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## A system for gene cloning and manipulation in the yeast *Candida glabrata*

(AIDS; Immunocompromised; mutant; pathogenic fungi; plasmid; *Torulopsis*; transformation)

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### SUMMARY

The opportunistic pathogenic yeast, *Candida (Torulopsis) glabrata*, is an asexual imperfect fungus that exists largely as a haploid. Besides being a clinically important pathogen, this yeast also provides a model system for understanding basic biological mechanisms such as metal-activated metallothionein-encoding gene transcription. To facilitate molecular genetic studies in *C. glabrata*, we isolated a strain auxotrophic for uracil biosynthesis. The *ura*<sup>-</sup> mutation could be functionally complemented by the *URA3* gene of *Saccharomyces cerevisiae*, consistent with a defect in the *C. glabrata* *URA3* gene in this strain. We also found that the centromere-based *S. cerevisiae* plasmid pRS316 could stably transform and replicate in multiple copies in *C. glabrata*. In contrast, high-copy-number *S. cerevisiae* plasmids containing the 2 $\mu$  circle autonomous replication sequence were not able to replicate productively in *C. glabrata*. We cloned the *C. glabrata* *URA3* gene, encoding orotidine-5'-phosphate decarboxylase, by complementation of a *ura3*<sup>-</sup> strain of *S. cerevisiae*. The deduced amino-acid sequence is highly similar to that of the *URA3* protein from *S. cerevisiae*. *C. glabrata* *URA3* provides a genetic locus for targeted gene integration in *C. glabrata*. Integrative plasmids were constructed based on the cloned *C. glabrata* *URA3* and are applicable for directed insertions of genes of interest at the *ura3* locus through homologous recombination.

### INTRODUCTION

*Candida (Torulopsis) glabrata* is among several clinically isolated yeast species of the *Candida* genus.

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Abbreviations: A, absorbance (1 cm); aa, amino acid(s); Ap, ampicillin; ARS, autonomously replicating sequence(s); bp, base pair(s); *C.*, *Candida*; *E.*, *Escherichia*; EMS, ethyl methanesulfonate; 5-FOA, 5-fluoroorotic acid; GCG, Genetics Computer Group (Madison, WI, USA); *K.*, *Kluyveromyces*; kb, kilobase(s) or 1000 bp; MCS, multiple cloning site(s); *MT-IIa*, tandemly amplified genomic locus encoding one of the *MT-II* isoforms of the *C. glabrata* metallothionein gene family; nt, nucleotide(s); ORF, open reading frame; *S.*, *Saccharomyces*; SC, synthetic complete media; SC-ura, SC lacking uracil; *URA3*, gene encoding orotidine-5'-phosphate decarboxylase; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside; YPD, rich media containing 1% yeast extract/ 2% peptone/ 2% dextrose.

Although less virulent than *Candida albicans*, *C. glabrata* has been demonstrated to be the pathogen of opportunistic infections such as vaginitis, endophthalmitis and persistent *Torulopsis glabrata* fungaemia (Redondo-Lopez et al., 1990; Odds, 1987; Damani and Webb, 1988). Clinically, this species has been found in many human or animal organs including the gastrointestinal and genitourinary tracts, stomach, skin and urinary tract (White, 1976; Frye et al., 1988; Sinnott et al., 1987). *C. glabrata* is the second most frequently isolated species from vagina in both asymptomatic females and patients suffering from yeast vaginitis (Redondo-Lopez et al., 1990). Recently there have been increasing concerns over *C. glabrata* because it can cause systemic infections in immunosuppressed patients, where it may be life-threatening. *C. glabrata* infections were reported to be a factor in the death of diabetes (Hickey et al., 1983) and cancer patients (Aisner et al., 1976), and Moniaci et al. (1988) reported

a case of *C. glabrata* infection in a patient with AIDS. Studies of the body's defense against *Candida* infection reveal that the human tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) can enhance the fungicidal activity against *C. glabrata*, a process mediated by human neutrophils (Ferrante, 1989). However, the molecular mechanism of TNF- $\alpha$  in stimulating this potential antifungal activity of neutrophils remains to be determined. Because of the metabolic similarities between fungi and their eukaryotic hosts, efforts to develop efficient antifungal agents are largely hampered.

In order to study the molecular mechanisms underlying pathogenic as well as other biological processes of *C. glabrata*, the development of genetic tools and molecular biology methods is essential. The advantage of using *C. glabrata* to study the pathogenicity of the *Candida* species is that this organism is unicellular and exists as a haploid (Whelan, 1987), rendering it more tractable for the isolation of mutants and for manipulation of the *C. glabrata* genome. Recently, a gene cloning system using a DNA fragment carrying a *C. glabrata* autonomous replication function has been described (Mehra et al., 1992).

The aim of the present study was to develop genetic tools that can be used for gene manipulation in *C. glabrata*, and these include (1) the isolation of a *C. glabrata* mutant strain harboring a uracil biosynthetic mutation in the *ura3* locus, (2) the isolation and sequencing of the *C. glabrata URA3* gene, which provides a genetic locus for targeted gene integration as demonstrated with the *C. glabrata AMT1* gene and (3) the use of episomal plasmids that can conveniently shuttle between *C. glabrata*, *S. cerevisiae* and *Escherichia coli*.

## EXPERIMENTAL AND DISCUSSION

### (a) Isolation of a *ura3*<sup>-</sup> auxotrophic strain and cloning of the *C. glabrata URA3* gene

To generate a *C. glabrata* strain suitable for genetic transformation, a strain bearing a mutation in the gene encoding orotidine 5'-phosphate decarboxylase was isolated. The *C. glabrata* strain (85/038) was described previously (Zhou and Thiele, 1991) and used as the parental wt strain (a gift from Dr. P. Magee). One stable *ura*<sup>-</sup> mutant was identified after EMS mutagenesis, purified for single colonies and designated 'Q' (Fig. 1). The *ura*<sup>-</sup> phenotype of the Q mutant strain can be complemented by the *S. cerevisiae URA3* gene on an episomal plasmid pRS316 as described in section b, or in a single integrated copy (Zhou et al., 1992), consistent with a mutation in the *C. glabrata* orotidine-5'-phosphate decarboxylase-encoding gene, which is functionally equivalent to *URA3* from *S. cerevisiae* (Rose et al., 1984).

To obtain a genetically defined locus for gene targeting by homologous recombination, a genetic selection approach was implemented to isolate the *C. glabrata URA3* gene by complementation of a *S. cerevisiae ura3*<sup>-</sup> strain. This selection is based on the suggestion that *C. glabrata* and *S. cerevisiae* are very close evolutionarily (Barns et al., 1991) and that *C. glabrata* genes can function properly in *S. cerevisiae* (Zhou and Thiele, 1991; Zhou et al., 1992). A *C. glabrata* genomic DNA library was constructed and was transformed into the recipient *S. cerevisiae* strain DTY7 (*leu2-3, -112* and *ura3-52*). A plasmid, designated U1(b), was recovered based on its ability to complement the *ura3*<sup>-</sup> phenotype of DTY7. Restriction analysis indicated that U1(b) contains a 3.7-kb insert. Furthermore, subcloning and complementation studies identified a 1.0-kb *XhoI-SspI* fragment within the 3.7-kb insert that enables DTY7 cells to grow on SC-ura media, suggesting that the functional *C. glabrata URA3* gene is located within this fragment (Fig. 1a). The nt sequence of the *C. glabrata URA3* gene was subsequently determined and a continuous ORF was identified using the GCG computer program (Fig. 1b). The *C. glabrata URA3* gene encodes a polypeptide of 256 amino acids that shares extensive homology with the *URA3* gene product from *S. cerevisiae*, demonstrating 82% protein sequence identity (Rose et al., 1984). Fig. 2 shows the aa sequence comparison between *C. glabrata* and *S. cerevisiae URA3* proteins. Furthermore, the *C. glabrata URA3* protein is 85% and 74% identical to the functionally analogous proteins from the yeasts *Kluyveromyces lactis* and *C. albicans*, respectively (Shuster et al., 1987; Ernst and Losberger, 1989). This protein sequence similarity provides a basis for effective complementation of the *S. cerevisiae ura3*<sup>-</sup> auxotroph by *C. glabrata URA3* gene product and vice versa (Zhou et al., 1992).

### (b) Episomal plasmids for gene transfer in *C. glabrata*

The ability to stably introduce genetic information into *C. glabrata* by plasmid-mediated transformation is critical to the development of this organism as a facile experimental system. We observed that the low-copy centromere-based *S. cerevisiae* plasmid pRS316 (Sikorski and Hieter, 1989) can efficiently transform *C. glabrata Q* (*ura3*<sup>-</sup>) strain, forming large colonies on SC-ura media after one to two days of incubation. This plasmid contains the *ARS4*, *URA3* and *CEN6* sequences of *S. cerevisiae* and exists as a single or low-copy episomal plasmid in baker's yeast (Sikorski and Hieter, 1989). Plasmid pRS316 can be readily recovered from *C. glabrata* by transforming total genomic DNA of the *C. glabrata* recipient cells into *E. coli* and isolating plasmid DNA from individual Ap-resistant *E. coli* transformants. This suggests that pRS316 exists in *C. glabrata Q* cells as an extra-



chromosomal, self-replicating plasmid. Furthermore, transformants with pRS316 can be cured of the plasmid by selection on 5-FOA medium. All isolates resistant to 5-FOA revert to uracil auxotrophy, as expected, consistent with a lack of integration of the plasmid into the *C. glabrata* genome (data not shown).

The copy number of the pRS316 plasmid was determined in two *C. glabrata* strains: Q and the *amt1-1* strain, in which the *C. glabrata* *AMT1* copper-activated transcription factor gene was insertionally inactivated (Zhou et al., 1992). By comparing the counts (cpm) of the linearized plasmid bands to the authentic *AMT1* gene DNA restriction fragments in Southern blotting analysis, we calculated the ratios from each comparison, which indicate that pRS316 is maintained at from 10 to 31 copies in Q or *amt1-1* cells (Fig. 3). These results confirm that the pRS316 plasmid retains episomal replication function in *C. glabrata*, although it is currently unclear why the single copy *S. cerevisiae* plasmid, pRS316, exists as a high-copy plasmid in *C. glabrata*. Another episomal plasmid, pW25, that contains multiple copies of the *MT-IIa* gene on the plasmid pRS426 (Sikorski and Hieter, 1989), was constructed and also found to be able to replicate in the *C. glabrata* Q strain. The existence of a weak autonomously replicating sequence *ARS* activity associated with the *MT-IIa* gene was recently reported (Mehra et al., 1992). However, the small colony size of transformants harboring only one copy of the *MT-IIa* gene on pRS426 suggests that the *MT-IIa* gene has poor *ARS* function relative to that of pRS316. The *S. cerevisiae* high copy plasmid pRS426, which contains 2 $\mu$  plasmid DNA sequences, was also transformed into the *C. glabrata* Q strain. The recipient cells grew as microcolonies on SC-ura plates, indicating that pRS426 replicates poorly in *C. glabrata*. This suggests that the *S. cerevisiae* 2 $\mu$  sequence can not foster efficient replication in *C. glabrata*. The existence of extra-chromosomal 2 $\mu$ -like circular DNA has not been reported in *C. glabrata*.

### (c) Integrative transformation and homologous recombination at the *C. glabrata* *ura3* locus

Since the plasmid U1(b) can not self-replicate in *C. glabrata*, we used it as an integrative plasmid to target integration of genes at the *C. glabrata* *ura3* locus (Fig. 4a). Following integrative transformation using the *AMT1* gene carried on the linearized U1(b) plasmid, our Southern blotting analysis detected the appearance of the diagnostic 10.7-kb (*Pst*I digestion) or 2.2-kb and 0.7-kb (*Stu*I + *Spe*I digestion) restriction fragments of the *URA*<sup>+</sup> transformant (Fig. 4b, c), indicating that U1(b) plasmid has successfully delivered the *AMT1* gene to the *C. glabrata* *ura3* locus. Two pRS425-based integrative plasmids with the *URA3* gene carried on different DNA restriction

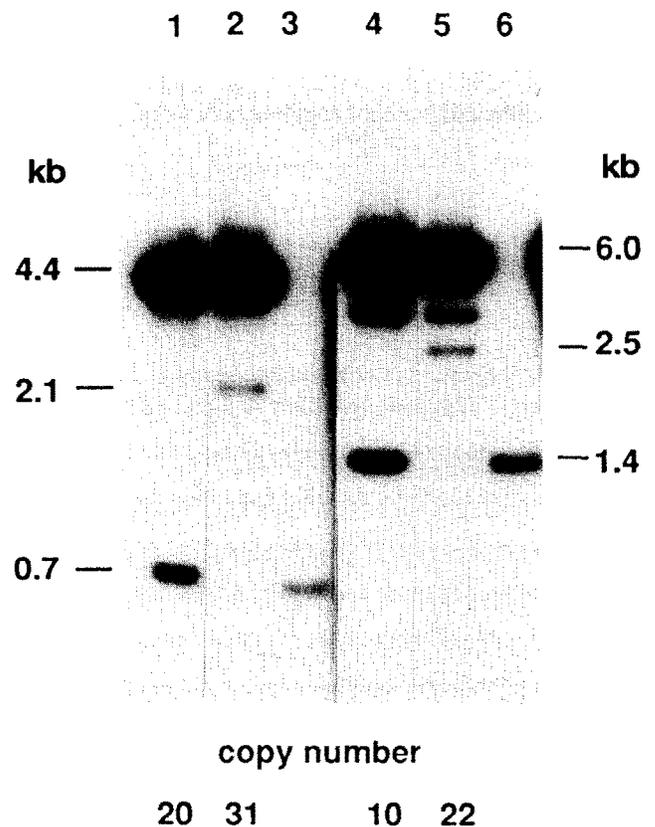


Fig. 3. Southern blotting analysis to determine the copy number of the pRS316 plasmid in *C. glabrata*. The *C. glabrata* Q (lane 1 and 4) and *amt1-1* (Zhou et al., 1992) (lanes 2 and 5) strains were transformed with the pRS316 plasmid that contained a 613-bp *Bgl*III-*Sty*I fragment of the *AMT1* gene (5' portion) which was fused in-frame to the *E. coli* *lacZ* gene. Genomic DNA from the recipient cells, or from the Q cells without episomal plasmid (lanes 3 and 6) was prepared and digested to completion with the restriction enzymes *Xba*I + *Xho*I + *Bgl*III (lanes 1-3) or *Pst*I + *Bgl*III (lanes 4-6). Southern blotting (1.0% agarose gel) was carried out as described (Ausubel et al., 1987) using a 613-bp <sup>32</sup>P-labeled *Bgl*III-*Sty*I fragment containing the 5' portion of the *AMT1* gene as the hybridization probe (Zhou and Thiele, 1991). The copy number of the plasmid was determined by comparing respectively, the cpm of <sup>32</sup>P associated with the 4.4-kb or 6.0-kb plasmid DNA fragments with that of the chromosomal *AMT1* bands (0.7 or 1.4 kb in the Q strain, 2.1 or 2.5 kb in the *amt1-1* strain) using a Beta-gen scanner. The sizes of each fragment (in kb) corresponding to the extrachromosomal plasmid and the authentic *AMT1* gene restriction fragments are indicated. The number below each lane represents the copy number of the pRS316 plasmid, relative to the single endogenous *AMT1* locus.

fragments were tested for their efficiency of integrative transformation and homologous recombination. The plasmid carrying the 3.7-kb *Sau*3A fragment [U1(b)] transformed more efficiently than that containing only a 1.0-kb *Xho*I-*Ssp*I fragment which only encompasses the *URA3* ORF. This observation suggests a positive correlation between the length of homologous DNA and efficient chromosomal recombination in *C. glabrata*, however, further experimentation must be conducted to ascertain the validity of this observation.

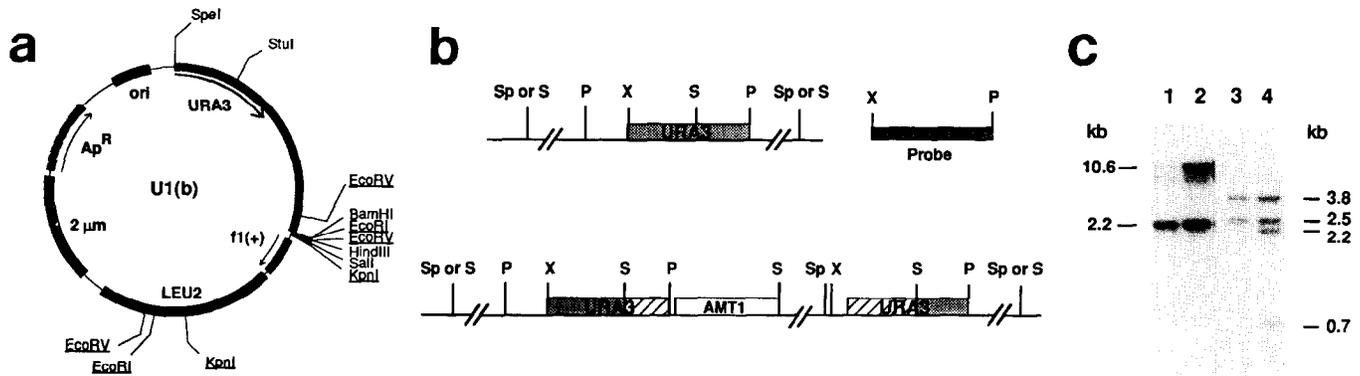


Fig. 4. Gene targeting at the *C. glabrata ura3* locus. (a) *C. glabrata* integrative plasmid U1(b). A 3.7-kb *C. glabrata* genomic fragment containing the *URA3* gene was cloned into the *Bam*HI site of the plasmid pRS425 to make the plasmid U1(b) (10.6-kb). The restriction sites that can be used for subcloning genes of interest into the U1(b) plasmid are indicated. The *Eco*RI, *Eco*RV and *Kpn*I sites underlined are not unique but can also be used as cloning sites, resulting in the inactivation of the *LEU2* gene. Prior to transformation, the plasmid may be linearized by digestion with *Stu*I, which has a unique restriction site within the *URA3* coding sequence, to facilitate homologous recombination. (b) Schematic representation of the *URA3* locus prior and after plasmid integration. (c) Southern blotting verified the integration. Integrative plasmid U1(b), which carries the *AMT1* gene, was first digested to completion with *Stu*I. The linearized plasmid was transformed into Q. Genomic DNA from Q or a *URA*<sup>+</sup> transformant were digested to completion with *Pst*I (lanes 1 and 2) or *Stu*I+*Spe*I (lanes 3 and 4), and fractionated on a 1% agarose gel. Southern blotting was performed (Ausubel et al., 1987) using a 1.5-kb <sup>32</sup>P-labeled *Xho*I+*Pst*I DNA restriction fragment encompassing the *URA3* ORF as a hybridization probe. The sizes of each DNA restriction fragment are indicated in kb. P, *Pst*I; S, *Stu*I; Sp, *Spe*I; X, *Xho*I.

#### (d) Conclusions

(1) An auxotrophic strain of *C. glabrata*, carrying a non-functional *ura3* locus was isolated by EMS mutagenesis from the wt strain 85/038.

(2) The *C. glabrata URA3* gene was cloned and sequenced. It encodes a 265-aa polypeptide which is highly similar to the *URA3* gene product from the baker's yeast *S. cerevisiae* and other yeasts. The *URA3* gene provides a defined genetic locus for targeted gene integration in *C. glabrata*.

(3) The episomal plasmid pRS316, initially constructed as a low copy replicating plasmids in *S. cerevisiae*, was demonstrated to replicate efficiently in the *C. glabrata ura3*<sup>-</sup> strain. This plasmid can shuttle between *C. glabrata*, *S. cerevisiae* and *E. coli*, and should prove useful for gene manipulations or phylogenetic studies in these two yeast systems.

(4) Modified or wt genes of interest which are cloned into the *URA3*-based integrative plasmids, such as U1(b), can be efficiently targeted to integrate at the *C. glabrata ura3* locus for genetic and functional analysis. Gene disruption, deletion and replacement can be achieved by constructing integrative plasmids or specific DNA restriction fragments carrying genes of interest and the appropriate selection marker such as *URA3*.

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