A New Type of Gene-Disruption Cassette with a Rescue Gene for *Pichia pastoris*

Tatsuro Shibui
Food Biotechnology Laboratory, School of Food Sciences, Nippon Veterinary and Life Science University, 1-7-1 Kyounamcho, Musashinoshi, Tokyo 180-8602, Japan

Hiroyoshi Hara
Food Biotechnology Laboratory, School of Food Sciences, Nippon Veterinary and Life Science University, 1-7-1 Kyounamcho, Musashinoshi, Tokyo 180-8602, Japan

DOI 10.1002/btpr.2541
Published online September 4, 2017 in Wiley Online Library (wileyonlinelibrary.com)

Pichia pastoris has been used for the production of many recombinant proteins, and many useful mutant strains have been created. However, the efficiency of mutant isolation by gene-targeting is usually low and the procedure is difficult for those inexperienced in yeast genetics. In order to overcome these issues, we developed a new gene-disruption system with a rescue gene using an inducible Cre/mutant–loxP system. With only short homology regions, the gene-disruption cassette of the system replaces its target–gene locus containing a mutation with a compensatory rescue gene. As the cassette contains the AOX1 promoter-driven Cre gene, when targeted strains are grown on media containing methanol, the DNA fragment, i.e., the marker, rescue and Cre genes, between the mutant-loxP sequences in the cassette is excised, leaving only the remaining mutant-loxP sequence in the genome, and consequently a target gene-disrupted mutant can be isolated. The system was initially validated on ADE2 gene disruption, where the disruption can easily be detected by color-change of the colonies. Then, the system was applied for knocking-out URA3 and OCH1 genes, reported to be difficult to accomplish by conventional gene-targeting methods. All three gene-disruption cassettes with their rescue genes replaced their target genes, and the Cre/mutant–loxP system worked well to successfully isolate their knock-out mutants. This study identified a new gene-disruption system that could be used to effectively and strategically knock out genes of interest, especially whose deletion is detrimental to growth, without using special strains, e.g., deficient in nonhomologous end-joining, in P. pastoris. © 2017 American Institute of Chemical Engineers *Biotechnol. Prog.*, 33:1201–1208, 2017
Keywords: Pichia pastoris, *phosphoribosylaminoimidazole carboxylase, ADE2, orotidine-5′-phosphate decarboxylase, URA3, α-1,6-mannosyltransferase, OCH1, Cre/loxP, AOX1 promoter*

Introduction

Pichia pastoris was initially identified and developed as a potential source of single-cell protein (SCP) about 50 years ago,[1,2] since it exhibited the ability to assimilate abundant methanol made from petroleum and achieve high cell densities in continuous culture. However, the cost of sources of methanol was increased by the oil crisis at that time. Therefore, SCP production by *P. pastoris* from methanol was no longer viable and ceased.

As *P. pastoris* is a eukaryotic organism that is easier to handle than mammalian cells, and has the potential to produce large quantities of foreign proteins and ability to make post-translational modifications,[3] it has become increasingly used for both research and the production of recombinant proteins with diagnostic and therapeutic applications,[4,5] and has become a highly successful protein expression system.

Correspondence concerning this article should be addressed to T. Shibui at tshibui@nvlu.ac.jp

Many genetically manipulated forms of *P. pastoris* have been reported.[5,6] However, in contrast to model yeasts, such as *Saccharomyces cerevisiae* that has a very efficient gene-targeting system, the efficiency of gene targeting is low in *P. pastoris*. This generally leads to difficulty in the reproduction of the gene-disrupted mutants reported by others, and prevents further understanding of the gene functions and roles in *P. pastoris*. Especially, disruption of the *OCH*1 gene is markedly less reproducible, and the locus was believed to be difficult for targeting[7] as the reason is most likely the growth defect of the *OCH1* deletion strain. Recently, isolation of the *OCH1*-gene deletion mutants of *P. pastoris* with adroit methods was reported by Krainer et al.[8] and Chen et al.[9]

Site-specific recombinase systems, such as *FLP-FRT*[10] and *Cre/lox*P,[11] have often been used for gene targeting, since marker genes can be easily removed from their host genome by a site-specific recombination mediated by those recombinases.[12,13] Among them, *Cre/mutant–lox*P systems have the advantage of irreversible recombination.[14] After the DNA fragment between the *mutant-lox*P sites has been excised, there is no longer a sequence in the genome that is

Table 1. DNA Primers for PCR used in Construction and Analysis of the Gene-Disruption Mutant

Name of Oligo DNAs	Sequence
For cloning the rescue gene in the vector	
Ade2 5′ F[1]	5′-**CTTTTTTTTCTGTAC**AAATCAGTTCTAGCTGGCCTCC-3′
Ade2 3′ R[2]	5′-**AATTCGCGTCTGTAC**TGTGTACTATTATATATACATC-3′
Ura3 5′ F[1]	5′-**CTTTTTTTTCTGTACA**TAACCACCTTTGACGAATTGAC-3′
Ura3 3′ R[2]	5′-**AATTCGCGTCTGTAC**TTTGCTGGCTACTCCTTGAGTC-3′
Och1 5′ F[1]	5′-**CTTTTTTTTCTGTACA**GCTTGGGGTAATAGATAGATG-3′
Och1 3′ R[2]	5′-**AATTCGCGTCTGTAC**TTCTTTGATGATTGGTGTTGGG-3′
For the gene-disruption cassette	
Ade2 Pr.1F[3]	5′-CCAAGAATCGTAGAAACGATTAAAAAACTTCCAAACTCTC*AAAGGTTTGGAAAAGTACCG*-3′
Ade2 Pr.1R[4]	5′-CTATTATATATACATCTATTAAAAAACAATACTAAATTCA*CACAAGGTTGGTGCTACGCG*-3′
Ura3 Pr.1F[3]	5′-AGTTAACATCAACCTGATCAAAGGGATAGATACCTAGACAAA*AGGTTTGGAAAAGTACCG*-3′
Ura3 Pr.1R[4]	5′-AATCGCCCGAAACAATTGATCCCCTGTACATACTTGTAAT*CACAAGGTTGGTGCTACGCG*-3′
Och1 Pr.1F[3]	5′-GTTCCACAATTTCCAGTGTTCGTAGCAAATATCATCAGCCA*AAGGTTTGGAAAAGTACCG*-3′
Och1 Pr.1R[4]	5′-ATTCTCTAATCTCGCTATATCTATTTTACTCTAGCTTTCT*CGGAAATAAACAAACACGCG*-3′
For analysis of genome DNA	
Ade2 Pr.2F[5]	5′-CCTCGAACTATTCCTTGGC-3′
Ade2 Pr.2R[6]	5′-TATCATGTCTGCATCGGGAC-3′
Ura3 Pr.2F[5]	5′-CTGCAGAAATGGGGAGATAA-3′
Ura3 Pr.2R[6]	5′-ACTAGTGGTTTTCTGGGGGT-3′
Och1 Pr.2F[5]	5′-ATTGGCTTTATTTGTTTGTCC-3′
Och1 Pr.2R[6]	5′-TGCTTCTTGGTGTTTGTGTTCCG-3′
Pr.ZeoR[7]	5′-CTACGAACACTGGAAATTGTGG-3′
Pr.Aox1R[8]	5′-TTTGATCTTCTCAAGTTGTCG-3′
For sequencing analysis	
Ade2 Pr.SF[9]	5′-GCACGCTTCTTTCTTATCGACG-3′
Ade2 Pr.SR[10]	5′-AATTCTGACATCCTTTTAGAGC-3′
Ura3 Pr.SF[9]	5′-GAACTCTTGTTGTTCTTTAGCG-3′
Ura3 Pr.SR[10]	5′-CATTCCACCAGTTGGTGAGC-3′
Och1 Pr.SF[9]	5′-ATCAGACTTTGATTTGATGAGG-3′
Och1 Pr.SR[10]	5′-TGATAGTCAGGGATTTCGCG-3′

[1,2]The bold letters are the vector sequences required for cloning of the PCR-amplified *rescue* genes (Gene Accession No. FN392321 for *ADE2,* Gene Accession No. AF321098 for *URA3,* and Gene Accession No. E12456 for *OCH1*) by the cloning system using *Poxvirus* DNA polymerase 5′ → 3′ exonuclease activity. [3,4]The underlined sequences are the 5′ and 3′ untranslated regions (UTR) of the rescue genes. Italic sequences are the vector sequences required for PCR-amplification of the genes between the L- and R-*lox*P sequences. [5,6,9,10]Sequences are located in the 5′ and 3′ UTRs of the rescue genes in the *P. pastoris GS115* genome. [7,8]Sequences are located in the zeocin resistance gene and *AOX1* promoter regions in the gene disruption cassette, respectively.

recognized by *Cre* recombinase. The gene-targeted strains are consequently free of *Cre* recombinase-reactive sequences. We previously reported reusable *P. pastoris* vectors using one of those mutant systems.[15]

On the other hand, the improvement of PCR technology has enabled us to amplify longer DNA fragments with high-fidelity, although concatenating many shorter fragments to construct a longer DNA fragment by overlapping PCR is still not always successful. The construction of gene-disruption cassettes by PCR has become a convenient and time-saving method for the preparation of DNA fragments for transformation. Short flanking regions of 30–50 bp that are included in the 5′ portion of PCR primers are sufficient for integration at the precise genome locus via homologous recombination in *S. cerevisiae*.[12,16,17]

In this paper, we describe PCR-construction of gene-disruption cassettes that consist of *Cre*/*mutant-loxP* and rescue genes, and the strategic isolation of deletion mutants of complete coding regions of target genes.

Materials and Methods

Bacterial and yeast strains

Escherichia coli, JM 109, competent cells used for plasmid construction were purchased from Toyobo Biochemicals (Japan). *Pichia pastoris, GS115,* for the construction of the *ADE2*, *URA3,* and *OCH1* deletion mutants was from Life Technology (USA).

Kits for DNA manipulation

The KOD FX polymerase kit used for colony-PCR of *P. pastoris* was purchased from Toyobo Biochemicals (Japan). The cloning kit, which utilizes the unique properties of the 5′→3′ exonuclease activity of *Poxvirus DNA polymerase*,[18] was from Clonetech (Infusion Cloning Kit, USA).

PCR used for the construction of plasmids and disruption cassettes was performed with hi-fidelity DNA polymerase (Prime Max PCR Kit, Clonetech, USA), according to the supplier's manual.

DNA purification kits were purchased from Nippon Genetics, Gbm (Japan).

Oligo DNAs and plasmid

Oligo DNAs used for PCR amplification were obtained from Medical Bio-Laboratory (Japan), and their sequences are shown in Table 1.

The plasmid pInt2 Cre containing the *AOX*1-driven *Cre gene* and mutant *lox*P sequence was previously described.[15]

Culture media

LB agar medium (Life Technology, USA) supplemented with ampicillin (Meiji Pharmaceuticals, Japan) at 40 mg/L (LB Amp agar) was used for plasmid construction. YPD medium containing 1% yeast extract (Difco, USA), 2% Bact-peptone (Difco, USA), and 0.2% glucose [(WAKO, Japan), and YPD agar containing 1.5% Bacto-agar Difco were used for *P. pastoris* cultivation. YPDSZ YPD containing 1M] sorbitol, zeocin 100 mg/L (WAKO, Japan), and 1.5%

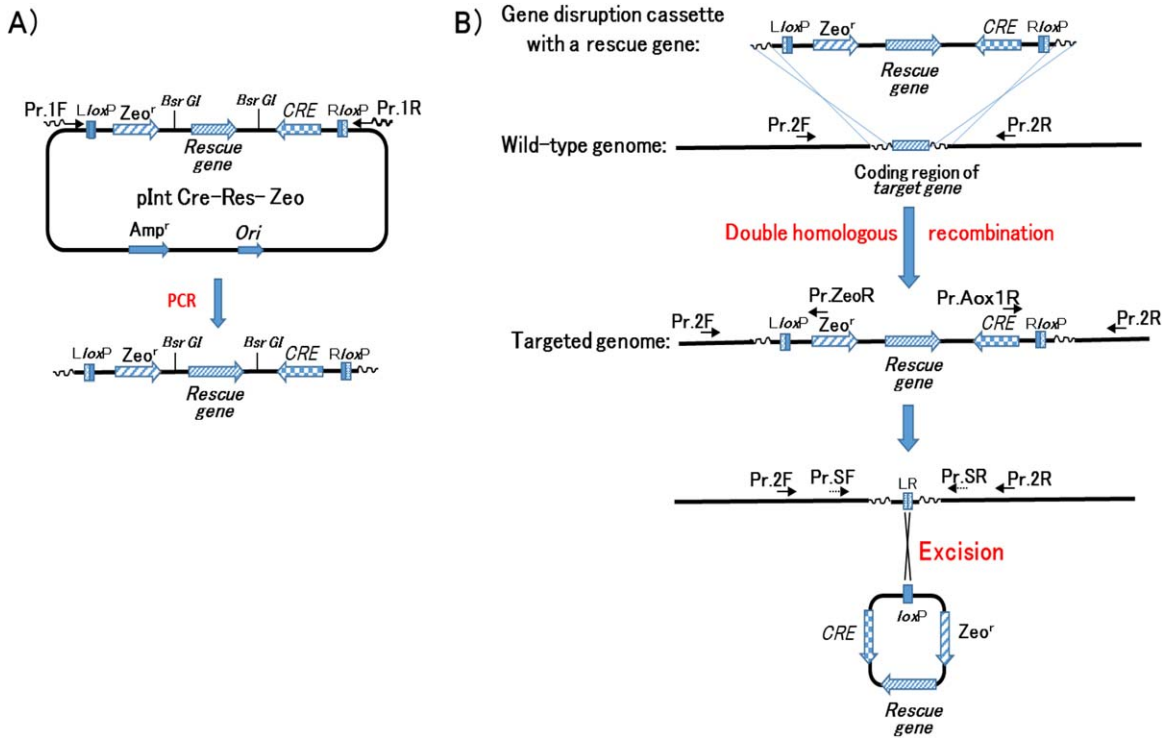

Figure 1. Schematic workflow of construction of a disruption cassette with a rescue gene and knockout of the target gene.

(A) PCR construction of a gene-disruption cassette with its rescue gene. Primers Pr. 1F and Pr. 1R, which contain 40-bp homologous sequences at their 5′ ends, are used for construction of a disruption cassette with its rescue gene by PCR. (B) Gene targeting of the gene-disruption cassette with its rescue gene and removal of the marker and rescue and *CRE* genes from the targeted genome by *Cre* recombinase. A double homologous recombination event replaces the coding region of the target gene with the disruption cassette with its rescue gene. Growth of the gene-targeted cells on media containing methanol induces the production of *Cre* recombinase that recognizes the L and R *lox*P sites, and excises the sequence between them, leaving only a mutant LR sequence in their genome. *Abbreviations*: Pr.1F and Pr.1 R, PCR primers used for construction of the gene-disruption cassette with its rescue gene; Pr.2F and Pr.2R, PCR primers used for genome analyses; Pr.SF and Pr.SR, PCR primers used for sequencing; L*lox*P and R*lox*P, mutant *lox*P sequences: Zeo^r, zeocin resistance gene; *CRE*, *Cre* recombinase expression gene driven by *AOX*1 promoter; Amp and Ori, ampicillin resistance gene and replication origin from pUC18, respectively. Sequences of the primers used for gene-disruptions of *ADE2*, *URA3,* and *OCH1 genes* are listed.

Bacto-agar was used for the selection of transformed cells after electroporation. YPDA [YPD containing 0.002% adenine (WAKO, Japan), and 1.5% Bacto-agar according to the Pichia-Pink^{TM} Expression System manual (Invitrogen, USA)] was for the cultivation of *ADE2* deletion mutants. YPDU [YPD containing 0.004% uracil (WAKO, Japan), and 1.5% Bacto-agar] was for the cultivation of *URA3* deletion mutants. Minimal methanol medium (YNBMM)[6] containing 6.7% Yeast Nitrogen Base with amino acids (Difco, USA), 0.5% methanol, and 1.5% agar was used for induction of the *AOX1*-driven *Cre* gene expression. YNBMM A [YNBMM containing 0.002% adenine (WAKO, Japan)], YNBMMU [YNBMM containing 0.04% uracil (WAKO, Japan)], and YNBMM were used for the isolation of *ADE*2, *URA3,* and *OCH*1 deletion mutants, respectively. Minimal dextrose medium (YNBMD)[6] containing 6.7% yeast nitrogen base with amino acids (Difco, USA), 0.5% glucose, and 1.5% agar was used for the initial cultivation of *OCH1*-deletion mutants. YNBMDA (YNBDM containing 0.002% adenine) and YNBMDU (YNBMD containing 0.004% uracil) were used for the initial cultivation of *ADE2*- and *URA3*-deletion mutants, respectively. YNBMDU + FOA [YNBMDU containing FOA (5-Fluoroorotic acid) 1 mg/mL] was used for screening *ura3* mutants. FOA was purchased from WAKO (Japan).

Transformation of P. pastoris

P. pastoris cells were transformed by electroporation according to the manual provided by Life Technology. Transformants were selected on YPDSZ plates. In cases of *ADE2*- or *URA3*-gene-disruption cassettes without rescue genes, adenine or uracil was added to YPDSZ plates as appropriate. Under our conditions, about 1,000 colonies appeared on YPDZ plates per 1 μg of PCR-amplified DNA constructs with incubation at 28°C for 48 h.

Sequencing analysis of the genome of the isolated clone

DNA fragments that contained the deleted regions of *ADE2, URA3* and *OCH1*-coding sequences were amplified by colony-PCR using the primers Ade2 Pr.2 F and Ade2 Pr.2 R, Ura3 Pr.2 F and Ura3 Pr.2 R, and Och1 Pr.2 F and Och1 Pr.2 R, shown in Table 1, with the KOD FX DNA polymerase kit, respectively. The amplified DNA fragments were purified with the DNA purification kit (Nippon Genetics, Japan). Sequencing of the DNA fragments was performed in both forward and reverse directions using the primers Ade2 Pr.S F and Ade2 Pr.S R, Ura3 Pr.S F and Ura3 Pr.S R, and Och1 Pr.S F and Och1 Pr.S R, respectively, by Eurofin (Japan) (see Figure 1B and Table 1).

Results and Discussion

Gene-disruption cassettes without rescue genes

Using a reusable vector, pInt2 Cre, we firstly constructed disruption cassettes without rescue genes by PCR with the

primers Ade2 Pr.1F and Ade2 Pr.1R, Ura3 Pr.1F and Ura3 Pr.1R, and Och1 Pr.1F and Och1 Pr.1R in Table 1 for the isolation of *ADE2*, *URA3,* and *OCH1* deletion mutants, respectively.

For *ADE2* mutants, approximately 1,000 colonies that appeared on the YPDSZ+ 0.002% adenine plates were visually screened. However, no obvious color-changed colonies, i.e., red,[19] were observed. Then, three hundred of those colonies were screened for their adenine requirement using YNBMD and YNBMDA plates. None of the 300 colonies showed an adenine requirement. An attempt to isolate adenine mutants using this cassette without the rescue gene was unsuccessful.

For *URA3* mutants, by electroporation with 1 μg of the gene-disruption cassette, about 1,000 colonies appeared on YPDSZ+ 0.004% uracil plates with incubation at 28°C for 48 h. Among them, four hundred colonies were picked up, and screened on YNBMD or YNBMDU plates. Those colonies were all non-auxotrophic for uracil. Then, we used YNBMDU + FOA (5-fluoroorotic acid) plates for the positive screening of uracil-minus mutants. Using 1 μg of the cassette, several tiny colonies appeared about 10 days after electroporation. After cloning on YNBMDU + FOA plates,12 of the clones were checked for the *URA3* gene by PCR with the primers Ura3 Pr2F and Ura3 Pr2R, listed in Table 1. All of them showed a DNA fragment with a length of the wild-type *URA3* gene (data not shown). They were considered spontaneous FOA-resistant mutants. Thus, the isolation of *URA3* mutants by the disruption cassette without its rescue gene was also not successful.

For the *OCH1* gene, by electroporation with 1 μg of the gene-disruption cassette about 1,000 colonies appeared on YPDSZ plates with incubation at 28°C for 48 h. Approximately four hundred colonies among them were picked up and directly screened by PCR using the primers Och1 Pr2F and Och1 Pr2R, listed in Table 1. All of them showed the length of the wild-type DNA fragment, and isolation of the mutants using the disruption cassette without the rescue gene also failed.

Construction of gene-disruption cassettes with rescue genes

An outline of the general construction scheme is presented in Figure 1. Rescue genes, *ADE2, URA3,* and *OCH*1 genes, were PCR-amplified from the *GS115* genome using the primers Ade2 5′ F and Ade2 3′ R, Ura3 5′ F and Ura3 3′ R, and Och1 5′ F and Och1 3′ R, shown in Table 1, respectively. Those rescue genes were then cloned into a *Bsr* GI site in pInt2 Cre, located between the zeocin resistance and *AOX1* promoter-driven *Cre* genes, to construct the plasmids pAde2, pUra3, and pOch1. They are described as pInt Cre-Res-Zeo in Figure 1A. Then, a gene-disruption cassette with a rescue gene for *ADE2*, *URA3,* or *OCH1* was PCR-constructed from pAde2, pUra3, or pOch1, using the primers Ade2 Pr.1F and Ade2 Pr.1R, Ura3 Pr.1F and Ura3 Pr.1R, and Och1 Pr.1F and Och1 Pr.1R, shown in Table 1, respectively. PCR-constructs of those cassettes were checked by agarose gel electrophoresis. Then, the cassettes were purified with the DNA purification kit, and used for electroporation.

Since promoter regions of those rescue genes were not defined, we cloned the DNA fragments containing about 700 bp upstream of each coding region. From our results described below, the amplified DNA fragments effectively covered their promoter regions. The lengths of those gene-disruption cassettes were 6.2, 6.4, and 6.0 kbp for *ADE2, URA3,* and *OCH1* cassettes, respectively. Those DNA lengths were considered to be near the upper limit for the precise and efficient amplification of DNA fragments by PCR without marked adjustment of the PCR conditions set by the manufacturer.

Isolation of ADE2 gene-targeted clones using a gene-disruption cassette with the ADE2 rescue gene

About 1 μg of the gene-disruption construct with the rescue gene was used for electroporation of *GS115*. More than 1,000 colonies appeared on YPDSZ plates with incubation at 28°C for 48 h. Among them, 70 colonies were selected, and their genomes were checked for *ADE2* gene-targeting by colony PCR using two sets of primers, Ade2 Pr.2 F and Pr.Zeo R for amplification of the 5′ portion of the targeted gene, and Pr.Aox1 R and Ade2 Pr.2 R for its 3′ portion. Sequences of the primers are listed in Table 1. Two colonies were positive with the 5′ portion primers. One of them was also positive with the 3′ portion primers. The 5′ and 3′ portion-positive clone (*ADE2* parental strain) further showed a DNA fragment whose *ADE2*-coding region was replaced with the disruption cassette by PCR with the primers Ade2 Pr.2 F and Ade2 Pr2.R, shown in Table 1 (lane 2 in Figure 3A). The gene-disruption cassette replaced the *ADE2* gene at an efficacy of 1/70. The parental strain was subjected to single colony isolation three times for further use. As for the gene-disruption efficiency of *ADE2*, Du et al.[20] reported that, on using 1.7- and 1.0-kbp homologous sequences for the 5′- and 3′-ends of their gene-disruption cassette, respectively, they isolated only one pink colony, although they did not report how many colonies they visually screened.

Isolation of ADE2 deletion mutants with induction of the cre/mutant–loxP system

In our previous study,[15] we examined the excision rate of our *Cre*/*mutant–lox*P system. A total of 96% of the cells lost zeocin resistance on incubation at 30°C for 6 h in methanol-containing medium. In this study, in order to visually check the morphological changes of the colonies, YNBMM plate-culture was used for induction of the *Cre*/*mutant–lox*P system.

The *ADE2* parental strain cells isolated in the previous section formed normal white colonies on YNBMD plates on day 2 at 28°C. When they were spread on YNBMMA plates, their *Cre*/*mutant–lox*P system was induced, and dark red or orange-colored colonies appeared on the plates (data not shown). From one of the colored colonies, we isolated the *ADE2* deletion mutant. The parental colonies on YNBMD plates were also slightly pink after incubation for 4–7 days at 28°C. This indicated that the slight induction of *Cre* recombinase occurred on YNBMD plates, even though the *AOX1* promoter was reported to be very tightly regulated.[21] Therefore, a 1.2-kbp DNA fragment of the *ADE2*-deletion gene was also amplified from the parental cells grown on the YNBMD plates, and also detected on agarose gel electrophoresis along with the parental 6.4-kbp DNA fragment (see lane 3 in Figure 2A). Although those deletion mutants were spontaneously derived from parental cells on YNBMD plates, they could not grow further on the plate without adenine; thus, a very small portion of cells of the deletion

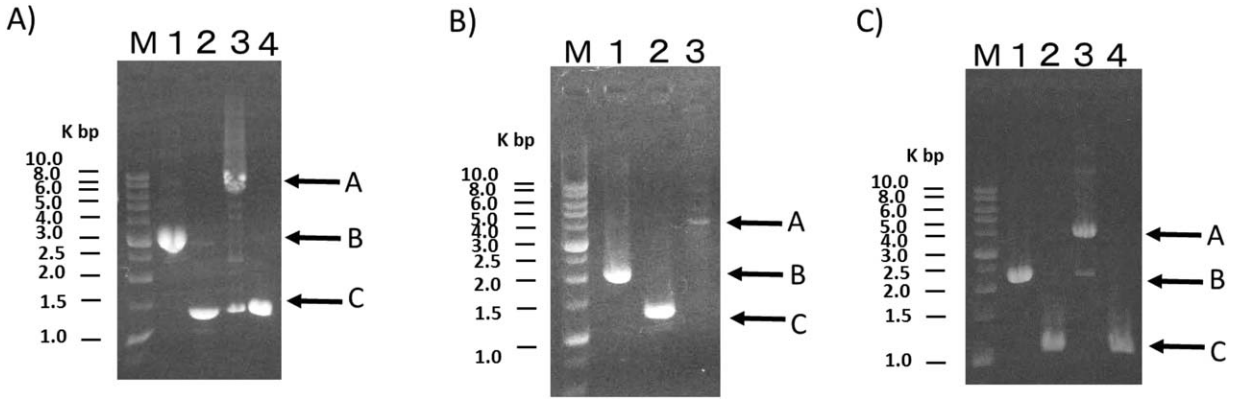

Figure 2. Genomic DNA analysis of isolated clones by PCR.

(A) *ADE2* strains. Lane M: DNA molecular marker, lane 1: GS115 (wild-type), lane 2: *ADE2*-deletion, Lane 3: GS115 + Zeo^r + *ADE2* (rescue) gene + *CRE* (parental strain), lane 4: The larger colony appeared on YPDA. Arrows A–C indicate the +Zeo^r + *ADE2* (rescue) gene, wild-type *ADE2,* and *ADE2*-deletion DNA fragments, respectively. PCR was carried out with primers, Ade2 Pr2.F and Ade2 Pr2.R. (B) *URA3* strains. Lane M: DNA molecular marker, lane 1: GS115 (wild-type), lane 2: *URA3*-deletion, lane 3: GS115 + Zeo^r + *URA3* (rescue) gene + *CRE* (parental strain). Arrows A, B, and C indicate the +Zeo^r + *URA3* (rescue) gene + *CRE*, wild-type *URA3,* and *URA3*-deletion DNA fragments, respectively. PCR was carried out with primers, Ura3 Pr2.F and Ura3 Pr2.R. (C) *OCH1* strains. Lane M: DNA molecular marker, lane 1: GS115 (wild-type), lane 2: *OCH1*-deletion, lane 3: GS115 + Zeo^r + *OCH1* (rescue) gene + *CRE* (parental strain), lane 4: The larger colony appeared on YPD. Arrows A–C indicate the +Zeo^r + *OCH1* (rescue) gene + *CRE*, wild-type *OCH1,* and *OCH1*-deletion DNA fragments, respectively. PCR was carried out with primers, Och1 Pr2.F and Och1 Pr2.R.

mutant were considered to contribute to the slight color changes of the colonies, and detection of the deleted DNA fragment on PCR analysis with agarose gel electrophoresis.

The *ADE2*-deletion mutant isolated on the YNBMDA plates initially did not grow well on rich YPD plates plus adenine (YPDA plates). Du *et al.*[20] also reported that their *ade2* clone exhibited a slow growth phenotype on the media supplemented with adenine. However, on day 2 at 28°C after inoculation, several colonies appeared on the plates, showing growth but at a slower rate than that of the wild-type on YPDA plates on subcultivation.

According to the results on the growth of our *ade2* mutant described above, the failure to isolate the *ade2* mutant using the cassette without the rescue gene might be due to poor growth of the initial *ade2* mutants on the YPDSZ medium containing adenine. It is possible that we missed the mutant colonies as they grew very slowly on the YPDSZA plates.

Isolation of URA3 gene-targeted clones using a gene-disruption cassette with the URA3 rescue gene

As the system worked well for isolation of the *ade2*-deletion mutant, it was applied to obtain *ura3*-deletion mutants. *GS115* was transformed by a *URA3* gene-disruption cassette with the rescue gene, i.e., the *URA3* gene. About 1 μg of the gene-disruption construct with the rescue gene was used for the electroporation of *GS115*. Approximately 1,000 colonies appeared on YPDSZ plates with incubation at 28°C for 48 h. Among them, 60 colonies were selected to check their genome in the same way as *ADE2*. Six colonies were positive with the 5′ portion primers Ura3 Pr.2 F and Pr.Zeo R, shown in Table 1. Among them, one colony (the *ura3* parental strain) was also positive with the 3′ portion primers Pr.Aox1 R and Ura3 Pr.2 R, shown in Table 1. The parental strain was subjected to single colony isolation three times for further use. By PCR with the primers Ura3 Pr.2 F and Ura3 Pr.2 R, shown in Table 1, the clone also showed a *URA3* gene fragment that had been replaced with the disruption cassette (Figure 2B, lane 2). The efficacy of *URA3* gene replacement by the cassette with the rescue gene was 1/60.

Isolation of a URA3 deletion mutant

The *URA3* parental strain isolated was firstly streaked on YNBMMU plates to induce the *Cre/mutant–lox*P system. Unlike the case of the *ade2* parental strain, since morphological changes of colonies were not observed on YNBMMU plates, one of the colonies that appeared on the plates was streaked again on YNBMMU plates. On the 8th day of incubation at 28°C, many tiny colonies were recognized along with a few normal-sized colonies on the plate (data not shown). After cloning of those tiny colonies, their *URA3* gene regions were checked with PCR using the primers Ura3 Pr.2 F and Ura3 Pr.2 R, shown in Table 1 (Figure 2B). All showed deletion of the *URA3* gene. Growth of our *URA3* mutant was very slow on YNBMDU and YPDU plates, even though those plates were supplemented with an adequate amount of uracil (0.004%), which was consistent with the reports on *ura3 P. pastoris* by Du et al.[17] and Nett et al.[22] As predicted from them, our ura3 mutant could also grow on YNBMDU containing FOA that inhibits the growth of *ura3* wild-type strains (data not shown), which means that this strain can also be used for the negative selection of *URA3*-gene vectors on YNBMDU + FOA plates.

We tried to compare the gene-disruption efficacy of our *URA3* gene-disruption system with other *URA3* gene-disruption systems. However, *ura3 P. pastoris* in the previous reports[17,20,23] comprised spontaneous mutants isolated on FOA media. Thus, we were not able to compare our efficiency of gene-disruption directly. As for the related gene *URA5*, the *ura5 P. pastoris* isolation rate using a gene-disruption cassette with 0.9- and 1.1-kbp homologous sequences at the 5′ and 3′ ends was 30 in 10,000 colonies that appeared on selection plates.[23]

From our results and those of others,[7] the failure of our first attempt to isolate *ura3 P. pastoris* without the rescue gene was possibly due to the significantly reduced growth of the mutants. However, in our system, the parental strain grew well with the help of a rescue gene, and we could efficiently isolate the *URA3* deletion mutants even though they grew very slowly.

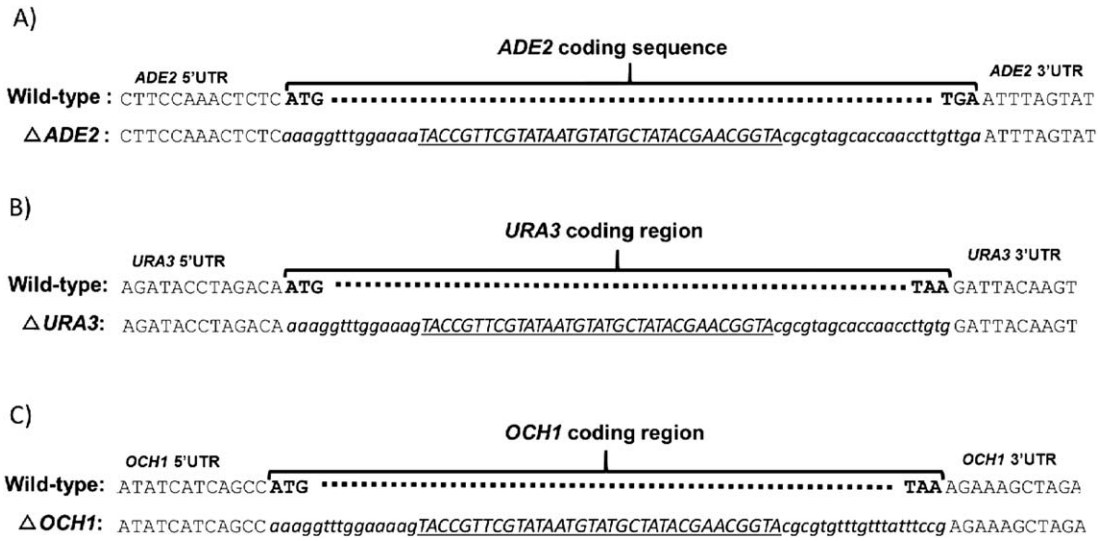

Figure 3. Nucleotide sequences of the targeted regions of the isolated deletion mutants.

(A) *ADE2*-deletion mutant. Wild-type: *ADE2* wild-type genome. △*ADE2*: *ADE2*-deletion mutant genome. (B) *URA3*-deletion mutant. Wild-type: *URA3* wild-type genome. △*URA3: URA3*-deletion mutant genome. (C) *OCH1*-deletion mutant. Wild-type: *OCH1* wild-type genome. △*OCH1: OCH1*-deletion mutant genome. The entire coding region of each wild-type gene was replaced by the sequences in italics that were generated from the disruption cassettes. Underlined capital letters are the resulting sequence (LR) of recombination between the mutant L and R *lox*P by *Cre* recombinase.

Isolation of OCH1 gene-targeted clones using a gene-disruption cassette with the OCH1 rescue gene

The system was further applied for the isolation of *OCH1* deletion mutants. After electroporation using 1 μg of an *OCH1* gene-disruption cassette with the rescue gene, 1,000 colonies appeared on YMDSZ plates for 48 h at 28°C. Forty colonies were selected and had their *OCH1* genome checked in the same way as described in the previous sections. Four were positive with the 5′ portion primers Och1 Pr.2 F and Pr.Zeo R, shown in Table 1. One of them (the *och1* parental strain) was also positive with the 3′ portion primers Pr.Aox1 R and Och1 Pr.2 R, shown in Table 1. The parental strain was subjected to single colony isolation three times for further use. By PCR with the primers Och1 Pr.2 F and Och1 Pr.2 R, shown in Table 1, this clone also showed an *OCH1* locus fragment that was replaced by the cassette (Figure 2C lane 2, arrow A). Since the parental clone was singly isolated completely, a minor band seen at about 2.2 kbp in Figure 2C lane 3 was considered to be a nonspecifically amplified one. The efficacy of isolating the *och1* parental strain was 1/40.

Isolation of OCH1-deletion mutants

The *OCH1* parental strain isolated in the previous section was firstly streaked on a YNBMM plate. One of the colonies that appeared on the plate was selected, and its cells were streaked again on YNBMM plates. After 6 days at 28°C, many small colonies appeared around a few normal-sized colonies on the plate (data not shown). The *OCH1* regions of those small colonies were checked with PCR using the primers Och1 Pr.2 F and Och1 Pr.2 R, shown in Table 1, and they were confirmed to be an *OCH1*-deletion mutant.

Consistent with previous reports on *och1*-deficient *S. cerevisiae*[24] and *P. pasoris*[8,9] strains, those colonies showed slow growth and a rough surface. They initially grew poorly on YPD plates. However, after 7 days, colonies with faster growth appeared among poor-growth ones. The number of those faster-growth colonies increased daily. One of those faster-growth colonies was picked up, and its *OCH1* gene locus was checked by PCR. It was confirmed that cells of the faster-growth colony had the same deleted *OCH1* locus as the original deletion-mutant on YNBMM (Figure 2C, lane 3), and were not contaminated cells. Their growth was faster than the original, but still slower than that of the wild-type *P. pastoris*.

Chen *et al.*[9] reported the isolation of their *OCH1* deletion mutant with enhancement of the gene targeting efficiency of *P. pastoris* by increasing genetic redundancy. They constructed an episomal *OCH1*-expression plasmid, and introduced it into *P. pastoris* prior to disruption of the chromosomal *OCH1* gene. Their disruption cassette, which was separated by cutting with restriction enzymes from a plasmid with the *OCH1* locus, contained 874 and 852 bp homologous sequences at the 5′ and 3′ ends, respectively, and was introduced into *P. pastoris* harboring the *OCH1* episomal plasmid. With the help of the episomal redundant gene, chromosomal *OCH1*-targeted cells grew normally, and were efficiently isolated. Their targeting efficiency was 6/60, which was higher than in our cassette′s case. Then, their episomal plasmid was removed by the expression of *maz f* in the episomal plasmid to isolate *och1 P. pastoris*. They also reported that the cell had abnormally slow growth. Their system requires the construction of an episomal expression plasmid, and its introduction into *P. pastoris* prior to gene-disruption. This is more laborious than using our gene-disruption cassette with a rescue gene.

Krainer *et al.*[8] reported the use of a high homologous recombination mutant strain, *ku70* deletion,[25] to facilitate the isolation of precisely targeted strains of *och1*. They successfully isolated a few *och1, ku70* strains, and they recognized abnormal colony shapes of their *och1* mutants. Then, they applied those abnormal shapes for isolation of the deletion mutants from a wild-type strain. They used about 1.5-kbp homologous sequences at both ends of their gene-disruption cassette. They did not describe how may colonies were

visually screened in the case of the wild-type strain. However, they mentioned that, prior to the visual screening, they picked up 100 clones to directly check their genome by PCR; however, they could not find precisely integrated clones. From their results and those of others,[7] the efficiency of isolating *OCH1*-target strains is considered to be very low using simple gene-targeting constructs.

Efficacy of gene-targeting of the gene-disruption cassettes with rescue genes

Efficacies of correct gene-targeting with our cassettes were 1/70, 1/60, and 1/40 for *ADE2*, *URA3*, and *OCH1*, respectively. These results showed that the gene-targeting efficiency of our gene-disruption cassettes with short homologous sequences (40 bp) in *P. pastoris* was lower than reported in *S. cerevisiae*.[16] Longer homologous sequences were used to improve the integration efficiency in *P. pastoris*.[8] The construction of gene-disruption cassettes with longer (about 1.0 kbp at both ends) homologous sequences is often conducted by 3-fragment-fusion PCR. Since the length of our gene-disruption cassettes was about 6.2 kbp, we considered that it would be difficult to attach about 1.0-kbp homologous fragments at both ends of our cassettes, and prepare a sufficient amount of precisely amplified constructs of >8.0 kbp by ligating the 3-fragment with PCR without fine adjustment of the PCR conditions. However, as the next step, we are going to reconstruct our system to be able to attach longer homologous sequences at both ends to improve the targeting efficacy.

Effect of the rescue genes

Our *ade2, ura3,* and *och1 P. pastoris* showed reduced growth on YPD medium even when supplemented appropriately. However, their parental strains grew as normally as the wild-type. The rescue genes effectively functioned in the targeted cells. Thus, the rescue genes could at least help gene-targeted clones to grow as efficiently as wild-type cells. Although the system required an extra excision step to remove the rescue genes, our results showed that the step was very efficient and reliable. This indicates that our gene-disruption cassettes with rescue genes is especially useful for the disruption of genes that may be detrimental to growth.

Sequences of the deletion regions of the isolated mutants

By sequencing the target region of each clone, we confirmed that the disruption cassettes with rescue genes precisely replaced each coding locus, and the region between the L- and R-*lox*P sites (zeocin resistance, rescue, and *Cre* expression genes) in those parental strains was precisely excised from their genome at these *mutant-lox*P sites by *Cre* recombinase (Figure 3). The LR sequence left behind in the genome after recombination at these sites is no longer recognized by the recombinase,[14,26] and it will not be an obstacle upon the further use of the strains for a recombinant protein production system using the *Cre*/*mutant–lox*P system, as we previously reported.[15]

Conclusions

- Using the *Cre*/*mutant–lox*P system, a new gene-disruption cassette system with a rescue gene for *P. pastoris* was developed, and the cassettes constructed by the system replaced the targeted locus.
- The *Cre*/*mutant–lox*P system in the cassette worked well in the targeted strain, and the zeocin resistance, rescue, and *Cre* expression genes between the L and R *lox*P sites were excised from genomes of the targeted strains upon induction of the *Cre* gene, and deletion mutants of the target-genes were successfully created.
- Sequencing analysis of the deleted loci confirmed the gene-replacement of the cassette and the precise removal of the DNA fragment between the mutant *lox*P sites.
- Mutants with the complete deletion of *ADE2*, *URA3,* or *OCH1* coding regions were isolated using this system.
- Construction of our disruption cassettes with rescue genes is so simple that the system could also be applicable for strategically obtaining a mutant strain whose mutation is not lethal, but has a significant biological impact on cell growth.

Literature Cited

1. Braude R, Hosking ZD, Mitchell KG, Plonka S, Sambrook IE. Pruteen, a new source of protein for growing pigs. I. Metabolic experiment: utilization of nitrogen. *Livestock Prod Sci.* 1977;4: 79–78.
2. Clare JJ, Rayment FB, Ballantine SP, Sreekrishna K, Romanos MA. High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology (NY)* 1991;9:455–460.
3. Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S, Gerngross TU. Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science* 2006;313:1441–1443.
4. Cregg JM, Cereghino JL, Shi J, Higgins DR. Recombinant protein expression in *Pichia pastoris*. *Mol Biotechnol.* 2000;16:23–52.
5. Li T, Cheng J, Hu B, Liu Y, Quian G, Liu F. Construction, production, and characterization of recombinant scFv antibodies against methamidophos expressed in *Pichia pastoris*. *World J Microbiol Biotechnol.* 2008;24:867–874.
6. Alani E, Cao L, Kleckner N. A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains. *Genetics* 1987;116:541–545.
7. Vervecken W, Kaigorodov V, Callewaert N, Geysens S, De Vusser K, Contreras R. *In vivo* synthesis of mammalian-like, hybrid-type N-glycans in *Pichia pastoris*. *Appl Environ Microbiol.* 2004;70:2639–2646.
8. Krainer FW, Gmeiner C, Neutsch L, Windwarder M, Pletzenauer R, Herwig C, Altmann F, Glieder A, Spadiut O. Knockout of an endogenous mannosyltransferase increases the homogeneity of glycoproteins produced in *Pichia pastoris*. *Sci Rep.* 2013;3:3279.
9. Chen Z, Sun H, Li P, He N, Zhu T, Li Y. Enhancement of the gene targeting efficiency of non-conventional yeasts by increasing genetic redundancy. *PLoS One* 2013;8:e57952.
10. Zhu XD, Sadowski PD. Cleavage-dependent ligation by the FLP recombinase. Characterization of a mutant FLP protein with an alteration in a catalytic amino acid. *J Biol Chem.* 1995;270: 23044–23054.
11. Sauer B. Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol.* 1987;7:2087–2096.
12. Guldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 1996;24:2519–2524.
13. Xu QR, Yan L, Lv QZ, Zhou M, Sui X, Cao YB, Jiang YY. Molecular genetic techniques for gene manipulation in *Candida albicans*. *Virulence* 2014;5:507–520.
14. Araki K, Araki M, Yamamura K. Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res.* 1997;25:868–872.

15. Shibui TH, Sakaguchi D. A new efficient vector for repeated use in *Pichia pastoris*. *Int J Res Stud Biosci*. 2014;3:72–79.
16. Manivasakam P, Weber SC, McElver J, Schiestl RH. Micro-homology mediated PCR targeting in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 1995;23:2799–2800.
17. Lin Cereghino GP, Lin Cereghino J, Sunga AJ, Johnson MA, Lim M, Gleeson MA, Cregg JM. New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*. *Gene* 2001;263:159–169.
18. Zhu B, Cai G, Hall EO, Freeman GJ. In-fusion assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations. *Biotechniques* 2007;43:354–359.
19. Ugolini S, Bruschi CV. The red/white colony color assay in the yeast *Saccharomyces cerevisiae*: epistatic growth advantage of white ade8-18, ade2 cells over red ade2 cells. *Curr Genet*. 1996;30:485–492.
20. Du M, Battles MB, Nett JH. A color-based stable multi-copy integrant selection system for *Pichia pastoris* using the attenuated ADE1 and ADE2 genes as auxotrophic markers. *Bioeng Bugs*. 3:32–37.[]
21. Goncalves AM, Pedro AQ, Maia C, Sousa F, Queiroz JA, Passarinha LA. *Pichia pastoris*: a recombinant microfactory for antibodies and human membrane proteins. *J Microbiol Biotechnol*.23:587–601.[]
22. Nett JH, Hodel N, Rausch S, Wildt S. Cloning and disruption of the *Pichia pastoris* ARG1, ARG2, ARG3, HIS1, HIS2, HIS5, HIS6 genes and their use as auxotrophic markers. *Yeast* 2005; 22:295–304.
23. Nett JH, Gerngross TU. Cloning and disruption of the PpURA5 gene and construction of a set of integration vectors for the stable genetic modification of *Pichia pastoris*. *Yeast* 2003;20: 1279–1290.
24. Zhou J, Zhang H, Liu X, Wang PG, Qi Q. Influence of *N*-glycosylation on *Saccharomyces cerevisiae* morphology: a Golgi glycosylation mutant shows cell division defects. *Curr Microbiol*. 2007;55:198–204.
25. Naatsaari L, Mistlberger B, Ruth C, Hajek T, Hartner FS, Glieder A. Deletion of the *Pichia pastoris* KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. *PLoS One* 2012;7:e39720.
26. Shibui T, Hara H, Sakaguchi D. Engineering a *Pichia pastoris* strain capable of incorporating repeated DNA constructs with the same selection marker. *CIBTech Journal of Biotechnology* 2015;4:1–11.

Manuscript received Feb. 24, 2017, and revision received July 7, 2017.