Mycoscience (2010) 51:28–33
DOI 10.1007/s10267-009-0001-7

FULL PAPER

Deletion of *CnLIG4* DNA ligase gene in the fungal pathogen *Cryptococcus neoformans* elevates homologous recombination efficiency

Kiminori Shimizu · Hao-Man Li · Eric V. Virtudazo · Akira Watanabe · Katsuhiko Kamei · Masashi Yamaguchi · Susumu Kawamoto

Received: 18 May 2009 / Accepted: 4 July 2009 / Published online: 23 December 2009

Abstract In eukaryotes from yeasts to human, DNA double-strand breaks are repaired by nonhomologous end-joining (NHEJ) or homologous integration (HI). In the human pathogenic yeast *Cryptococcus neoformans*, gene manipulation by HI does not occur frequently because ectopic integration by NHEJ is predominant, and it has been necessary to screen 30–100 transformants per experiment to obtain transformants with the desired genotypes. To overcome this problem, we constructed a strain in which one of the NHEJ-related genes, *CnLIG4*, was deleted. *CnLIG4* encodes a homologue of the human DNA ligase IV involved in the last step of DNA repair by NHEJ. Gene targeting in the *URA5* locus of a *URA5*-lacking strain TAD1 with *URA5* gene fragments having 1-kb flanking sequences achieved 80% HI efficiency, which is higher than that of the wild-type control (50%). Growth phenotypes and virulence were not attenuated by deletion of the *CnLIG4* gene. Such results suggest that the *CnLIG4* knockout strain created in this study provides an additional alternative for the molecular genetics study of *C. neoformans*.

Keywords Gene manipulation · Homologous integration · Molecular genetics · Nonhomologous end-joining

K. Shimizu (✉) · H.-M. Li · E. V. Virtudazo · A. Watanabe · K. Kamei · M. Yamaguchi · S. Kawamoto
Medical Mycology Research Center, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8673, Japan
e-mail: kshimizu@faculty.chiba-u.jp

Introduction

Cryptococcus neoformans (anamorph of *Filobasidiella neoformans* in Tremellales) is one of the most commonly isolated fungal species in clinical cases (Casadevall and Perfect 1998). Its infection is often associated with therapeutic immunosuppression, neoplastic disease, and acquired immunodeficiency syndrome (AIDS). It is believed that fungal infection occurs initially by inhalation of desiccated yeast cells or basidiospores into the lungs through the trachea, followed by dissemination into a number of internal organs, especially the central nervous system, thereby causing meningitis. Occurring not only in immunocompromised individuals, *C. neoformans* can occasionally cause disease in a healthy host that otherwise is not susceptible to infection by any other fatal fungal pathogens.

Targeted gene disruption by homologous recombination is a powerful tool to understand the basic molecular biology of fungal species. Transformation and deletion of genes of interest have also been developed in *C. neoformans*. However, homologous recombination does not occur with satisfactory efficiency. Recently, in the filamentous fungus *Neurospora crassa*, genes involved in nonhomologous end-joining (NHEJ) were knocked out to increase homologous integration (HI) frequency (Ninomiya et al. 2004). Those genes, *MUS-51* and *MUS-52* [homologues of *YKU70* and *YKU80* (*KU* genes) of *Saccharomyces cerevisiae*, respectively], are predicted to be essential for the NHEJ process. Inactivation of this process leads to inefficient heterologous integration, resulting in the higher appearance of transformants with HI, i.e., gene integration into the desired locus of the genome. Highly efficient homologous recombination events have also been reported in *KU* gene(s)-deficient

mutants in a number of fungal species including *Aspergillus fumigatus*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. sojae*, and *Candida glabrata* (da Silva Ferreira et al. 2006; Krappmann et al. 2006; Nayak et al. 2006; Pöggeler and Kück 2006; Takahashi et al. 2006; Meyer et al. 2007; Ueno et al. 2007; Villalba et al. 2008). In *C. neoformans*, Goins et al. (2006) reported that gene targeting efficiency was elevated by deletion of *CKU70* or *CKU80* (*YKU70* and *YKU80* homologues, respectively). However, efficient HI was achieved only with relatively long flanking sequences.

More recently, Ishibashi et al. (2006) reported that the absence of the DNA ligase gene *MUS-53* (human DNA ligase IV homologue) in *N. crassa*, which is also involved in NHEJ, leads to more efficient HI compared to *MUS-51*- and/or *MUS-52*-deficient strains. When the *MUS-53* gene was inactivated, a highly efficient desired HI was observed with only 100-bp flanking sequences, whereas 500 bp was necessary for satisfactory HI rates in *MUS-51* or *MUS-52* mutants. In *A. oryzae*, as in *N. crassa*, HI frequency is elevated up to 80–100% when the *LigD* gene (homologue of *MUS-53*) is disrupted (Mizutani et al. 2008), whereas *KU* (homologue of *MUS-51* or *MUS-52*) disruptants achieve up to 70% HI (Takahashi et al. 2006).

In the present study, we examined the HI efficiency of the *MUS-53* homologue (designated as *CnLIG4*) disruptant in *C. neoformans*. The *CnLIG4* deletion strain was not deficient in growth in vitro and in virulence in vivo. The HI frequency of the deletion strain with 500-bp or 1-kb flanking sequences was higher than the wild-type control but less satisfactory compared to the previously reported knockout strains of *CKU70* and/or *CKU80* gene(s) (Goins et al. 2006). However, HI was observed even with 100-bp flanking regions, possibly providing another option for *C. neoformans* genetic manipulation with less effort.

Materials and methods

Strains and media

Cryptococcus neoformans strains B-4500 (Kwon-Chung et al. 1992) and TAD1 (Drivinya et al. 2004) were maintained on a YPD [1% yeast extract, 1% polypeptone, 1% glucose (w/v)] plate at 25°C for use. For transformation, YPD plates were supplemented with 1.2 M sorbitol. Nourseothricin (100 μg/ml) was added to YPD plates for selection of transformants. TLHM12 (Δ*CnLIG4*, Δ*URA5*), TLHM15 (Δ*CKU70*, Δ*URA5*), TLHM14 (Δ*CKU80*, Δ*URA5*), and TLHM20 (Δ*CnLIG4*) were constructed in this study (see following).

Structural analysis and construction of *CnLIG4* deletion strains

To clarify the structure of the *C. neoformans CnLIG4* gene, cDNA was constructed and both ends of mRNA were analyzed with GeneRacer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The structural information for the *CnLIG4* gene was deposited in the DDBJ database (AB505024). Based on the structural information, primers CnLIG4-5.UP (5′-TGGGGAAAAGT TTGATGTTGTGTG-3′), CnLIG4-5.LP (5′-AGCTCACAT CCTCGCAGCCTAGCAAAAGTGACTCTATTCACTTT TGAACCAGTCAGAGAAAGGT-3′), CnLIG4-3.UP (5′-CT AGTTTCTACATCTCTTCTCGTGTTAATACAGATAAAC CTCAGTGGTATCAGACCGCCTAATC-3′), and CnLIG4-3.LP (5′-TGTCAACCCCTGGTGCTGGATCTC-3′) for gene knockout were designed, where the underlined nucleotides correspond to the nourseothricin-resistant marker (*NAT*) gene of pCH233 (Idnurm et al. 2004). CnLIG4-5.UP and CnLIG4-5.LP, and CnLIG4-3.UP and CnLIG4-3.LP, were used to amplify 1-kb flanking regions of the *CnLIG4* coding sequence, respectively, by using *C. neoformans* genomic DNA as a template. Primers NAT.UP (5′-TGA ATAGAGTCACTTTTGCTAG-3′) and NAT.LP 5′-GGTTT ATCTGTATTAACACGA-3′) were used to amplify the *NAT* gene by using pCH233 as template. Three polymerase chain reaction (PCR) fragments obtained as already described were then mixed and used to generate a target gene knockout cassette by the overlap PCR procedure (Davidson et al. 2002), with primers CnLIG4-5.UP and CnLIG4-3.LP. The generated knockout cassette was introduced biolistically into *C. neoformans* cells. Transformants were screened on YPD with nouseothricin, and the proper integration event was confirmed by PCR with primers CnLIG4.UUP (5′-AAA GGGAGAGTTGGAGTACCGATT-3′) and CnLIG4.LLP (5′-CGGAGTAACAGTGGAAGCAGGAGT-3′), sequences of which were derived from upstream or downstream of CnLIG4-5.UP or CnLIG4-3.LP, respectively (Fig. 1). Primers NAT.UP and NAT.LP were also used for confirmation of the *CnLIG4* deletion (Fig. 1). TLHM15 and TLHM14 were also constructed by knocking out *CKU70* or *CKU80* genes in TAD1 (Δ*URA5*), respectively, according to the method described by Goins et al. (2006).

HI frequency assay

Primers URA5.UP1 (5′-GACTACTGACGTAGTATTAT CGGT-3′) and URA5.LP1 (5′-CGGCCACATATCGAT GATCACGA-3′), URA5.UP2 (5′-AGGAGGTAAACTG GGAGATAGGTG-3′) and URA5.LP2 (5′-TCTCCAATAC GATGGCCATAGGGA-3′), URA5.UP3 (5′-CTGTCTCT GAAGCAAGACTAGCGA-3′) and URA5.LP3 (5′-GG CCACGGACCAACCCTGGCACAT-3′), and URA5.UP4

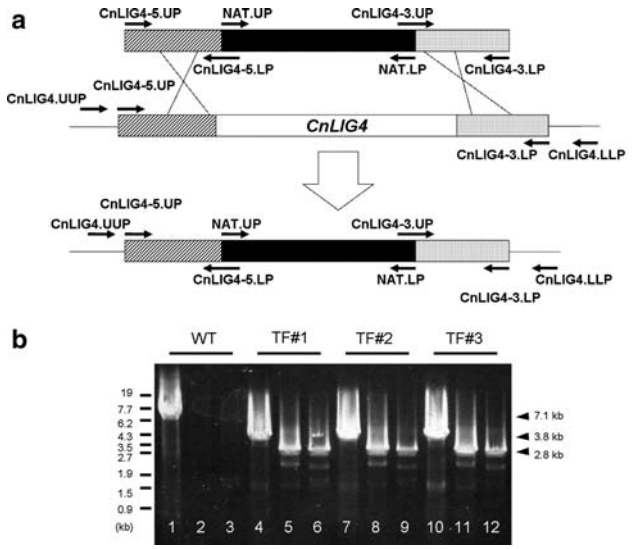

Fig. 1 Construction scheme and confirmation of Δ*CnLIG4* strains of *Cryptococcus neoformans*. **a** Scheme of replacement of *CnLIG4* gene (5.1 kb) with nourseothricin-resistant marker (*NAT*) gene cassette (*closed box*, 1.8 kb). Upstream (*striped box*, 1.0 kb) and downstream (*shaded box*, 1.0 kb) flanking sequences of *CnLIG4* gene were fused with the *NAT* gene by polymerase chain reaction (PCR) and used for transformation. Positions of 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 wild-type control B-4500 (*WT*) and three successful disruptants (*TF#1–3*) were PCR amplified with primers CnLIG4.UUP and CnLIG4.LLP (*lanes 1*, *4*, *7*, *10*), or CnLIG4.UUP and NAT.LP (*lanes 2*, *5*, *8*, *11*), or NAT.UP and CnLIG4.LLP (*lanes 3*, *6*, *9*, *12*). Note that the primers CnLIG4.UUP and CnLIG4.LLP reside outside the flanking sequences used for *CnLIG4* disruption

(5′-GGGATGGTATTGAAGACGATCCAG-3′) and URA 5.LP4 (5′-CTTGTGTCCAGATCCGGCCATT-3′) were used to amplify the *URA5* gene with flanking sequences of 1 kb, 500 bp, 100 bp, or 50 bp, respectively, at both ends. These PCR fragments were used to transform TAD1, TLHM12, TLHM15, or TLHM14 to assess the HI frequency. Primers URA5.UUP (5′-AATTAAACTCTCC GCCATATCCTC-3′) and URA5.LLP (5′-CTCTAGTAT CGCTCGACTGTCTCA-3′) were used to amplify the entire *URA5* gene region to determine whether the transformed *URA5* gene fragments were introduced by HI or NHEJ (Fig. 2).

Growth and virulence assay

Ten milliliters of YPD medium was inoculated with exponentially growing cells to optical density (OD) ($\lambda = 660$ nm) = 0.1 in a 100-ml flask. Cells were incubated at either 30 or 37°C with shaking at 120 rpm. At 4, 6, 8, 10, or 12 h after inoculation, OD ($\lambda = 660$ nm) was measured. Experiments were repeated three times. The wild type and the *CnLIG4* gene disruptant of *C. neoformans*

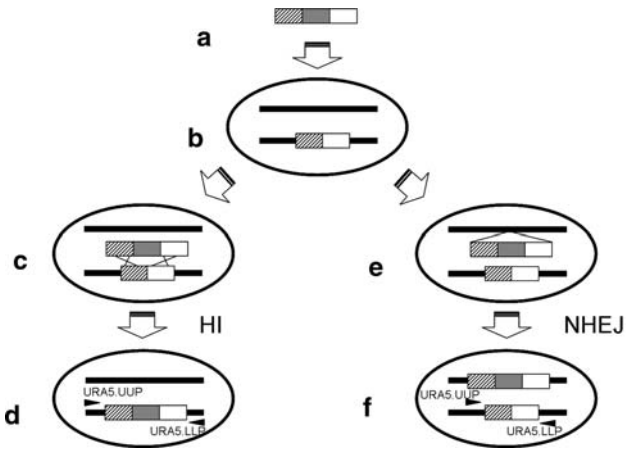

Fig. 2 Diagram of homologous integration (HI) efficiency assay used in this study. *URA5* gene (*shaded box*) with upstream (*striped box*) and downstream (*open box*) flanking sequences (**a**) were transformed into cells in which the *URA5* gene is eliminated (**b**). When the introduced fragment (**a**) is integrated into the genome by HI (**c**), the resulting transformant raises a 3.8-kb PCR fragment with primers URA5.UUP and URA5.LLP because the region contains the entire *URA5* gene (**d**). When nonhomologous end-joining (NHEJ) occurs (**e**), however, a 2.0-kb PCR fragment will be amplified because the region lacks the *URA5* gene (**f**)

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 colony-forming units (CFU) *Cryptococcus* cells. Five 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.

Results

Structure of *CnLIG4* gene and construction of the *CnLIG4* deletion strain

By a BLAST search against the *C. neoformans* genome database (http://www-sequence.stanford.edu/group/C.neoformans/overview.html) for *N. crassa MUS-53*, a single *MUS-53* homologue was found, designated as *CnLIG4*. Based on the alignment obtained by the homology search, we designed primers for 5′- and 3′-RACE (rapid amplification of cDNA ends) to identify both the start and end of the *CnLIG4* transcript. We then PCR amplified the entire cDNA for *CnLIG4*, and the PCR fragment was sequenced to analyze the structure of the *CnLIG4* gene. It was revealed that the *CnLIG4* gene consists of a 3237-bp open reading frame interrupted by 29 introns coding for 1079 amino acid residues. The deduced amino acid sequence of the *CnLIG4* gene showed high homology to other fungal

orthologues of human DNA ligase IV (39% identity with DNA ligase IV of *Coprinopsis cinerea* and 35% with MUS-53 of *N. crassa*).

We then transformed a *C. neoformans URA5* deletion strain TAD1 with a *CnLIG4* gene disruption cassette created by PCR as described in Materials and methods. We screened about 100 nourseothricin-resistant transformants by PCR with primers CnLIG4.UUP and CnLIG4.LLP, and 3 of them turned out to be successful disruptants for the *CnLIG4* gene. The genotypes for *CnLIG4* gene disruption were confirmed by PCR with CnLIG4.UUP and CnLIG4.LLP, by which the parental strains gave a 7.1-kb fragment whereas *CnLIG4* gene disruptants gave 3.8-kb amplification (see Fig. 1). PCR amplification specific for gene disruptants with primers CnLIG4.UUP and NAT.LP, or NAT.UP and CnLIG4.LLP, was also observed in these transformants (2.8 kb; see Fig. 1). One of those disruptants, TF#1, designated as TLHM12, was chosen for further analysis.

HI frequency assay

TAD1 (Δ*URA5*) and TLHM12 (Δ*CnLIG4*, Δ*URA5*) were transformed with *URA5* gene fragments with various sizes of flanking sequences (see Fig. 2). When gene fragments with flanking regions of 1 kb or 500 bp at both ends were used, HI events occurred more frequently in the Δ*CnLIG4* strain compared to the wild type (Fig. 3). However, when the flanking sequences were shortened to 100 bp, only 1 of 20 was a transformant with HI in the Δ*CnLIG4* strain. Furthermore, no HI was observed when the homologous regions were shortened to 50 bp. As it has been described that, in *C. neoformans*, deletion of the *CKU70* or *CKU80* gene (homologues of *MUS-51* and *MUS-52*, respectively) results in the elevation of HI frequency (Goins et al. 2006), we also constructed knockout strains of those genes in the TAD1 background to directly compare the HI frequency in the same genotypic background. As summarized in Table 1, HI frequencies in the Δ*CKU70* or Δ*CKU80* background were slightly higher than the Δ*CnLIG4* background. One of the transformants obtained during the HI frequency assay where *URA5* was reintroduced into the original *URA5* locus was designated as TLHM20 and used for growth and virulence assays.

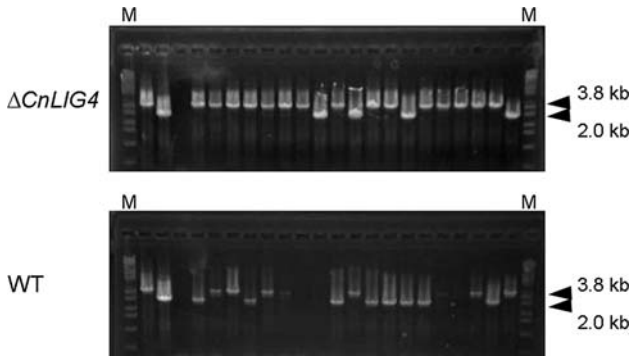

Fig. 3 Agarose gel electrophoresis of PCR fragments amplified with primers URA5.UUP and URA5.LLP. Δ*URA5* strains with Δ*CnLIG4* or wild type (*WT*) background were transformed with *URA5* gene harboring 1-kb flanking sequences. Transformants by HI raise 3.8-kb fragments whereas those by NHEJ give 2.0-kb fragments. More results are summarized in Table 1. *M* molecular marker

Table 1 Frequency of homologous recombination in *Cryptococcus neoformans* Δ*CnLIG4*, Δ*CKU70*, or Δ*CKU80* strains

Strains	Flanking sequences			
	1 kb (%)	500 bp (%)	100 bp (%)	50 bp(%)
WT	7/15 (47)	3/20 (15)	0/20 (0)	0/20 (0)
Δ*CnLIG4*	15/19 (79)	13/20 (65)	1/20 (5)	0/20 (0)
Δ*CKU70*	22/23 (96)	n.d.	2/16 (13)	n.d.
Δ*CKU80*	18/20 (90)	n.d.	0/16 (0)	n.d.

WT wild type, *n.d.* not determined

CnLIG4 is not required for normal growth and virulence

B-4500 (wild type) and TLHM20 (Δ*CnLIG4*) were grown at 30 or 37°C in a liquid YPD, and their doubling times were calculated (Table 2). At both temperatures, the mutant strain grew as the wild-type control, suggesting that the *CnLIG4* gene is not required for normal growth in a liquid culture. Growth was also compared on a solid medium, but no detectable difference was seen. Melanin and a polysaccharide capsule, well-recognized virulent factors of *C. neoformans*, were unchanged in the deletion strain. The wild-type and the Δ*CnLIG4* strains were inoculated to ICR mice via tail vein injection. As shown in Fig. 4, the Δ*CnLIG4* strain killed mice equally as did the wild-type control ($P = 0.500$), suggesting that the *CnLIG4* gene is not required for *C. neoformans* intrinsic virulence in the murine model.

Table 2 Doubling times (h) of a *C. neoformans* Δ*CnLIG4* strain

Strains	Temperature (°C)	
	30	37
B-4500 (wild type)	1.9 ± 0.2^{a}	2.4 ± 0.1^{b}
TLHM20 (Δ*CnLIG4*)	2.0 ± 0.2^{a}	2.5 ± 0.1^{b}

[a, b] These values are not significantly different (*t* test values are $P = 0.57$ and 0.50, respectively)

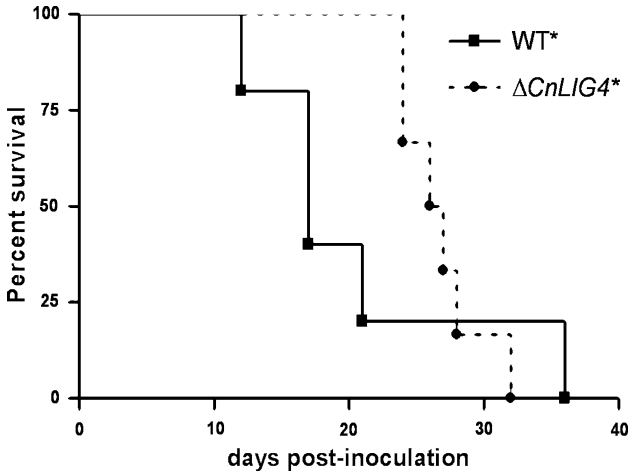

Fig. 4 Virulence of *CnLIG4* disruptant. ICR mice were infected with 2×10^6 cells of B-4500 (*WT*) or TLHM12 (Δ*CnLIG4*) by tail vein injection. Percentage of survival (%) was monitored for 36 days after inoculation. $*P = 0.500$ (not significant)

Discussion

In this study, we structurally analyzed the DNA ligase coding gene *CnLIG4*, specifically involved in NHEJ, and created a *CnLIG4* knockout strain for efficient targeted gene manipulation. The Δ*CnLIG4* strain showed a similar growth rate and virulence compared to the wild-type strain, suggesting that the *CnLIG4* gene is not important for regular growth and full expression of virulence in *C. neoformans*. However, the Δ*CnLIG4* strain was found to be more efficient in HI events when transformed with gene fragments having various lengths of homologous sequences. For example, when transformed with a fragment having 1-kb or 500-bp homologous regions on both ends, HI occurred more frequently compared to the wild-type control (see Table 1). On the other hand, HI with a fragment having 100- or 50-bp homologous sequences was not very successful regardless of the presence of the *CnLIG4* gene.

In the *CnLIG4* knockout strain of *C. neoformans*, many transformants appeared no matter how short the flanking sequences were, but HI seemed to require much longer homologous regions. Even when transformed with a fragment harboring 1-kb homologous sequences in *C. neoformans*, only 80% of the entire transformants were those of HI. However, in the *MUS-53* knockout strain of *N. crassa*, nearly 100% of the transformants were those of HI when a gene fragment with 100-bp flanking sequences was used, and no transformation event was observed when the flanking sequences were shortened to 50 bp, strongly suggesting that the *MUS-53*, an orthologue of *CnLIG4*, is necessary for NHEJ (Ishibashi et al. 2006). These results suggest that the DNA ligase IV coding genes in *C. neoformans* and *N. crassa* contribute differently to NHEJ. In addition, *MgLig4* of *Magnaporthe grisea* has been shown to be involved in NHEJ only in part; the HI efficiency of the knockout strain was relatively higher than that of the wild-type strain, and the event was gene dependent (Kito et al. 2008). It is considered that the mechanism of double-strand break repair, which influences HI efficiency, may be widely different even within the fungal kingdom.

Our results also suggest that the *CnLIG4* knockout strain of *C. neoformans* is slightly less efficient for HI events compared to the disruptants of *KU* genes. In *M. grisea*, it has been shown that the HI event frequency in *KU* gene (*MgKU80*) disruptants depends on the target gene, and a polyketide synthase/nonribosomal peptide synthase gene *ACE1* could not be targeted in the Δ*MgKU80* background, while other genes were efficiently replaced by HI (Villalba et al. 2008). Villalba et al. concluded that the *ACE1* gene locus has an intrinsically lesser probability for gene replacement. In this study, we compared the HI frequency of only the *URA5* gene, and the *URA5* gene was targeted more efficiently in the Δ*CKU70* or Δ*CKU80* background. However, because of the complexity of the mechanism for the DNA repair system, there could be other genes in which the Δ*CnLIG4* disruptant allows more efficient gene targeting, thus providing an additional option for molecular genetics study in *C. neoformans*.

In *C. neoformans*, Ku proteins (products of *CKU70* and *CKU80*) and DNA ligase IV (product of *CnLIG4*) seem to contribute to the DNA repair system by NHEJ, at least through partly different pathways, as their phenotypes for HI frequency were slightly different. A double-knockout strain for *CnLIG4* and *CKU70* or *CKU80* genes might be a solution to improve HI frequency with a gene fragment having much shorter flanking sequences (e.g., 100 bp). We are currently creating such strains, which, we expect, may become a more powerful tool for molecular genetics research in *C. neoformans*.

Acknowledgments This study was in part supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18790302) to K.S.

References

Casadevall A, Perfect JR (1998) *Cryptococcus neoformans*. ASM Press, Washington, DC

da Silva Ferreira ME, Kress MR, Savoldi M, Goldman MH, Hartl A, Heinekamp T, Brakhaqe AA, Goldman GH (2006) The *akuB*^{KU80} mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. Eukaryot Cell 5:207–211

Davidson RC, Blankenship JR, Kraus PR, de Jesus Berrios M, Hull CM, D'Souza C, Wang P, Heitman J (2002) PCR-based strategy to generate integrative targeting alleles with large regions of homology. Microbiology 148:2607–2615

Drivinya A, Shimizu K, Takeo K (2004) Construction of a complete *URA5* deletion strain of a human pathogenic yeast *Cryptococcus neoformans*. Jpn J Med Mycol 45:1–6

Goins CL, Gerik KJ, Lodge JK (2006) Improvements to gene deletion in the fungal pathogen *Cryptococcus neoformans*: absence of Ku proteins increases homologous recombination, and co-transformation of independent DNA molecules allows rapid complementation of deletion phenotypes. Fungal Genet Biol 43: 531–544

Idnurm A, Reedy JL, Nussbaum JC, Heitman J (2004) *Cryptococcus neoformans* virulence gene discovery through insertional mutagenesis. Eukaryot Cell 3:420–429

Ishibashi K, Suzuki K, Ando Y, Takakura C, Inoue H (2006) Nonhomologous chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4 homolog) in *Neurospora*. Proc Natl Acad Sci USA 103:14871–14876

Kito H, Fujikawa T, Moriwaki A, Tomono A, Izawa M, Kamakura T, Ohashi M, Sato H, Abe K, Nishimura M (2008) MgLig4, a homolog of *Neurospora crassa* Mus-53 (DNA ligase IV), is involved in, but not essential for, non-homologous end-joining events in *Magnaporthe grisea*. Fungal Genet Biol 45:1543–1551

Krappmann S, Sasse C, Braus GH (2006) Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. Eukaryot Cell 5:212–215

Kwon-Chung KJ, Edman JC, Wickes BL (1992) Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect Immun 60:602–605

Meyer V, Arentshorst M, EI-Ghezal A, Drews A-C, Kooistra R, van den Hondel CAMJJ, Ram AFJ (2007) Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J Biotechnol 128:770–775

Mizutani O, Kudo Y, Saito A, Matsuura T, Inoue H, Abe K, Gomi K (2008) A defect of LigD (human Lig4 homolog) for nonhomologous end joining significantly improves efficiency of gene-targeting in *Aspergillus oryzae*. Fungal Genet Biol 45:878–889

Nayak T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR (2006) A versatile and efficient gene-targeting system for *Aspergillus nidulans*. Genetics 172:1557–1566

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. Proc Natl Acad Sci USA 101:12248–12253

Pöggeler S, Kück U (2006) Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian *ku70* ortholog. Gene (Amst) 378:1–10

Takahashi T, Masuda T, Koyama Y (2006) Enhanced gene targeting frequency in *ku70* and *ku80* disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. Mol Genet Genomics 275:460–470

Ueno K, Uno J, Nakayama H, Sasamoto K, Mikami Y, Chibana H (2007) Development of highly efficient gene targeting system induced by transient repression of *YKU80* expression in *Candida glabrata*. Eukaryot Cell 6:1239–1247

Villalba F, Collemare J, Landraud P, Lambou K, Brozek V, Cirer B, Morin D, Bruel C, Beffa R, Lebrun M-H (2008) Improved gene targeting in *Magnaporthe grisea* by inactivation of *MgKU80* required for non-homologous end joining. Fungal Genet Biol 45:68–75