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**Original Article**

# Construction of a Complete *URA5* Deletion Strain of a Human Pathogenic Yeast *Cryptococcus neoformans*

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**Abstract**

*Cryptococcus neoformans* is an opportunistic human pathogen, which infects the central nervous system causing the fatal disease, meningitis. In order to understand the genetic background of this human pathogen, the basic molecular manipulation techniques of deletion, overexpression, and so on have been developed. *URA5*, a gene encoding orotate phosphoribosyltransferase, has frequently been used to introduce foreign gene fragments by complementing *ura5* mutant strains, which are not, however, stable; reversion to uracil prototroph is thus frequently observed on selective condition. The high possibility of reversion makes it inconvenient to use this mutation to identify appropriate transformants and thus, manipulation in molecular genetics. We report here the isolation of a stable *ura5* mutant of *C. neoformans*, designated as TAD1, by eliminating the *URA5* gene by homologous recombination using the biolistic DNA delivery system. The availability of the stable *ura5* mutant offers the advantage that no spontaneous reversion occurs so that a satisfactory rate of homologous recombination can be achieved. The strain will allow efficient genomic analysis in *C. neoformans*.

**Key words**: *Cryptococcus neoformans,* homologous recombination, molecular genetics, orotate phosphoribosyltransferase gene, transformantion

## Introduction

*Cryptococcus neoformans* is an opportunistic human pathogen with worldwide distribution especially in the United States and in Europe. It infects the central nervous system causing meningitis, which is fatal if untreated. Although *C. neoformans* can occasionally cause disease in an apparently healthy host, it is primarily associated with therapeutic immunosuppression, neoplastic disease, and AIDS. Cryptococcocal infection, as well as many other fungal diseases, is thought to begin with inhalation of airborne fungal cells from an environmental source. The infectious strain may then remain in a dormant phase in the host. As soon as some kind of immune defect occurs, which for most is caused by AIDS, the fungus becomes able to multiply, disseminate and cause infection[1]. In some cases, individuals with no apparent immune defect can also develop cryptococcosis.

*Cryptococcus neoformans* has some excellent properties for genetic manipulation. For example, this fungus is usually haploid which makes it amenable to genetic analysis by sexual cross. In addition, this property also gives us chances to modify its genome by molecular techniques, such as target gene disruption, controllable expression of genes of interest, green fluorescent protein study to track target gene products and so on. Those properties have been exploited to analyze the contribution of a number of genes of *Cryptococcus*, some of which have been shown to be involved in its pathogenicity[1]. In order to conduct gene manipulation, two different transformation systems have been developed for *C. neoformans*: electroporation and biolistic delivery of gold microprojectiles decorated with DNA[2]. This second approach was developed to circum-

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vent difficulties in delivering exogenous DNA through the polysaccharide capsule. Both transformation systems relied on the use of genes involved in primary metabolisms, *URA5* or *ADE2*, as selectable markers[3-5]. Recipient strains for transformation were obtained either by selection of *ura5* auxotrophic strains on medium containing 5-fluoro-orotic acid (5-FOA)[6], or by the isolation of *ade2* mutants by UV exposure[7].

Biolistic transformation yields higher rates of integration and homologous recombination than electroporation in both serotype A and D strains[8]. It is essential that gene disruption alleles be constructed with DNA sequences that are isogenic to the recipient strain, because mismatches of DNA sequences may result in abortion of homologous recombination events presumably by the DNA repair system. In several studies, the frequency of homologous recombination that can be achieved with ~1000 bp of flanking sequence on each side of the selectable marker is in the range of 2% to 25%, with some exceptional cases at higher efficiency[9-14].

The strains of *C. neoformans* with mutations in *ura5* gene previously reported are not stable and reversion to uracil prototroph is frequently observed on selective medium[6]. The high probability of reversion makes it inconvenient to use this mutation to identify appropriate transformants and thus, manipulation in molecular genetics. At present, no stable *ura5* mutant is available as a tool for transformation in order to study the molecular genetics of *C. neoformans*.

In this study, we describe the isolation of a stable *ura5* mutant of *C. neoformans* by eliminating the *URA5* gene by homologous recombination using the biolistic DNA delivery system[2]. The availability of the stable *ura5* mutant offers the advantage that the spontaneous reversion does not occur and a satisfactory rate of homologous recombination is achieved. The strain allows efficient genomic analysis in *C. neoformans*.

## Materials and Methods

### Strains and media

*Cryptococcus neoformans* strain B-4500[15], a serotype D, and B-4500 FOA[16] were maintained on PDA (potato dextrose agar, Difco) slants at 30°C for use. For *C. neoformans* experiments, minimal medium (MIN; 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g ammonium sulfate, 20 g glucose per 1 liter) was used throughout the research unless otherwise stated. Twenty grams of agar was used for the solid medium. To isolate *URA5* deletion strains, MIN medium were supplemented with 1 g/*l* of 5-FOA (5-fluoroorotic acid) and 50 mg/*l* of uracil (MINFOA). After screening of 5-FOA resistant

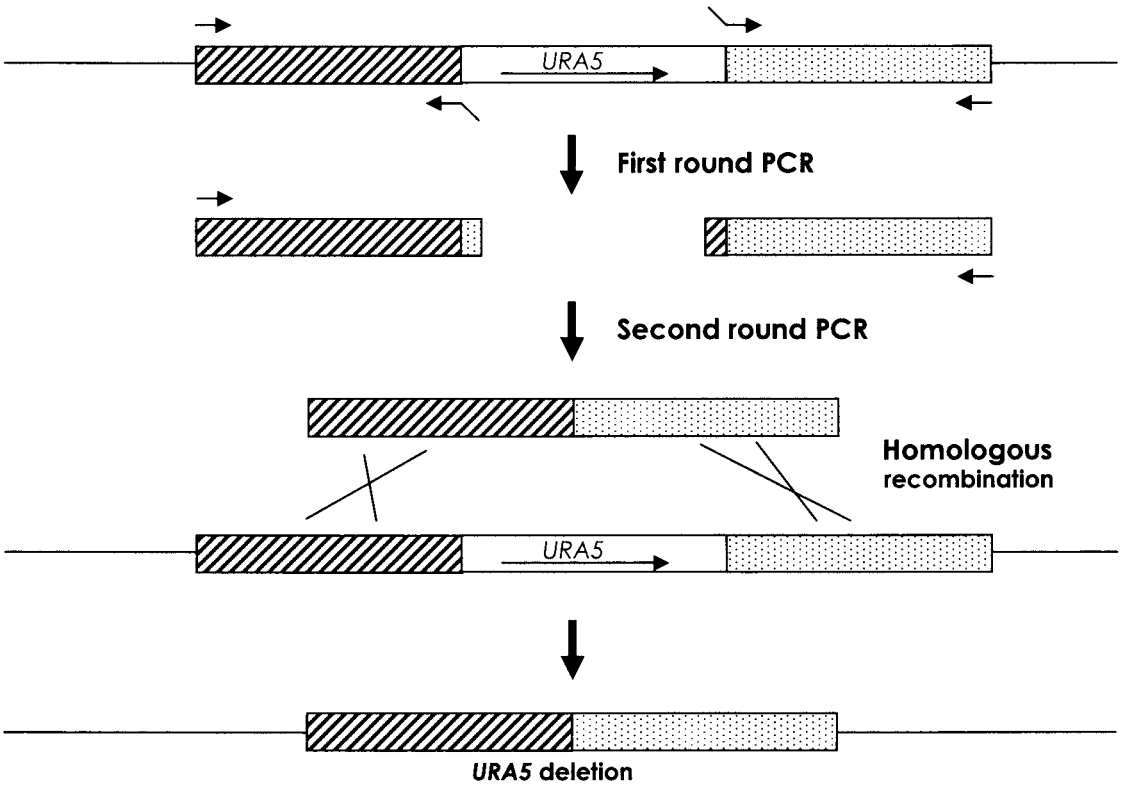


Fig. 1. The scheme to introduce *URA5* deletion in *Cryptococcus neoformans*. Both upstream (lined box) and downstream (shaded box) regions were PCR amplified followed by overlap PCR for transformation as described in Materials and Methods. The resulting transformant lacks the entire sequence of *URA5* gene.

strains, they were maintained on MIN supplemented with 50 mg/*l* uracil (MINURA). *URA5* deletion strains grew only on MINURA agar or on YPG (10 g yeast extract, 10 g polypeptone, 10 g glucose per liter) agar supplemented with 50 mg/*l* uracil.

**_URA5_ deletion construct**

Primers URA5-5.UP (5'-CTTGGTGGACTG-ATTGTGAT-3') and URA5-5.LP (5'-GAGTTT-GATTGGACGATTGGGAGGGATGGAGAGAC-3') were used to amplify the upstream flanking sequence of *URA5* gene (URA5-5), and URA5-3.UP (5'-GTCTCTCCATCCCTCCCAATCGTC-CAATCAAACTC-3') and URA5-3.LP (5'-CT-CCCCACCTTCCCACTTCC-3') were used to amplify the downstream flanking sequence of the *URA5* gene (URA5-3). The underlined sequences correspond to the oligonucleotides for overlap connection by PCR. All PCR amplifications were performed using iCycler (Bio-Rad) with ExTaq polymerase (Takara). The initial two PCR fragments (URA5-5 and URA5-3) were amplified by 35 cycles of 15 sec at 96°C, 15 sec at 55°C and 2 min at 72°C, following the initial denaturation for 4 min at 96°C and followed by the final extension for 5 min at 72°C. The PCR fragments were run on a 1% agarose gel and recovered from the gel with a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The two obtained fragments (URA5-5 and URA5-3) were then mixed to the equal mole, and used for the second PCR amplification. The second PCR was amplified by the same cycles except that the time period for extension was lengthened to 4 min. About a 2.3 kb fragment was yielded, and was purified with a QIAquick Gel Extraction Kit (Qiagen) following the agarose gel electrophoresis. The scheme of the *URA5* deletion is outlined in Fig. 1. In order to check the structure of the *URA5* loci, URA5.UP (5'-GAGCGAAGTTGCT-CGACC3') and URA5.LP (5'-TTGCCTCCAG-GAGGTGG-3') were used for PCR amplification.

**Transformation**

For *C. neoformans* transformation, the biolistic DNA delivery strategy was employed as described by Davidson *et al.*[8]. Cells were cultured until OD ($\lambda$=660) reached 1, which is the late log phase of this fungus, in YPG medium at 30°C with shaking at 110 rpm. Cells were collected by centrifugation and resuspended in sterilized water to the concentration of $5\times10^8$ cells per 1 m*l*. Two hundred microliters of this suspension was spread on MINFOA agar supplemented with 1 M sorbitol. These plates were then bombarded with DNA-coated gold beads in a vacuum chamber with a gas pressure of 1350 *l*b./in$^2$ with a helium-driven biolistic system (BioRad) and then incubated at 30°C for 3-5 days. Possible transformants which grew on MINFOA were then transferred to fresh MINFOA agar plates to obtain single isolates.

**Growth assay**

Fifty ml of MIN or MINURA medium was inoculated with $10^8$ cells of each strain in a 100 m*l* flask. Cells were incubated at 30°C with shaking at 110 rpm. At the time point of 2, 5, 9, 14 and 22 hours after inoculation, 3 m*l* of each culture was taken out for measurement. ODs were measured at $\lambda$=660 nm. Experiments were repeated 3 times.

**Genomic DNA isolation**

*Cryptococcus neoformans* strains were cultured in 2 m*l* of YPG liquid medium, which was supplemented with uracil when needed. The cells of overnight culture were harvested into 2 m*l* microcentrifuge tubes by centrifugation, washed once with 1 m*l* of the sterilized water and frozen at −80°C for 15 min. The cells were lyophilized for 3 hours. After complete lyophilization, they were then mixed with approximately 200 $\mu l$ of 0.45mm glass beads and vortexed vigorously until fine powder was created. Then, 0.5 m*l* phenol-chloroform and 0.5 m*l* TENTS buffer (10 mM TrisHCl, pH7.5; 1 mM EDTA, pH8; 100 mM NaCl; 2% Triton X-100; 1% SDS) were added, mixed gently and centrifuged at 4°C for 20 min at 13.2 rpm. The aqueous phase was transferred to a new microcentrifuge tube and DNA was precipitated by adding 3 M sodium acetate (pH 5.2) and ethanol. DNA was then pelleted by centrifugation, washed with 70% ethanol, dried and resuspended in 30 $\mu l$ of Tris-HCl (10 mM, pH 8) containing 10 $\mu l$/m*l* RNase. After incubation at 65°C for 15 min to digest RNA, the samples were stored at −20°C.

## Results and Discussion

**Deletion of _URA5_ gene of _C. neoformans_**

By biolistic delivery of the DNA fragment constructed by the overlap PCR technique (see Materials and Methods and Fig. 1) into *C. neoformans* B-4500 (wild type), we obtained about 200 possible transformants. They were tested for resistance against 5-FOA or auxotroph to uracil, and all the candidates were found resistant to 5-FOA and to require uracil, which is a typical phenotype of strains lacking genes encoding

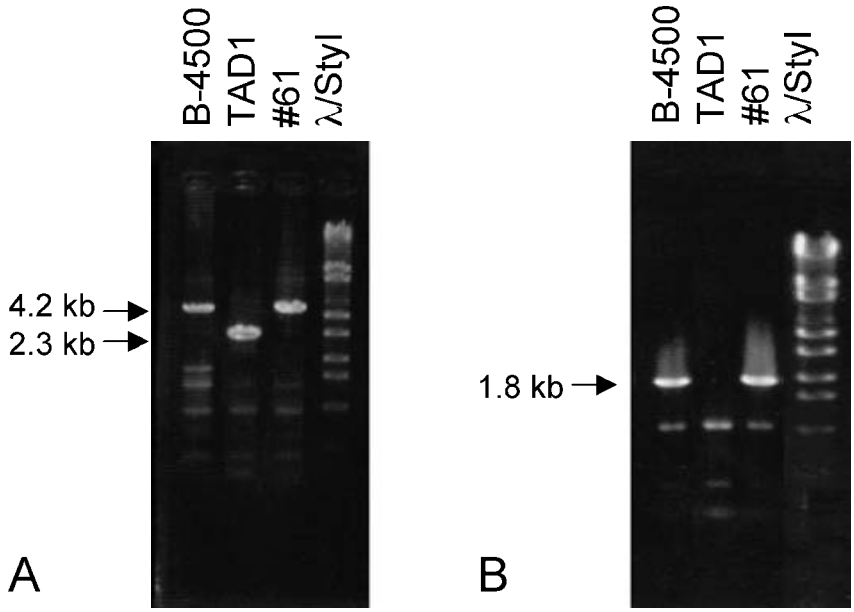


Fig. 2. Difference of structure in *URA5* deletion strain. PCRs were performed as described in the text. A, PCR with primers URA5-5.UP and URA5-3.LP; B, PCR with primers URA5.UP and URA5.LP.

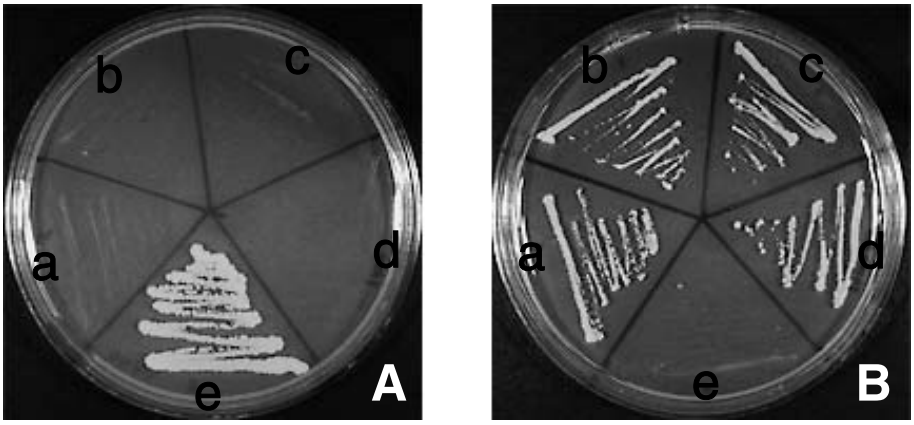


Fig. 3. Growth of three stable transformants (a, TAD1; b, TAD2; c, TAD3), a spontaneous *ura5* mutant B-4500 FOA (d) and a wild type B-4500 (e) at 30°C for 7days. A, MIN agar; B, MINFOA agar.

orotate phosphoribosyltransferases or orotidine monophosphate pyrophosphorylases[3, 6]. However, a control plate without bombarding DNA, also bore a significant number of colonies, and we then analyzed the structure of their genomes. Genomic DNA was extracted from the transformants obtained above, and was used for PCR with primers URA5-5.UP and URA5-3.LP. If the strain has a wild type allele of *URA5*, a 4.2 kb fragment would be amplified while a mutant one would raise a 2.3 kb fragment. We tested 200 strains obtained from biolistic transformation, and 37 of them were found likely to have only mutant alleles of *URA5* (Fig. 2A), while others raised a 4.2 kb fragment (#61 in Fig. 2). We determined the nucleotide sequence of the fragment amplified with URA5-5.UP and URA5-3.LP in those transformants, and identified that the structure of the amplified fragment was actually lacking the entire *URA5* gene, as we expected. We also performed PCR with primers URA5.UP and URA5.LP to amplify the entire *URA5* gene, and those which showed amplification of shorter fragments with URA5-5.UP and URA5-3.LP lacked amplification of *URA5* gene, whereas a wild type control or a transformant which had a wild type allele of *URA5* (#61 in Fig. 2) allowed its amplification (Fig. 2B). Three transformants lacking the complete *URA5* gene isolated in this study were designated as TAD1, TAD2, and TAD3, respectively (see Fig. 3).

In this study, only 37 out of 200 possible transformants turned out to have an expected homologous recombination, and the rest still kept the wild type *URA5* gene, at least as speculated by the length of the PCR product amplified with URA5-5.UP and URA5-5.LP. Because of its ease of screening, the pyrimidine

metabolic pathway was targeted to introduce auxotroph for uracil or uridine in many fungal species[6]. They were simply isolated by plating wild type cells onto media containing 5-FOA and pyrimidine. Thus, it might be possible that the strains appearing on MINFOA which had *URA5* PCR fragment of wild type size were spontaneous mutants resistant to 5-FOA.

**Stability of *URA5* deletion strains**

One of the major problems in manipulating *C. neoformans ura5* mutant strains in molecular genetics study is the high occurrence of reversion on selective media such as MIN. We tested to confirm our expectation that the transformants were actually stable and did not bear any revertant. We thus inoculated TAD1, a transformant obtained in this study, and B-4500 FOA, natural mutant strain isolated from MINFOA. Both strains ($1\times10^8$ or $1\times10^9$ cells per plate) were plated on MIN agar and incubated for 14 days at 30°C. B-4500 FOA raised 3-10 reversions per plate, which was close to the previous observation made by Kwon-Chung *et al.*[6]. In the TAD1 strain, as expected, no reversion was observed, since TAD1 lacks the entire *URA5* gene which may allow a spontaneous reverse mutation.

**Growth experiment**

We further asked if the newly constructed transformants have an ability to grow normally except for uracil auxotroph. We inoculated a deletion strain TAD1 and the parent strain B-4500 in either MIN or MINURA to compare the growth rate during certain time periods; Fig. 4 shows a comparison of growth among those strains. In MIN, TAD1 which is auxotrophic to uracil, did not grow, or showed minimum proliferation (Fig. 4A). On the other hand, in MINURA, TAD1 showed a similar growth rate to the wild type control, suggesting that the supplement of uracil completely suppressed the growth deficiency of TAD1 (Fig. 4B). We repeated the same experiment with different deletion strains (TAD2 and TAD3), and obtained the same results (data not shown).

**Transformation efficiency of TAD1**

We next questioned whether TAD1, the newly created URA5$^-$ strain, is actually useful for molecular genetics manipulation. We designed a PCR overlap construct to disrupt *S. cerevisiae BCK1* gene homolog in *C. neoformans* (unpublished data). Briefly, the deletion construct was made to insert *URA5* gene as a selectable marker within the open reading frame of *BCK1* homolog; detail of the construct will be published elsewhere. When B-4500 FOA was used as a recipient for transformation, 8 out of 28 possible transformants seemed to have the expected genome structure by a targeted homologous recombination (about 30%). On the other hand, with TAD1, 6 out of 12 turned out to have disruption of the *BCK1* homolog (50%). Thus, with TAD1, it is more efficient to obtain a transformant with an expected integration than with B-4500 FOA. Our interpretation of this difference includes: 1) TAD1 did not bear reversion to restore *URA5* gene function while B-4500 FOA did, as described in our experiment above; 2) TAD1 did not have any homologous sequence to *URA5* marker gene whereas B-4500 FOA did, so that B-4500 FOA allowed the *BCK1* disruption construct more frequently to integrate into its endogenous *URA5* region. Although we did not analyze the state of integration in the transformants obtained in this experiment with *BCK1* disruption construct, we conclude that the newly created *URA5* deletion strain would be a powerful tool in manipulation of genes in *C. neoformans*.

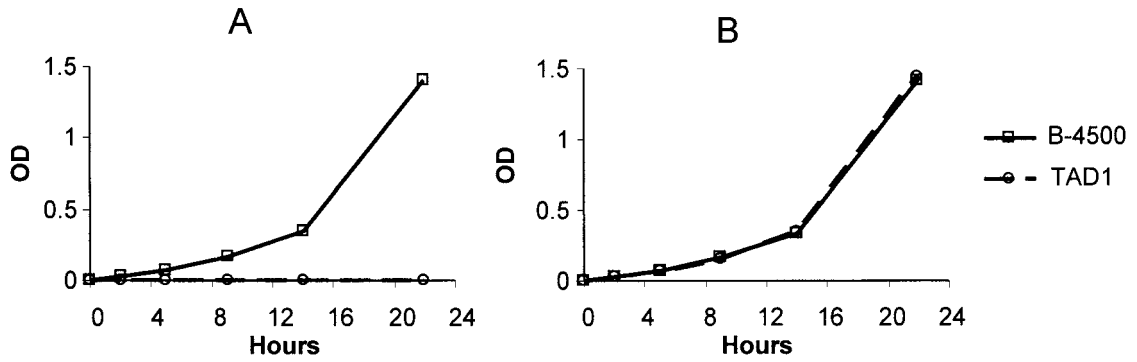


Fig. 4. Growth rate of the stable transformant TAD1 and the wild type *C. neoformans* at 30°C. A: MIN liquid media; B: MINURA liquid media.

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