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Original Article

Analysis of Serotype AD Strains from F1 Progenies between Urease-positive- and Negative-strains of *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is a pathogenic basidiomycete with a defined sexual cycle involving mating between haploid yeast cells with a transient diploid state. We examined F1 progeny from a crossing between the urease-negative strain (environmental isolate, serotype A, mating type α, haploid) and a tester strain (B 3502 from NIH of USA; urease-positive, serotype D, mating type a, haploid) for serotype, mating type, ploidy and urease activity, and performed partial sequencing of the urease gene. Phenotypes of the F1 progeny and results of SSCP analyses suggested that the serotype AD strain of the F1 progeny is a hybrid of the parental serotype A and D strains.

Key words: *Cryptococcus neoformans,* sequencing, serotype AD, mating, SSCP (single-strand conformational polymorphism), urease

Introduction

The basidiomycetous yeast *Cryptococcus neoformans* causes meningitis in both immunocompromised and immunocompetent patients. At present, two varieties have been recognized: *C. neoformans* var. *neoformans* (serotypes A, D, and AD) and *C. neoformans* var. *gattii* (serotypes B and C). It was recently proposed that serotype A was separated from *C. neoformans* var. *neoformans* into a new variety called *C. neoformans* var. *grubii*[1]. This classification, however, has not been completely agreed upon among mycologists and requires further investigation[2, 3].

We previously reported the relations among serotype, mating type, and ploidy in *C. neoformans*[4]. In that report, we suggested that the serotype AD strain of *C. neoformans* is a hybrid of serotypes A and D, and several other reports have also discussed the origin of serotype AD. Boekhout *et al*. showed that serotype boundaries did not fully coincide with the genotypic groups, but the genotype corresponding to serotype AD was the result of the hybridization of reproductive events[3]. Lengeler *et al*. concluded that serotype AD strains of *C. neoformans* were unusual aneuploid or diploid strains that result from matings between serotypes A and D strains[5]. Cogliati *et al*. investigated the origin of the strains identified as diploid by genotyping by PCR fingerprinting, determination of mating type crossing on V8 juice medium, PCR of *MF* and *STE20* genes, dot blot hybridization, and sequencing of *MFa* and *MF*α genes. Finally, they suggested those diploid isolates were able to recombine by sexual reproduction[6]. Xu *et al*. cloned and sequenced a portion (537 bp) of the laccase gene, and discussed the origins and evolution of serotype AD[7].

The urease activity and mating ability of *C. neoformans* is well characterized. The urease activity is an important characteristic for identification, and the mating ability is useful for genetic research. Cox *et al*. sequenced and disrupted the urease gene of *C. neoformans*, and suggested that urease activity is involved in the pathogenesis of cryptococcosis[8]. The determination of urease activity using Christensen's urea agar is very simple. In the genetic study, more than one phenotypic marker is needed. In the present study, we chose mating type, serotype, urease activity and ploidy because they are the most typical and usual char-

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acteristics of *C. neoformans*. We examined F1 progeny from a cross between a urease-negative strain[9] (environmental isolate, serotype A, mating type α, haploid) and a tester strain (B 3502 from the NIH in the USA; urease-positive, serotype D, mating type a, haploid) for serotype, mating type, ploidy and urease activity and obtained partial sequence of the urease gene. With additional data of this study, we tried to prove that the AD serotype of F1 progeny is a hybrid of the parental serotypes A and D strains.

Material and Methods

Mating study: *C. neoformans* IFM 5845 (B 3502; urease-positive, serotype D, mating type a, haploid) and IFM 41469 (urease-negative, serotype A, mating type α, haploid) as parental strains were mated on hay-cube infusion agar. F1 progeny were isolated as described previously[4].

Serotyping, ***urease activity***, ***ploidy***: Serotyping was carried out by slide agglutinating tests with factor antisera (Crypto Check, Iatron Laboratories, Inc., Tokyo, Japan). Urease activities were checked by Christensen's urea agar (Eiken Chemical Co., Ltd., Tokyo, Japan). Ploidy was determined by flow cytometry as described previously[10].

DNA extraction: Genomic DNA was extracted with Dr. GenTLE^{TM} (from yeast) (Takara Bio Inc., Shiga, Japan).

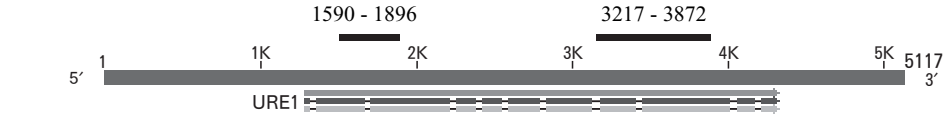
PCR and sequencing of urease gene: PCR was performed with Ready-To-Go^{TM} PCR Beads (Amersham Biosciences AB, Uppsala, Sweden) with an initial denaturation step of 94℃ for 10 min followed by 30 cycles of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min, extension at 72℃ for 2 min, and a final extension at 72℃ for 10 min. The PCR products were purified and sequenced with an ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) with a Thermo Sequenase^{TM} II Dye Terminator Cycle Sequencing Kit (Amersham Biosciences AB). Primers listed in Fig. 1 were designed from NCBI data (*URE1*, Accession No. AF006062).

RNA isolation and amplification of cDNA of urease gene (RT-PCR): Total RNA was prepared from cells according to the hot-phenol method[11]. From the isolated total RNA, cDNA was synthesized according to the manufacturer's instructions with the SuperScript^{TM} Preamplification System (Invitrogen, Carlsbad, CA, USA). The urease gene was amplified as under the previous conditions by PCR with the Ure-Bs and Ure-Ca primers (Fig. 1). PCR products were separated by electrophoresis on 1% agarose gels in 1 × TBE, stained with ethidium bromide and visualized with an UV illuminator at 360 nm.

SSCP (single-strand conformational polymorphism) analysis: Two microliters of PCR product was mixed with 18 μl of loading buffer (98% formamide, 10 mM EDTA, 0.15% bromophenol blue, and 0.15% xylene cyanol), denatured at 94℃ for 2 min, and then chilled on ice immediately. Four microliters of denatured sample was loaded on 10% MDE Hydrolink acrylamide gels (Takara Bio Inc.) and run in 0.6 × TBE buffer for 3 h at constant power (250 V) at 5℃. Gels were stained with ethidium bromide and visualized with an UV illuminator at 360 nm.

Results

Serotypes, ploidies, mating types and urease activities of the parental strains (IFM 5845 and 41469) and the F1 progeny are listed in Table 1. The phenotypes of the haploid strains among the F1 progeny were classified into three types: paternal, maternal, and recombinant. Three of five diploid strains (IFM 45395, 45448, and 45501) showed both types (paternal and maternal), the



Primer	Sequence (5' to 3')	bps	Position
Ure-As	AGG CAC TTT CCC CGA TGG TGT	307	1590th - 1896th
Ure-Aa	AAC TTG AAC GGG CCT GTC TC		
Ure-Bs	ACG TCG AGA GTA CCT TGG CC	294	3217th - 3511th
Ure-Ba	GAG TCG GCA AAG GCA ATG TCC	656	
Ure-Cs	GGA CAT TGC CTT TGC CGA CTC	383	3490th - 3872th
Ure-Ca	CCC AAG CAA TAA CAC CGC CCT TG		

Fig. 1. List of the primers and their position on the *URE1* gene.
This figure is modified from NCBI data (Accession No. AF006062).

Table 1. Characteristics of parental strains and their F1 progenies

IFM No.	Serotype	Ploidy	Mating type	Urease
45388	D	haploid	a	+
45390	D	haploid	a	−
45392	D	haploid	U**	−
45395	AD	diploid	aα	+
45412	D	haploid	a	−
45419	A	haploid	α	+
45425	D	haploid	a	+
45430	D	haploid	a	+***
45435	D	haploid	a	+
45437	D	diploid	a	+***
45440	A	haploid	α	+
45442	A	haploid	α	−
45447	A	haploid	α	−
45448	AD	diploid	aα	+
45452	A	haploid	α	−
45455	D	haploid	a	+
45483	D	haploid	a	+
45501	AD	diploid	aα	+
45526	D	haploid	a	+
45532	A	haploid	α	+
45541	A	diploid	α	−
5845*	D	haploid	a	+
41469*	A	haploid	α	−

*: parental strains
**: untypable
***: weakly positive

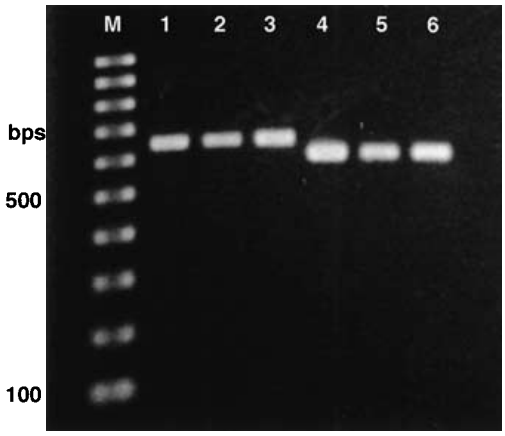


Fig. 2. PCR profiles of 656-bp bands from urease-positive and -negative strains.
Lanes 1 and 4, IFM 5844 (urease-positive, serotype D, MATα); lanes 2 and 5, IFM 5845 (urease-positive, serotype D, MATa); lanes 3 and 6, IFM 41469 (urease-negative, serotype A, MATα). Lanes 1, 2, 3, genomic DNA; lanes 4, 5, 6, cDNA

remaining two strains (IFM 45437 and 45541) showed either one or the other (paternal or maternal). Six recombinant strains were divided into two groups, serotype D, urease-negative and serotype A, urease-positive. Recombination occurred between serotype and urease activity, whereas there was no recombination between serotype and mating type.

For detection of the *URE1* gene by PCR, all primer pairs tested (Fig. 1) amplified a single band in all strains (three typical strains are shown in Fig. 2, lanes 1 to 3) even if urease activity was negative. The results of RT-PCR (Fig. 2, lanes 4 to 6) showed expression of *URE1* mRNA in a urease-negative strain (IFM 41469). The aligned sequences of 307 bps amplified PCR bands of the *URE1* gene from the parental strains and all of the different phenotypic strains from their parental strains are shown in Fig. 3. Sequencing analysis revealed no differences between urease-positive and urease-negative strains. Among the parental strains, 19 nucleotide substitutions were detected. In IFM 45395 (serotype AD, diploid, mating type aα, urease-positive), there were several letters "N" that means "cannot identify". For example, Fig. 4 shows the comparison of aligned sequences of a 294-bps amplified band and the electropherogram of IFM 45395. At the nucleotides (nt) 191 and nt 192, double peaks, which symbolized "C" and "T", overlapped each other in their electropherogram. Another "N" position similarly overlapped "G" and "A" or "C" and "G". Even if the sites where substitutions occurred between the parental strains do not show "N" in their electropherogram, their peaks presented both paternal and maternal sequences, as shown in Fig. 4 at nt 230. This phenomenon was not detected in other diploid strains without serotype AD (IFM 45437, 45541).

SSCP analysis of a 294-bp fragment of the *URE1* gene is shown in Fig. 5. In IFM 5845 and 41469, each strain contained two independent bands that corresponded to the four bands of IFM 45395. In other words, two pairs of similarly sized but not the same sequenced fragments were amplified from IFM 45395, which is serotype AD.

Discussion

Until now, some genetics studies of *C. neoformans* have used auxotrophic strains such as *ade2*, *ura5* and *lys1*[12, 13]. It appears that crosses of auxotrophic parents tend to produce diploid progeny because of complementation. In the present study, we used wild type prototrophic parental strains. In our crosses, the number of diploid F1 progeny was not as high as that with crosses of auxotrophic parents, but the diploid state was more stable than that with auxotrophic parents. In recombinant haploid F1, we hypothesized that the serotype-related gene and the mating type-related gene were located on the same chromosome, but that the *URE1* gene was not (Table 1).

We were unable to determine why the urease activity of IFM 41469 was negative. In the present study, we did not observe sequence characteristics

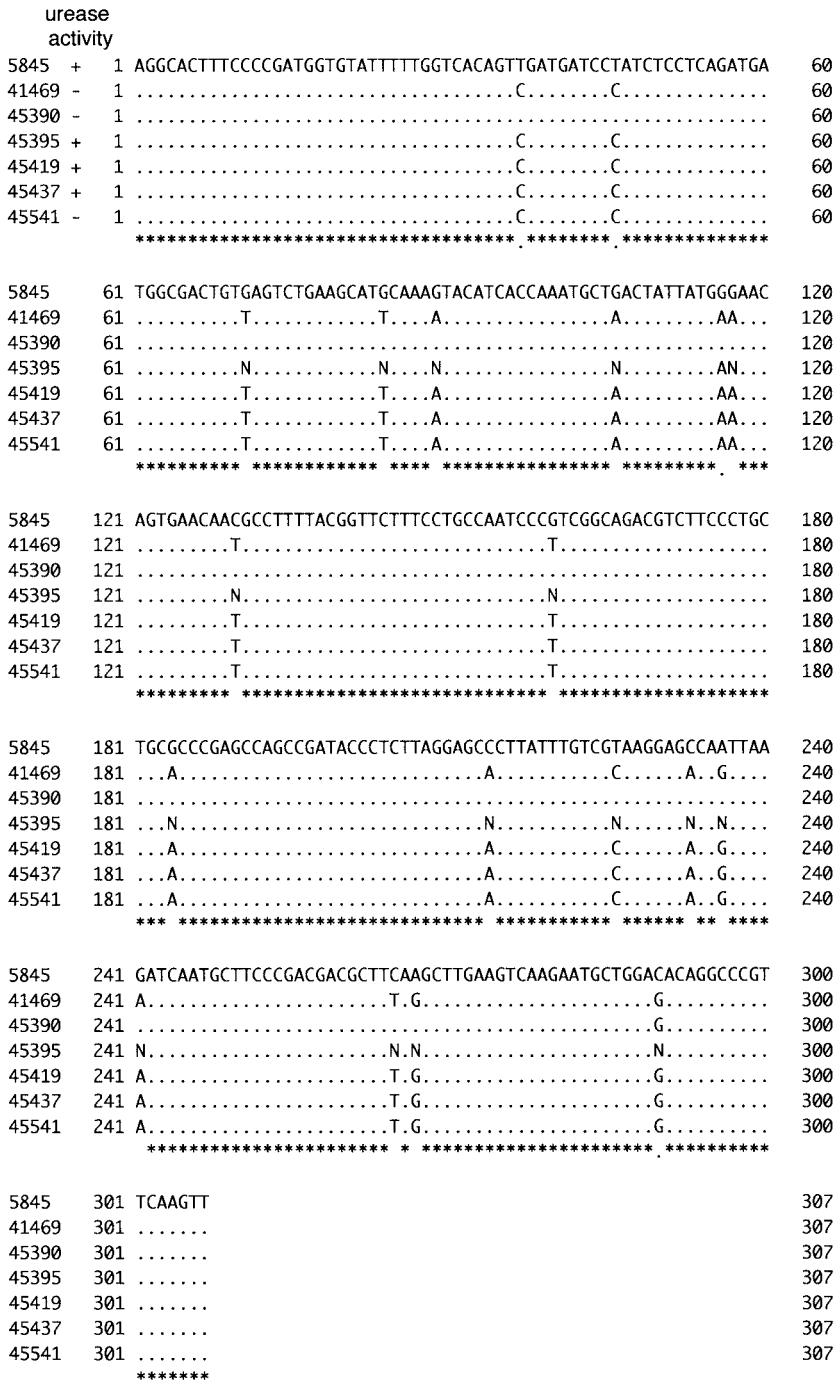


Fig. 3. Aligned sequences of the 307-bp PCR product from the *URE1* gene.

specific to urease-negative strains, and the *URE1* gene appeared normal in the urease-negative strain. However, we examined only a limited sequence, from nt 1590 to 1896 in Fig. 3 and from nt 3217 to 3872 (data not shown), and further analyses of additional sequences are necessary. In IFM 45390 (urease-negative, serotype D, mating type a, haploid), "G" at nt 290 indicates the possibility of the recombination from nt 268 to 289 (Fig. 3). Within the tested region (nt 1590 to 1896, nt 3217 to 3872), there was no relation to urease-positive and -negative. Assuming this recombination is fact, the differences of the sequence concerned with urease activity will be found in from nt 1897 to 3216 or below nt 3873. If there are no problems in the primary sequence, post-transcriptional modifications or problems may confer the urease-negative phenotype. The SSCP analyses (Fig. 5) revealed that F1 diploid AD strains inherited the entire genome of each parent.

On the basis of these data, we speculate that diploid serotype AD strains from crosses of serotypes A and D strains are the result of irregular meiosis. Further morphological evidence, such as

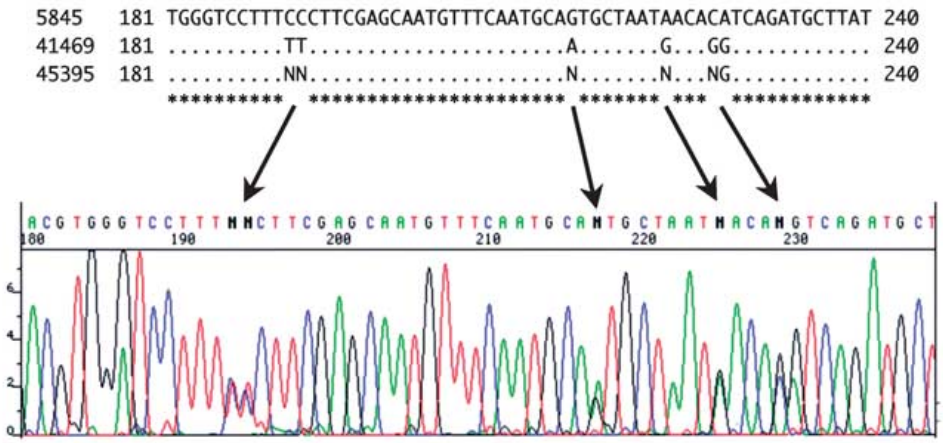


Fig. 4. Aligned sequences of the 294-bp band amplified from the *URE1* gene and electropherogram of IFM 45395. Arrows indicate corresponding base positions. N, nucleotide unclear.

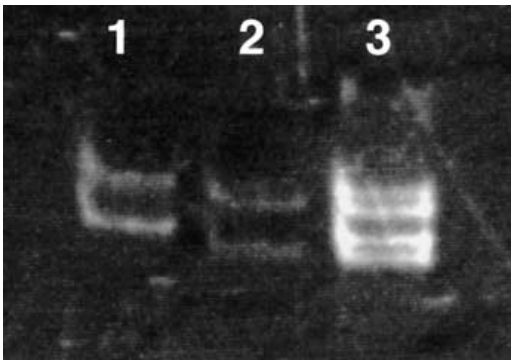


Fig. 5. SSCP of the 294-bp fragment of *URE1*. Lane 1, IFM 41469 (serotype A); lane 2, IFM 5845 (serotype D); lane 3, IFM 45395 (serotype AD).

nuclear staining, will be helpful; however, it was difficult to do morphological analyses in the present study because the frequency of the diploid strain was extremely low.

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