluconazole, which is the first drug administered in clinical situations (2); thus, candidiasis caused by C. glabrata is associated with high mortality (17). Despite this increase in incidence, little is known about the molecular basis of C. glabrata virulence in contrast to that of C. albicans.

C. glabrata offers some advantages for the study of fungal pathogenesis. It is more closely related to Saccharomyces cerevisiae than to C. albicans (2, 12), suggesting that more of the wealth of information accumulated by decades of study of S. cerevisiae might be transferable to C. glabrata. C. glabrata is a haploid fungus, whereas C. albicans is diploid, making gene targeting in C. albicans at least twice as difficult as that in C. glabrata (4). The whole genome sequence of C. glabrata has been completed, thus allowing the comprehensive identification of all genes (10). Taking these attributes together, C. glabrata has now come of age as a feasible model organism for the genome-wide study of pathogenicity in fungi and for the identification of antifungal drug targets.

Gene targeting is one of the most important approaches for analysis of gene function. Genome-wide functional analyses are facilitated by efficient gene targeting using constructs flanked at each end with short homologous sequences, the lengths of which are typically 40 to 60 bp. These short homologous sequences can be easily produced by single-step PCR using a pair of oligonucleotide DNA primers containing homologous flanking sequences. However, targeted integration with short homologous sequences is relatively inefficient in C. glabrata, a characteristic it shares with higher eukaryotes and most fungi except S. cerevisiae (8, 22, 28, 40). S. cerevisiae has highly efficient gene targeting, with exogenous DNA fragments correctly integrated into the targeted locus with an efficiency close to 100% and requiring only short target sequences of 40 bp in length (3, 40). As a consequence, a complete set of gene knockout strains of S. cerevisiae has been successfully constructed (13).

The targeted integration of a DNA fragment requires homologous recombination, which is one of the repair mechanisms for DNA double-strand breaks (DSB). Nonhomologous end joining (NHEJ) is another mechanism of DSB repair, but it does not require such homologous DNA sequences (9, 30). We observed that in C. glabrata, NHEJ seems to predominate...
at integration events involving DNA fragments with flanking short homologous sequences. Therefore, we reasoned that if the activity of NHEJ could be suppressed, then the frequency of homologous recombination should increase. Several genes, such as KU70, KU80, and LIG4 and their homologues, have been identified as key genes for NHEJ in eukaryotes, including mammals, plants, and fungi. Recently, mutants have been constructed in which the genes homologous to human KU70 or KU80 were disrupted in Cryptococcus neoformans with a single transformation. Drastically improved efficiency of gene targeting was found in these Ku mutants. It has also been shown that when NHEJ was disrupted in C. glabrata from infected mice, homogenized kidney was spread on agar plates consisting of 1.5% (wt/vol) agar, 0.5% (wt/vol) yeast extract, 1% (wt/vol) peptone, 1% (wt/vol) dextrose, 1,000 units/ml penicillin G, and 100 µg/ml streptomycin sulfate (Wako Chemicals, Japan). For the repression of genes of interest, inactivation of the tetracycline regulatable promoter (Tet-P) was carried out in appropriate medium with 20 µg/ml doxycycline (ICN Biomedicals Inc.) dissolved in 50% ethanol alcohol to prepare 2 mg/ml stock solution.

**Materials and Methods**

**Strains and media.** All strains in this study are described in Table 1. C. glabrata strains were grown routinely at 37°C on YPD (1% [wt/vol] yeast nitrogen base without amino acids, 2% [wt/vol] dextrose) media. Various growth supplements were added to SD medium as described in a standard experimental manual (6). For the screening of sulfortemuron methyl (SM) resistance as was performed for HET80, the cells were plated onto SD plates plus 20 µg/ml histidine with 20 µg/ml SM (Wako, Inc.; dissolved in dimethyl sulfoxide to prepare 2 mg/ml stock solution). For the selection of nourseothricin (NStC)-resistant strains, cells were plated onto YPD plates containing 100 µg/ml NStC (HKJ Jena, Germany; 10 mg/ml in water as stock solution). In order to test for the recovery of live C. glabrata from infected mice, homogenized kidney was spread on agar plates consisting of 1.5% (wt/vol) agar, 0.5% (wt/vol) yeast extract, 1% (wt/vol) peptone, 1% (wt/vol) dextrose, 1,000 units/ml penicillin G, and 100 µg/ml streptomycin sulfate (Wako Chemicals, Japan). For the repression of genes of interest, inactivation of the tetracycline regulatable promoter (Tet-P) was carried out in appropriate medium with 20 µg/ml doxycycline (ICN Biomedicals Inc.) dissolved in 50% ethanol alcohol to prepare 2 mg/ml stock solution.

*Escherichia coli* DH5α was used for DNA manipulation. General recombinant DNA procedures were performed as described previously (15, 36).

**Primers.** For a list of the primers used in this study, see Table S1 in the supplemental material.

**Plasmid construction.** The plasmids used in this study are listed in Table 2. All plasmids were based on pBluescriptII SK (+) (Stratagene).

| Plasmid number | Description of plasmid | Source or reference |
|----------------|------------------------|---------------------|
| pBluescriptII SK (+) | Cloning vector | Stratagene |
| pCGH07 | Carries CgHIS3 marker and Tet-P | 28 |
| pKULV2-1 | YKU80-ILV2 region cloned into pBluescriptII SK (+) | This study |
| pILV2-7 | YKU80 ORF eliminated from pKULV2 | This study |
| pBS-SF-4 | SAT1 flipper cloned into pBluescriptII SK (+) | This study |
| pKU-self.5 | pKULV2-1 containing the BglIII site and Nhel site | This study |
| pKU-self(ORF2) | pKULV2-1 containing the BglIII site and Nhel site | This study |
| pSFS2A | Contains FRT site, FLIP, and CaSAT1 as SAT1 flipper | 34 |
| pKUSF18 | SAT1 flipper integrated into pKULV2-1 | This study |
| pKUSForf2-10 | SAT1 flipper integrated into pKULV2-1 | This study |

**Table 1. Strains used in this study**

| Strain | Parent | Genotype | Source or reference |
|--------|--------|----------|---------------------|
| 2001H | 2001U | Δhis3::ScURA3 Δura3 | 21 |
| 2001T | 2001U | Δtrp1::ScURA3 Δura3 | 21 |
| 2001HT | 2001TU | Δhis3::ScURA3 Δtrp1 Δura3 | 21 |
| ACG4 | 2001HT | his3 trp1 pSADH::hisR::GAL::AD:TRP1 | This study |
| tADE2-38 | ACG4 | his3 trp1 pSADH::hisR::GAL::AD:TRP1 97::ADE2-CgHIS3 | This study |
| HET80 | ACG4 | yka80 ILV2(P182L) | This study |
| ACG4-SF | ACG4 | his3 trp1 pSADH::hisR::GAL::AD:TRP1 SAT1 flipper | This study |
| HETS146 | ACG4 | his3 trp1 pSADH::hisR::GAL::AD:TRP1 YKU80::SAT1 flipper | This study |
| HETS202 | HET80 | his3 trp1 pSADH::hisR::GAL::AD:TRP1 yka80::SAT1 flipper | This study |
| ACG4-SFP | ACG4-SF | his3 trp1 pSADH::hisR::GAL::AD:TRP1 FRT | This study |
| POP-SF1467 | HET-SF146 | his3 trp1 pSADH::hisR::GAL::AD:TRP1 YKU80::FRT | This study |
| POP-SF221 | HET-SF22 | His3 trp1 pSADH::hisR::GAL::AD:TRP1 YKU80::FRT | This study |
| KUE100 | 2001H | his3 yka80::SAT1 flipper | This study |
| KUE200 | 2001HT | his3 trp1 yka80::SAT1 flipper | This study |
| KPOP-11538 | KUE100 | his3 yka80::FRT cyb2::CgHIS3 | This study |

**Table 2. Plasmids used in this study**

| Plasmid | Description | Source or reference |
|---------|-------------|---------------------|
| pKU-VL1-1 | A 6-kb DNA fragment, including YKU80-ILV2 regions, was amplified by PCR using primer pair preKu80HindIII and preILV2HindIII and genomic DNA of tADE2-38 as a template DNA, including pSFS2AR5(Nhe-Sac) and plasmid pSFS2A (34) as a template DNA, including the SAT1 flipper. The PCR product was digested with KpnI and SacI and treated toward the outside of the ORF in pKU1LV2-1. The PCR product was circularized by self-ligation, and the ORF of YKU80 was removed from the plasmid to produce pILV2-7. | This study |
| pBS-SF-A | PCR was carried out with pKU1LV2-1 and the primer pair of preKuK2 and preLIV2F2. The primers anneal to sites flanking the open reading frame (ORF) of YKU80 toward the outside of the ORF in pKU1LV2-1. The PCR product was digested with KpnI and SacI and treated toward the outside of the ORF in pKU1LV2-1. | This study |
inserted into pBluescript SK(−) that had been cleaved by KpnI andSacI to generate pBS-SF-4.

pKU-self.5. The plasmid pKU-self.5 was constructed to add BglII andNheI sites on YKU80. PCR was carried out with the primer pair of pYKU80ORF2 (Bgl) and pYKU80ORF2 (Nhe) and plasmid pKU1LV2-1 as a template. The PCR products were treated with T4 polynucleotide kinase and circularized by self-ligation to construct pKU-self.5. PCR was carried out with the primer pair of pYKU80ORF2 (Bgl) and pKU80ORF2 (Nhe-Bgl) and plasmid pKU1LV2-1 as a template. PCR products were digested and circularized by self-ligation.

pKUSF-18 and pKUSFOrf2-10. pBS-SF4 was digested with BglII and NheI and the inserted DNA, including the SATI flipper, isolated from the plasmid. The isolated fragment was then ligated with plasmid pKU-self.5 and pKUSF-18 (Takara, Japan) according to the manufacturer’s protocol. To quantify mRNA, RNA manipulations for quantitative real-time RT-PCR.

DNA amplification was performed using KOD-plus-DNA polymerase (Toyobo) and GoTaq green master mix (Promega) or ready-to-go PCR beads (Amersham Biosciences) according to the manufacturers’ protocols. BigDye Terminator cycle sequencing kits (version 3.1; Applied Biosystems, Inc.) and an Applied Biosystems model 3100 automated capillary sequencer were used for nucleotide sequencing. Other standard recombinant-DNA procedures were performed as described previously (15, 36).

RNA manipulations for quantitative real-time RT-PCR. For RNA extraction, the yeast RNA method was performed as described previously (18). For quantitative real-time reverse transcription-PCR (RT-PCR), mRNA was purified from total RNA extracted with the Oligotex-dt (Super) mRNA purification kit (Takara, Japan) according to the manufacturer’s protocol. To quantify mRNA, we used the Syber RT-PCR kit (Takara) for the synthesis of cDNA and real-time PCR. The real-time PCR for ACTI was performed with primers ACTI 5′F and ACTI 249R, that for YKU80 was performed with primers YKU80 622F and YKU80 798R (see Table S1 in the supplemental material) using an ABI PRISM 7000 (Applied Biosystems).

5′ RACE. 5′ RACE (rapid amplification of cDNA ends) was performed using the GeneRacer kit (Invitrogen). Briefly, total RNA from parent strain ACG4 was treated with calf intestinal phosphatase and then treated with tobacco acid pyrophosphatase to remove the 5′ cap structure from intact, full-length mRNA. The GeneRacer RNA oligonucleotide, with a known priming site, was ligated to the 5′ end of the mRNA by using T4 RNA ligase. The ligated mRNA was reverse transcribed into cDNA using random primers (Promega), to obtain 5′ ends. The first-strand cDNA strands were amplified using the GeneRacer 5′ nested primer and primer kdyku80checkR1. Purified RACE PCR products were cloned into the pcRII-TOPO vector (Invitrogen) for sequencing.

C. glabrata transformation. The C. glabrata transformation protocol is a slightly modified version of the transformation protocol described in previous studies (20). Cells were streaked on a YPD agar plate and grown for 2 to 3 days at 37°C. A few colonies were then incubated in 10 ml of YPD liquid medium and cultured overnight with shaking at 37°C. The cells were resuspended in 10 ml of fresh YPD (starting optical density at 600 nm (OD600) of 0.4) and shaken to an OD600 of 1.0, approximately 2 to 3 h at 37°C. The cells were harvested by centrifugation at 6,000 × g for 5 min. The pellets were rinsed with 10 ml of TE buffer and harvested by centrifugation. The cells were resuspended in 10 ml of 0.15 M lithium acetate dissolved in TE buffer (LoAc/TE) and shaken lightly for 1 h at 30°C. The cells were again harvested and resuspended in 400 µl of 0.15 M LiOAc. Sixty microliters of the suspension was dispensed to a new 1.5-ml tube, supplemented with 5 to 10 µg (3 µl) of the disruption cassettes and 20 µg (2 µl) of carrier DNA (salmon sperm DNA [Wako]), boiled before use for 10 min and chilled for 10 min to denature double-strand DNA and mixed gently. This solution was incubated for 30 min at 37°C and then added to 120 µl of 52.5% polyethylene glycol 4000·0.15 M LiOAc and mixed entirely by pipetting. The cells were incubated for 45 min at 37°C. After mixing carefully, the cells were heat shocked by incubating for 45 min at 42°C and then spread onto appropriate selection plates and incubated at 37°C for a few days. For NSTC-resistant strains like HETS202, the cells were harvested and resuspended in 1 ml of fresh YPD liquid medium after the 42°C heat shock and this suspension was shaken at 37°C for 12 or 24 h. The cells were then harvested, washed in YPD liquid medium, and resuspended in 400 µl of YPD liquid medium. The suspension was spread onto four YPD plates containing 100 µg/ml of NSTC, and the plates were incubated at 37°C for 1 day.

Strain construction. Strain TADE2-38, in which ADE2 expression is able to be controlled by the Tet-P, was constructed from ACG4 (26) by the integration of the Tet-P cassette into the ADE2 promoter locus (Table 1). The Tet-P cassette was amplified by PCR with the following primers and template: pGCHadecF2 and pGCTeTade2R1 as primers and plasmid DNA pGTCGH (26) as a template. TADE2-38 was screened on an SD plate without histidine and then streaked on an SD plate containing adenine and doxycycline to verify that the red colony color resulted from the knockdown of ADE2 expression. To confirm the correct integration of the Tet-P cassette, PCR was carried out using the primers pCgADE2checkF1 and pCgADE2checkR1.

A disruption cassette was amplified by PCR using pLV2-7 as a template and using preKU80HindIII and pLV2mR2 as primers (see Table S1 in the supplemental material). This cassette included the point mutation in ILV2 that changed amino acid 182 (proline) to leucine. The cassettes were used for the transformation of parent strain ACG4 and integrated into the YKU80-ILV2 locus by homologous recombination after the cassette entered into the cell. The transformant designated HETS80 was screened on plates containing 20 µg/ml SM, and genomic DNA was extracted in order to check for the YKU80 deletion. The verification of YKU80 deletion was performed by PCR with three sets of primers: check PCR 1 used preKU80checkR2 and pKU80checkF2, check PCR 2 used preKU80checkR1 and pMLV2checkR1, and check PCR 3 used preKU80HindIII and Ku80checkmidR3. SATI flipper in C. glabrata. The SATI flipper module was 4.3 kb long and contained the following DNA elements: the NSTC resistance gene SATI, FLPI encoding a site-specific recombinase, and two FLP recombination target (FRT) sites (21) that are recognized by Flp1 recombinase, which can excise the utility of the module in C. glabrata. ACO4 was transformed with the SATI flipper, leading first to illegitimate integration. The transformant ACG4-SF was confirmed by PCR and grown under permissive conditions (YPD plate without NSTC). NSTC-sensitive clones were screened by subsequent testing for growth under restrictive conditions on YPD plates with NSTC. Those clones have lost the SATI flipper, as confirmed by PCR analysis. To examine the efficiency of the SATI flipper module in C. glabrata, cells of HETS202, in which the SATI flipper module was integrated, were cultured in the permissive liquid medium overnight at 37°C, spread on YPD plates, and then replicated on the restrictive plates (YPD, including 100 µg/ml of NSTC). Only 0.5% of the colonies could grow on the restrictive medium. Twelve colonies that could not grow on the selective plates were checked by PCR, and all of the strains produced a PCR product that proved the loss of the SATI flipper.

Knockdown system using SATI flipper. To knock down the YKU80 gene, the YKU80 knockdown cassette (Y-kd cassette) was integrated just upstream of either the first or the second ATG codon, yielding HETS146 and HETS202, respectively. The two Y-kd cassettes comprising the SATI flipper flanked by homologous sequences were amplified by PCR using the following templates and primers. For the amplification of the first Y-kd cassette, pKUSF-18 was used as a template, and kdyKUSF-1 and kdyKUSF-4 were used as primers. For the amplification of the second Y-kd cassette, pKUSForf2-10 was used as a template, and preKU80HindIII and pMLV2mR2 were used as primers. After the selection of transformants on YPD plates containing 100 µg/ml NSTC, PCR analysis was performed to confirm the site-specific integration of these Y-kd cassettes. Check PCR 1 used primers preSFS-A2AF2 (PacI) and kdyKU80checkR1; check PCR 2 used primers preKU80checkR2 and ku80checkmidR3. YKU80 knockdown strains for gene disruption, HETS202, KUE100, and KUE200, were constructed by using C. glabrata strains 2001H and 2001HT (21).

POP-SF467 and POP-SF221 were isolated by the following procedure. Transformants derived from HETS146 and HETS202 were incubated in YPD liquid medium without NSTC at 37°C for 24 h with shaking. Under this condition, the FLP-mediated excision of the SATI flipper occurs automatically. The cells were spread onto YPD agar plates without NSTC and incubated at 37°C for 24 h. The colonies were replicated to YPD plates with and without 100 µg/ml NSTC. Clones, which did not grow on the plate containing NSTC, were confirmed to have excised the SATI flipper by PCR. Check PCR 3 used primers pFR1 F1 and kdyKU80checkF1; check PCR 3 used primers kdyKU80checkR1 and kdyKU80checkmidR1. YKU80 knockdown strains for gene disruption, HETS202, KUE100, and KUE200, were constructed by using C. glabrata strains 2001H and 2001HT (21).
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FIG. 1. Gene targeting efficiency depends on the length of the homologous flanking sequence. The numbers shown on the right side describe the number of correct integrants per total number of colonies tested. The lengths of homologous tags are shown on the left side. The designation 80-500 means that an 80-bp flanking sequence and a 500-bp flanking sequence were attached to the 5’ and 3’ sides, respectively, of the DNA cassette. The efficiency of gene targeting accompanied by homologous recombination was measured by determining the frequency of integration of a DNA cassette bearing a Tet-P into the endogenous promoter region of ADE2. When the Tet-P is integrated into the correct locus, the colonies turn red on plates supplemented with doxycycline. As in S. cerevisiae, red colony color is caused by inactivation of the ADE2 gene in C. glabrata (19). Closed and open boxes represent the gene targeting efficiency of HET80 and ACG4, respectively.

Evaluation of DNA damage in Yku80 mutants in vitro. To measure the sensitivity of strains to DNA-damaging agents, we employed the microdilution method based on the CLSI (formerly NCCLS) standard M27-A (27). Drop titer tests and spread titer tests for sensitivity were also carried out. Cells from an overnight culture were harvested, rinsed with YPD, and then adjusted to an OD600 of 0.3. This suspension was diluted 100 times and spread onto two plates. These plates were exposed to 0, 20, 40, or 60 J/m2 254-nm UV rays (CL-1000 UV cross-linker [UV Products]) and then incubated at 37°C for 2 days. The spread titer test to measure the sensitivity to UV rays was performed as follows. Cells of an overnight culture were harvested, rinsed with YPD, and adjusted to an OD600 of 4. This suspension was diluted 10-4. Fifty microliters of the diluted suspension was spread onto two plates. These plates were exposed to 0, 20, 40, or 60 J/m2 254-nm UV rays (CL-1000 UV cross-linker [UV Products]) and then incubated at 37°C for 1 day. The CFU were determined on each exposed plate, and the survival rate (%) was calculated by the following formula:

\[
\text{Survival rate} = \frac{\text{CFU on each exposed plate}}{\text{CFU on the unexposed plate}} \times 100\%
\]

Infection experiment. Seven male CD-1 (ICR) mice, 5 weeks old and weighing 28.5 to 30.9 g, were used for virulence studies for each group. Cyclophosphamide (Shionogi & Co., Ltd., Japan) and hydrocortisone sodium phosphate (Banyu Pharmaceutical Co., Ltd., Japan) were concomitantly administered to the lateral tail vein at doses of 200 mg/kg of body weight and 125 mg/kg, respectively. Three days after the first administration, 100 mg/kg and 125 mg/kg of cyclophosphamide and hydrocortisone, respectively, and 1 × 106 cells of C. glabrata were administered into the lateral tail veins of the mice. The same amounts of the immunosuppressive agents were administered once every 4 days. The cells were suspended with saline to a final volume of 250 μl prior to their administration to the mice. Out of the seven mice allocated for each date, the heaviest and the lightest mice were removed and the five remaining mice were etherized and killed by cervical dislocation. Both kidneys were extirpated, homogenized, diluted with saline, and spread onto agar plates to determine the number of CFU of C. glabrata in the kidneys. This analysis was carried out at days 1, 7, and 14 after the injection of C. glabrata. The animal experiment complied with all relevant guidelines and policies of the Animal Welfare Committee of the Faculty of Medicine of Chiba University, Japan.

RESULTS

Length of homologous sequence required for efficient gene targeting to the correct locus in ACG4. The length requirement for the homologous sequence to induce correct gene targeting was measured in ACG4 (Fig. 1). The DNA cassette consists of the Tet-P, the HIS3 gene as a selection marker, and homologous sequences ranging in size from 40 to 1,000 bp on either side. When the flanking sequences were 40 bp, no correctly targeted integrants were identified. The highest efficiency of gene targeting was 63.5% when the length of the flanking sequences was 500 bp. The efficiency was decreased to 16.3% when the homologous sequence of the 5’ side was 60 bp, even if the 3’ side had a 500-bp homologous sequence (Fig. 1). Unexpectedly, when the length of the homologous sequences
was 1,000 bp, the targeting efficiency was lower than the efficiency when the length was 500 bp. From those results, it was suggested that efficient gene targeting required a few hundred base pairs of homologous flanking sequence in ACG4.

**Effect of carrier DNA concentration on transformation efficiency.** Denatured salmon sperm DNA is used as carrier DNA for experiments with *S. cerevisiae* and *K. lactis* (14, 22). The requirement for the amount of carrier DNA for efficient gene targeting in *C. glabrata* was investigated in this work. The efficiency was measured by the integration rate of the Tet-P cassette into the *ADE2* promoter with a 200-bp homologous sequence. The highest efficiency was identified when 20 μg of carrier DNA was used. About two-times-higher efficiency was achieved when using this amount of carrier DNA than when no carrier DNA was used.

**Construction of the YKU80 deletion mutant for efficient gene targeting.** A search of the *C. glabrata* genomic sequences in Genolevures (http://cbi.labri.fr/Genolevures/) revealed that one gene has homology to *S. cerevisiae* YKU80. It is designated YKU80 (CAGL0K03443g) in *C. glabrata*. The amino acid sequences deduced from these genes shared 39% identity and 58% similarity. A DNA cassette was designed to delete YKU80 and simultaneously confer resistance to sulfometuron methyl. Recipient strain ACG4 was transformed with the cassette to construct yku80 mutant HET80. To confirm construction of the yku80 deletion, PCR analysis was performed using three sets of primers (Fig. 2).

**Gene targeting efficiency with short homologous sequences in HET80.** The yku80 deletion mutant HET80 was evaluated for gene targeting efficiency (Fig. 1). The DNA cassette, including the Tet-P with the homologous flanking sequences ranging from 40 to 200 bp, was integrated into the promoter region of *ADE2*. The efficiency of gene targeting was 5.1% with 40 bp of homologous flanking sequence in HET80, while this length of homologous sequences provided no correct integration in ACG4. Likewise, with 80-bp flanking homologous sequences, the efficiency of gene targeting was 8.6% in HET80, approximately sevenfold more efficient than in ACG4. The efficiency rose to 80% with 500 bp of homologous flanking sequence in HET80. The relative increase in the rate of gene targeting efficiency caused by YKU80 deletion was greater with the shorter length of homologous flanking sequence in HET80 (Fig. 1).

To evaluate the targeting efficiency for various targets in *C. glabrata*, DNA cassettes for targeting to 27 different loci were produced and introduced into HET80. Between 5 and 30 colonies of the transformants were checked for the correct integration site by PCR. The average efficiency was 13% with 60 bp of homologous flanking sequence. These results suggest that gene targeting in HET80 was effective at various loci.

**Susceptibilities of yku80 mutant to DNA-damaging agents.** To characterize the yku80 deletion mutant of *C. glabrata*, the effects of temperature and DNA-damaging agents were examined in HET80. Since *C. glabrata* grows well at 37°C, 37°C and
42°C were chosen as permissive and restrictive temperatures, respectively. Whereas the growth seemed slightly delayed at 42°C in HET80, no distinct growth defect relative to the wild-type strain was identified. No valid differences between HET80 and ACG4 were recognized in the mortality of cells exposed to UV irradiation, MMS, H2O2, or hydroxyurea (Fig. 3). These results suggest that the YKU80 deletion mutant of C. glabrata is not disabled in general cellular functions or DNA repair.

Construction of YKU80 knockdown strain with insertion of SAT1 flipper. To employ the beneficial effects of YKU80 inactivation on gene targeting without any complicating effects, including possible synthetic sickness or lethality, it is necessary to construct a strain in which the expression of YKU80 is restored following transient knockdown during transformation. To realize such a convenient strain, the module SAT1 flipper was integrated just before the first ATG of the annotated open reading frame of YKU80, creating the strain HETS146. Unfortunately, the gene targeting efficiency was greatly decreased in this strain (Fig. 4 and 5). This was an unexpected result, and we constructed another strain, HETS202, in which the SAT1 flipper was integrated just before the second ATG, which is 81 nucleotides downstream from the first ATG on the same frame. During incubation under non-selective conditions, HETS146 and HETS202 lost the SAT1 flipper and produced POP-SF1467 and POP-SF221, respectively.

Evaluation of gene targeting efficiency in the YKU80 knockdown strains. The efficiency of gene targeting was measured by the integration into the promoter region of ADE2 of a DNA cassette containing the Tet-P with a flanking 200-bp homologous sequence. With the use of HETS146 and POP-SF1467, the efficiency rates were 1.97 and 27.4%, respectively, unexpectedly lower when the recipient included the SAT1 flipper than after it lost it. On the other hand, using HETS202 and POP-SF221, the rates were 40.3 and 25.0%, respectively. These results indicate that the expression of YKU80 was repressed...
when the SAT1 flipper was located before the second ATG and restored when it was lost (Fig. 5).

Transcription initiation site of YKU80. To confirm the site of the promoter region of YKU80, the initiation site of transcription of YKU80 was investigated by a 5’ RACE experiment. The sequences of nine clones, including the 5’ RACE products, were determined. This showed that all transcription was initiated downstream of the first ATG (data not shown). These results indicated that the first ATG is not an initiation codon of YKU80.

Evaluation of the yku80 knockdown effect on gene targeting to various loci. The gene targeting efficiency was 3.5% (4/114) in HETS202 with 40 bp of homologous sequence corresponding to the ADE2 promoter region. To extend these results to other loci, the gene targeting efficiency was measured at 15 loci using KUE200 in which the SAT1 flipper integrated upstream of the second ATG. The DNA cassettes included, in all cases, 56 bp of homologous flanking sequence on either side of the HIS3 selective marker. After each transformation, 3 to 30 colonies were checked by PCR. The efficiency of gene targeting was approximately 5 to 10% in three genes and higher than 30% in other genes. The results suggest that gene targeting with short homologous sequences in yku80 knockdown strains is effective at multiple loci.

Effect of the remaining FRT on repression of YKU80 transcription. After the SAT1 flipper is removed from the genome, the FRT sequence, which is a 34-bp short palindrome, remains in the sequence upstream of the second ATG of YKU80 in POP-SF221. The effect on gene expression caused by the FRT remaining in the 5’ end of the YKU80 ORF was quantified by real-time PCR. Since much more mRNA of YKU80 is transcribed during stationary growth phase than during exponential growth phase (K. Ueno et al., unpublished data), mRNA was harvested from the cells at stationary phase. The amount of mRNA relative to ACG4 is shown in Fig. 6. The YKU80 mRNA level was 0.38-fold in HETS202, but POP-SF221 had 1.09-fold, whereas no mRNA was identified in HET80. Thus, no effect of FRT on transcription was identified in POP-SF221 in comparison to its wild-type progenitor.

Mutation rate measured by spontaneous 5-FOA resistance in HETS202. It is an important criterion of a transformation host strain that the strain be genetically stable, without elevated mutation frequency occurring during maintenance. In order to confirm the genetic stability of the YKU80 knockdown strain, the mutation rate of HETS202 was investigated. The mutation rates in HETS202 and ACG4 (as a control) were measured as the frequency of 5-fluoroorotic acid (5-FOA) resistance. The strains were subcultured on YPD plates containing 100 μg/ml NSTC 10 times, and the number of CFU was determined on YPD plates containing 0.2% 5-FOA. The mutation rates of ACG4 and HETS202 were (2.97 ± 1.38) × 10⁻⁷ and (4.15 ± 1.08) × 10⁻⁷ (P = 0.31), respectively, indicating no significant difference in mutation rate caused by the repression of YKU80.

In vivo survival of strains carrying FRT in YKU80 promoter. To evaluate the virulence of strains in which FRT remains before the ORF of YKU80, an in vivo experiment was carried out. Cells of ACG4 and POP-SF221 and KPOP11538 were administered into the lateral tail veins of mice. KPOP11538 has a cyb2 mutation and was used as a control because growth defects have been previously identified in vitro (Ueno, unpublished). The fungal burdens of ACG4 and POP-SF221 were transiently decreased at day 7 and increased at day 14, while the fungal burden of KPOP11538 gradually decreased and eventually fell below the limit of detection. The pattern of the survival curve was not different for ACG4 and POP-SF221 (Fig. 7). These results indicate that FRT located in the YKU80 promoter region has no effect on the survival of C. glabrata in vivo.

DISCUSSION

To facilitate systematic C. glabrata mutant construction in a high-throughput manner, a new recipient strain that permits efficient gene targeting is required. As a first step, we demonstrated that the targeting efficiency with short homologous sequences is increased in a yku80 deletion mutant. This made the strain useful for efficient gene targeting, but leaving the gene crippled could give misleading results in disruptants. In S. cerevisiae, many genetic interactions of YKU80 with other genes, including synthetic lethal combinations, have been re-
ported (29, 33). There may be some effects, including unknown synthetic sickness or lethality, induced by YKU80 inactivation. Therefore, YKU80 should be restored to full activity for the analysis of the specific function of each gene after the mutant construction. Cotransformation of the DNA cassette for Tet-P integration and a DNA cassette for YKU80 restoration did not succeed (data not shown), so we decided to construct a strain in which the expression of YKU80 could be repressed during transformation for optimal gene targeting and the expression easily restored after the gene targeting.

The SAT1 flipper is an FLPL-mediated pop-out system that is efficiently removed from chromosomal sites in C. albicans (34). In this work, we have used the SAT1 flipper for the repression of YKU80 expression in C. glabrata. The SAT1 flipper has not previously been reported to be useful for gene manipulation in C. glabrata. The maltose promoter is not likely to regulate transcription in C. glabrata as well as in C. albicans, because the DNA cassette SAT1 flipper was frequently lost in medium, including glucose. Even when the cells were cultured in YPD, including NStC, 26.3% of them lost the SAT1 flipper. The high frequency of the pop-out is convenient for this work, since a step in which the strain is plated on maltose medium to induce the pop-out can be skipped. For other uses, however, the promoter needs to be replaced with a controllable promoter in order to obtain better regulation of FLPL. These results suggest that the MAL2 promoter in the SAT1 flipper cassette can’t be repressed in medium, including glucose, since it is adequately leaky to generate derivatives in which the SAT1 flipper is removed.

In yku80 mutants of S. cerevisiae, a severe growth defect at 37°C had been identified due to telomere shortening, among other causes (1, 5, 9, 11). These cells also exhibit hypersensitivity to DNA-damaging agents, such as MMS, but not to UV radiation or hydroxyurea (5, 16, 37). In the yku80 mutant of C. glabrata, no obvious growth delay was identified at 37°C or 42°C. No sensitization to MMS, H2O2, UV radiation, or hydroxyurea was identified in this study (Fig. 3). These results suggest that DNA repair and other essential cell functions are not critically affected by the deletion of yku80 in C. glabrata. It seems likely that DSB repair is supported by another pathway(s) independent of YKU80, such as microhomology end joining (23). In Schizosaccharomyces pombe, although sensitivity to MMS did not occur in Ku single mutants, NHEJ was almost suppressed, which was verified by a plasmid repair assay (24, 39).

The single deletion of yku80 in C. glabrata had no obvious effect on the growth rate, DNA repair ability, and mutation rate (Fig. 3). The SAT1 flipper could be maintained in YKU80 knockdown strains under selection with 100 μg/ml of NStC and then automatically lost from the cells under nonselective conditions. This construct allows efficient gene targeting and restoration of the expression of YKU80 by a single transformation. Although the FRT has been left at the promoter region of YKU80 after the SAT1 flipper was excised from chromosome, the FRT has no detectable effect on the survival of C. glabrata in vivo (Fig. 7). The YKU80 knockdown strain, HETS202, is a new recipient strain that enables rapid construction of desired recombinants via high-efficiency gene targeting; furthermore, strains deploying NHEJ regulation, e.g., HETS202, will facilitate gene manipulation and contribute to comprehensive gene function analyses, elucidating pathogenicity and facilitating the identification of antifungal drug targets in C. glabrata.

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