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Conversion of homothallic yeast to heterothallism through *HO* gene disruption

W. H. van Zyl*, E. J. Lodolo, M. Gericke**

Division of Food Science and Technology, CSIR, P.O. Box 395, Pretoria 0001, South Africa

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Abstract. A simple method was developed for the conversion of homothallic *Saccharomyces cerevisiae* yeast strains to heterothallism through *HO* gene disruption. An integrative *ho::neo* disrupted allele was constructed by cloning a dominant selectable marker, the bacterial *neo* gene, within the *HO* gene. Transformation of a homothallic diploid *S. cerevisiae* strain with plasmid DNA containing the *ho::neo* allele yielded G418-resistant yeast transformants in which one of the *HO* alleles was replaced by the disrupted *ho::neo* allele. Meiotic tetrad analysis of four-spored asci from these G418-resistant transformants gave rise to haploid heterothallic and diploid homothallic tetrad progeny. The presence of the *ho::neo* and *HO* alleles in the heterothallic and homothallic progeny was confirmed by Southern-blot analysis.

Key words: *S. cerevisiae* – Homothallism/heterothallism – *HO* gene disruption – G418 resistance

Introduction

Strains of the yeast *Saccharomyces cerevisiae* have been in use in the baking, brewing, and distilling industries long before the advent of modern genetic and DNA manipulation technology. These industrial *S. cerevisiae* strains have, by and large, been obtained through trial and selection over a long period of time. Most of these industrial strains have been selected, apart from other specific characteristics, for stability and constancy of industrial properties. This unintentionally resulted in these strains often being polyploid or aneuploid with no, or poor, mating abilities (Kielland-Brandt et al. 1983; Snow 1983; Evans 1990).

* *Present address:* Microbiology Department, Stellenbosch University, Stellenbosch 7600, South Africa

** Present address: Research and Development, The South African Breweries, P.O. Box 782178, Sandton 2146, South Africa

Correspondence to: W. H. van Zyl

The emphasis on industrial yeast improvement has shifted to genetic improvement. However, industrial *S. cerevisiae* strains are very unsusceptible to formal genetic analysis and improvement. Developments in this area have lagged considerably behind the vast progress made in fundamental yeast genetics at Universities and other research laboratories. Meiotic tetrad analysis, the generation of mutants and hybrids, as well as plasmid transformation technology, were developed on laboratory *S. cerevisiae* strains. The application of these techniques to industrial strains has not been very successful. One of the main reasons is that most wild-type and industrial *S. cerevisiae* strains are homothallic and therefore remain polyploid, in contrast to the heterothallic laboratory strains that can be maintained in the haploid phase (Nasmyth 1982; Johnston 1990).

In haploid homothallic strains high-efficiency switching between **a** and α cell types inevitably results in mating, producing the diploid phase. The *HO* gene codes for an endonuclease that introduces a double-stranded nick at the mating-type locus of homothallic *S. cerevisiae* strains, initiating the mating-type switch. The haploid phase in homothallic strains thus only exists transiently after spore germination. However, most heterothallic *S. cerevisiae* strains contain a defective *HO* gene (*ho* allele) that prevents mating-type switching from occurring. The lack of endonuclease production in heterothallic haploid *S. cerevisiae* strains allows maintenance of the haploid phase (Herskowitz 1988).

Different approaches have been taken to convert homothallic industrial yeasts to heterothallism. Hisatomi et al. (1986) used mutagenesis to isolate heterothallic mutants from homothallic yeast strains. Bakalinsky and Snow (1990) reported the introduction of the heterothallic (*ho*) allele into wine yeast strains through spore-to-cell hybridization. In this report, we describe a simple, yet effective, method to disrupt the wild-type *HO* gene on one of the chromosomes of homothallic *S. cerevisiae* strains, including prototrophic industrial strains. Heterothallic progeny can subsequently be generated from such recombinant homothallic strains through meiotic

tetrad analysis. The great advantage of this method is that only the stability of the haploid phase of an industrial *S. cerevisiae* strain is altered without necessarily affecting the gene pool and, therefore, the unique characteristics of the strain (Bakalinsky and Snow 1990).

Materials and methods

Strains and media. The *Escherichia coli* strain JM101 [*supE, thi Δ(lac-proAB)*/F′ (*traD36, proAB*$^+$, *lacI*^{q}, *lacZΔM15*)] was used as host for plasmid constructions. The diploid *S. cerevisiae* strain MT13 (*MAT***a**/*MATα*, *HO*/*HO*, *HMLα*/*HMLα*, *HMR***a**/*HMR***a**, *his4*/*his4*, *ura3*/*ura3*, *lys1*/*lys1*; Okamoto and Iino 1981), obtained as type strain CCRC 21666 from the Culture Collection and Research Center, FIRDI, Hsinchu, Taiwan, ROC, was used as homothallic recipient for plasmid transformations. Media used for *E. coli* cultivation are described by Silhavy et al. (1984) and for *S. cerevisiae* cultivation by Rose et al. (1988). Selection for G418 resistance was done on YPD plates supplemented with 400–800 μg/ml of G418 (Geneticin, Sigma). The mating ability of yeast strains obtained in this study was determined by pairing with the haploid strains DC14 (**a**, *ho*, *his1*) and DC17 (*α*, *ho*, *his1*), respectively. Successful mating was assessed by counter-selection on synthetic medium without amino acids (Rose et al. 1988).

DNA isolation and manipulations. The plasmids pFR10 (derivative of plasmid pAK37; Klar 1987) and pKX34 (Lang-Hinrichs et al. 1989) were used as DNA sources of the *HO* gene and *neo* gene, respectively. Plasmid DNA was purified from *E. coli* cultures according to Silhavy et al. (1984). Genomic DNA was isolated from *S. cerevisiae* strains according to Hoffman and Winston (1987). Plasmid manipulations were performed by standard protocols as described by Sambrook et al. (1989). The necessary molecular biological enzymes and DNA linkers were purchased from Boehringer Mannheim and used as recommended by the manufacturer.

DNA transformations. E coli transformations were done according to Silhavy et al. (1984) and yeast transformations with the lithium acetate method of Gietz and Schiestl (1991). Selection for G418 resistance was performed as described by Lang-Hinrichs et al. (1989).

DNA content. The cellular DNA of stationary-phase cells (5×10^9 cells per sample) was extracted with perchloric acid and the DNA content determined in duplicate according to the diphenylamine method described by Stewart (1975). Herring sperm DNA (Boehringer Mannheim) was used as a DNA standard and *S. cerevisiae* Y294, a genetically known strain (Fedor-Chaiken et al. 1990), as a haploid reference strain.

Southern-blot analysis. DNA hybridizations were performed as described by Sambrook et al. (1989). Pre-digested yeast chromosomal DNA was separated in 0.8% agarose gels and transferred to nitrocellulose membranes (Immobilon™-NC, Millipore). [^{32}P]-labeled DNA probes were prepared according to the random primer labeling procedure described by Feinberg and Vogelstein (1984). Hybridizations were performed in Church buffer as described by Church and Gilbert (1984).

Results

Construction of the integrative ho::neo allele

The bacterial *neo* gene from Tn5 codes for aminoglycoside phosphotransferase that confers resistance to the antibiotic substance G418 in yeast, thus allowing selection for DNA containing the *neo* gene in polyploid prototrophic yeast transformants (Jimenez and Davies 1980). A defective allele of the *HO* gene was constructed by inserting the *neo* gene within the *HO* gene. This would allow dominant selection for the disrupted allele when introduced into homothallic yeast.

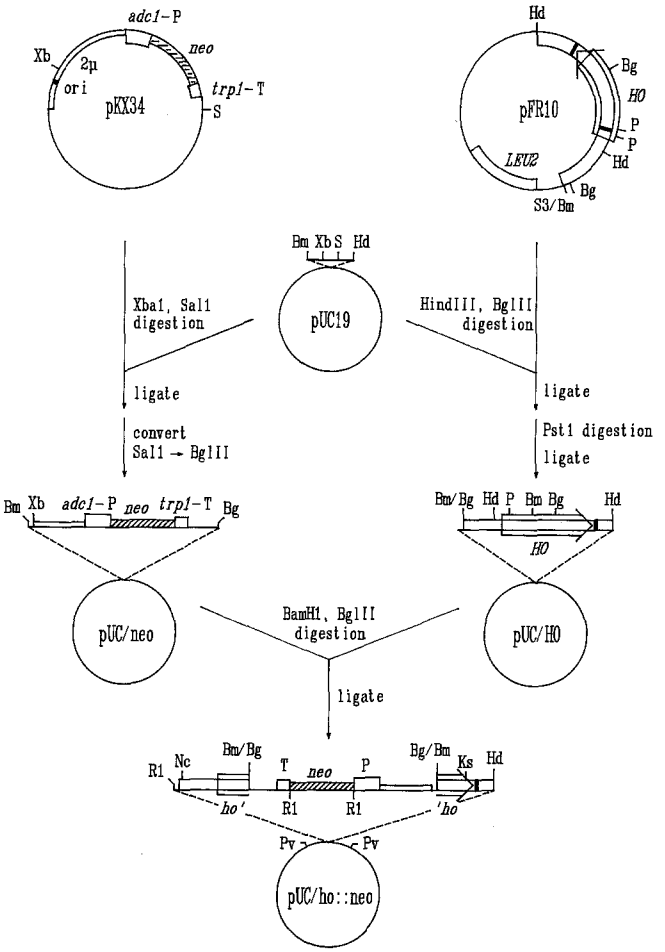

Fig. 1. The construction of plasmid pUC/ho::neo for *HO* gene disruption. Yeast sequences are *boxed*, the *neo* gene is indicated with a *hatched box* and vector DNA with a *line*. The locations of ARS sequences are indicated with *filled boxes*. Restriction site abbreviations: *Bm*, *Bam*HI; *Bg*, *Bgl*II; *R1*, *Eco*RI; *Hd*, *Hin*dIII; *Ks*, *Ksp*I; *Nc*, *Nco*I; *P*, *Pst*I; *Pv*, *Pvu*II; *S*, *Sal*I; *S3*, *Sau*3A; and *Xb*, *Xba*I

Kearsey (1984) searched for chromosomal replicators in yeast and identified a strong ARS (autonomous replicating sequence) adjacent to the *HO* gene (called the *HO* ARS). Subsequently, the *HO* gene was cloned and sequenced by Russell et al. (1986). They identified the boundaries of the *HO* gene, as well as potential ARS sequences within and immediately adjacent to the 3′ end of the gene. The ARS sequences could be eliminated in such a way that homothallic yeast could be transformed with an integrative *ho::neo* disrupted allele.

The construction of the integrative *ho::neo* allele is illustrated in Fig. 1. The *HO* gene was isolated as both a 1.3-kb *Hin*dIII/*Bgl*II and a 1.8-kb *Bgl*II fragment from the plasmid pFR10 and reconstructed as a 1.3-kb *Bgl*II/*Hin*dIII fragment into the *E. coli* plasmid pUC19. The potential ARS sequence between the two unique *Pst*I sites within the *HO* gene was deleted by removing the

short 96-bp *Pst*I fragment from the cloned *HO* gene, yielding plasmid pUC/*HO*. The *neo* gene, sandwiched between the constitutive yeast *adc1* promoter and *trp1* terminator, was cloned as a *Xba*I/*Sal*I fragment from plasmid pKX34 (Lang-Hinrichs et al. 1989) into pUC19. Subsequently, the *Sal*I site was converted to a *Bgl*II site through the addition of *Bgl*II linkers, yielding plasmid pUC/*neo*. The *adc1-neo-trp1* expression cassette was retrieved from plasmid pUC/*neo* as a 3.9-kb *Bam*HI/*Bgl*II fragment and introduced into the *HO* gene in plasmid pUC/*HO* by replacing the 391-bp fragment between the *Bam*HI and *Bgl*II sites within the *HO* gene. The resulting plasmid pUC/*ho*::*neo* still contained the *STB* (*REP3*) sequence of 2μ, as well as the *HO* ARS and could therefore persist episomally (Armstrong et al. 1989).

HO gene replacement on the chromosome

Gene replacement of one of the *HO* genes in the homothallic *S. cerevisiae* strain MT13 was facilitated by transforming the yeast with plasmid DNA containing the integrative *ho*::*neo* allele. The integrative *ho*::*neo* allele was isolated as a linear DNA fragment from plasmid pUC/*ho*::*neo* by digesting the plasmid at the unique *Nco*I site in the 5′ flanking sequence upstream of the *HO* gene and the *Ksp*I site at the 3′ end of the *HO* gene. The *Ksp*I site is located between the *HO* gene and the *HO* ARS, thereby separating the *ho*::*neo* fragment from the *HO* ARS (Fig. 1). The remaining 1,300 and 600 bp *HO* sequences that flank the integrative *ho*::*neo* allele should promote integration of the *ho*::*neo* disrupted allele into the chromosome at the *HO* locus through homologous recombination (Rothstein 1983), replacing one of the *HO* genes with the defective *ho*::*neo* allele.

Approximately 5 µg of pUC/*ho*::*neo* plasmid DNA (pre-digested with *Nco*I and *Ksp*I), together with 200 µg of denatured salmon sperm carrier DNA, were used per yeast transformation. The transformed yeast cells were resuspendend in 200 µl of YPD and incubated for about 4 h at 30 °C to ensure proper expression of the *neo* gene. Subsequently, the cells were directly spread-plated on YPD plates supplemented with 400 µg/ml of G418 and incubated at 30 °C for 2–3 days until colonies developed. The homothallic *S. cerevisiae* strain MT13 was unable to grow at a G418 concentration of 400 µg/ml. Numerous G418-resistant yeast colonies were obtained and purified by re-streaking on YPD plates supplemented with 800 µg/ml of G418. The cellular location of the *ho*::*neo* allele (chromosomal or episomal) in a few of the G418-resistant transformants was determined by Southern-blot analyses (data not shown). The majority of these transformants contained the episomal plasmid pUC/*ho*::*neo*, presumably from in-vivo re-ligation of partially digested plasmid DNA that still contained the strong *HO* ARS sequence.

The high background of undesirable transformants containing the episomal plasmid pUC/*ho*::*neo* was eliminated by digesting plasmid pUC/*ho*::*neo* DNA with *Nco*I, *Ksp*I, and *Pvu*II. The latter restriction enzyme recognizes the two *Pvu*II sites in the pUC19 moiety of plasmid pUC/*ho*::*neo*. Transformation of strain MT13 with the *Nco*I/*Ksp*I/*Pvu*II-digested pUC/*ho*::*neo* plasmid DNA yielded 22 G418-resistant transformants. Southern-blot analysis was performed on the 22 transformants and 12 of these were found to contain the *ho*::*neo* allele integrated at one of the *HO* loci (data not shown).

Progeny analysis for heterothallism

Four of the twelve G418-resistant transformants containing the chromosomal *ho*::*neo* allele, were induced to sporulate on acetate sporulation medium (Rose et al. 1988). Sufficient sporulation occurred after 4 days and tetrad analyses were performed on a few four-spored asci from each of the transformants. The progeny of seven tetrad sets from these G418-resistant transformants was scored with regard to G418 resistance, mating ability, and cellular DNA content (ploidy determination). The results for two G418-resistant transformants, TR5 and TR7, and the progeny from one tetrad set of each are shown in Table 1. The tetrad sets gave rise to 50% G418-sensitive and 50% G418-resistant progeny, indicating 2:2 segregation of these phenotypes. The G418-sensitive tetrad progeny were unable to mate with strains DC14 and DC17. Their cellular DNA content values corresponded to that of the diploid strain MT13, confirming that they were diploid. However, the G418-resistant tetrad progeny were mating-competent (**a** or α maters, see Table 1). Their cellular DNA content values corresponded to that of the haploid *S. cerevisiae* strain Y294, confirming that these progeny remained haploid.

The presence or absence of the *ho*::*neo* allele at the *HO* chromosomal loci of strain MT13, transformant TR5, and the progeny from a meiotic tetrad set of transformant TR5 (see Table 1) was confirmed by Southern-blot analysis (Fig. 2A). The G418-sensitive strain MT13, as well as the G418-sensitive tetrad progeny, strains TR5-

Table 1. Analysis of G418-resistant yeast transformants and their meiotic progeny with regard to G418 resistance, mating ability and ploidy

Strain	G418 resistance (800 µg/ml)	Mating type	DNA content (fg/cell)	Ploidy
MT13	–	St[a]	35.52	2n
Y294			19.88	n
TR5	+	St	33.04	2n
TR5-1a	+	**a**	17.04	n
TR5-1b	–	St	33.62	2n
TR5-1c	+	α	18.44	n
TR5-1d	–	St	36.88	2n
TR7	+	St	34.05	2n
TR7-4a	+	α	19.12	n
TR7-4b	–	St	35.80	2n
TR7-4c	+	**a**	17.16	n
TR7-4d	–	St	36.65	2n

[a] St, unable to mate with mating-type tester strains DC14 and DC17

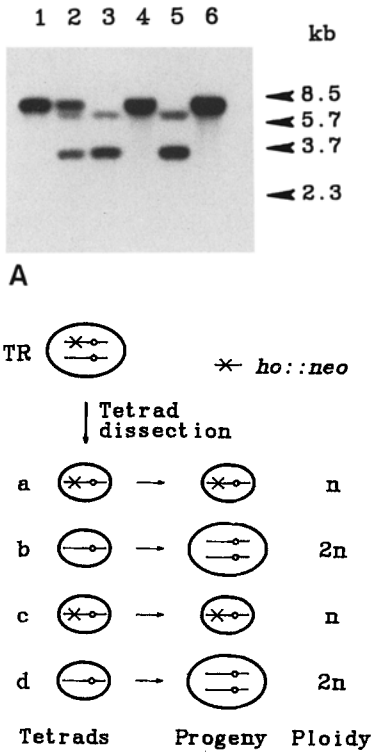

Fig. 2. **A** Southern-blot analysis of the *HO* chromosomal region. Chromosomal DNA isolated from *S. cerevisiae* strains MT13 (*lane 1*), TR5 (*lane 2*), TR5-1a (*lane 3*), TR5-1b (*lane 4*), TR5-1c (*lane 5*), and TR5-1d (*lane 6*), each digested with *Eco*RI, were probed with a 2.5-kb [^{32}P]-labeled *Hin*dIII fragment of the *HO* gene, derived from plasmid pUC/*HO* (see Fig. 1). Molecular sizes are indicated in kb. **B** Schematic lay-out of the conversion of homothallic yeast to heterothallism through *HO* gene disruption. The generation of homothallic and heterothallic progeny through meiotic tetrad analysis of an ascus from G418-resistant transformants TR5 or TR7 (see Table 1) is demonstrated

1b and TR5-1d, contained the wild-type *HO* allele on an *Eco*RI chromosomal fragment of about 7.0 kb, as highlighted by the [^{32}P]-labeled *HO* probe. In contrast, the G418-resistant tetrad progeny, strains TR5-1a and TR5-1c, contained the *ho*::*neo* allele, represented by two *Eco*RI chromosomal fragments of about 3.6 and 6.1 kb respectively. However, these strains lacked the 7.0-kb *Eco*RI fragment representing the wild-type *HO* allele. The presence of two additional *Eco*RI restriction sites within the *ho*::*neo* allele (see plasmid pUC/*ho*::*neo* in Fig. 1) was responsible for the two *Eco*RI chromosomal fragments observed in the G418-resistant tetrad progeny. The weaker radioactive signals observed for the 6.1-kb chromosomal fragment were the result of the limited overlapping homology between this chromosomal region and the 2.5-kb *Hin*dIII *HO* gene fragment used as a [^{32}P]-labeled probe (See Fig. 2A). When subtracting the 2.1-kb *adc1* promoter region from the 3.6-kb *Eco*RI fragment observed, the remaining 1.5-kb *HO* region concurred with the predicted size of the corresponding *Bgl*II to *Eco*RI *HO* region of *S. cerevisiae*, as determined by Nasmyth (1985). These results not only confirm the chromosomal location of the *ho*::*neo* allele, but also the actual replacement of the wild-type *HO* allele with the *ho*::*neo* allele at the *HO* locus. The G418-resistant transformant TR5 contained both the *HO* and *ho*::*neo* alleles, as indicated by the presence of all three *Eco*RI chromosomal fragments (Fig. 2A, *lane 2*). This confirmed that only one of the two *HO* alleles in transformant TR5 was replaced by the *ho*::*neo* allele.

Discussion

A simple method for the conversion of homothallic *S. cerevisiae* yeast strains to heterothallism through *HO* gene disruption was developed. The bacterial *neo* gene, expressed from an *adc1-trp1* yeast expression cassette, was cloned within the *HO* gene open reading frame. Kearsey (1984) and Russell et al. (1986) reported the presence of ARS sequences within the *HO* gene region. These ARS sequences were deleted during the construction of the *ho*::*neo* disrupted allele in plasmid pUC/*ho*::*neo*. Pre-digestion of plasmid pUC/*ho*::*neo* with the restriction enzymes *Nco*I, *Ksp*I and *Pvu*II yielded the *ho*::*neo* allele as an integrative linear DNA fragment ideal for yeast transformation. The *neo* gene was used as a dominant selectable marker, conferring G418-resistance to successful yeast transformants. The efficacy of the method was demonstrated in the type strain MT13 (CCRC 21666), a genetically defined homothallic diploid *S. cerevisiae* strain. This homothallic strain was transformed with plasmid DNA containing the integrative *ho*::*neo* allele and G418-resistant transformants were readily recovered on rich medium (YPD), supplemented with 400 to 800 μg/ml of G418. By Southern-blot analysis, more than 50% of these transformants were found to be successful *ho*::*neo* integrants.

Four of the *ho*::*neo* integrants were allowed to sporulate and meiotic tetrad dissections were performed on four-spored asci. Analysis of the tetrad progeny clearly indicated 2:2 segregation of the *HO* and *ho*::*neo* alleles, those containing the *ho*::*neo* allele being G418-resistant. Southern-blot analysis on the G418-resistant transformant TR5 and its progeny indeed confirmed that one of the *HO* alleles was replaced with the *ho*::*neo* allele and that subsequent meiotic 2:2 segregation of the *HO* and *ho*::*neo* alleles occurred in the tetrad progeny. Mating ability and cellular DNA-content assays performed on the tetrad progeny clearly showed that the G418-sensitive tetrad progeny which received the *HO* allele were diploid and unable to mate with haploid mating-tester strains. These progeny remained homothallic and therefore mating-type-switching competent. The generation of **a** and α mating-type cells resulted in diploidization and the **a**/α diploid cells formed were unable to mate with haploid mating-tester strains. On the contrary, the mating ability and DNA-content assays showed that the G418-resistant tetrad progeny which received the *ho*::*neo* allele remained haploid and were able to mate with haploid mating-tester strains. The G418-resistant progeny were heterothallic and therefore unable to switch mating types. These results clearly demonstrated the successful employment of the integrative *ho*::*neo* allele to generate heterothallic progeny from homothallic *S. cerevisiae* strains by *HO* gene disruption. The segregation of the *HO* and *ho*::*neo* alleles during the meiotic tetrad analysis and the

generation of homothallic and heterothallic tetrad progeny from the analyzed tetrad set of transformant TR5 (see Table 1 and Fig. 2A) is demonstrated with a schematic lay-out in Fig. 2B.

The simplicity of this method, and the fact that only the *HO* gene is disrupted without disturbing the rest of the gene pool, makes it a very powerful tool for the formal genetic study of homothallic yeast strains, including industrial strains. The generation of heterothallic progeny from industrial yeast would make them more amendable to controlled strain hybridization (crosses) or to genetic manipulations to combine or introduce desirable strain characteristics. When heterothallic progeny need to be void of bacterial sequences, the latter can also be removed via gene replacement (Rothstein 1983). One such an approach can be the isolation of *ura3* variants of the heterothallic haploid progeny by 5-floro-orotic acid resistance (FOA^R) selection (Boeke et al. 1987), followed by the subsequent replacement of the *ho*::*neo* allele with an *ho*::*URA3* allele through homologous recombination.

A drawback of the *ho*::*neo* genetic tool would be its dependence on yeast strains to be sporulation-competent with subsequent production of viable spores. However, when yeast strains produce few viable spores, it should be possible to enrich for such spores by ether treatment (Rockmill et al. 1991) prior to selection for G418 resistance.

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