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FULL PAPER

Non-homologous end joining pathway of the human pathogen *Cryptococcus neoformans* influences homologous integration efficiency but not virulence

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Abstract The efficiency of gene targeting by integration through homologous recombination (homologous integration, HI) in the human pathogen *Cryptococcus neoformans* remains unsatisfactory. In order to achieve a much more efficient gene targeting system in *C. neoformans*, a new double knockout strain in genes involved in the non-homologous end joining (NHEJ) pathway was constructed. HI frequency was elevated by as much as approximately fivefold in the single or double knockout strains in NHEJ genes, and the frequency depended on the gene targeted. None of the NHEJ gene knockouts showed significant differences in regular growth, sensitivity to DNA-damaging drugs or UV, and virulence compared to the wild-type control, suggesting that the NHEJ pathway does not play a significant role in these biological stresses in *C. neoformans*. It was also suggested that the genes analyzed in this study are components of a single NHEJ pathway, as the mutants (including the double mutant) displayed the same phenotypes.

Keywords DNA ligase IV · Gene manipulation · KU protein · Molecular genetics

Introduction

Cryptococcus neoformans (anamorph of *Filobasidiella neoformans* in Tremellales) is one of the most life-threatening fungal pathogens (Casadevall and Perfect 1998). Infection with this pathogen is often associated with immunodeficiency caused by therapeutic immunosuppression, neoplastic disease, and AIDS. *Cryptococcus* infection is believed to occur initially by inhalation of desiccated yeast cells or basidiospores, followed by dissemination to the central nervous system to cause meningitis. This fungus occasionally causes disease not only in immunocompromised patients, but even in healthy individuals without apparent pre-existing diseases.

Since the completion of the genome sequence of *C. neoformans*, a number of genes have been targeted and deleted, inactivated or disrupted in this pathogen in order to assess their functions or its involvement in pathogenicity and basic biology. To reduce the cost of, the time needed and the effort required to perform genetic manipulation in *C. neoformans*, strains lacking genes involved in non-homologous end joining (NHEJ) have been developed (Goins et al. 2006; Shimizu et al. 2010). In these strains, gene targeting efficiency has been elevated to 70–90% depending on homologous sequence length, but a significant number of transformants due to NHEJ still appear in any transformation experiments.

In other fungal species, highly efficient gene-targeting has been achieved by inactivating *KU* genes (homologs of human *KU70* and *KU80*, or *Saccharomyces cerevisiae YKU70* and *YKU80*), components of the NHEJ pathway. These fungi include *Neurospora crassa* (Ninomiya et al. 2004), *Kluyveromyces lactis* (Kooistra et al. 2004) *Aspergillus nidulans* (Nayak et al. 2006), *A. fumigatus* (da Silva Ferreira et al. 2006; Krappmann et al. 2006), *A. oryzae* (Takahashi et al. 2006), *A. sojae* (Takahashi et al. 2006), *Sordaria macrospore* (Pöggeler and Kück 2006), *A. niger* (Meyer et al. 2007), *Botrytis cinerea* (Choquer et al. 2008), *Candida glabrata* (Ueno et al. 2007), *Magnaporthe grisea* (Villalba et al. 2008), *A. parasiticus* (Chang 2008),

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Trichophyton mentagrophytes (Yamada et al. 2009) and *Hypocrea jecorina* (Guangtao et al. 2009). Another component of NHEJ, a DNA ligase IV, has been also inactivated in some filamentous fungi, such as *N. crassa* (Ishibashi et al. 2006), *A. oryzae* (Mizutani et al. 2008), *M. grisea* (Kito et al. 2008) and *Pichia ciferrii* (Schorsch et al. 2009). Compared to these, *C. neoformans* appears to be a rather laborious organism for gene targeting.

In order to elevate gene-targeting efficiency in *C. neoformans*, we explored the mechanism of NHEJ in this species by genetic analysis. Our results suggest that the NHEJ process in *C. neoformans* involves both KU protein and DNA ligase IV in a single and nonoverlapping pathway, and that the NHEJ mechanisms for KU and DNA ligase IV appear to be independent.

Materials and methods

Strains and media

The *Cryptococcus neoformans* strains used in this study are listed in Table 1. All strains were maintained on a YPD (10 g yeast extract, 10 g polypeptone, 10 g glucose per 1 l) plate at 25°C for use. For transformation, YPD or YNB [1.7 g yeast nitrogen base without amino acids and ammonium sulfate (Difco), 5 g ammonium sulfate, 20 g glucose per 1 l] plates were supplemented with 1.2 M sorbitol. Nourseothricin (100 μg/ml) or G418 (200 μg/ml) was added to YPD plate for selection of nourseothricin or G418-resistant transformants. Adenine (250 μg/ml) was supplemented for the selection of Δ*ade2* transformants. L-Deoxyphenylalanine (L-DOPA) medium was prepared as described by Chaskes and Tyndall (1975) to screen for melanin-deficient Δ*lac1* transformants.

Strain constructions

The primers used in this study are summarized in Table 2. In order to introduce Δ*cnlig4* into the Δ*cku70* background to create a double knockout strain, the primers CnLIG4-5.UP and CnLIG4-3.LP were used to amplify a gene knockout cassette using a genomic DNA of TLHM12 as template. The knockout cassette was introduced biolistically into TLHM15. Transformants were screened on YPD with G418, and proper integration was confirmed by PCR with the primers CnLIG4.UUP and CnLIG4.LLP, as described previously (Shimizu et al. 2010). The primers NAT.UP and NAT.LP were also used to confirm *cnlig4* deletion (see Fig. 1). To create the *lac1* (a laccase-coding gene involved in melanin biosynthesis) deletion strain TLHM29, the primers LAC1-5.UP, LAC1-3.LP, LAC1-3.UP and LAC1-3.LP were used to amplify LAC1 flanking sequences from B4500 genomic DNA, and the primers M13FWD and M13REV were used to amplify the URA5 marker gene from pKIS117.1 as a template. Three PCR fragments raised as above were then mixed and fused by PCR with the primers LAC1-5.UP and LAC1-3.LP to generate the Δ*lac1* construct as described previously (Shimizu et al. 2010), and the gene knockout cassette was biolistically introduced into TLHM15. To construct the *ade2* (a gene involved in adenine biosynthesis) deletion strain TLHM30, the primers ADE2-5.UP, ADE2-5.UP, ADE2-3.LP, ADE2-3.UP, ADE2-3.LP, M13FWD and M13REV were used to generate the Δ*ade2* construct as described above, and the gene knockout fragment was also biolistically introduced into TLHM15.

HI frequency assay

To introduce the *URA5* gene for homologous integration (HI) frequency assay, the primer pairs URA5.UP1 and

Table 1 Strains used in this study

Strain	Genotype	Source
B4500	Wild type (WT)	Kwon-Chung et al. (1992)
TAD1	Δ*ura5*	Drivinya et al. (2004)
TLHM12	Δ*cnlig4*::*NAT*, Δ*ura5*	Shimizu et al. (2010)
TLHM14	Δ*cku80*::*NEO1*, Δ*ura5*	Shimizu et al. (2010)
TLHM15	Δ*cku70*::*NEO1*, Δ*ura5*	Shimizu et al. (2010)
TLHM16	Δ*cnlig4*::*NAT*, Δ*cku80*::*NEO1*, Δ*ura5*	This study
TLHM22	Δ*cnlig4*::*NAT*	Shimizu et al. (2010)
TLHM24	Δ*cku70*::*NEO1*	This study
TLHM32	Δ*cku80*::*NEO1*	This study
TLHM31	Δ*cnlig4*::*NAT*, Δ*cku70*::*NEO1*	This study
TLHM29	Δ*lac1*::*URA5*, Δ*cku70*::*NEO1*, Δ*ura5*	This study
TLHM30	Δ*ade2*::*URA5*, Δ*cku70*::*NEO1*, Δ*ura5*	This study

Table 2 Primers used in this study

Primer	Sequence
LIG4.5UP	5′-TGGGGAAAAGTTTGATGTTGTGTG-3′
LIG4.3LP	5′-TGTCAACCCCTGGTGCTGGATCTC-3′
LIG4.UUP	5′-AAAGGGAGAGTTGGAGTACCGATT-3′
LIG4.LLP	5′-CGGAGTAACAGTGGAAGCAGGAGT-3′
NAT.UP	5′-GGTTTATCTGTATTAACACGA-3′
NAT.LP	5′-TGAATAGAGTCACTTTTGCTAG-3′
LAC1-5.UP	5′-GGGAATGGGTGTACGTTAGT-3′
LAC1-5.LP	5′-GCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTC GTTTTACTACTGTGAGCGTCGGTATAG-3′
LAC1-3.UP	5′-TAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCT AAGACCGCTCGGGTTGGAAGAA-3′
LAC1-3.LP	5′-TCTGTCAGGAGCTTGTTCAG-3′
ADE2-5.UP	5′-AAGGCTGATAGAGGAATTGT-3′
ADE2-5.LP	5′-GCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC TTTGTTACAAGAGATGCTCG-3′
ADE2-3.UP	5′-TAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCT ATCAACGGTTTTATGCTGGT-3′
ADE2-3.LP	5′-AGAGAACGGGAGGGGAAAAG-3′
M13FWD2	5′-CGCCAGGGTTTTCCCAGTCACGAC-3′
M13REV	5′-AGCGGATAACAATTTCACACAGGA-3′
URA5.UP1	5′-GACTACTGACGTAGTATTATCGGT-3′
URA5.LP1	5′-TCGGCCACATATCGATGATCACGA-3′
URA5.UP3	5′-CTGTCTCTGAAGCAAGACTAGCGA-3′
URA5.LP3	5′-GGCCACGGACCAACCCTGGCACAT-3′
URA5.UUP	5′-AATTAAACTCTCCGCCATATCCTC-3′
URA5.LLP	5′-CTCTAGTATCGCTCGACTGTCTCA-3′
LAC1-5.UP100	5′-GCATCTTCCCACTATCAACT-3′
LAC1-3.LP100	5′-AGTTAACCAAAAAGTCCGTCA-3′
LAC1.UUP	5′-CAAATTGGGAGACGTGCGCT-3′
LAC1.LLP	5′-GGCCTTTGATTTCGTTCGCT-3′
ADE2-5.UP100	5′-ATAATTTGTGCCTGACTGGC-3′
ADE2-3.LP100	5′-TTGAAGGGTGTTGCTATTATAA-3′
ADE2.UUP	5′-TGAGAAGATGAAGGGGCGAA-3′
ADE2.LLP	5′-GAATGGAATTAGTGCCGATC-3′

The underlined nucleotides correspond to the *URA5* marker gene of pKIS117.1

URA5.LP1 or URA5.UP3 and URA5.LP3 were used to amplify DNA fragments containing a functional *URA5* gene with either 1000 or 100 bp flanking sequences, and B4500 genomic DNA was used as a template. NHEJ mutant strains were transformed with the PCR fragments, and about 20 transformants from each experiment were chosen to determine whether the transformed *URA5* gene fragments were introduced by HI or NHEJ. For this assay, the primers URA5.UUP and URA5.LLP were used as described previously (Shimizu et al. 2010; see Fig. 2). To knock out the *LAC1* gene for HI frequency assay, the primer pairs LAC1-5.UP and LAC1-3.LP or LAC1-5.UP100 and LAC1-3.LP100 were used to amplify the Δ*lac1* cassette, containing the *URA5* marker gene, with either 1000 or 100 bp flanking sequences, and TLHM29 genomic DNA was used as a template. The gene fragments were then introduced into NHEJ mutant strains and the uracil prototrophic transformants that arose were transferred onto L-DOPA plates. White and dark colonies were counted as HI or NHEJ transformants. PCR with the primers LAC1.UUP and LAC1.LLP was also conducted to confirm the targeted gene integration (see Fig. 3). For *ADE2* gene deletion to assess HI frequency, the primer pairs ADE2-5.UP and ADE2-3.LP or ADE2-5.UP100 and ADE2-3.LP100 were used to amplify the Δ*ade2* cassette containing the *URA5* marker gene with either 1000 or 100 bp flanking sequences, and TLHM30 genomic DNA was used as template. The gene knockout cassettes were then introduced into NHEJ mutant strains and uracil prototrophic transformants were incubated for 1 week to facilitate the accumulation of

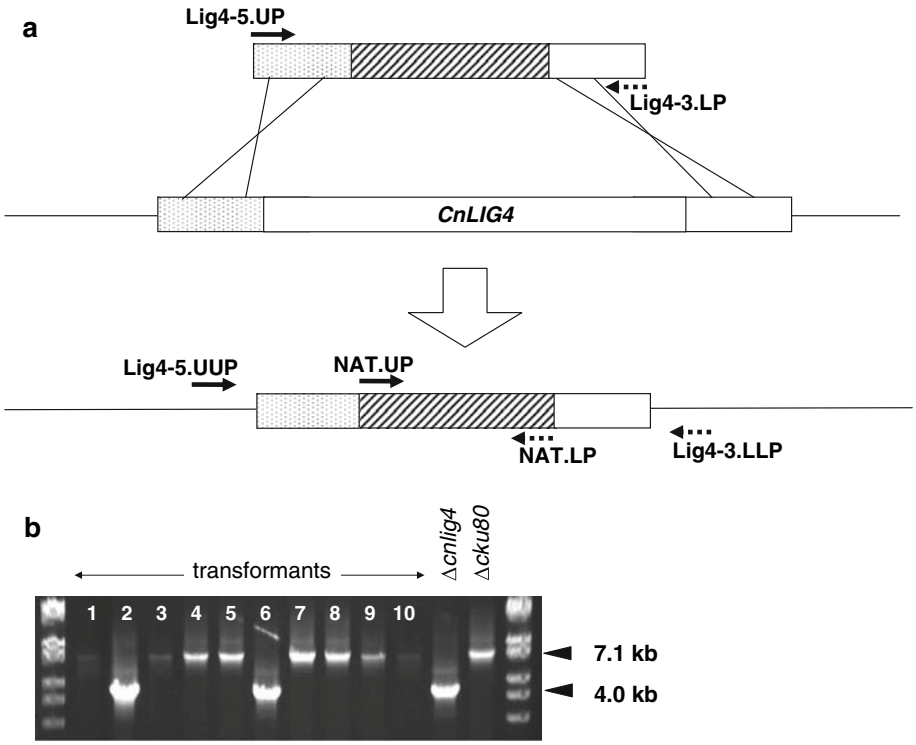

Fig. 1 Construction of Δ*cnlig4* into Δ*cku80* double deletion strain. **a** Scheme for the replacement of the *CnLIG4* gene (5.1 kb) with the *NAT* gene cassette (*striped box* 1.8 kb). The *CnLIG4* gene knockout construct containing the upstream (*dotted box* 1.0 kb) and downstream (*open box* 1.0 kb) flanking sequences of the *CnLIG4* gene fused with *NAT* was PCR amplified using TLHM12 (Δ*cnlig4*) genomic DNA as a template and used for transformation. The positions of the primers used for *CnLIG4* gene disruption are also indicated. **b** Agarose gel electrophoresis of amplified DNA fragments in the *CnLIG4* regions. Genomic DNA of the recipient control TLHM14 (Δ*cku80*), TLHM12 (Δ*cnlig4*) and 10 transformants obtained were PCR amplified with the primers CnLIG4.UUP and CnLIG4.LLP. Two transformants were successful *CnLIG4* gene disruptants (*lanes 2* and *6*), as the PCR fragments are identical to that of TLHM12 (Δ*cnlig4*). Note that the primers CnLIG4.UUP and CnLIG4.LLP reside outside the flanking sequences used for *CnLIG4* disruption

the red pigment, which is a sign of *ADE2* gene inactivation (Ueno et al. 2007). Red and white colonies were counted as HI and NHEJ transformants, respectively. PCR with primers ADE2.UUP and ADE2.LLP was also performed to confirm the targeted gene integration (see Fig. 4).

Chemical and UV sensitivity test

Cells from an overnight culture were harvested, rinsed with 0.1 M phosphate-buffered saline (pH 7.4), and the cells were enumerated with a hemacytometer to adjust the cell concentration. To measure the sensitivity of mutants to DNA-damaging agents, cells were suspended in liquid YNB medium to a concentration of 10^2 cells/ml, and 100 µl of the suspension were placed onto each well in a 96-well plate. The reagents were then added to the wells to final concentrations of 0, 0.003, 0.006, 0.013, 0.025, 0.005 and 0.1% for methyl methane sulfonate (MMS), and 0, 2.5, 5, 10, 20, 41, 81, 163, 325 and 650 mM for hydroxyurea (HU), respectively. The plates were incubated at 30°C for 3 days, and growth was measured by a Sunrise Thermo spectrophotometer (TECAN, Austria) at an optical density of $\lambda = 620$ nm. For the UV sensitivity test, 100 cells were plated onto YPD plates and exposed to 254-nm UV rays of either 0, 200, 400, 800 or 1600 J/m^2. The plates were incubated at 30°C for 3 days, and the number of colonies that appeared was counted.

Virulence assay

The wild-type and NHEJ mutants of *C. neoformans* were injected into mice and survival curves were drawn. Five-week-old male Crj:CD-1 (ICR; Charles River) mice were inoculated intravenously with 2.0×10^6 CFU *Cryptococcus* cells. Five or 6 animals were used for each experiment. Survival data were statistically analyzed by the log-rank test (PRISM 4.0; GraphPad Software). The animal protocol used for this study was approved by the Animal Use Committee, Chiba University.

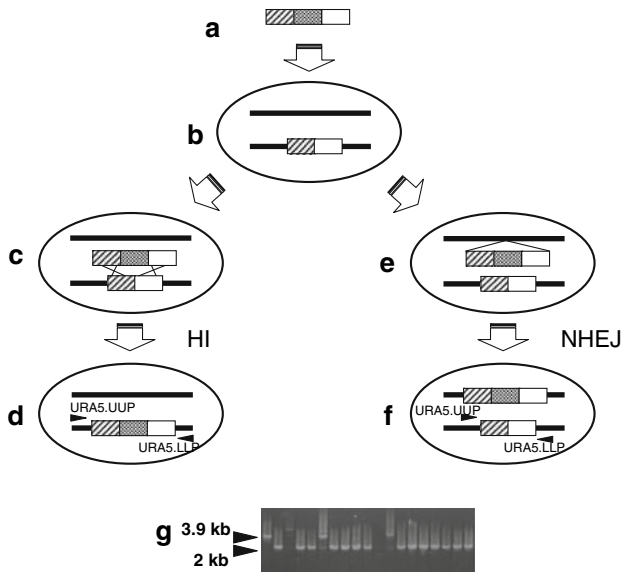

Fig. 2 HI frequency assay with the *URA5* gene. Diagram of the HI efficiency assay used in this study (*a–f*). The *URA5* gene (*dotted box*) with upstream (*striped box*) and downstream (*open box*) flanking sequences (*a*) was transformed into cells in which *URA5* gene is eliminated (*b*). When the introduced fragment (*a*) is integrated into the genome by HI (*c*), the resulting transformant raises a 3.9 kb PCR fragment with the primers URA5.UUP and URA5.LLP, because the region contains the entire *URA5* gene (*d*). But when NHEJ occurs (*e*), a 2.0 kb PCR fragment will be amplified, as the region lacks the *URA5* gene (*f*). An example of agarose gel electrophoresis of PCR fragments by URA5.UUP and URA5.LLP is shown in (*g*). The amplified fragments of 3.9 kb result from HI, and those of 2.0 kb result from NHEJ

Results

Construction of the double knockout strain and characterization of the NHEJ mutants of *C. neoformans*

In order to create a double knockout strain, we introduced the Δ*cnlig4* cassette into TLHM14 (Δ*cku80*). We screened 10 transformants, and 2 of them turned out to be successful disruptants (Fig. 1). The strains grew normally on a solid plate and in a liquid shake culture compared to the NHEJ wild-type or Δ*cnlig4* or Δ*cku70* single deletion strains, respectively (data not shown). In order to characterize the effects of DNA-damaging agents and UV, NHEJ mutants were grown in the presence of MMS or HU, or exposed to UV (Fig. 5). No obvious differences between the wild-type and NHEJ mutants were found under any condition tested, suggesting that none of the components of the NHEJ pathway are essential for regular growth or DNA repair.

HI frequencies are elevated in NHEJ mutants

TAD1 (Δ*ura5*), TLHM12 (Δ*cnlig4*, Δ*ura5*), TLHM14 (Δ*cku80*, Δ*ura5*), TLHM15 (Δ*cku70*, Δ*ura5*) and TLHM16 (Δ*cku80*, Δ*cnlig4*, Δ*ura5*) were transformed with the *URA5* gene with 100 or 1 kb flanking sequences on both ends (see Fig. 5a). When the DNA with 100 bp flanking regions was used, no HI event was observed in any strain. When the fragment with 1 kb flanking sequences was used, the HI frequency in the Δ*cnlig4* strain was elevated to approximately 1.6 times higher than that of the wild-type control (Table 3). In addition, the laccase gene *LAC1* and the adenine biosynthetic gene *ADE2* were targeted to assess the HI frequency in the NHEJ mutants by using the gene disruption cassette with 100 or 1 kb flanking sequences (see Figs. 3b, 4b). As shown in Fig. 3a (*LAC1*) and Fig. 4a (*ADE2*), successfully targeted transformants (results of HI) could be visually distinguished from ectopically integrated transformants (results of NHEJ). As summarized in Tables 4 and 5, the HI frequency was elevated to 1.9 (Δ*cku70*)–4.9 (Δ*cku80*) times higher than that of the wild-type control TAD1 when LAC1 was targeted, but it was almost comparable to that of the control strain when *ADE2* was targeted.

We then investigated whether HI frequency depends on the target gene by transforming a *C. neoformans* Δ*cku70* mutant strain with various gene knockout cassettes. As shown in Table 6, the HI frequency varies from 13% (*CnUBP6*) to 80% (*CnUBI4*), suggesting that some genes are more efficiently targeted than others.

NHEJ pathway is not required for virulence of *C. neoformans*

The wild-type, Δ*cnlig4*, Δ*cku70*, Δ*cku80* and the double knockout (Δ*cku80*, Δ*cnlig4*) strains were inoculated into ICR mice via tail vein injection. Figure 6 shows the survival curves of mice infected with each strain. All of the strains were equally virulent to the wild-type strain ($p = 0.80$ for WT vs Δ*cnlig4*, $p = 0.40$ for WT vs Δ*cku70*, $p = 0.41$ for WT vs Δ*cku80*, $p = 0.23$ for WT vs Δ*cku80*, Δ*cnlig4*). These results suggest that no NHEJ genes are required for *C. neoformans* to be virulent against mice. The *p* values for all of the pairwise comparisons among mice infected with mutant strains were calculated, and all of them were >0.14, which strongly indicates that all of the strains used in this virulence assay are equally virulent in the murine model.

Discussion

We demonstrated in a previous report that by deleting the *CnLIG4* gene encoding a DNA ligase IV involved in NHEJ, the HI efficiency of *C. neoformans* was elevated to 80%. We also showed that the HI frequency reached up to 90% in *C. neoformans cku70* or *cku80* mutant strains. In

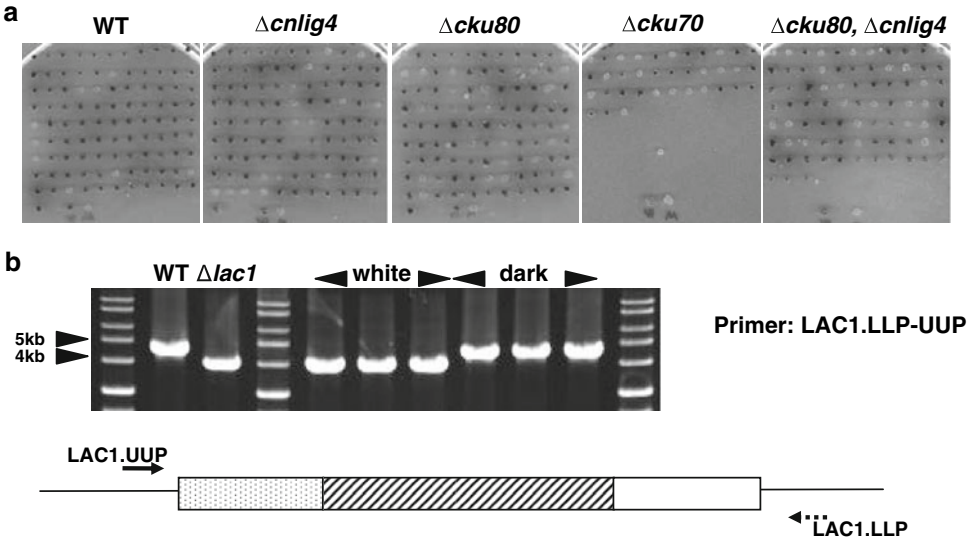

Fig. 3 HI frequency assay with the *LAC1* gene. Transformants that appeared on transformation plates were transferred onto L-DOPA medium plates and incubated at 25°C for 7 days. After the incubation, the white and dark colonies were counted (**a**). Some of the white and dark colonies were used for PCR with the primers LAC1.UUP and LAC1.LLP. Successful *LAC1* deletion transformants (*white colonies*, result of HI) bore 4 kb fragments, whereas exogenous integration transformants (*dark colonies*, result of NHEJ) bore 5 kb fragments (**b**). *URA5* marker gene, *striped box* 2.0 kb; upstream, *dotted box* 1.0 kb; downstream, *open box* 1.0 kb flanking sequences

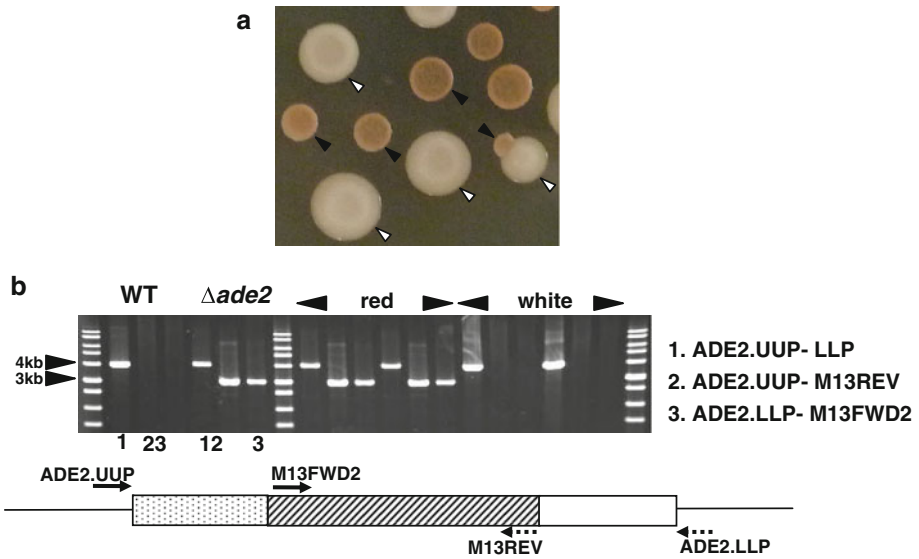

Fig. 4 HI frequency assay with the *ADE2* gene. Transformants that appeared on transformation plates were kept at 25°C for 7 days. After the incubation, the white and red colonies (indicated by *open* and *closed arrowheads*, respectively) were counted (**a**). Some of *white* and *red colonies* were used for PCR with the primers ADE2.UUP and ADE2.LLP, ADE2.UUP and M13REV, or ADE2.LLP and M13FWD2. Successful *ADE2* deletion transformants (*red colonies*, result of HI) bore 4 kb fragments by the primers ADE2.UUP and ADE2.LLP, and 3 kb fragments by the primer pairs ADE2.UUP and M13REV, or ADE2.LLP and M13FWD2. Unsuccessful transformants (*white colonies*, result of NHEJ) also gave 4 kb fragments by the primers ADE2.UUP and ADE2.LLP, but they did not give PCR fragments by ADE2.UUP and M13REV or by ADE2.LLP and M13FWD2 (**b**). *URA5* marker gene, *striped box* 2.0 kb; upstream, *dotted box* 1.0 kb; downstream, *open box* 1.0 kb flanking sequences

this study, we conducted a genetic analysis by creating a double knockout strain in both the *cnlig4* and the *cku70* genes and comparing its phenotypes with other single knockout strains. As all of the single and double knockout strains showed similar phenotypes in terms of growth, sensitivities to UV and DNA damaging chemicals, virulence and HI efficiencies, the genes analyzed in this study appear to be components of the single pathway of NHEJ. In *N. crassa*, the HI efficiency of the *mus-51* (*YKU70* homolog) mutant is significantly lower than that of the

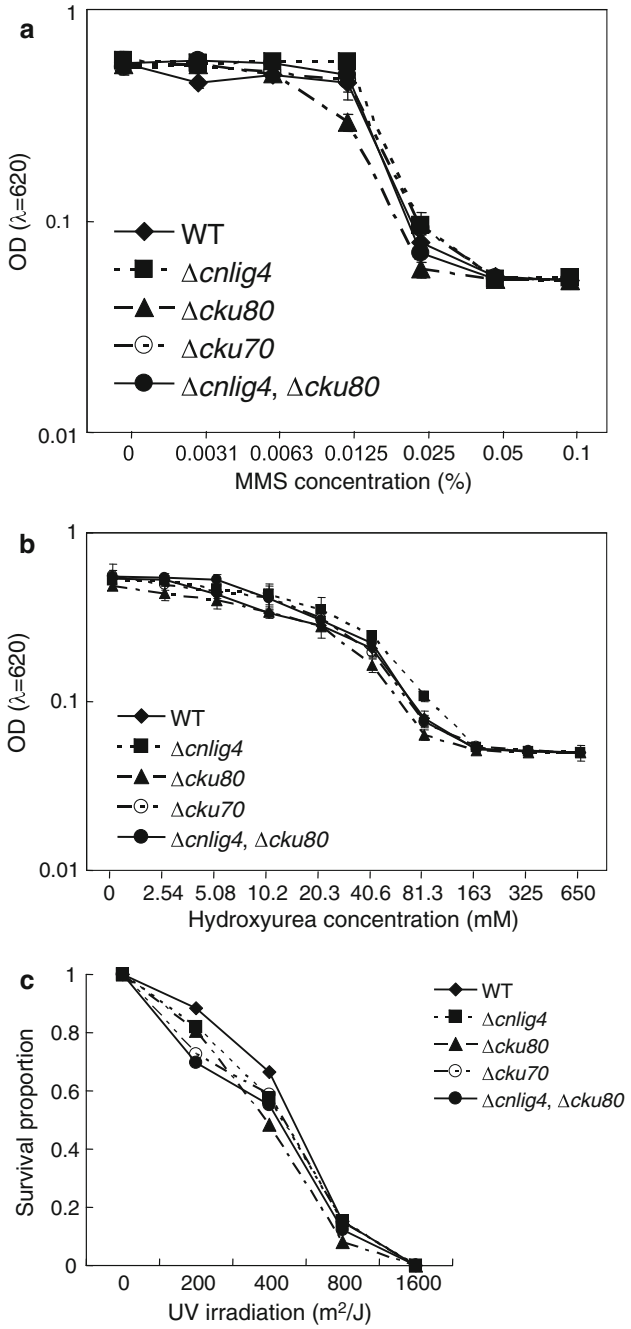

Fig. 5 Sensitivity of NHEJ mutants to DNA-damaging chemicals and UV. The *C. neoformans* NHEJ mutant strains were grown in the presence of various concentrations of methyl methane sulfonate (*MMS*, **a**) or hydroxyurea (*HU*, **b**). These mutants were also exposed to various strengths of UV (**c**). All experiments were performed in triplicate, and standard deviations are indicated

mus-52 (*LIG4* homolog) mutant. Thus, the presence of a minor pathway responsible for NHEJ is suggested, which is controlled by MUS-11 (Rad52 homolog), a major component of DSB repair by homologous recombination (HR) (Ishibashi et al. 2006). A homolog of the Rad52 gene was found by a BLAST search in the genome of *C. neoformans*. Although its involvement in NHEJ has not yet been elucidated, the RAD52 in *C. neoformans* may not have such an obvious role in NHEJ as in *N. crassa*, based on our observation that the phenotypes of the mutants are quite similar to each other. To better understand the DSB repair mechanisms in *C. neoformans*, the role of components in HI including RAD52 should be determined.

Our results also indicate that CKU70, CKU80 and CnLIG4 are not responsible for sensitivity to DNA-damaging drugs or UV. In some fungal species such as *A. fumigatus* (Krappmann et al. 2006), *A. nidulans* (Nayak et al. 2006), *A. parasiticus* (Chang 2008) or *C. glabrata* (Ueno et al. 2007), it has been reported that the homologs of *YKU70* or *YKU80* genes are not responsible for UV or DNA-damaging chemical sensitivities. In other cases, including *A. oryzae* (Mizutani et al. 2008), *N. crassa* (Ishibashi et al. 2006) and *T. mentagrophytes* (Yamada et al. 2009), their involvement in resistance to DNA-damaging agents has been reported. Our results suggest that the involvement of the NHEJ pathway to DSB repair caused by physical or chemical stimulation in *C. neoformans* is one of the former cases. Since there seems to be no correlation between these differences and fungal phylogenetic relationships (James et al. 2006), the evolution of NHEJ pathways in fungi appears to be complicated. For growth in regular conditions, no fungal species has been reported to be influenced by the deletion of NHEJ component(s), as in the case of *C. neoformans*. In the plant-pathogenic fungus *B. cinerea*, *BcKu70* and *BcKu80* knockout strains are just as virulent towards the host plant as the wild-type control (Choquer et al. 2008). These results show that the NHEJ pathway in fungal pathogens may not be important for fully expressing their pathogenicities in either animals or plants.

In general, DSBs are repaired through two major recombination pathways: HR and NHEJ (Aylon and Kupiec 2004). HR requires homologous DNA sequences, whereas NHEJ does not. In DSB repair through the NHEJ, DSBs are recognized by KU proteins and rejoined through direct ligation by DNA ligase IV regardless of genetic information from their original sequences. In *C. neoformans*, even though genes encoding KU proteins and/or DNA ligase IV were eliminated, ectopic and undesired integration frequently occurred during transformation experiments, suggesting that KU- or DNA ligase IV-independent mechanisms that allow random integration should be functioning in this fungus. In the genome of *C. neoformans*, there are at least 2 additional genes that likely code for DNA ligases, and their activities may allow foreign DNA to be integrated ectopically even when DNA ligase IV encoded by *CnLIG4* is inactive. The functions of these ligase coding genes are currently being analyzed in order to determine their involvement in NHEJ. A better understanding of the details

Table 3 HI frequencies of NHEJ mutant strains with respect to the *URA5* gene

Strain	NHEJ genotype	Flanking size = 1000 bp		Flanking size = 100 bp	
		HI frequency	Relative value of HI frequency	HI frequency	Relative value of HI frequency
TAD1	WT	10/22 (45%)	1.0	0/20 (0%)	0
TLHM12	Δ*cnlig4*	16/22 (73%)	1.6	0/23 (0%)	0
TLHM14	Δ*cku80*	11/23 (48%)	1.1	0/16 (0%)	0
TLHM15	Δ*cku70*	12/22 (55%)	1.2	0/7 (0%)	0
TLHM16	Δ*cku80*, Δ*cnlig4*	10/23 (43%)	1.0	0/18 (0%)	0

Table 4 HI frequencies of NHEJ mutant strains with respect to the *LAC1* gene

Strain	NHEJ genotype	Flanking size = 1000 bp		Flanking size = 100 bp	
		HI frequency	Relative value of HI frequency	HI frequency	Relative value of HI frequency
TAD1	WT	9/100 (9%)	1.0	0/100 (0%)	0
TLHM12	Δ*cnlig4*	25/100 (25%)	2.8	3/100 (3%)	∞
TLHM14	Δ*cku80*	16/36 (44%)	4.9	2/67 (3%)	∞
TLHM15	Δ*cku70*	17/100 (17%)	1.9	1/84 (1%)	∞
TLHM16	Δ*cku80*, Δ*cnlig4*	25/80 (28%)	3.1	1/32 (3%)	∞

Table 5 HI frequencies of NHEJ mutant strains with respect to the *ADE2* gene

Strain	NHEJ genotype	Flanking size = 1000 bp		Flanking size = 100 bp	
		HI frequency	Relative value of HI frequency	HI frequency	Relative value of HI frequency
TAD1	WT	76/177 (43%)	1.0	0/160	0
TLHM12	Δ*cnlig4*	105/165 (64%)	1.5	0/142	0
TLHM14	Δ*cku80*	257/403 (64%)	1.5	0/87	0
TLHM15	Δ*cku70*	22/49 (45%)	1.0	0/66	0
TLHM16	Δ*cku80*, Δ*cnlig4*	5/13 (38%)	0.9	0/58	0

Table 6 HI frequencies of the Δ*cku70* strain with respect to various target genes

Target gene	Gene ID	HI frequency
CnUBP6	CNG01690	1/8 (13%)
CnUCH37	CND01170	2/8 (25%)
CnRPN10	CNA04640	3/6 (50%)
CnPHO1	CNG00033	6/15 (40%)
CnPHO2	CNK00554	2/7 (29%)
CnPHO80	CNBK0910	3/8 (38%)
CnPHO3	CNK01690	5/9 (56%)
CnUBI4	CNA03330	4/5 (80%)

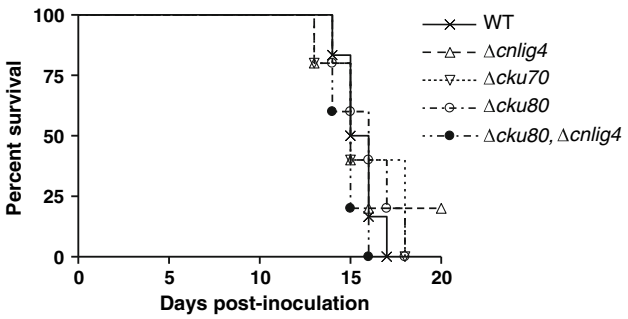

Fig. 6 Virulence of NHEJ mutants. ICR mice were infected with 2×10^6 cells of B4500 (WT), TLHM22 (Δ*cnlig4*::*NAT*), TLHM24 (Δ*cku70*::*NEO1*), TLHM32 (Δ*cku80*::*NEO1*) and TLHM31 (Δ*cnlig4*::*NAT*, Δ*cku80*::*NEO1*) by tail vain injection. Percent survival (%) was monitored for 20 days after inoculation

of the mechanism of DSB repair and NHEJ will greatly facilitate molecular studies in *C. neoformans*.

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