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Deletion of the DNA Ligase IV Gene in *Candida glabrata* Significantly Increases Gene-Targeting Efficiency

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Candida glabrata **is reported as the second most prevalent human opportunistic fungal pathogen in the United States. Over the last decades, its incidence increased, whereas that of *Candida albicans* decreased slightly. One of the main reasons for this shift is attributed to the inherent tolerance of *C. glabrata* toward the commonly used azole antifungal drugs. Despite a close phylogenetic distance to *Saccharomyces cerevisiae*, homologous recombination works with poor efficiency in *C. glabrata* compared to baker's yeast, in fact limiting targeted genetic alterations of the pathogen's genome. It has been shown that nonhomologous DNA end joining is dominant over specific gene targeting in *C. glabrata*. To improve the homologous recombination efficiency, we have generated a strain in which the *LIG4* gene has been deleted, which resulted in a significant increase in correct gene targeting. The very specific function of Lig4 in mediating nonhomologous end joining is the reason for the absence of clear side effects, some of which affect the *ku80* mutant, another mutant with reduced nonhomologous end joining. We also generated a *LIG4* reintegration cassette. Our results show that the *lig4* mutant strain may be a valuable tool for the *C. glabrata* research community.**

Candida spp. are part of the commensal microbiota in most humans. In healthy individuals they do not cause disease, but in immunocompromised patients they can result in systemic infections with a high mortality rate (1–3). The most commonly isolated *Candida* species is *C. albicans*, but whereas its incidence is slightly decreasing, the incidence of another species, *C. glabrata*, is on the rise (4). One of the underlying causes is that *C. glabrata* is inherently tolerant to azole antifungals, the most commonly used family of drugs against *Candida* spp. (4–7). Because of this, more and more research groups have started to work on this pathogen. To identify virulence factors or the genetic information that explains why this species is tolerant toward azoles, it would be important to have a genome-wide deletion strain collection. For the closely related species *Saccharomyces cerevisiae*, such a collection already has been available to the research community for more than 1 decade, and it has contributed to increased understanding of the cell biology of this species (8). A major problem is that homologous recombination in *C. glabrata* is far less efficient than in *S. cerevisiae*. In *S. cerevisiae*, efficient gene targeting requires typically 40 bp of homologous flanking sequence at each end, and gene disruption cassettes can easily be made by PCR using 60- to 80-bp oligonucleotides. However, like many other *Candida* species, in *C. glabrata*, much longer flanking sequences of up to 400 bp on each side are needed. This can also be performed by PCR, using a two-step PCR approach, in which 5′ and 3′ flanking sequences are first amplified and the products obtained are then used as primers to amplify a marker gene to replace one's favorite gene in the genome. This method was first developed for *C. albicans* (9) and was recently used to generate a collection of 619 deletion strains in the *C. glabrata* ATCC 2001 background (10). Compared to *C. albicans*, however, this method still results in many false positives when performed with *C. glabrata*, i.e., many transformants carrying nonspecific genomic integrations of the disruption cassette are obtained. Integration of foreign DNA into the genome requires either functional nonhomologous end-joining (NHEJ) or homologous recombination (HR), the two mechanisms to repair the double-stranded DNA breaks (11, 12). HR targets DNA by homologous sequences, while NHEJ does not (11, 13). The low gene-targeting efficiency of *C. glabrata* is due to a very efficient NHEJ and less efficient HR.

The process of NHEJ is less complicated in yeasts than in mammalian cells, where it requires many proteins. In yeast, NHEJ depends on the combined action of *KU* genes and DNA ligase IV (13, 14). Deletions of genes homologous to the human genes KU70 and KU80 were shown to decrease the number of NHEJ events, resulting in an increase in correct gene targeting, in *Neurospora crassa* (15), *Kluyveromyces lactis* (16), *Cryptococcus neoformans* (17), and some *Aspergillus* spp. (18–20). Transcriptional repression of *KU80* in *C. glabrata* also resulted in an increased gene-targeting efficiency (21). However, *KU* genes are also involved in telomere length maintenance and regulation of gene silencing at telomeres. Telomere sequences are enriched for genes involved in adhesion to biotic and abiotic substrates, which is one of the main virulence factors of *C. glabrata* (22). One family of adhesins, the *EPA* gene family, plays a major role in this process, and it is well established that Ku70/Ku80 are involved in the regulation of subtelomeric silencing of some *EPA* genes, so that modification of this part of the NHEJ mechanism may have undesired consequences affecting the physiology of *C. glabrata* (23). Another component involved in the NHEJ process is ligase IV (Lig4). This enzyme was

Received 21 December 2014 **Accepted** 28 May 2015

Accepted manuscript posted online 5 June 2015

Citation Cen Y, Fiori A, Van Dijck P. 2015. Deletion of the DNA ligase IV gene in *Candida glabrata* significantly increases gene-targeting efficiency. Eukaryot Cell 14:783–791. doi:10.1128/EC.00281-14.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00281-14.

doi:10.1128/EC.00281-14

TABLE 1 Strains used in this study

Strain	Genotype or description	Reference or source
2001HT	*his3*Δ *trp1*Δ (made from CBS138)	28
AFG1 (lig4-A)	*lig4*::*HIS3 trp1*Δ isolate A (made from 2001HT)	This study
AFG2 (lig4-B)	*lig4*::*HIS3 trp1*Δ isolate B (made from 2001HT)	This study
CYC001	*ade2*::*SAT1 his3*Δ *trp1*Δ isolate A	This study
CYC002	*ade2*::*SAT1 his3*Δ *trp1*Δ isolate B	This study
CYC003	*ade2*::*SAT1 lig4*::*HIS3 trp1*Δ isolate A	This study
CYC004	*ade2*::*SAT1 lig4*::*HIS3 trp1*Δ isolate B	This study
CYC005	*arg8*::*SAT1 his3*Δ *trp1*Δ isolate A	This study
CYC006	*arg8*::*SAT1 lig4*::*HIS3 trp1*Δ isolate A	This study
CYC007	*arg8*::*SAT1 lig4*::*HIS3 trp1*Δ isolate B	This study
CYC008	*cna1*::*SAT1 his3*Δ *trp1*Δ isolate A	This study
CYC009	2001HT *ku80*::*SAT1* isolate A	This study
CYC010	2001HT *ku80*::*SAT1* isolate B	This study
CYC011	2001HT *ku80*::*SAT1* isolate C	This study
KUE200	2001HT *ku80*::*SAT1*	21

previously shown, in other organisms, to be more specific for the NHEJ process, so its deletion may result in fewer side effects (12, 13, 24–27). To test whether deletion of *LIG4* in *C. glabrata* results in strongly improved gene targeting without any unwanted phenotype, we generated a deletion in this gene (CAGL0E02695g), and we show that the corresponding mutants display a strong increase in correct gene targeting. Differently from the *ku80* mutants, no detectable side effects could be observed on growth, DNA stress tolerance, or antifungal drug resistance. *Lig4* mutants show apparently no phenotype that should be of concern to the research community, other than an increase in the gene-targeting efficiency. Despite this, we also generated a *LIG4* reintegration construct for reconstitution of *LIG4* in its original locus once the desired genetic alterations have been performed. Thus, we provide a useful strain and plasmid that can serve as a basis for targeted engineering in this human fungal pathogen.

MATERIALS AND METHODS

Yeast strains, primers, and media. The *C. glabrata* strains used in this study are listed in Table 1. *C. glabrata* cells were grown in either liquid YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete (SC; 1.7 g/liter Difco yeast nitrogen base without ammonium sulfate, 0.79 g/liter complete supplement mixture [CSM; MP Biomedicals], 5 g/liter ammonium sulfate, supplemented with 2% dextrose) medium. For selection of transformants, L-histidine, adenine, or L-arginine was omitted from the SC medium. For solid media, 1.5 g/100 ml Difco agar granulated was supplemented to the liquid medium mentioned.

Full-length gene sequences were obtained from the Candida Genome Database (http://www.candidagenome.org/). The primers used in this study are listed in Table S1 in the supplemental material. These sequences were compared to those in the Candida Genome Database using BLAST in order to determine their specificity.

Construction of *LIG4* deletion strains and *KU80* deletion strains. We used the fusion PCR method to generate the deletion of *LIG4* (9). The 500-bp 5′ flanking region was amplified from genomic DNA using primers Cglig4-a and Cglig4-b. The 500-bp 3′ flanking site was amplified using primes Cglig4-c and Cglig4-d. The *ScHIS3* marker was amplified using primers Schis3-for and Schis3-rev with plasmid pRS423 (GenBank accession number U03454) as the template. The whole deletion cassette was amplified by fusion PCR using the three amplified fragments due to the complementary tails present in Schis3-for and Cglig4-b and in Schis3-rev and Cglig4-c, respectively. The cassette was transformed in *C. glabrata* strain 2001HT (28) by electroporation (0.2-cm cuvette, 1.5 kV) as previously described (29). Genomic DNA from selected transformants was isolated using the FastPrep method. For the identification of correct *lig4*Δ strains, genomic DNA, prepared using the FastPrep method, was used as the template for PCR to check for correct deletion of *LIG4* as well as for its correct replacement by the *HIS3* marker. Primers cglig4-ko-check-for/Schis3-rev and cglig4-ko-check-rev/Schis3-for were used to test the replacement of *LIG4* by *ScHIS3* gene. Primers cglig4-ko-check-for/cglig4-check-inrev and primers cglig4-ko-check-rev/cglig4-check-infor were used to test the loss of *LIG4* from the genome. Genomic DNA from the wild-type strain was used as a control.

We have used the same approach to generate the *KU80* deletion strain. The 500-bp 5′ flanking region was amplified using primers Ku80-a and Ku80-b. The 500-bp 3′ flanking side was amplified using primers Ku80-c and Ku80-d. The *SAT1* marker was amplified using primers KU80-SAT-for and KU80-SAT-rev from plasmid pSFS2 (GenBank accession number AY524979). The whole deletion cassette was amplified by fusion PCR using the three amplified fragments due to the complementary tails present in KU80-SAT-for and Ku80-b and in KU80-SAT-rev and Ku80-c, respectively. Primers KU80-CHECK-FOR/L4-REV-3 and KU80-CHECK-REV/L4-FOR-9 were used to test the replacement of *KU80* by the *SAT1* gene. Primers KU80-CHECK-FOR/KU80-CHECK-INREV and KU80-CHECK-REV/KU80-CHECK-INFOR were used to test the loss of *KU80* from the genome. Apart from our own *ku80* mutants, we also used a deletion strain (KUE200) that was previously generated and kindly provided to us by H. Chibana (Japan) (21).

Construction of *ADE2*, *ARG8*, and *CNA1* deletion strains. To delete the *ADE2* gene, we used 40-bp and 100-bp flanking regions fused with the *SAT1* marker. The *SAT1* marker deletion cassettes were amplified either with cgade2-ko-sat-for and cgade2-ko-sat-rev or with 100ADE2-ko-sat-for and 100ADE2-ko-sat-rev from plasmid pSFS2. Amplified DNA fragments were then transformed with electroporation as described above. Putative correct transformants were identified by color and adenine auxotrophy and by colony PCR (30). Primers cgade2-ko-check-for/cgade2-check-inrev and cgade2-ko-check-rev/cgade2-check-infor were used to test the loss of *ADE2*. Primers cgade2-ko-check-for/Cgade2-ko-sat-rev and cgade2-ko-check-rev/Cgade2-ko-sat-for were used to test for the correct replacement of *ADE2* by the *SAT1* marker gene.

A similar approach was used to generate *ARG8* and *CNA1* deletion strains. We used 40-bp flanking sites to generate the deletion cassettes. Primers ARG-KO-FOR and ARG-KO-REV and primers CNA1-KO-FOR and CNA1-KO-REV were used to amplify the *SAT1* marker from plasmid pSFS2 to generate the *ARG8* and *CNA1* deletion cassettes, respectively.

Primers ARG-CHECK-FOR/ARG-CHECK-INREV and ARG-CHECK-REV/ARG-CHECK-INFOR were used to test the loss of *ARG8*. Primers ARG-CHECK-FOR/L4-REV-3 and ARG-CHECK-REV/L4-FOR-9 were used to test for the correct replacement of *ARG8* by the *SAT1* marker gene.

Primers CNA1-CHECK-FOR/CNA1-CHECK-INREV and CNA1-CHECK-REV/CNA1-CHECK-INFOR were used to test the loss of *CNA1*. Primers CNA1-CHECK-FOR/L4-REV-3 and CNA1-CHECK-REV/L4-FOR-9 were used to test for the correct replacement of *CNA1* by the *SAT1* marker gene.

Generation of the *LIG4* reintegration construct. The *LIG4* reintegration construct was made using the Gibson Assembly master mix (provided by New England BioLabs) and assembled in the bacterial vector pUC18 (GenBank accession number L09136). The *LIG4* open reading frame (ORF) together with a 500-bp promoter sequence was amplified by PCR using primers Plig4-for-EcoRI-1 and Plig4-rev-2. The *ScADH1* terminator was inserted downstream of the *LIG4* fragment and amplified by primers ADH1-ter-for and ADH1-ter-rev from plasmid pBEVY-T (GenBank accession number AF069723). The nourseothricin marker gene *SAT1* was amplified by primers Plig4-SAT1-for and Plig4-SAT1-rev from plasmid pSFS2, while the 500-bp *LIG4* terminator was amplified by primers PLIG4-TER-FOR and PLIG4-TER-EcoRI-REV. Plasmid pUC18 was digested with restriction enzymes BamHI and EcoRI. The five linear

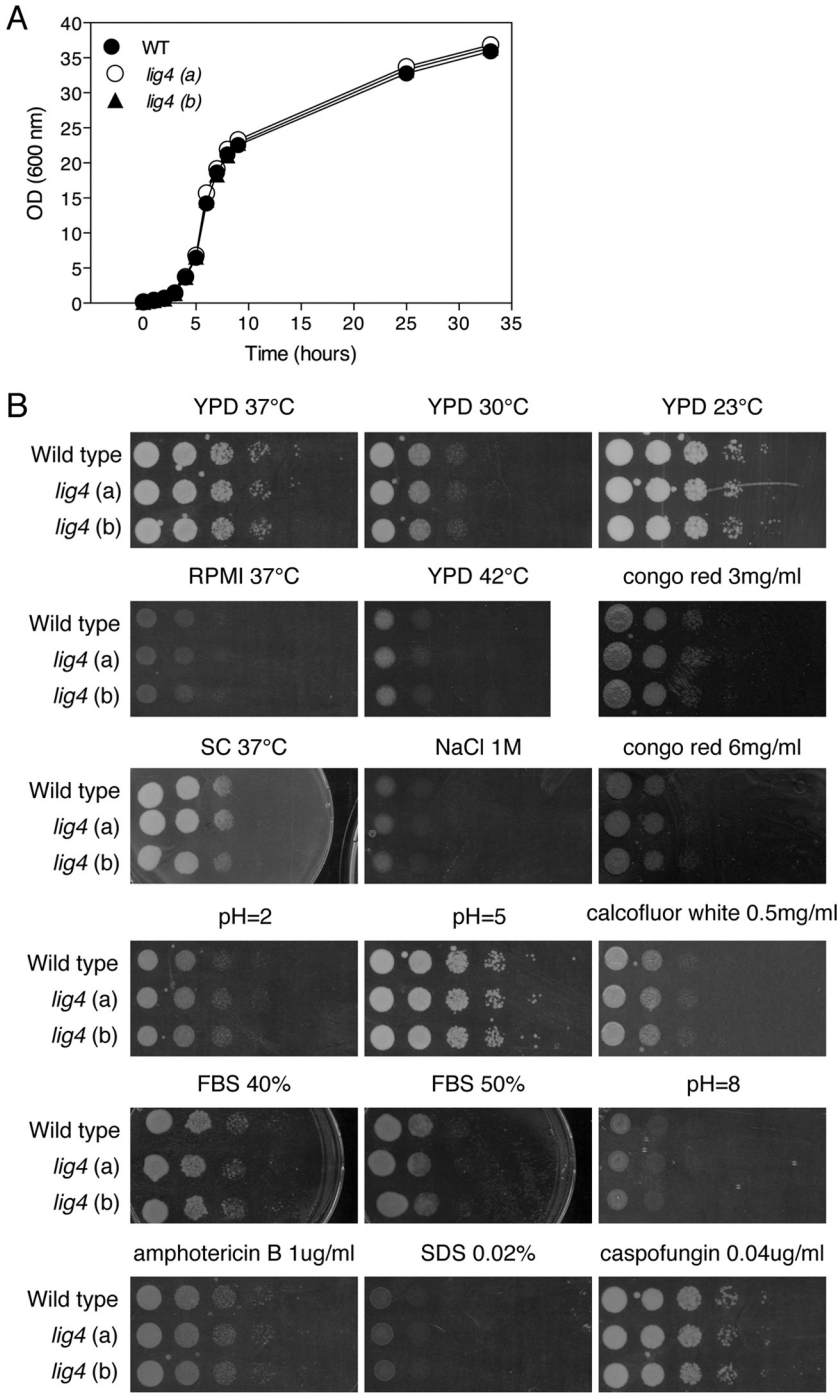

FIG 1 Deletion of *LIG4* does not affect growth or stress tolerance of *C. glabrata* cells. (A) Growth curve of wild-type strain and the two *lig4* mutants (AFG1 and AFG2) in YPD medium at 37°C. (B) The growth and stress tolerance of the wild type and two independent *lig4* mutants were tested by spot assays under different conditions/stresses. Cells were assayed in different media (YPD, RPMI, SC) at 37°C and at different temperatures and pHs and on YPD supplemented with the indicated compounds at 37°C. Spot assays were done by 10-fold serial dilutions.

fragments were assembled using the Gibson Assembly kit, and the resulting plasmid was checked by sequence analysis.

To further increase the efficiency of the reintegration cassette, we inserted a fragment in the EcoRI site that contains the recognition sequence of the CspCI restriction endonuclease, which is a double digesting enzyme, and that was amplified using the oligonucleotides LIG4-REIN-CSPCI-FOR and LIG4-REIN-CSPCI-REV and pUC18 as the template. Digestion by CspCI will result in the complete removal of nonhomologous DNA (EcoRI restriction site), leaving homologous flanking sites.

Southern blotting. Southern blotting was performed to determine the level of ectopic integration. Briefly, genomic DNA from overnight cultures of the *ade2*, *arg8*, *cna1*, and *ku80* mutant strains as well as the wild-type strain was isolated and digested for 14 h with EcoRI, EcoRV, EcoRI, and KpnI, respectively. A 0.8% agarose gel was run overnight at 40 V, and

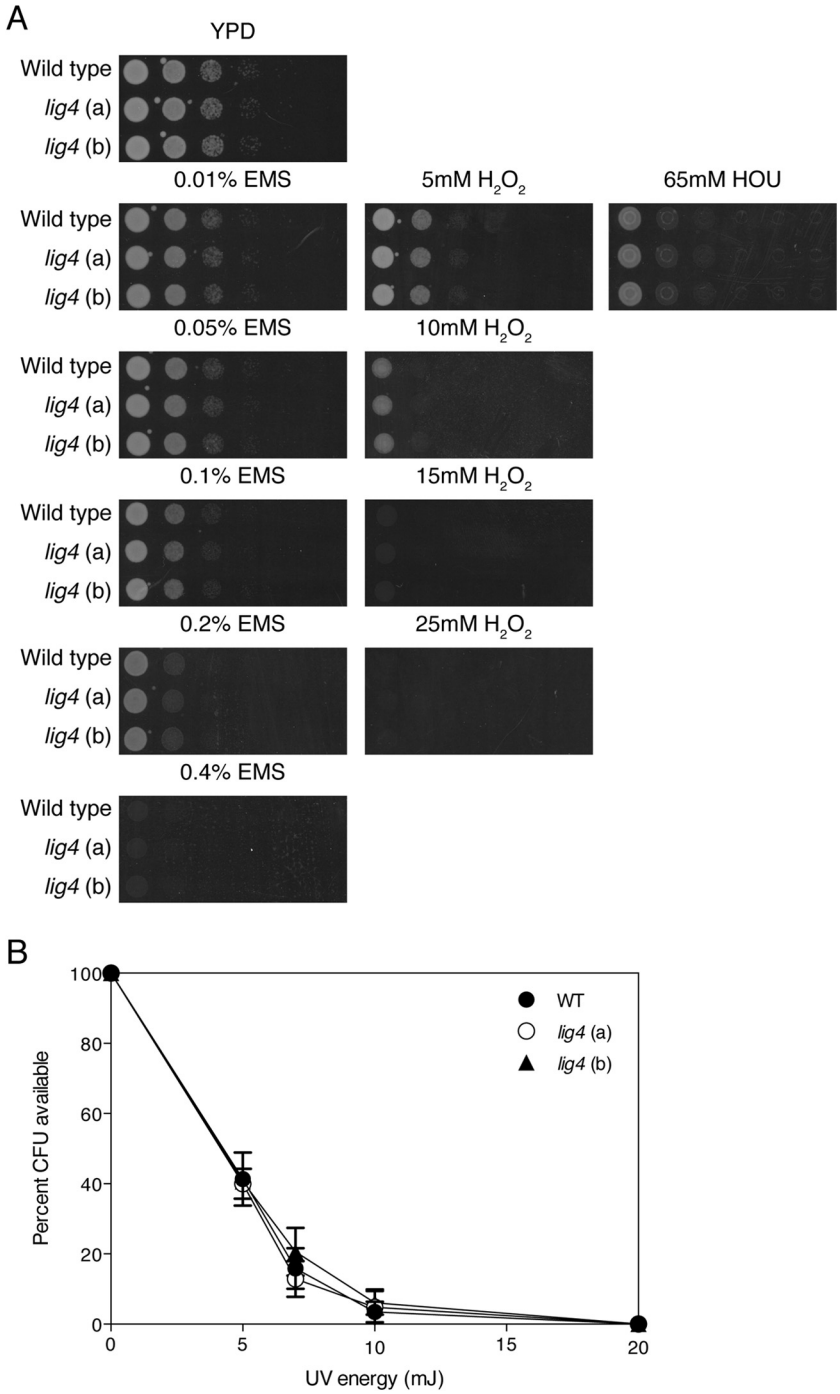

FIG 2 DNA damage conditions affect the *lig4* mutant in the same way as the wild-type strain. (A) Spot assays (10× serial dilutions) of wild-type and *lig4* mutant cells on YPD plates containing different concentrations of mutagenic chemicals. Plates were incubated for 15 h at 37°C. (B) Survival of *C. glabrata* wild-type and *lig4* mutant cells upon exposure to UV light. To calculate the survival rates, the number of CFU on the exposed plates was divided by the number of CFU on the unexposed plates and multiplied by 100. Cell viability was calculated based on three independent experiments.

the DNA was transferred to a Hybond-NX membrane (Amersham Biosciences). The different probes were generated by PCR using ADE-sb-for and ADE-sb-rev, ARG8-sb-for and ARG8-sb-rev, CNA1-sb-for and CNA1-sb-rev, and KU80-sb-for and KU80-sb-rev, respectively. The probes (200 bp to 300 bp) were located in the flanking region of the deleted genes (*ADE2*, *ARG8*, *CNA1*, and *KU80*, respectively). Primers L4-FOR-8 and L4-REV-2 were used to amplify the probe for detection of the *SAT1* marker. Generation of the probe and hybridization were performed using the AlkPhos direct-labeling reagents (Amersham GE Healthcare) according to the instructions in the manual. The signals were detected using the CDP-Star detection reagent (Amersham, GE Healthcare).

Real-time PCR analysis. For expression analysis, overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.2 in YPD and

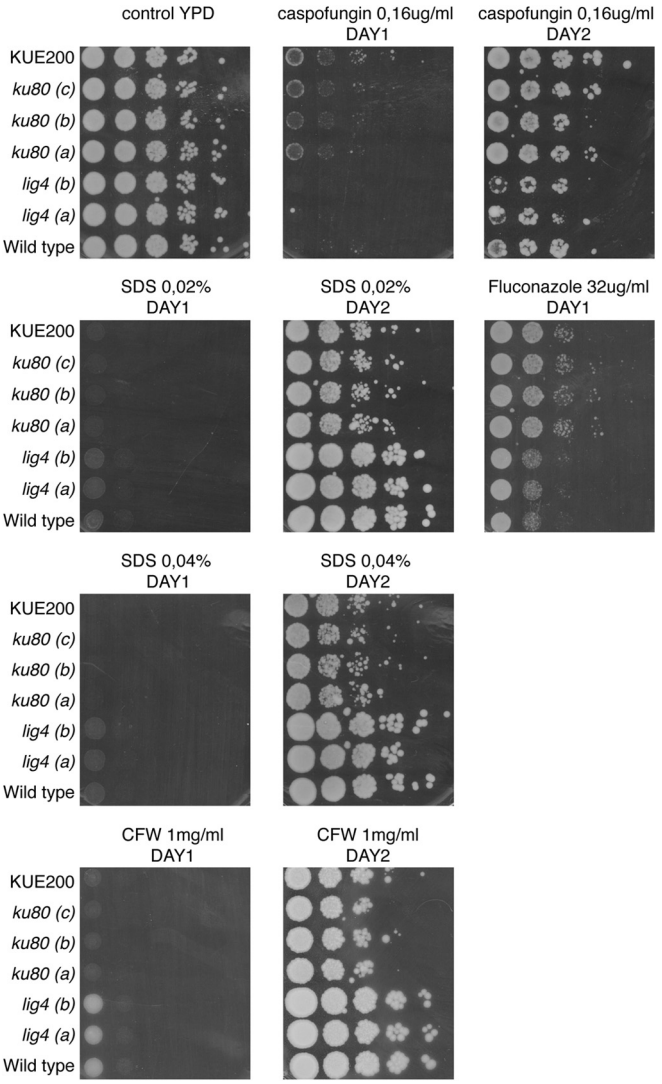

FIG 3 Deletion of *KU80* affects cell membrane and cell wall stress tolerance of *C. glabrata* cells, whereas deletion of *LIG4* has no effect. Two independent *lig4* mutants (a and b), three independent *ku80* mutants (a, b, and c), and the published ku80 mutant (21) were grown in overnight cultures, diluted using serial 10-fold dilutions, and plated on YPD medium containing caspofungin, fluconazole, SDS, and calcofluor white (CFW) at the indicated concentrations. Plates were incubated at 37°C for the indicated times.

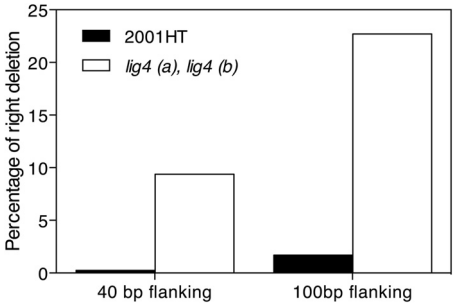

FIG 4 Gene-targeting efficiency is strongly increased in the *lig4* mutant. The wild type and the *lig4* (*a*) mutant were transformed with DNA fragments containing either 40- or 100-bp flanking sequences to target a deletion cassette to the *ADE2* locus. The percentage of correct *ADE2* deletion strains to the total number of transformants is shown.

were harvested after either 4 h (exponential phase) or 24 h (stationary phase) of incubation at 37°C. RNA was extracted by the TRIzol RNA isolation reagent (Life technologies). The amount of RNA was quantified by a NanoDrop spectrophotometer (ND-1000; Life Science). After DNase treatment, the RNA concentration was adjusted to a final concentration of 1 μg/μl. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed in 96-well plates using the Step One Plus real-time PCR system (Applied Biosystems) and the Go *Taq* qPCR Master Mix (Promega). Five microliters of 0.4 ng/μl cDNA samples and 15 μl of Master Mix (containing the primers) were added to the plates. Real-time PCRs were performed at 95°C for 2 min, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Samples were then kept at 95°C for 15 s and 60°C for 1 min. Finally, samples were subjected to incubations at increasing temperatures from 60°C to 95°C and kept 15 s at 95°C. *ACT1* was used as a control gene, and normalized data were then used to calculate the relative gene expression levels. Data for each target gene were calculated as the fold change in comparison with the reference gene *ACT1* using the $\Delta\Delta C_T$ quantification method (where C_T is threshold cycle) (31).

The sequences of the primers are given in Table S1 in the supplemental material. The quantitative PCR results were obtained from 3 independent biological repeats.

Growth and stress experiments. To determine whether the absence of Lig4 had no effect on growth and stress tolerance (especially DNA stress), we tested two independent *lig4* mutants under different growth conditions, including different media (YPD, SC, and RPMI [RPMI 1640 from Sigma]), as well as upon applying different types of stress to the cells such as different temperatures (23°C, 30°C, 37°C, and 42°C), different pHs (pH 2, 5, or 8), different concentrations of SDS (0.02% and 0.04%), calcofluor white (CFW; 0.5 mg/ml and 1 mg/ml), Congo red (3 mg/ml and 6 mg/ml), amphotericin B (1 μg/ml), caspofungin (0.04 μg/ml and 0.16 μg/ml), sodium chloride (1 M), fetal bovine serum (40% and 50%), ethyl methanesulfonate (EMS; 0.01%, 0.05%, 0.1%, 0.2%, and 0.4%), H_2O_2 (5 mM, 10 mM, and 25 mM), and hydroxyurea (HOU; 65 mM), and different doses of UV, and we compared (some of) the phenotypes with those obtained for a wild-type strain as well as with those obtained for two independent *ku80* mutants and the published KUE200 strain. The stress tolerance assays were done by spot assays with a starting OD_{600} of 1 and plating 10-fold serial dilutions using YPD agar media under different conditions or mixed with corresponding concentrations of stress reagents. Different pH YPD agar media were buffered by 150 mM HEPES. For UV stress assays, overnight cultures were diluted to an OD_{600} of 0.2 in YPD medium. Further 10-fold dilutions were applied using YPD. One hundred microliters of the suspensions was spread onto YPD agar plates, and different UV energy doses were applied (using the Bio-Rad GS Gene Linker UV Chamber, C-L program), while the control plates were kept unexposed to UV light. Plates were incubated for 24 h at 37°C, and the numbers of surviving cells were determined by counting the CFU.

MICs using the broth dilution assay and Etest strips. The MIC was determined by broth dilution assay according to the CLSI standard reference method M27-S4 (32). Briefly, cells were harvested from overnight YPD culture and diluted to around 2,500 CFU/ml (verified by plating serial dilution on YPD plates) with RPMI medium (RPMI 1640). One hundred microliters of the diluted culture was added to each well. MIC plates were incubated at 37°C for 24 h. The MIC_{50} values were defined as the lowest concentration of the antifungal (either fluconazole, caspofungin, or amphotericin B) that caused a ≥50% decrease in OD_{600}. The broth dilution assays were repeated 3 times independently.

Fluconazole MICs were also determined using Etest strips (AB Biodisk) on RPMI-glucose plates. Cells were harvested from overnight cultures in YPD, diluted to an OD_{600} of 0.2, and plated using sterile swabs. Plates were incubated at 37°C for 48 h.

TABLE 2 Correct transformation efficiency in wild-type *C. glabrata* and two independent *lig4* mutant strains[a]

Gene deletion tested and batch no.[b]	No. of correct gene deletions/total no. of transformants		
	Wild type	*lig4* (a)	*lig4* (b)
ADE2			
Batch1	1/105	1/3	0/1
Batch2	0/71	0/0	0/2
Batch3	0/31	0/2	0/2
Batch4	0/29	0/6	1/5
Overall	1/236	1/11	1/10
ARG8			
Batch1	1/332	0/1	1/4
Batch2	0/17	0/2	0/0
Batch3	0/40	0/0	0/1
Batch4	0/45	0/0	1/2
Overall	1/434	0/3	2/7
CNA1			
Batch1	0/37	0/0	0/0
Batch2	0/33	0/0	0/0
Batch3	0/39	0/1	0/0
Batch4	0/120	0/0	0/0
Batch5	0/196	0/1	1/3
Batch6	0/121	0/0	0/1
Overall	0/546	0/2	1/4

[a] For each experiment, the wild-type strain and the two *lig4* mutants were incubated in 50 ml YPD medium at an OD_{600} of 0.2 and grown at 37°C to an OD_{600} of 1.5. Competent cells harvested from one such culture were defined as one batch.
[b] Data are from at least four independent transformation rounds in the wild-type background and in the two independent *lig4* mutants.

RESULTS AND DISCUSSION

To obtain a *LIG4* deletion strain, we used a deletion construct consisting of a 500-bp promoter and 500-bp terminator sequence flanking the *ScHIS3* gene. Among the ~1,000 histidine prototrophic colonies generated by transformation of this construct and verified by colony PCR, two clones showing correct integration of the disruption cassette were found. This confirms that in *C. glabrata* (at least in the standard strain 2001HT), there is an extremely low HR and a high NHEJ efficiency, as in nearly all transformants the deletion cassette was integrated in a wrong place in the genome.

As previously used approaches to improve homologous recombination, such as deletion of *KU70/KU80*, result in side effects such as changes in subtelomeric silencing (23), we tested the two independent *lig4*Δ mutants (*lig4-A* and *lig4-B*) under different growth and stress conditions using spot assays (Fig. 1). Deletion of *LIG4* has no effect on growth at different temperatures, at different pHs, or in different growth media. Similarly, the *lig4*Δ mutants show no altered sensitivity to cell wall and salt stress or to antifungals when added in the medium (Fig. 1). We also determined the MIC values for amphotericin B, caspofungin, and fluconazole, and they were also not changed in the two independent *lig4* mutants compared to the wild-type strain and were 1 μg/ml, 0.1 μg/ml, and 16 μg/ml, respectively. As NHEJ is crucial for cell response to DNA damage, we also tested the susceptibility of the mutants to a number of conditions such as UV light, EMS, H_2O_2, and HOU (Fig. 2). The two independent *LIG4* deletion strains show the same sensitivity toward all these stresses as the wild-type strain, suggesting that absence of Lig4 does not interfere with cell growth and stress tolerance, at least phenotypically under all tested stress conditions. The expression of adhesion-encoding genes is an important virulence factor of *C. glabrata*, and it was previously shown that the *ku80* mutants resulted in differences in the expression of some of these genes (23). *EPA6* and *EPA7* are induced in *ku80* mutants, whereas the expression levels of *EPA1* and *EPA2* are unaltered. In order to compare the expression of the *EPA* genes in our *lig4* mutants with that in *ku80* mutants in the same background, we have generated our own set of *ku80* mutants. Expression of the four *EPA* genes in exponential or stationary phase was not significantly different between the wild type and the *lig4* mutant, and also not in the *ku80* mutant (see Fig. S1 in the supplemental material). A possible explanation for the normal *EPA* gene

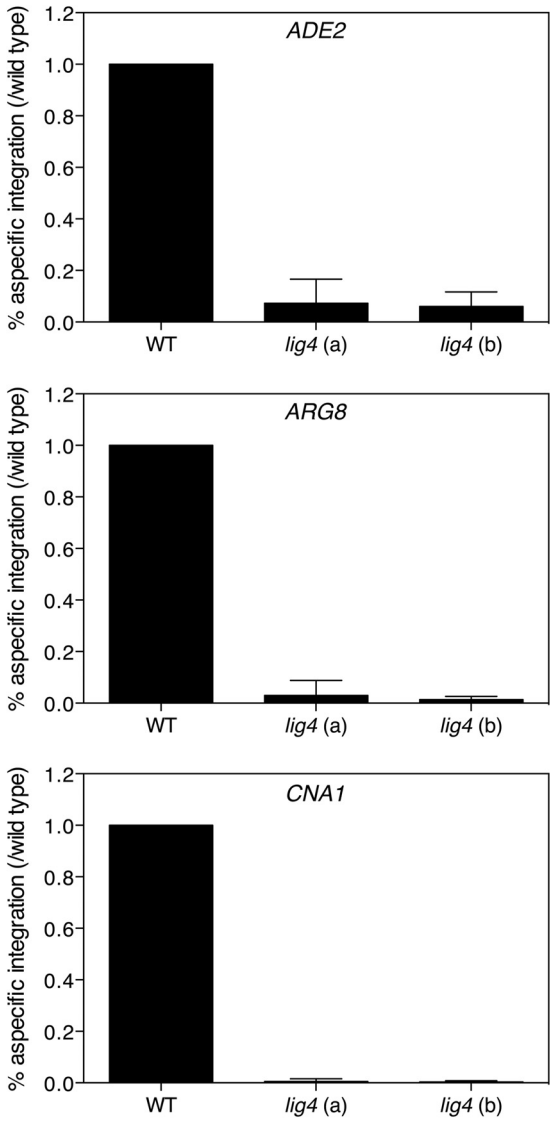

FIG 5 Relative aspecific integration is much lower in the *lig4* mutants than in the wild-type strain. Relative aspecific integration values, calculated as the number of false-positive transformants of each batch/number of false-positive transformants of the wild-type strain of the batch, for deletion cassettes targeting *ADE2*, *ARG8*, or *CNA1* are shown. $P < 0.05$ for comparison of the wild type to the *lig4* mutants.

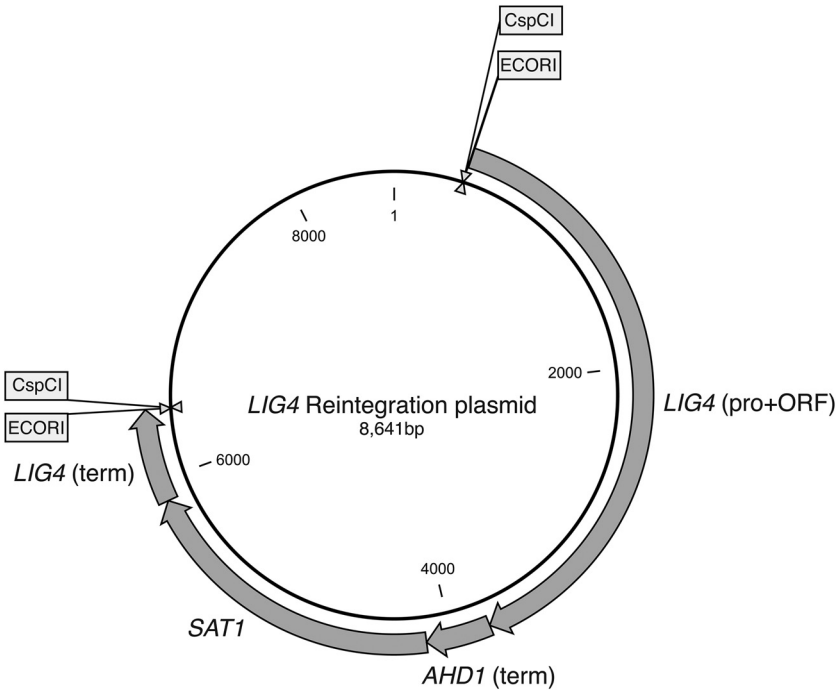

FIG 6 Map of the *LIG4* reintegration plasmid. Digestion of this plasmid with EcoRI or CspC1 will target the *LIG4* gene to its original locus in the genome.

expression in our *ku80* mutants compared to the previously published data is the strain background. Our *ku80* mutants were generated in ATCC 2001, whereas the published data were obtained in the BG2 background. Apart from differences in expression of *EPA* genes, no other phenotypes of the *ku80* mutants were previously described. In our effort to validate the *lig4* mutants as much as possible, we identified environmental conditions that resulted in phenotypes for the *ku80* mutants but not for the *lig4* mutants. The *ku80* mutants showed a clear phenotype when growing on media containing membrane or cell wall stress reagents (Fig. 3). Compared to the wild type and the *lig4* mutants, the three independent *ku80* mutants as well as the published *ku80* mutant (KUE200) (21) showed an increased sensitivity toward SDS and CFW and an increased tolerance against fluconazole, not against caspofungin. MIC analysis for caspofungin confirmed these data. For fluconazole, however, the difference in sensitivity was confirmed using two different approaches. MIC determinations using the broth dilution method showed that the *ku80* mutants had an increased MIC for fluconazole (32 μg/ml) compared to all other strains and that there was no difference in MIC between the wild-type strain and the *lig4* mutants. This was confirmed using E-strips with fluconazole. The wild-type strain and the *lig4* mutant showed an MIC of 8 μg/ml, whereas the *ku80* (a) and KUE200 mutant strains have an MIC of 16 μg/ml (see Fig. S2 in the supplemental material).

Our phenotypic analysis shows that the *lig4* mutant strain behaves exactly as does the wild type for all conditions that we have tested and therefore may be a very good strain to use for gene targeting, as it may strongly decrease the number of transformants that need to be checked to obtain the correct desired strain.

To determine whether the absence of Lig4 results in an improved gene-targeting efficiency, the wild-type strain and the two *lig4*Δ mutants were transformed with the *SAT1* marker flanked by either 40 bp or 100 bp of promoter and terminator sequences of the target genes. As a proof of principle, we have chosen to delete the *ADE2* gene. Deletion of *ADE2* results in pink to red colonies because of the accumulation of a red compound in the cells (33). All transformants were screened for colony color as well as for adenine auxotrophy. For the constructs with the 100-bp flanking sequence, we obtained 120 transformants in the wild-type background and 22 in the *lig4*Δ background. Interestingly, only 2 transformants were found to be correct (based on color and adenine auxotrophy) in the wild-type background whereas 5 were correct in the *lig4*Δ mutant. These seven colonies together with 20 randomly picked white colonies from each transformation were checked with colony PCR for loss of *ADE2* and replacement by the marker gene *SAT1*. Only the seven red colonies turned out to be the correct ones. Similar results were obtained when the flanking sequences were further reduced to 40 bp, which is the typical size of flanking sequences used in *S. cerevisiae* for gene-targeting approaches. In this case, 1 of the 368 transformants in the wild-type background and 3 of 32 transformants in the *lig4*Δ strain were correct. These results show that the efficiency of correct targeting is about 35 times higher in the *lig4*Δ strain than in the wild-type strain, when using 40-bp flanking sequences (Fig. 4). Using 100-bp flanking regions, the gene targeting efficiency was even higher in the *lig4*Δ mutant (Fig. 4).

To validate the use of the *lig4*Δ mutant for improved gene targeting, we repeated the experiment with *ADE2* several independent times and also included two other genes, *ARG8* and *CNA1*, which are located on different chromosomes. *ARG8* encodes an enzyme involved in arginine biosynthesis, so the deletion of *ARG8* results in auxotrophy for arginine. Cna1 is a subunit of the calcineurin phosphatase, and deletion of *CNA1* has no easy detectible phenotype. Therefore, all *CNA1* transformants were screened by colony PCR using primers CNA1-CHECK-FOR and CNA1-

CHECK-INREV (see Table S1 in the supplemental material). Correct gene targeting for the three genes was further tested by colony PCR for loss of the target genes and their replacement by the *SAT1* marker gene. Table 2 lists the numbers of transformants that we obtained and the numbers of correct deletion strains we selected and verified. The number of overall transformants clearly shows that the *lig4* mutants resulted in a strongly reduced nonhomologous integration in comparison to the wild-type strain. The number of correct deletion strains remains more or less the same for the wild type and for the *lig4* mutants. This also implies that Lig4 does not interfere with the homologous recombination pathway and is specific for the NHEJ pathway. To better demonstrate the increased gene targeting efficiency in the *lig4*Δ background compared to the wild-type strain, we calculated the relative aspecific integration (number of false-positive transformants of each batch/number of false-positive transformants of the wild-type strain of the batch) (Fig. 5). Finally, we confirmed correct deletion of the target genes using Southern blot analysis (see Fig. S3 in the supplemental material). The Southern blot also shows that there is no ectopic integration in any of the checked deletion strains, either in the wild-type background or in the *lig4* mutant background.

To avoid any phenotype caused by the absence of *LIG4*, we also generated a reintegration cassette that can be used to reintegrate the *LIG4* gene at its original locus. We have generated this cassette using the Gibson Assembly kit as described in Materials and Methods (Fig. 6). To target the *LIG4* gene to its original locus, an EcoRI or CspCI digestion (the latter is a double-digester enzyme and will result in a complete homologous DNA fragment, possibly further increasing efficiency) of this plasmid followed by transformation and selection on nourseothricin will restore proper expression of this ligase gene at its original locus. Both the strain and the reintegration plasmid will be available for the *Candida* research community.

ACKNOWLEDGMENTS

We acknowledge Taiga Miyazaki and Hironobu Nakayama for their generous gift of *C. glabrata* strain 2001HT and Hiroji Chibana for kindly providing us the *C. glabrata* strain KUE200. We thank Ilse Palmans and Cindy Colombo for excellent technical assistance and Nico Vangoethem for help with the preparation of figures.

This work was supported by a Ph.D. scholarship (file no. 2010833102) from the China Scholarship Council to Y.C. and by the Fund for Scientific Research Flanders (FWO project VS.036.14N).

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