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A new disruption vector (pDHO) to obtain heterothallic strains from both *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*

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Summary. Yeasts are responsible for several traits in fermented beverages, including wine and beer, and their genetic manipulation is often necessary to improve the quality of the fermentation product. Improvement of wild-type strains of *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* is difficult due to their homothallic character and variable ploidy level. Homothallism is determined by the *HO* gene in *S. cerevisiae* and the Sc-*HO* gene in *S. pastorianus*. In this work, we describe the construction of an *HO* disruption vector (pDHO) containing an HO disruption cassette and discuss its use in generating heterothallic yeast strains from homothallic *Saccharomyces* species. [**Int Microbiol** 2011; 14(4):201-206]

Keywords: *Saccharomyces cerevisiae* · *Saccharomyces pastorianus* · homothallic · heterothallic · gene deletion

Introduction

The manipulation of yeast by humans dates back some 7000 years, when beer and wine were first produced [6]. Nowadays, most of the yeast varieties used in industry derive from wild-type strains, although they have been selected over long periods of time in order to improve the final products [1,9,15]. Formerly, mixtures of several species were used in fermentations whereas today industrial fermentations are performed by the addition of 'starter cultures' in order to obtain homogeneous final products. As a general rule, the yeast *Saccharomyces cerevisiae* serves as the starter culture in the production of wine and beer, mostly based on its ethanol tolerance. Another species, *Saccharomyces pastorianus,* is used to produce lager beer because of its ability to ferment at low temperatures [2,4,7,9]. Both *S. cerevisiae* and *S. pastorianus* are *Saccharomyces sensu stricto* species, i.e., they belong to the genus *Saccharomyces* and are so closely related that, in some cases, they can hybridize and produce viable fertile progeny [11]. Wild-type strains of these species are generally diploid or polyploid, homothallic, heterozygous, poorly sporulating cells, and they are often genetically unstable. The above-mentioned characteristics make it difficult to perform genetic manipulations, which are necessary to obtain a final

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product, such as wine or beer, with homogeneous properties [1,2,9,10,13,14].

Genetic improvement of wild-type *S. cerevisiae* strains is difficult because of their homothallism trait and their ploidy level. When a homothallic haploid is generated by meiosis, it quickly switches its "a" or "α" types, subsequently mating and generating a diploid identical to the parental cell. The *HO* gene, responsible for homothallism, codes for a site-specific endonuclease that recognizes the mating-type locus and produces a double-stranded break. Hence, DNA repair results in a switch in the *MAT* locus affecting the "a" or "α" cassettes. Heterothallic strains contain a defective *HO* gene that prevents *MAT* locus switching. These strains can be improved by sexual breeding because, after meiosis, they remain in the haploid state and can be mated with the desirable strain [5,17,19]. Homothallism is present in several industrially relevant species of *Saccharomyces sensu stricto,* such as *S. cerevisiae* and *S. pastorianus* [11]. *S. pastorianus*, a natural hybrid of *S. cerevisiae* and *Saccharomyces bayanus*, carries two types of chromosomes, one from *S. cerevisiae* and the other from *S. bayanus*, and therefore has two *HO* gene types [4,16,20]. The Sc-*HO* gene (*S. cerevisiae* type *HO*) derives from *S. cerevisiae*, whereas the Lg-*HO* gene (lager-fermenting yeast-specific *HO*) comes from *S. bayanus*. The two *HO* genes are functional and located in their respective chromosomal type [18]. Although both types provide the homothallic character, deletion of the Lg-*HO* gene renders the yeast heterothallic, despite the remaining Sc-*HO* gene [8,18].

Here we describe the design of a vector with a disruption cassette for the *HO* gene (based on the *S. cerevisiae* gene) that renders *S. cerevisiae* and *S. pastorianus* more amenable to genetic manipulation and improvement. This cassette worked well on both *S. cerevisiae* and *S. pastorianus*, as we succeeded in obtaining heterothallic, competent mating strains for both yeast species.

Materials and methods

Strains and media. Wine wild-type strain 145A211 and two genetic strains, CSH84 L(α) and CSH89L(a), from *S. cerevisiae*, and the brewery strain Weihenstephan 34/70 from *S. pastorianus* were used. *Escherichia coli* TOP10 (Invitrogen) was grown in Luria Bertani (LB) (tryptone 1 %, yeast extract 0.5 %, CaCl 1 %) medium at 37 ºC. *S. cerevisiae* and *S. pastorianus* were grown in YPD (dextrose 2 %, yeast extract 1 %, peptone 2 %). Yeast strains harboring auxotrophies were grown in selective dropout (SD) (yeast extract without amino acids 0.67 %, glucose 2 %) medium supplemented with the required amino acid(s). When another carbon source was used for galactose incorporation, YPD and SD were substituted by YPGal and SGal, respectively. All yeast cultures were maintained at 30 ºC. When cultured in sporulation medium SPOI (potassium acetate 1 %, yeast extract 0.1 %, glucose 0.05 %), the strains were maintained at 23 ºC for 5 days. Solid media were prepared by the addition of 2 % agar. When necessary, the media were supplemented with G418 (200 μg/ml; Sigma-Aldrich).

Nucleic acid manipulations. Genomic DNA was isolated from an overnight yeast culture using the Genomic Purification Kit (Promega). Enzymatic digestion was done using restriction enzymes from Takara. PCRs were carried out with the primers listed in Table 1. DNA was amplified using the proofreading polymerase ACCUZYME™ DNA polymerase (Bioline). In PCRs, to verify correct integration and deletion of the cassette, the BIOTAQ™ DNA polymerase (Bioline) was used (Table 1).

pDHO vector construction. Plasmid pDHO (plasmid for *HO* disruption) was designed to direct loxp-KanMX-loxP cassette integration into the *HO* gene by homologous recombination (Fig. 1). Two fragments of the *HO* gene were obtained by PCR amplification of yeast genomic DNA. Primers HO1f and HO1r yielded an 80-bp DNA fragment (HO1) corresponding to nucleotides 16–96 of the *HO* gene, flanked by *Pst*I (5′) and *Sal*I (3′) restriction sites, which were included in the primers. Primers HO2f and HO2r gave an amplicon of 137 bp (HO2), corresponding to nucleotides 1572–1654 of the *HO* gene, flanked by *Bam*HI and *Eco*RV (5′) and *Eco*RI (3′) restriction sites. The G418 resistance cassette loxP-KanMX-loxP was PCR-amplified from plasmid pUG6, using the loxpF and loxpR primers, which contained *Bam*HI and *Sal*I, and *Bam*HI and *Eco*RV restriction sites, respectively. The

Table 1. List of primers

HO1f	AAAA**CTGCAG**CGACTATTCTGATGGCTAACGG
HO1r	ACGC**GTCGAC**GTGCCATCTGCGCACATAACG
HO2f	CGC*G***GATCC**TGC**GATATC**TGCAAGTATGTACCAGAAGC
HO2r	CCG**GAATTC**CACTCTGGTCCTTTAACTG
loxpf	CGC**GGATCC**GCGGAG**GTCGAC**AACCCTTAATATAAC
loxpr	CGC**GGATCC**GCG**GATATC**ACCTAATAACTTCGTATAGC

Restriction sites BamHI, EcoRV, EcoRI, and PstI are shown in bold letters.

HO1 and HO2 fragments were then digested with *Pst*I and *Eco*RI, respectively, and inserted into pUC19, thus generating the pHO12 plasmid. This plasmid was subsequently digested with *Bam*HI and *Eco*RV. The loxP-KanMX-loxP disruption cassette was digested with the same enzymes followed by ligation into pHO12, between HO1 and HO2, thus generating plasmid pDHO.

Yeast transformation. Yeast transformations were performed with lithium acetate, using either 2 µg of plasmid DNA or 3–5 µg of PCR product. Selection was in YPD supplemented with the appropriate antibiotic or in SD media lacking the corresponding amino acid.

Resistance cassette elimination. The vector used in these experiments was YEp351-Cre-Cyh, which contains a galactose-inducible cre-loxP system, as describe by Delneri et al. [3].

Tetrad analysis. Tetrads were obtained from either homothallic diploid strains or heterothallic diploids generated by mating the appropriate strains. The diploids were maintained in SPOI medium for 7 days at 30 ºC, until an appropriate number of asci containing four ascospores were available. Asci were collected and treated with 0.5 µg/ml lyticase (Sigma-Aldrich) at 30 ºC for 15 min. Lyticase-treated asci were dissected using a Micromanipulator II (Allen Benjamin Inc.) coupled to a Nikon SE microscope.

Results and Discussion

The pDHO vector. Yeast genetic manipulation can be carried out by clonal selection, mutation, hybridization, cloning, and transformation. The combination of all of these techniques has increased the possibilities to artificially introduce diversity in yeasts [9]. Yeast genes can be deleted or disrupted by homologous recombination between a disrupting cassette and the genomic DNA. In such cases, the gene is

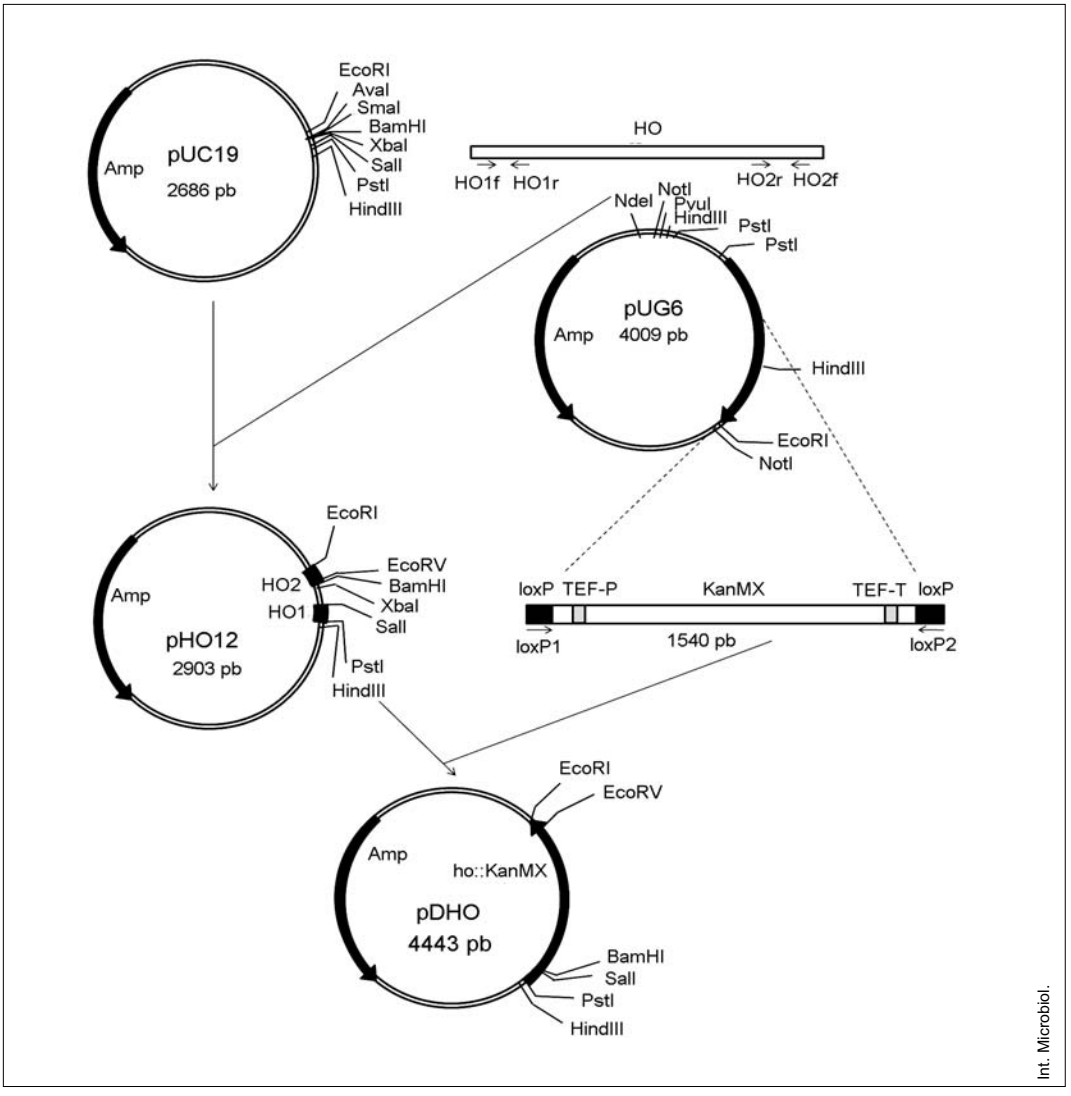

Fig. 1. pDHO vector construction.

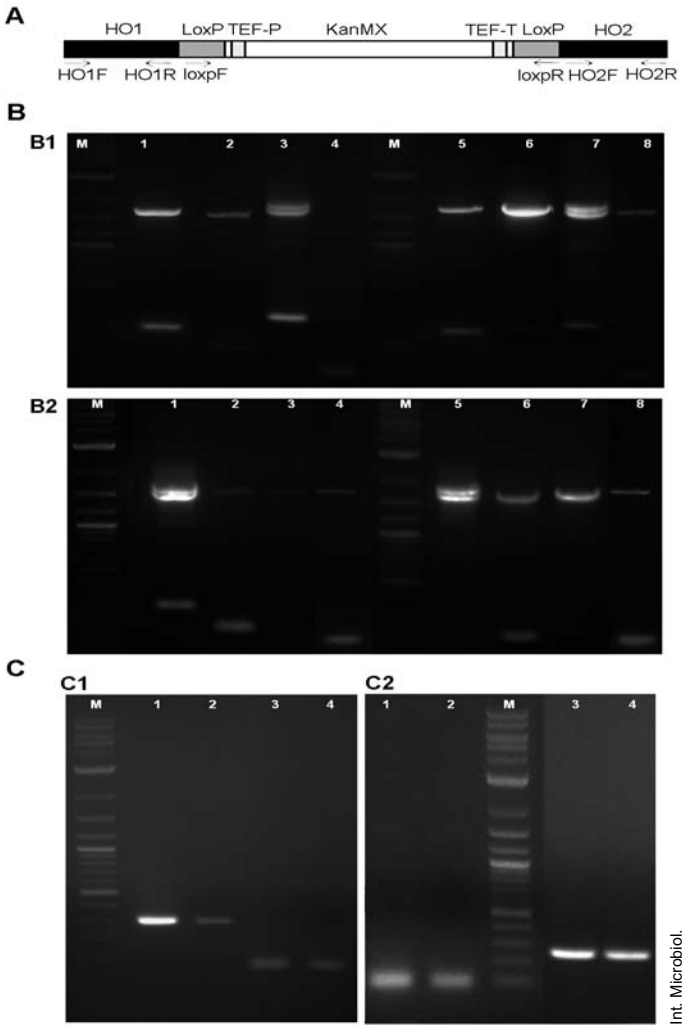

Fig. 2. PCR analyses of the yeast clones generated to confirm correct cassette integration and gene deletions. (**A**) Schematic representation of the HO-KanMX cassette, with the location of the different PCR primers used for the analyses indicated. (**B**) Correct integration of the cassette into the yeast DNA was verified by PCR amplification using different combinations of primers. (**B1**) *S. cerevisiae* 145A211 clone1 (lanes 1–4) and clone 2 (lanes 5–8), were amplified with primers HO1f/HO2r (lanes 3 and 7); HO1f, loxpr (lanes 1 and 5); loxpf/HO2r (lanes 2 and 6); and loxpf/lopr (lanes 4 and 8). (**B2**) *S. pastorianus* Weihenstephan 34/70 clone1 (lanes 1–4) and clone 2 (lanes 5-8) were amplified with primers: HO1f/HO2r (lanes 1 and 5); HO1f/loxpr (lanes 2 and 6); loxpf/HO2r (lanes 3 and 7); and loxpf/loxpr (lanes 4 and 8). (**C**) PCR-amplified chromosomal DNA from two of the Δ*ho* clones generated from *S. cerevisiae* 145A211 and *S. pastorianus* Weihenstephan 34/70, to confirm removal of the *HO* and sc-*HO* genes, respectively. PCR amplification was carried for: (**C1**) *S. cerevisiae* 145A211 clone1 (lanes 1 and 3) and clone 2 (lanes 2 and 4) with primers: HO1f/HO2r (lanes 1 and 2) and loxpf/loxpr (lanes 3 and 4). (**C2**) *S. pastorianus* Weihenstephan 34/70. Clone1 (lanes 1 and 3) and clone 2 (lanes 2 and 4) were amplified with primers: HO1f/HO2r (lanes 3 and 4) and loxpf/loxpr (lanes 1 and 2). Lane M corresponds to the molecular weight marker 2-Log DNA Ladder (GE Healthcare).

replaced by the cassette, which usually consists of a resistance or prototrophic marker gene flanked by two regions corresponding to the right and left extremes of the disrupted gene [12,22]. Here we report the construction of a vector (pDHO) containing a disrupting cassette (Fig. 1) that causes the specific deletion of the *HO* (Genbank X90957.1) and Sc-*HO* genes (Genbank AB027449.1) present in *S. cerevisiae* and *S. pastorianus*, respectively.

Amplification of HO1 and HO2 fragments and their subsequent cloning into pUC19 resulted in a new plasmid, pHO12. Amplification of the cassette loxP-KanMX-loxP from the pUG6 plasmid and its cloning into pHO12 between HO1 and HO2 gave rise to the disruption vector pDHO, containing the HO-KanMX disruption cassette, which disrupts the homothallism in *HO* from *S. cerevisiae* and Sc-*HO* from *S. pastorianus*, which can be obtained from the pDHO vector by PCR or by enzyme digestion.

Generation of heterothallic strains. We used the HO-KanMX disruption cassette, obtained by PCR using the primers HO1F/HO2R, to transform *S. cerevisiae* wine strain 145A211 and *S. pastorianus* Weihestephan 34/70 lager strain. This resulted in 100 *S. cerevisiae* transformants/µg DNA and about 40 *S. pastorianus* transformants/µg DNA. Two G418-resistant transformants containing the HO-KanMX cassette were selected for each strain. Disruption of the *HO* and Sc-*HO* genes and the correct insertion of the cassette were confirmed by PCR, using the primers pairs sets described in Table 1. Amplification of the expected size PCR fragments was obtained, as seen in Fig. 2A, B. Primers HO1F/HO2R generated, in all cases, two PCR fragments: one of 1761 bp, corresponding to the wild-type gene, and another of 1879 bp, corresponding to the gene disrupted with the cassette. The presence of two bands indicated that only one allele was disrupted and therefore that the G418-resistant clones were heterozygous for

sc-*HO* and *HO* (sc-*HO*/Sc-*ho*::KanMX and *HO*/*ho*::KanMX), since recombination of the disruption cassette occurred only in one chromosome of the pair [3].

To obtain a homozygous disrupted strain, the sc-*HO*/Sc-*ho*::KanMX and *HO*/*ho*::KanMX transformants were induced to sporulate in SPOI medium, followed by the selection of G418-resistant spore clones. As gene segregation was 2:2, half of the spores were resistant to G418 and therefore *ho*::KanMX and Sc-*ho*::KanMX. These resistant spore clones were cultured in sporulation medium but no asci were obtained, confirming the heterothallic nature of the *S. cerevisiae* and *S. pastorianus* strains generated.

As genetically engineered yeast strains harboring exogenous DNA are not permitted in industrial use, the resistance KanMX cassette was removed. For this purpose, we selected a disrupted ho::KanMX clone from *S. cerevisiae* and another from *S. pastorianus* and transformed them with the vector YEp351-Cre-Cyh. The transformants obtained (Δ*ho*), lacking the exogenous *KanMX* gene, were selected based on their inability to grow in the presence of G418. To confirm the removal of the exogenous cassette, the resulting constructs were PCR-amplified. As seen in Fig. 2C, amplification with the HO1/HO2 primer pair produced only a DNA fragment of the expected size (HO1 plus the HO2 fragments). Additionally, no PCR product was obtained when primers loxpf/loxpr were employed, confirming elimination of the Kan resistance cassette.

Mating ability of the heterothallic strains generated. To verify the mating competence of the heterothallic cells generated above, we mated six spore clones each for *S. cerevisiae* and *S. pastorianus* with two *S. cerevisiae* strains, CSH84L(α) and CSH89L(a). In all mating assays, diploid cells were obtained with only one of the mating type strains (either α or a), thus establishing the mating type of the generated *S. cerevisiae* and *S. pastorianus* spores. Like the strains derived from 145A211 and Weihestephan 34/70, they lacked auxotrophic markers. The presence of zygotes and the sporulation ability of the cells obtained confirmed the occurrence of mating and therefore the heterothallism of the Δ*ho* strains in both species. Although *S. pastorianus* contains two *HO* gene types, Sc-*HO* and Lg-*HO*, heterothallic competent mating strains could be obtained simply by deletion of the Sc-*HO* gene. The pDHO vector described herein is thus a useful tool for obtaining heterothallic strains from both *S. cerevisiae* and *S. pastorianus*. These heterothallic strains can, in turn, be used to improve industrial strains by either traditional breeding techniques or genetic engineering.

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Competing interests: None declared.

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