Construction of a Recyclable Genetic Marker and Serial Gene Deletions in the Human Pathogenic Mucorales *Mucor circinelloides*

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**ABSTRACT** *Mucor circinelloides* is a human pathogen, biofuel producer, and model system that belongs to a basal fungal lineage; however, the genetics of this fungus are limited. In contrast to ascomycetes and basidiomycetes, basal fungal lineages have been understudied. This may be caused by a lack of attention given to these fungi, as well as limited tools for genetic analysis. Nonetheless, the importance of these fungi as pathogens and model systems has increased. *M. circinelloides* is one of a few genetically tractable organisms in the basal fungi, but it is far from a robust genetic system when compared to model fungi in the subkingdom Dikarya. One problem is the organism is resistant to drugs utilized to select for dominant organisms in the basal fungi, but it is far from a robust genetic system when compared to model fungi in the subkingdom Dikarya. One problem is the organism is resistant to drugs utilized to select for dominant markers in other fungal transformation systems. Thus, we developed a blaster recyclable marker system by using the *pyrG* gene (encoding an orotidine-5′-phosphate decarboxylase, ortholog of *URA3* in *Saccharomyces cerevisiae*). A 237-bp fragment downstream of the *pyrG* gene was tandemly incorporated into the upstream region of the gene, resulting in construction of a *pyrG-dpl237* marker. To test the functionality of the *pyrG-dpl237* marker, we disrupted the *carRP* gene that is involved in carotenoid synthesis in *pyrG*− mutant background. The resulting *carRP::pyrG-dpl237* mutants exhibit a white colony phenotype due to lack of carotene, whereas wild type displays yellowish colonies. The *pyrG* marker was then successfully excised, generating *carRP-dpl237* on 5-FOA medium. The mutants became auxotrophic and required uridine for growth. We then disrupted the calcineurin B regulatory subunit *cnbR* gene in the *carRP::dpl237* strain, generating mutants with the alleles *carRP::dpl237* and *cnbR::pyrG*. These results demonstrate that the recyclable marker system is fully functional, and therefore the *pyrG-dpl237* marker can be used for sequential gene deletions in *M. circinelloides*.

*Mucor circinelloides* is a basal fungus belonging to the phylum Mucoromycota (Spatafora et al. 2016). *M. circinelloides* is one of the etiological agents of mucormycosis. Mucormycosis is an opportunistic fungal infection recently recognized as an emerging infectious disease (Kauffman 2004; Brown 2005; Chayakulkeeree et al. 2006; Lanternier et al. 2012a). Fungi in the order Mucorales are the causal agents of mucormycosis, and include *Mucor* spp., *Rhizopus* spp., *Lichtheimia* (previously *Absidia*) spp., *Apophysomyces* spp., *Cunninghamella* spp., *Rhizomucor* spp., and others (Chayakulkeeree et al. 2006; Ibrahim and Spellberg 2006; Neblett Fanfair et al. 2012). Recent data indicates a significant increase in mucormycosis due to a rising number of immunocompromised patients with conditions such as diabetes, HIV/AIDS, hematologic malignancies, hematopoietic stem cell/solid organ transplantation, or trauma (Ribes et al. 2000; Marr et al. 2002; Kontoyiannis et al. 2005; Roden et al. 2005; Spellberg et al. 2005). Mucormycosis is the second most common mold infection among patients with hematological malignancies and transplants, and is associated with high mortality rates of ~50% for all mucormycosis infections and >90% in disseminated infections (Ribes et al. 2000; Roden et al. 2005; Lanternier et al. 2012b). Even after recovery, patients often suffer from...
permanent disfiguration in the affected areas due to surgical debride-
ment as a frequent treatment modality (Kwon-Chung and Bennet
1992; Kontoyiannis et al. 2005; Roden et al. 2005; Spellberg et al.
2005; Kontoyiannis and Lewis 2006).

M. circinelloides has served as a system to study virulence and
pathogenesis of mucormycosis in animal host models. For example,
various Mucor isolates produce spores of different sizes, and the size of
spores contributes to virulence as larger spores are more virulent than
smaller spores (Li et al. 2011). The difference in virulence may be due to
a difference in the interactions of larger and smaller spores with mac-
rophages whereby larger spores germinate inside macrophages, and
smaller spores remain dormant inside macrophages (Li et al. 2011).
The morphogenesis of M. circinelloides also contributes to virulence, in
which the filamentous form of the fungus is more virulent than the
yeast form (Lee et al. 2013). In addition, the different morphogenic
states (spores/hyphae vs. yeast) result in different host-pathogen inter-
actions (Lee et al. 2015). For example, Mucor spores arrest or delay
phagosome maturation upon phagocytosis by macrophages, whereas
Mucor yeast do not. Cytokine responses from immune cells differ be-
tween Mucor spores/hyphae and yeast. A recent study found that a
phospholipase D and myosin V family protein are required for full
virulence in Mucor through an RNAi-based genome screen (Trieu
et al. 2017).

M. circinelloides is also able to contaminate and spoil foods (Lazar
et al. 2014; Lee et al. 2014). M. circinelloides is known to produce the
toxin 3-nitropyridonic acid (3NP) (Hollmann et al. 2008), which
makes this fungus a potential cause of food poisoning. 3NP inhibits
mitochondrial succinate dehydrogenase, causing acute brain injury,
methemoglobinemia, dystonia after ingestion, and symptoms sim-
ilar to Huntington’s disease (Alston et al. 1977; Gabrielson et al.
2001; Brouillet et al. 2005). Interestingly, 3NP was responsible for
217 sugarcane poisoning outbreaks in China during the period
1972–1989, which involved 884 patients with 88 mortalities [reviewed
in Magan and Olsen (2004)]. Therefore, it is apparent that Mucor poses
a threat to public health.

M. circinelloides also serves as a model to study light sensing in
fungi. Asexual sporulation is triggered by light. Blue light activates
carotenoid biosynthesis and sporangiophere phototropism (Navarro
et al. 1999; Silva et al. 2006). Mucor also encodes homologs of the
Neurospora crassa white collar-1 (WC-1), which forms the white collar
complex with other factors that entrain the circadian clock (Liu and
Bell-Pedersen 2006). The functions of the three wc-1 genes have been
eclucidated in Mucor, in that they are all involved in light response
although each of them has distinct light transduction pathways (Silva
et al. 2006, 2008; Navarro et al. 2013).

M. circinelloides also has a conserved and active RNAi pathway that
governs the expression of nonintegrated transgenes (Nicolas et al.
2003), and therefore serves as a model to study RNA silencing in fungi.
Genetic analysis demonstrated that Dicer, Argonaute, and RNA-
dependent RNA polymerase play major roles in this RNA silencing,
and these three components are conserved in M. circinelloides (de Haro
et al. 2009; Calo et al. 2012; Billmyre et al. 2013; Cervantes et al.
2013). Interestingly, M. circinelloides exhibits a novel mechanism of drug
resistance evoked by RNAi-mediated silencing (Calo et al. 2014). In the
presence of the calcineurin inhibitor FK506, the fungus can silence the
target gene of FK506, which encodes FKBP12, to become drug resistant.
However, without selective pressure, the fungus will eventually deacti-
vate the silencing mechanism and become drug sensitive in an epige-
etic RNAi mediated process (Calo et al. 2014). A recent study found
that the noncanonical RdRP-dependent/Dicer-independent RNAi
pathway indeed inhibits the canonical RNAi-mediated drug resistant
pathways, suggesting a balance between two RNAi pathways in
M. circinelloides (Calo et al. 2017).

The recent oil crisis and environmental conservation initiatives have
prompted innovations in the production of biofuel. Research has focused on oilseed
and plant-based carbohydrates that can be converted into fatty acid esters by microorganisms (Kalscheuer
et al. 2006; Steen et al. 2010). An additional important biofuel
source is lipid-accumulating microorganisms, such as oleaginous
fungi and algae (Kosa and Ragauskas 2011). M. circinelloides is an
oleaginous microorganism that accumulates lipids as a storage
source (Vicente et al. 2009; Kosa and Ragauskas 2011; Rodriguez-
Frómeta et al. 2013) and produces a high level of linoleic acid, which
served as a basis for the first commercial microbial lipid (Ratledge
2004). Biodiesel produced by M. circinelloides satisfies the specifica-
tions set by American and most European standards (Vicente et al.
2009).

M. circinelloides is clearly a prominent organism in many aspects of
biology. Unfortunately, the genetics of this fungus are limited. Resis-
tance to drugs utilized to select for dominant markers in other fungal
transformations, including neomycin, nourseothricin, hygromycin,
zeomycin, carboxin, and glufosinate, is common (data not shown). This
precludes the use of these common dominant drug resistance markers
for Mucor. Only two auxotrophic markers (leuA- and pyrG-) in one
strain (Nicolas et al. 2007) and one auxotrophic marker (Met-) in
another strain (Anaya and Roncero 1991) are available in the back-
ground of the sequenced isolates (http://genome.jgi-psf.org/Mucci2/
Mucci2.home.html), which significantly limits the ability to disrupt
multiple genes for genetic analysis.

In this study, we developed a recyclable pyrG blaster marker for multiple gene deletions in M. circinelloides that is analogous to the
URA3 blaster marker for Candida albicans (Wilson et al. 2000). A
237-bp fragment of the 3’-downstream of the pyrG gene was tandemly
incorporated in the 5’-upstream of the pyrG to generate a pyrG-dpl237
(237 bp-pyrG-237 bp) marker. The marker was used to successfully
disconnect the carRP gene to generate an albino mutant (carRP::pyrG-
dpl237). Spontaneous excision of the pyrG marker in the carRP::pyrG-
dpl237 allele occurred; resulting in generation of carRP::dpl237
mutants. Additionally, the cnbR gene was disrupted by using pyrG
as a marker, demonstrating the pyrG blaster marker is fully functional
and can be used for multiple gene disruption.

MATERIALS AND METHODS

Fungal strains and growth conditions
The strains and plasmids used in this study are listed in Table 1. For
spore production, M. circinelloides strains were grown on yeast
peptone glucose agar (YPG, 3 g/liter yeast extract, 10 g/liter peptone,
20 g/liter glucose, 2% agar, pH 4.5) media at 26° in the light for 4 d.
To collect spores, deionized sterile water was added to the plates and
the spores were gently scraped with a cell spreader. For further
experiments, the number of spores was calculated with the auto-
mated cell counter TC20 (Bio-Rad) or a hemocytometer. YPD agar
was used to compare the phenotypes of yeast-locked cnbRΔ mutants
with wild type. For transformation, minimal media with casamino
acids (MMC; pH 3.2, 10 g casamino acids, 0.5 g yeast nitrogen base
without amino acids and ammonium sulfate, 20 g glucose, 1 mg
niacin, 1 mg thiamine, and 15 g agar in 1 liter dH2O) media contain-
ing 0.5 M sorbitol was used.

M. circinelloides spores are multinucleate, so vegetative selection
was required to isolate homokaryotic transformants. The initial hetero-
karyotic transformants were subjected to vegetative rounds of growth
Development of a recyclable pyrG marker

A 2002-bp DNA fragment containing the pyrG gene was amplified with M13 forward and reverse primers from the vector pTOPO-pyrG that we previously constructed (Lee et al. 2013). This plasmid was used as an initial plasmid to construct a 237-bp pyrG-dpl237 fragment (or pyrG-dpl237 allele). The 237-bp DNA fragment located downstream of the pyrG gene was amplified with the primers SCL635 and SCL636 (Table 2), which contain overhanging XbaI or NotI restriction enzyme recognition sites on either side. The amplified 237-bp fragment was cloned into pPyrG-TOPO digested with XbaI and NotI, generating the pSL13 (Table 1), which harbors a DNA cassette with 237-bp direct repeats on both sides of the pyrG gene.

Disruption of genes

To disrupt the carRP gene, we constructed a disruption cassette containing the pyrG blaster marker flanked by ~1 kb of 5’ and 3’ of the up- and downstream regions of the carRP gene via overlap PCR. The 5’ region was amplified with primers SCL639 and SCL640, and the 3’ region was amplified with primers SCL641 and SCL642. Genomic DNA of strain MU402 served as template. The pyrG blaster (pyrG-dpl237) marker was amplified with M13 forward and reverse primers. The three fragments were then subjected to an overlap PCR with nested primers SCL643 and SCL644, to generate a disruption allele. A mixture of 75 ng of each fragment was used as template. To disrupt the cnbR gene in the carRPΔ mutant background, the cnbR disruption cassette was amplified from plasmid pCnbR-KO (Lee et al. 2013) via PCR using a pair of primers, SCL286 and SCL287, with the Thermo Scientific DreamTaq DNA polymerase. The cnbR disruption construct was introduced into the MSL29 (leuA− pyrG− carRPΔ::dpl237) strain via electroporation as described previously (Lee et al. 2013). In brief, 10⁶ germinated spores were protoplasted in the presence of chitosanase (US Biological) and lyzing enzyme (Sigma). The protoplasts were resuspended in 0.5 M sorbitol solution and mixed with 3 μg of the disruption cassette DNA. Electroporation was performed in 0.2-cm cuvettes (pulse at 0.8 kV, 25 μF, 400 ohm) and the protoplasts were spread onto selective MMC (pH 3.2) media supplemented with 0.5 M sorbitol. From 16 independent transformations, we obtained five mutants that exhibit the yeast-locked phenotype. Two independently obtained mutants, MAG1 and MAG2, were used in this study (Table 1).

Table 1 List of strains and plasmids used in this study

| Name | Genotypes or Characteristics | Reference |
|------|-----------------------------|-----------|
| M. circinelloides strains | | |
| MU402 | leuA− pyrG− | Nicolas et al. (2007) |
| MSL7 | leuA− pyrG− cnbRΔ::pyrG | Lee et al. (2013) |
| MSL27 | leuA− pyrG− carRPΔ::pyrG-dpl237 | This study |
| MSL28 | leuA− pyrG− carRPΔ::dpl237 | This study |
| MSL29 | leuA− pyrG− carRPΔ::dpl237 | This study |
| MSL30 | leuA− pyrG− carRPΔ::dpl237 | This study |
| MAG1 | leuA− pyrG− carRPΔ::dpl237 cnbRΔ::pyrG | This study |
| MAG2 | leuA− pyrG− carRPΔ::dpl237 cnbRΔ::pyrG | This study |
| Plasmids | | |
| pCR21-TOPO | Ampicillin® Kanamycin® | Invitrogen |
| pTOPO-pyrG | 2002-bp pyrG fragment cloned into pCR21-TOPO | Lee et al. (2013) |
| pCnbR-KO | cnbR disruption construct cloned into pCR21-TOPO | Lee et al. (2013) |
| pSL13 | pyrG blaster marker in pCR21-TOPO | This study |

Southern blotting analysis

Southern blotting was performed to ensure the absence of the carRP gene, the presence of carRP-pyrG-dpl237, and the excision of the pyrG gene from the carRP::pyrG-dpl237 allele. Genomic DNA (30 μg) of the wild type and carRP::pyrG-dpl237 and carRP::dpl237 mutants was digested using either EcoRI or BclI enzyme, or both. The digested genomic DNA fragments were separated in a 1% agarose gel. The gel was then rinsed in deionized distilled water and placed in a denaturing solution (1.5 M NaCl, 0.5 M NaOH). The gel was then rinsed again with deionized distilled water and placed in a neutralization solution [1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA]. Capillary transfer of DNA into nylon membrane was performed for 18 hr. The membrane was then placed in a UV cross-linking chamber (Spectrolinker XL-1000 UV cross-linker with an autoexposure setting; Spectronics Co.) to cross-link DNA to the membrane. The probe was generated by PCR with primers SL1 and SL2. The probe was then labeled with biotin by using the Thermo Scientific Biotin DecaLabel DNA labeling Kit following the manufacturer’s instruction. The Thermo Scientific Biotin Chromogenic Detection Kit was used to detect the labeled probes on the membrane following the manufacturer’s instruction.

Excision of the pyrG gene from the pyrG blaster marker

Spores of the MSL27 (carRPΔ::pyrG-dpl237) strain were plated on MMC media containing 5-FOA (2.5 mg/ml), uridine (0.61 mg/ml), and uracil (0.56 mg/ml), to select mutants that still produced white colonies but required uridine to grow. The candidate mutants were passaged on MMC media containing uridine, uracil, and 5-FOA. Excision of the pyrG gene was verified by PCR and Southern blotting.

Data availability

All the strains and plasmids used in this study are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS AND DISCUSSION

Generation of pyrG blaster marker and disruption of the carRP gene

We developed a pyrG blaster recyclable marker system by using the pyrG gene (encoding orotidine-5’-phosphate decarboxylase, ortholog of URA3 in S. cerevisiae). To construct a pyrG blaster
DNA cassette that can be easily amplified by PCR, we adapted a 237-bp repeat as previously described in C. albicans (Wilson et al. 2000). In our previous study, we generated the pTOPO-pyrG plasmid harboring a 2002-bp DNA fragment containing the pyrG gene (Lee et al. 2013) (Figure 1A, top). The plasmid was used as an initial plasmid to construct a 237 bp-pyrG-237 bp (or pyrG-dpl237) allele. The 237-bp DNA fragment located at the downstream of the pyrG gene was amplified with primers that contain overhanging XbaI and NotI restriction enzyme recognition sites on each side. The amplified 237-bp fragment was cloned into pTOPO-pyrG digested with XbaI and NotI, generating the pSL13 (Figure 1A, bottom), which harbors a DNA cassette with 237-bp direct repeats on both sides of the pyrG gene. The tandem-repeated 237 bp on both sides of the pyrG gene enhances a spontaneous homologous recombination between them, resulting in excision of the pyrG gene. The marker gene therefore can be used for another gene disruption.

To test the functionality of the pyrG blaster marker, we targeted the carRP gene, which encodes a protein with dual enzyme activities (lycopene cyclase and phytoene synthase) that are involved in carotenoid synthesis in M. circinelloides (Velayos et al. 1997) (Figure 1B). Complete loss of the carRP gene resulted in white colony formation due to the lack of accumulation of carotenoids, whereas the wild type displayed yellowish colonies. This characteristic of the carRP mutants enabled us to screen homokaryotic homologous recombinant transformants by evaluating the colony color (Rodriguez-Frómeta et al. 2013). We obtained seven transformants from eight independent transformations. Progeny from two transformants displayed both white and yellow colonies. The white and yellow mixed progeny indicate that the nuclei of the transformants are heterogeneous, containing nuclei with carRPΔ and wild-type carRP alleles. Mucor spores are multineucleate and their hyphae are coenocytic (asceptate), so cycles of vegetative passage under selective conditions are therefore required to enrich recombinant nuclei. The transformant MSL27 (carRPΔ::pyrG-dpl237) stably produced exclusively white colony progeny, and this strain was used to test the recyclable pyrG marker.

We further validated that spores of the MSL27 strain contain only the carRPΔ allele, and not the wild-type allele. The primers SCL649 (P1) and SCL650 (P2) outside of the disruption cassette were used to test the genotype of the progeny. A 4020-bp PCR fragment was produced from the wild-type carRP allele, and a 4649-bp fragment was produced from the carRPΔ allele, which confirms that the MSL27 strain does not contain the wild-type carRP allele (Figure 2). The disruption of the carRP gene was further verified by a Southern blotting analysis (Figure 3). A single digestion with BclI and a double digestion with BclI and EcoRI were performed. The probe for the 5’ upstream of the carRP gene recognized 5011-bp fragment when the wild-type genomic DNA was digested with BclI and 1322-bp fragment when double-digested with EcoRI and BclI. On the other hand, the replacement of the carRP gene with pyrG-dpl237 introduced additional EcoRI and BclI recognition sites; the probe then recognized 2501-bp fragment when the genomic DNA of MSL27 (carRPΔ::pyrG-dpl237) was single-digested with BclI, and an 872-bp fragment when double-digested with BclI and EcoRI.

### Excision of pyrG gene in the carRPΔ::pyrG-dpl237 mutant

To obtain spontaneous mutants that excised the pyrG marker, spores of the MSL27 (carRPΔ::pyrG-dpl237) strain were placed on MMC media containing 5-FOA (2.5 mg/ml), uridine (0.61 mg/ml), and uracil (0.56 mg/ml), to select mutants that still produced white colonies but required uridine to grow. The mutants that grow in the presence of 5-FOA are likely homokaryotic because resistance to 5-FOA is recessive strain. Two candidates (MSL28 and MSL29) were obtained (5-FOA resistance frequency at 5.2 ± 1.3 progeny/10⁵ spores inoculated) and the absence of the pyrG marker gene was confirmed by PCR with the primers SCL649 (P1) and SCL650 (P2), in which a 2624-bp fragment was produced after excision of the pyrG gene, instead of the 4649-bp fragment from the carRPΔ::pyrG-dpl237 allele (Figure 2). We confirmed that the two carRPΔ::dpl237 strains, MSL28 and MSL29, produce white colonies (Figure 2). We also confirmed that the MSL28 and MSL29 (carRPΔ::dpl237) require uridine to grow on MMC media due to the excision of the functional pyrG gene. The excision of the pyrG gene was further verified by a Southern blotting analysis (Figure 3). When genomic DNAs of the two carRPΔ::dpl237 mutants were single-digested with BclI, the probe recognized a 3539-bp fragment, whereas in wild type and carRPΔ::pyrG-dpl237, the probe recognized a 5011-bp or a 2501-bp fragment, respectively. When double-digested with BclI and EcoRI, the probe recognized an 870-bp fragment in the carRPΔ::dpl237 mutants, whereas it recognized a 1322-bp or an 872-bp fragment in the wild type and carRPΔ::pyrG-dpl237.
fragment in wild type or the carRPΔ:pyrG-dpl237 mutant, respectively (Figure 3). These results confirm the pyrG gene was successfully excised from the locus and the pyrG gene can be used to disrupt another gene.

Disruption of the cnbR gene in a carRPΔ::dpl237 mutant
We proceeded to disrupt the calcineurin B regulatory subunit cnbR gene in the carRPΔ::dpl237 strain to verify that the recyclable marker system was fully functional. In our previous study, we found that the disruption of cnbR gene resulted in a yeast-locked phenotype, which makes screening for homologous recombinant progeny straightforward. The MSL29 strain (carRPΔ::dpl237) was subject to transformation to disrupt the cnbR gene. This resulted in 75 transformants from 16 independent transformations. We screened the transformants by PCR with the cnbR deletion-specific primers JOHE22226 and SCL566 (Table 2, P1 and P2 in Supplemental Material, Figure S1, respectively) as previously described (Lee et al. 2013). Five transformants were found to carry an allele with cnbR::pyrG (data not shown). Homokaryotic cnbR::pyrG mutants exhibit a yeast-locked phenotype (Lee et al. 2013). After two
rounds of vegetative passage on selective media, all five of them produced yeast-locked colonies along with wild-type colonies that were heterokaryotic containing nuclei either with \(cnbR\) or \(cnbR::\text{pyrG}\). The yeast-locked colonies were selected and it was confirmed that they carry \(cnbR::\text{pyrG}\) and \(\text{carRP}::\text{dpl237}\) by PCR (Figure S1). A disruption in the \(cnbR\) gene in the \(\text{carRP}::\text{dpl237}\) strain resulted in progeny that were both yeast locked and white in color (Figure 4). The results verified that the recyclable \(\text{pyrG}\) blaster marker is fully functional and can be used for a series of gene deletions in \(M.\ circinelloides\).

Conclusions

Compared to ascomycetes and basidiomycetes, basal fungal lineages have been understudied. This may be caused by a deficit in attention given to these fungi, as well as fastidious genetic analysis. However, the importance of these fungi as pathogens and model systems has increased. \(M.\ circinelloides\) is one of very few genetically amenable organisms belonging to the basal fungi, but it is far from a robust genetic system when compared to model fungi in the Dikarya. \(M.\ circinelloides\) is a human fungal pathogen of significant public health concern. The lack of robust genetic tools presents an obstacle for investigating the biology of this pathogen with an aim toward the development of effective antifungal drugs. This study generates the facile genetic tool, recyclable \(\text{pyrG}\) marker, which will drive fundamentally important biomedical research on this pathogen, while providing a broader and sustainable impact on the field by enabling detailed genetic analyses of related Mucoralean fungi of relevance to human health.
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