Fungal Genetics and Biology 107 (2017) 44–50

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regular Articles

Comparison of genome engineering using the CRISPR-Cas9 system in *C. glabrata* wild-type and *lig4* strains

Yuke Cen[1,2], Bea Timmermans[1], Ben Souffriau, Johan M. Thevelein, Patrick Van Dijck*

KU Leuven, Laboratory of Molecular Cell Biology, Kasteelpark Arenberg 31, bus 2438, 3001 Leuven, Belgium
VIB-KU Leuven Center for Microbiology, Leuven, Belgium

ARTICLE INFO

Keywords:
Candida glabrata
CRISPR-Cas9
Non-homologous end Joining

ABSTRACT

Candida glabrata is reported as the second most prevalent human opportunistic fungal pathogen in North America and is threatening patients all over the world. Its incidence is rising, while it has developed resistance to the most widely used antifungal drugs, necessitating new approaches based on better insight into the biology of the organism. Despite its close phylogenetic relationship with *Saccharomyces cerevisiae*, generating precise genomic alterations in this species is problematic. Previously we have shown that deletion of *LIG4*, which encodes an enzyme involved in Non-Homologous End Joining (NHEJ), strongly enhances the probability of obtaining correctly modified transformants. In this work we used the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated protein 9 (Cas9) system to genetically engineer the *C. glabrata* genome, targeting the genes *ADE2*, *MET15* and *SOK2*, located on different chromosomes. We used the CRISPR-Cas9 technology to replace the open reading frame (ORF) by the *SAT1* selective marker or introduced a premature stop codon in *ADE2* and *MET15,* as they are easily scored by their adenine or methionine auxotrophy, respectively. The *SOK2* gene was modified by insertion of a triple HA-tag sequence and the transformants were verified in a western blot. The CRISPR-Cas9 mediated targeting efficiency varies depending on the gene targeted and the genetic modification performed. We show that CRISPR-Cas9 mediated genome editing is more efficient than the conventional method in the wild-type strain, moreover it has the big advantage being marker-free. In previous work, we showed that the targeting efficiency is highly increased in the *lig4*Δ strain using the conventional way to delete genes in *C. glabrata*. Using the CRISPR-Cas9 system in this strain, the percentage of correct transformants is consistently higher compared to the wild-type strain. This indicates that using the *lig4* mutant as such is already a strong improvement, while the CRISPR-Cas9 gives the additional advantage of not leaving a scar or marker and that it therefore can be used to generate multiple modifications.

1. Introduction

The incidence of fungal infections has become a major problem in hospitals. Major risk factors include immunosuppression, the increased use of broad-spectrum antibiotics and fluconazole as prophylaxis and the frequent use of medical implants which can be colonized by the fungus to form a biofilm structure. In addition, some fungal species appear to become resistant against the widely used antifungal drugs fluconazole or the echinocandins (Lass-Flörl, 2009). *Candida* species are the most important cause of opportunistic fungal infections (Pfaller and Diekema, 2007). The most commonly isolated *Candida* species is *C. albicans*, but in the last couple of decades the incidence of infections caused by non-*albicans*-*Candida* (NAC) species has increased significantly. This is at least partially caused by *C. glabrata*, since it has become the second or third most frequently isolated *Candida* species depending on the geographical location of the survey (Rodrigues et al., 2014). Because of its emerging clinical importance, the number of researchers studying *C. glabrata* is increasing but the lack of a complete deletion collection is hampering the identification of alternative drug targets. Because the homologous recombination (HR) efficiency is low, especially compared to its close relative *Saccharomyces cerevisiae,* genetic manipulations are still a big challenge in *C. glabrata*. The conventional method of genome engineering in *C. glabrata* is using a selection marker with flanking sequences of up to 500 bp. This approach was recently used to generate a collection of 619 deletion strains in the ATCC2001 background by Schwarzmüller et al. (2014). Using the

* Corresponding author at: Laboratory of Molecular Cell Biology, KU Leuven, VIB-KU Leuven Center for Microbiology, Kasteelpark Arenberg 31, bus 2438, 3001 Heverlee, Belgium.
E-mail address: Patrick.vandijck@kuleuven.vib.be (P. Van Dijck).
[1] Both authors contributed equally to the work.
[2] Current address: Institute of Bioengineering, Zhejiang University of Technology, China.

http://dx.doi.org/10.1016/j.fgb.2017.08.004
Received 15 July 2017; Received in revised form 9 August 2017; Accepted 15 August 2017
Available online 16 August 2017

conventional method, a lot of colonies need to be checked since the occurrence of false positive transformants is common. Some efforts were taken to decrease the off-target rate of HR by reducing non-homologous end joining (NHEJ), by silencing *YKU80* gene or by deleting *LIG4* (Cen et al., 2015; Ueno et al., 2007). The *lig4*Δ mutant turned out to be very efficient and has no known side effects (Cen et al., 2015). The conventional approach of genome engineering results in the insertion of a selection marker, or at least a scar when the marker is flipped out, at the locus of interest and does not allow immediate site-directed mutagenesis of the genome.

The Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated system (Cas) has been widely applied from animals to plants. This has changed the method of genome engineering in the last few years remarkably (Doudna and Charpentier, 2014). Briefly, the CRISPR-Cas system employs a short guide RNA (gRNA) to direct the Cas endonuclease to a specific target site, after which a double-strand break (DSB) in the DNA is formed. In order to survive, the cell needs to repair the double-strand break. For artificial manipulation of the genome, a donor DNA consisting of a desired sequence and short sites flanking the DSB needs to be provided together with the gRNA and Cas endonuclease. Up to date, the type II CRISPR-Cas9 system using the bacterial Cas9 protein is the most simplified and adapted system for artificial DNA manipulation (Shalem et al., 2015). Recently, the application of this system in fungi, such as *S. cerevisiae* and *C. albicans*, has been reported (DiCarlo et al., 2013; Vyas et al., 2015) and while finalizing this manuscript, CRISPR-Cas9 was also shown to work in *C. glabrata* (Enkler et al., 2016). As a perspective, Hsueh-lui Ho and Ken Haynes already suggested to develop the CRISPR-Cas system in *C. glabrata*, especially in the *lig4*Δ strain to obtain a lower false positive rate (Ho and Haynes, 2015).

We adapted the *S. cerevisiae* CRISPR-Cas9 system for use in *C. glabrata*. Three genes, located on different chromosomes, were targeted with the CRISPR-Cas9 system and altered in three different ways: deletion of the ORF, introduction of a stop codon or insertion of a triple HA-tag. We show that the CRISPR-Cas9 system works in the strains used, but the efficiency varies depending on the genetic modification and the gene targeted. In general, the efficiency to delete genes was higher when a premature stop codon was introduced rather than when the ORF was replaced by the *SAT1* selection marker. We obtained a higher percentage of correct transformants in the wild-type strain compared to the conventional method. Using the CRISPR-Cas9 system, the genome can be edited without the introduction of a marker or any other scar sequence. This makes the CRISPR-Cas9 system an attractive tool for genome engineering of *C. glabrata*. Unexpectedly, we didn't see a significant increased transformation efficiency in the *lig4*Δ strain when using the CRISPR-Cas9 system. However, the percentage of correct transformants was consistently higher in the *lig4*Δ strain, indicating the importance of the NHEJ process after formation of the DSB.

2. Results

2.1. Construction of CRISPR-Cas9 plasmids for use in C. glabrata

As starting material, we used the plasmids for CRISPR-Cas9 from *S. cerevisiae* generated by DiCarlo et al. (2013). For Cas9 expression, vector pTEF-Cas9-KanMX (Fig. 1A) was constructed by introduction of the dominant KanMX cassette into vector p414-TEF1p-Cas9-CYC1t (DiCarlo et al., 2013). In this way, the plasmid can be selected for in the *C. glabrata* recipient strains ATCC2001HTL (Schwarzmüller et al., 2014) or 2001HT (Miyazaki et al., 2010) on minimal medium lacking tryptophan, as well as in prototrophic strains on medium supplemented with geneticin.

The gRNA vectors were constructed by insertion of the *SNR52* promoter, the 20 bp guide sequence, structural crRNA and *CYC1* terminator as well as the dominant hygromycin cassette in the recipient vector YCplac111 (Fig. 1B and Supplementary Fig. S1A). The resulting vectors, further referred to as YCp-gRNA-X with X the gene that is targeted, can be selected on minimal medium lacking leucine or on medium supplemented with hygromycin.

2.2. CRISPR-Cas9 mediated mutagenesis

Three *C. glabrata* genes, *ADE2* (CAGL0K10340g), *MET15* (CAGL0D06402g) and *SOK2* (CAGL0L01771g), located on different chromosomes, were targeted for CRISPR-Cas9 mediated mutagenesis. We also used three different modification strategies, using a single guide sequence for each targeted gene (YCp-gRNA-*ADE2,* YCp-gRNA-*MET15* or YCp-gRNA-*SOK2*). Two different approaches were employed to disrupt *ADE2* and *MET15* (Fig. 1C). To repair the DSB mediated by Cas9 and the gRNA, in one approach donor DNA was used that consisted of the dominant *SAT1* cassette flanked by 40 bp homologous to the 5′ and 3′ sequences the ORF respectively. In the other strategy, the donor DNA consisted of the insertion of an in frame stop codon, causing a premature end of translation. Both approaches were carried out in the wild-type strain and the *lig4*Δ strain. In the third approach, a triple HA-tag sequence was inserted into the *SOK2* ORF in the *C. glabrata lig4*Δ strain.

For each CRISPR-Cas9 mediated mutagenesis of the genome, two consecutive transformations were done. After first introducing the plasmid expressing Cas9, in a second transformation, the gRNA plasmid (YCp-gRNA-X) and donor DNA were co-transformed in the Cas9 expressing strain. After the second transformation, the cells were plated on geneticin (presence of the Cas9 plasmid) and hygromycin (presence of the YCp-gRNA-X plasmid) selection. *C. glabrata* was also transformed with either the Cas9 plasmid or the YCp-gRNA-X plasmid and donor DNA. In this case no colonies were present, indicating the necessity of having both plasmids to produce a functional system. Similar as was observed by Enkler et al. (2016), we also observed an increase in the duplication time in cells expressing Cas9. Depending on the gene targeted, transformants were checked by plating, colony PCR and/or sequencing as described in materials and methods.

2.3. The CRISPR-Cas9 system is more efficient than the conventional way to delete the ADE2 ORF in wild-type C. glabrata, but this effect is absent in the lig4Δ strain

Using the conventional way of replacing the *ADE2* ORF by the *SAT1* cassette, an efficiency of only 0.4% of correct transformants was found in Cen et al. (2015). In this work, we used the same construct as donor DNA together with the CRISPR-Cas9 system and 1.1% of the transformants (5/466) were found to be correct (Table 1). This difference is due to the DSB generated by Cas9 and the guide RNA. In the *lig4*Δ strain, 10% of the transformants was correct when the conventional method was applied (Cen et al., 2015). Using the CRISPR-Cas9 system, a modest increase in targeting efficiency (1.5% vs 1.1%) was seen in the *lig4*Δ strain compared to the wild-type strain (Table 1). This modest increase in CRISPR-Cas9 mediated targeting contrasts sharply with the major improvement in gene targeting resulting from the *lig4*Δ strain when the conventional gene targeting method was used (Cen et al., 2015). The difference in the targeting efficiency between the wild-type and *lig4*Δ strain is small but consistent, we therefore believe that the use of the *lig4*Δ strain in genome engineering is advantageous.

2.4. The targeting efficiency depends on the ORF targeted

To compare the genome modifications generated by CRISPR-Cas9, we used the same approach to disrupt *ADE2* and *MET15.* Table 1 shows the number of correct and the total number of transformants for each mutagenesis approach and strain used. It is quite clear that the efficiency to delete *MET15* is much higher compared to the *ADE2* locus, both in the wild-type and in the *lig4*Δ strain. When the *ADE2* ORF is replaced by *SAT1*, 5 out of 466 or 1.1% of the wild-type transformants