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Positional cloning in *Cryptococcus neoformans* and its application for identification and cloning of the gene encoding methylenetetrahydrofolate reductase

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ABSTRACT

Cryptococcus neoformans, a basidiomycetous human pathogenic yeast, has been widely used in research fields in medical mycology as well as basic biology. Gene cloning or identification of the gene responsible for a mutation of interest is a key step for functional analysis of a particular gene. The availability therefore, of the multiple methods for cloning is desirable. In this study, we proposed a method for a mapping-based gene identification/cloning (positional cloning) method in *C. neoformans*. To this end, we constructed a series of tester strains, one of whose chromosomes was labeled with the *URA5* gene. A heterozygous diploid constructed by crossing one of the tester strains to a mutant strain of interest loses a chromosome(s) spontaneously, which is the basis for assigning a recessive mutant gene to a particular chromosome in the mitotic mapping method. Once the gene of interest is mapped to one of the 14 chromosomes, classical genetic crosses can then be performed to determine its more precise location. The positional information thus obtained can then be used to significantly narrow down candidate genes by referring to the *Cryptococcus* genome database. Each candidate gene is then examined whether it would complement the mutation. We successfully applied this method to identify *CNA07390* encoding methylenetetrahydrofolate reductase as the gene responsible for a methionine-requiring mutant in our mutant collection.

1. Introduction

Cryptococcus neoformans is known not only as a human pathogen whose infection causes life-threatening encephalomeningitis mainly in immuno-compromised persons (Mitchell and Perfect, 1995) but also as a model organism among basidiomycete because of the ability to manipulate its life cycle (Kwon-Chung, 1975) along with its having a publicly available whole genome sequence database (Loftus et al., 2005). Molecular genetic methods developed with this organism have greatly facilitated the unveiling of the functions of genes involved in various phenomena displayed by this yeast.

Among the cloning methods, the complementation-based method using a gene library is most versatile as long as the host-vector system for introduction and retrieval of foreign DNA is available. The host-vector system of *C. neoformans* has been developed (Edman, 1992) and successfully been used in cloning some of the *Cryptococcus* genes involved in capsule formation (Chang and Kwon-Chung, 1994 and Chang and Kwon-Chung, 1998; Chang et al., 1996). However, there are some disadvantages with the host-vector system of *C. neoformans* due to its unique feature (Edman, 1992). Insertional mutagenesis is a widely used method for gene identification (Idnurm et al., 2004), however, since this procedure deals with only insertional mutations, some other method than those descried above for identification of the mutant genes is necessary for the comprehensive analysis of the function of a gene.

Mapping-based methods for gene identification/cloning are promising in organisms with a publicly available genome database. Once a newly isolated mutation in *C. neoformans* is located on a chromosome through genetic crosses, the candidate gene responsible for the mutation can be narrowed down with the help of *Cryptococcus* genome database. Although the precise chromosomal map of *C. neoformans* has been constructed (Forche et al., 2000; Marra et al., 2004), it has not been fully used for mapping of mutant genes. The difficulty in the application of the mapping-based approach to gene identification in *Cryptococcus* is due to the fact that this yeast has 14 chromosomes that must be tested to correctly assign a gene to a chromosome. Furthermore, available landmark

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genetic markers are rather scarce, requiring a great deal of effort (e.g. creating standard markers) to map a gene on a chromosome by genetic crosses. As far as chromosome assignment is concerned, *Saccharomyces cerevisiae*, whose genome is organized into 16 chromosomes, is also under a similar situation as *C. neoformans*. This difficulty, however, has been circumvented in *S. cerevisiae* through the use of the mitotic mapping method (Wakem and Sherman, 1990), which is based on the fact that integration of two-micron plasmid DNA causes instability of the chromosome possessing the two-micron plasmid DNA sequence (Falco et al., 1982). This chromosomal instability greatly facilitated the assignment of a mutation to a chromosome without the need for passing through the meiotic cycle.

The mitotic mapping method based on random loss of chromosomes was originally developed with filamentous fungi without sexual cycle (Pontecorvo et al., 1953). We exploited this phenomenon to assign a gene of *Cryptococcus* to a chromosome. *C. neoformans*, intrinsically a haploid yeast, can have a mitotic diploid state, which tends to lose chromosome(s) randomly. Taking advantage of this feature, we developed a mitotic mapping method in *C. neoformans* that would facilitate assignment of a mutant gene to a chromosome. We successfully applied this method to identify the gene responsible for an unmapped methionine-requiring mutant in our mutant collection.

2. Materials and methods

2.1. Strains and media

All yeast strains used in this study are derivatives of the *Cryptococcus neoformans* serotype D strain B4500 (*MAT*α) (Kwon-Chung et al., 1992) and listed in Table 1A. The methionine-requiring mutation in the CAT661 (*MAT*a *ura5 met-un*) and CAT662 (*MAT*α *ura5 met-un*) strains was obtained during our screening of auxotrophic mutants by *Agrobacterium tumefaciens*-mediated mutagenesis as described previously (Walton et al., 2005), but it was found that the mutation site was not linked to the insertion site of T-DNA. Gene disruption experiments were performed by biolistic transformation as described previously (Toffaletti et al., 1993). In brief, donor DNA was constructed by sandwiching the marker gene, such as *URA5*, *NAT* (nourseothricin-resistance), *HYG* (hygromycin-resistance), or *NEO* (G418-resistance) between about 1 kb each of 5′-UTR and 3′-UTR of the gene to be disrupted. In some experiments, the split marker method (Goins et al., 2006; Fu et al., 2006; Kim et al., 2009) was adopted. Media routinely used for cultivating yeast strains are YPG (nutrient medium) (1% each of Polypepton (Nihonseiyaku, Tokyo, Japan), Yeast extract (Becton–Dickinson, Md, USA), and glucose) and YNB (synthetic minimal medium) (0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate (Becton–Dickinson, Md, USA), 0.5% ammonium sulfate, 2% glucose). Hay cube infusion medium (5% Hay cube, 0.2% K_2HPO_4, pH 6.2) was used for inducing the meiotic processes (White and Jacobson, 1985). 5-FOA (5-fluoroorotic acid) (Wako Chem. Co., Tokyo, Japan) medium (Boeke et al., 1987) was used for counterselecting uracil prototrophic cells. Low-phosphate (Low-Pi) medium consists of 0.67% Yeast Nitrogen Base without inorganic phosphate and amino acids (Formedium Ltd, Hunstanton, UK), 200 μM KH_2PO_4, and 2% glucose and was used for scoring the repressible acid phosphatase production. Agar was added to 2% to solidify media. Cultivation temperature was 30 °C unless otherwise stated.

Table 1A
Strains used in this study.

Strain	Relevant genotype	Reference or source
B4500	*MAT*α wild-type	Kwon-Chung et al. (1992)
TLHM15	*MAT*α *ura5 cku70::NEO*	Li et al. (2010)
CAT2	*MAT*a *ura5*	This study
CAT122	*MAT*a *ura5 lys2 pho2*	This study
CAT177	*MAT*a/*MAT*α *ura5/ura5 lys2/++/ade2 cku70::NEO/cku70::NEO*	This study
CAT661	*MAT*a *ura5 met-un HYG*	This study
CAT662	*MAT*α *ura5 met-un HYG*	This study
CAT1081	*MAT*α *ura5 cna07930::URA5*[a]	This study
CAT1477	*MAT*α *ura5 ade2 cnc04430::URA5*[a]	This study
CAT1596	*MAT*α *ura5 cna07390::URA5*[b]	This study
CAT1609	*CAT177 cnl04820::NAT/+*	This study
CAT1619	*MAT*a *ura5 cnl04820::NAT*[c]	A segregant from CAT1609
X-406-2D	*MAT*α *ura5 cna07930::URA5*	A segregant from CAT661/CAT1081

[a] These disruptions caused no phenotypic change.
[b, c] Each of the *cna03790::URA5* and the *cnl04820::URA5* disruptants displayed the methionine-requiring phenotype.

2.2. Genetic analysis

Crosses between two parental strains (a *MAT*a strain and a *MAT*α strain) were conducted on a hay cube infusion plate by mixing the culture of the parents and incubating the plate at 25 °C under dark until basidiospores emerged. Basidiospores were isolated using a micromanipulator (Singer, UK). Diploid yeast-type cells were selected by the prototroph recovery method, where two parents were differently labeled with an auxotrophic marker and a mating mixture on hay cube infusion medium was scraped off and transferred to a YNB plate followed by incubation at 37 °C (Yan et al., 2007).

2.3. DNA manipulation

Genomic DNA was extracted by the method described by Cao et al. (2009) and used for template in PCR (polymerase chain reaction) experiments. PCR reactions were run as follows; 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 1.5–5 min depending on the length of DNA to be amplified. Primers for PCR experiments are listed in Table 1B.

2.4. Detection of acid phosphatase activity on a plate

Acid phosphatase activity on the colonies was detected by the staining method described previously (Toh-e et al., 1973). In brief, staining solution containing 0.5 mg α-naphthylphosphate per ml, 5 mg Fast Blue Salt B per ml, 50 mM acetate buffer (pH4.0), and 1% agar was poured on the colonies growing on a Low-phosphate plate. Colonies producing acid phosphatase are stained in deep red while acid phosphatase non-producing colonies remain white. Cells of *C. neoformans*, as with other basidiomycetes, produce substance that reacts with Fast Blue Salt B to produce reddish pigment (van der Walt and Hopsu-Havu, 1976; Shimizu et al., 2014), however, this

Table 1B
List of primers.

Primer	Sequence
1223	5′-ATAGACATGTTGGGCGAGTTTACTAATGG-3′
1821	5′-GATGAGGATGAGGCAGCAAAAGAG-3′
1822	5′-GTCATCTATACCACGTAGACACGT-3′
1826	5′-ATGCTGTTCCCCTTCTCGTCAG-3′
1829	5′-GGATAGAAAGGCAATGGCAGATGC-3′
1830	5′-CAGATCGCACTTGGACATATGGGAA-3′
1838	5′-TTGTCCGCTACATCCGTCAG-3′
1839	5′-TTGTACGCTCAATTTGGAGAAGAGTG-3′
1840	5′-CCTGAATCACACATGATCCTCCCTC-3′
1841	5′-GTTCCACAGGAGGAAGCGTTCGATATC-3′