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Going green in *Cryptococcus neoformans*: The recycling of a selectable drug marker

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ABSTRACT

Cryptococcus neoformans is an opportunistic fungal pathogen that primarily affects immunocompromised individuals. Reverse genetics is commonly used to identify and characterize genes involved in a variety of cellular processes. In *C. neoformans* there is a limited set of positive selectable markers available to make gene deletions or other genetic manipulations. This has hampered the application of reverse genetics in this organism. We have adapted the Bacteriophage P1 Cre-*loxP* system for use in *C. neoformans* and successfully excised and reused the same drug marker, G418, to make two sequential gene deletions, *lac1*Δ and *cap59*Δ, in the same strain. This tool will allow investigators to make multiple sequential gene deletions in the same strain, which should facilitate the analysis of multigene families.

1. Introduction

Cryptococcus neoformans is an opportunistic fungal pathogen that primarily affects immunocompromised hosts. Initially infection can result in pneumonia and develop into a systemic infection that travels through the central nervous system, crossing the blood brain barrier resulting in cryptococcal meningitis. Infection with this organism is an AIDS defining illness (Casadevall and Perfect, 1998). *C. neoformans* grows well as haploid yeast cells, and has proved to be amenable to genetic manipulation. Additionally, with the sequencing of the cryptococcal genomes, JEC21, B3501 (Loftus et al., 2005), and H99 (jointly sequenced by Duke University, http://cgt.genetics.duke.edu, and the Broad Institute, http://www.broad.mit.edu/annotation/fungi/cryptococcus_neoformans) the use of reverse genetics in determining a gene products' involvement in cell integrity, cellular growth, known virulence factors, or virulence of *C. neoformans* is steadily increasing. Reverse genetics was used in the construction of a targeted gene deletion library that contains greater than 2000 gene deletions (Liu et al., 2008). These deleted candidate genes may prove to be involved in many of the previously mentioned processes.

Efficient selection of transformed cells requires a marker, and in *C. neoformans* both auxotrophic markers and positive selectable drug markers have been used successfully to generate deletion or mutant strains. The use of auxotrophic markers requires development of strains with the appropriate genetic background; therefore, they are not as suitable as positive drug markers for manipulation of clinical isolates or otherwise wild type strains. Currently, four positive selectable drug markers are used: nourseothricin (NAT) (McDade and Cox, 2001), phleomycin, geneticin (G418), and hygromycin (Hua et al., 2000). The product of the nourseothricin acetyltransferase gene provides resistance to the aminoglycoside antibiotic nourseothricin (NAT) that interrupts protein synthesis (McDade and Cox, 2001). Phleomycin is a copper-containing antibiotic that acts by interfering with DNA synthesis (Reiter et al., 1972). G418 is an aminoglycoside antibiotic that binds to the "A" site of the small ribosomal subunit; this action causes the ribosome to incorrectly read the genetic code (Yoshizawa et al., 1998). Hygromycin (HYG) has a similar mode of action to that of G418 by attacking the 30S ribosomal unit and thus interrupting protein translation (Broderson et al., 2000).

Using these four markers, many genes have been analyzed for multiple phenotypes, including virulence in animal models. The markers are also used for selection of gene modifications, such as the addition of epitope tags, point mutations, and swapping of promoter sequences. Additionally, they have been very effective when manipulating combinations of up to four genes. Strains with multiple gene deletions or modifications have been generated by sequential gene deletion: for example, using hygromycin, nourseothricin, and geneticin, three peroxiredoxins a.k.a. the thiol peroxidases (*TSA1*, *TSA3*, *TSA4*) were sequentially deleted to produce a triple *TSA* deletion strain (Missall et al., 2004). These markers have also been used to create multiple deletions through a combination

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of sequential gene deletion and genetic crosses: for example, a quadruple deacetylase deletion strain lacking all three chitin deacetylases, *CDA1*, *CDA2*, and *CDA3*, and the fungal polysaccharide deacetylase, *FPD1* was made using all four markers and mating (Baker et al., 2007). Whether choosing to make multiple gene deletions through sequential gene deletion, mating, or a combination of the two, distinct markers for each gene are required. Because of the limited number of positive selectable markers for use in *C. neoformans* it can be difficult to utilize these strategies in large gene families such as the chitin synthases, which has more than four members (Banks et al., 2005).

In this study we demonstrate the use of the Cre-*loxP* system, originally described in Bacteriophage P1 (Sauer and Henderson, 1988), in *C. neoformans*. The Cre-*loxP* recombinase system includes two short asymmetric DNA sequences termed *loxP* sites (approximately 34 base pairs including two inverted repeats) and the Cre-recombinase protein (38 kDa). The Cre protein covalently binds to two short *loxP* sites and catalyzes recombination within them; any intervening DNA sequence is looped out and excised. The Cre-recombinase is the only protein required for this action.

The Cre-*loxP* system has been usurped for use in several organisms. For instance, two teams of investigators have used the Cre-*loxP* system in mammalian systems. Araki et al. (2002) successfully incorporated the *CRE* gene into the germ line of mice (Araki et al., 2002). This created mouse lines that could be used for future gene deletions through gene trapping and targeting. Smith et al. (1995) used this system in embryonic stem cells of mice to mediate chromosomal rearrangement during the expression of Cre-recombinase (Smith et al., 1995). The Cre-*loxP* system has also been used in the construction of recombinant adenoviral vectors (Hardy et al., 1997). To create recombinant viral vectors in adenoviruses for gene delivery, the Cre-*loxP* system has been placed into strain ψ5 of the adenovirus, with *loxP* sites flanking each side of the packaging site. When Cre-recombinase is expressed, the packaging site is deleted (Hardy et al., 1997).

The Cre-*loxP* recombination system has also been used in many fungal systems. It was successfully introduced into the non-pathogenic yeast, *Saccharomyces cerevisiae* (Sauer, 1987). By flanking the *LEU2* gene with *loxP* sites this gene was successfully deleted during the transient expression of Cre-recombinase (Sauer, 1987). Additionally, the Cre-*loxP* system has been used in several other fungal systems, a few being: *Yarrowia lipolytica*, *Neotyphodium coenophialum*, *Neotyphodium uncinatum*, *Epichloë festucae*, *Aspergillus nidulans*, *Schizosaccharomyces pombe*, and *Kluyveromyces marxianus* (Fickers et al., 2003; Florea et al., 2009; Iwaki and Takegawa, 2004; Ribeiro et al., 2007; Watson et al., 2008). Furthermore, the Cre-*loxP* system was optimized for the deletion of targeted genes and marker recycling in the human fungal pathogen *Candida albicans* (Dennison et al., 2005).

Here we report the use of the Cre-*loxP* system for marker recycling in *C. neoformans*. Specifically, we describe the reuse of the G418 drug marker to produce a strain with two unique gene deletions. Using a sequential deletion strategy, we successfully deleted the laccase gene, *LAC1* (Wang et al., 1995), and the capsular gene, *CAP59* (Chang and Kwon-Chung, 1994), using the same positive selectable marker.

2. Materials and methods

2.1. Fungal strains and media

KN99α strain of *C. neoformans* serotype A was used as the wild type strain (Nielsen et al., 2003), and all deletions were made in this background. Strains were grown on rich medium, YPD (1% yeast extract, 2% bacto-peptone, and 2% dextrose) or YPG (1% yeast extract, 2% bacto-peptone, and 2% galactose). Selective YPD or YPG media contained 100 μg/ml nourseothricin (Werner BioAgents, Jena-Cospeda, Germany) or 200 μg/ml geneticin (G418) (Invitrogen, Carlsbad, CA).

2.2. PCR and cloning conditions for G418-loxP deletion construct

To add *loxP* sites to the geneticin drug marker we designed two primers that included the *loxP* sequence: the first primer consisted of 24 nt of the 5′ portion of the Actin promoter (P_{Actin}) that was added to the 3′ end of primer #319 reported by Guldener et al. (1996), #319-Actin 5′-TCGACAACCCTTAATATAACTTCGTATAATGTATGCTATACGAAGTTATTAGGTCTAGAaggatgtgagctggagagcggggc-3′. The second primer consisted of 24 nt of the 3′ end of the *NMT1* terminator (T_{NMT}) that was added to the 5′ end of primer #322 (Guldener et al., 1996), #322-NMT ACTACCTAATAACTTCGTATAGCATACATTATACGAAGTTATTATTAAGGGTTCTCGAGAGCTgggggacagatgatatccgacaag-3′. In both primer sequences the *loxP* sites are underlined. PCR reactions consisted of 50 pmols of each primer and 30 ng of pMH12-T vector template (G418 marker). Conditions were 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 4 m, cycled 35 times with a final extension of 72 °C for 10 m. The PCR amplicon of 2.7 kb was gel purified using QIAquick gel extraction kit per manufactures instructions (QIAGEN, Valencia, CA). The fragments were cloned into the pCR2.1 vector and One Shot® TOP10 chemically competent *Escherichia coli* using the TOPO TA Cloning® kit per manufactures instructions (Invitrogen, Carlsbad, CA). Inclusion of *loxP* sites flanking the G418 drug marker was confirmed by sequencing and the resulting vector was designated, pJL517 (Fig. 1).

2.3. PCR and Cloning conditions for the CRE construct for use in C. neoformans

We designed a Cre-recombinase construct driven by an endogenous galactose inducible promoter, P_{GAL7} (Fig. 1). The galactose promoters work by an inducible (galactose) and repressible (glucose) system (Ruff et al., 2009). The inclusion of the *GAL7* promoter allowed for the transient expression of the Cre-recombinase on medium containing galactose, YPG (Fig. 1). Because it has been shown that using endogenous terminators greatly increases the stability of a transcript in *C. neoformans* (Hua et al., 2000) an endogenous terminator, T_{GAPDH}, was included in this construct. An overlap PCR method (Davidson et al., 2002) was used to create the *CRE*-recombinase construct. Vector pSDM3155, kindly provided by J.J. Hooykaas, supplied the *CRE* template (Schrammeijer et al., 2003). Plasmid pSDM3155 does not contain a start or stop codon, therefore both were included in the design of the overlap primers 5-, 6-, 7-, and 8-GAL7CRE (Table 1). PCR amplifications were carried out as described above. Primers 6- and 7-GAL7CRE were used to amplify a 1 kb fragment containing *CRE* with a start and stop codon. Primers 3- and 8-GAL7CRE and 5- and 10-GAL7CRE were used to amplify the P_{GAL7} and T_{GAPDH} terminator fragments, respectively. The template for these reactions was 100 ng genomic DNA from the *C. neoformans* strain, JLCN587. This strain contains a NAT drug resistance construct attached upstream of a *GAL7* promoter. The *CRE* coding sequence was fused downstream of the *GAL7* promoter and upstream of the *GAPDH* terminator using primers 3- and 10-GAL7CRE. This construct, NAT:P_{GAL7}: *CRE*:T_{GAPDH}, was cloned into a vector that we designated, pJL519 (Fig. 1). The pJL519 vector containing the *CRE*-recombinase construct was verified by sequencing (Retrogen, San Diego, CA).

2.4. Generation of deletion constructs

Overlap PCR gene deletion technology was used to generate gene-specific deletion cassettes of *LAC1* or *CAP59*. Each reaction included a G418 cassette flanked by *loxP* sites (see above). Primers