Construction of *pro1* Reconstituted Strain of *Cryptococcus neoformans*

by

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Abstract

Cryptococcus neoformans (*Cn*) is a pathogenic yeast that causes cryptococcosis. The yeast infects the respiratory system where it can disseminate into the blood stream, causing fungal meningitis. Previous research identified a transcription factor, PRO1, that may be involved in dissemination. A *pro1*knockout strain showed reduced hyphal growth and a diminished capsule, an important virulence factor. To determine if PRO1 is involved in dissemination, we created the reconstituted strain, in which the gene is put back into the knock out strain with an antibiotic resistance marker. Polymerase chain reaction (PCR) was used to create an overlap construct that was introduced into the genome through biolistic transformation. Future studies will test the reconstituted strain to determine if the knock out strain has reverted back to the wild type phenotype: hyphal growth should be restored, the yeast will have a normal polysaccharide capsule, and the strain will be capable of disseminating into the bloodstream.

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INTRODUCTION

Cryptococcus neoformans (*Cn*) is a pathogenic yeast that can cause cyptococcal meningitis. The yeast originates through the respiratory system where it can disseminate into the blood stream and then cause fungal meningitis. While *Cn* has been known as a pathogen for almost a hundred years, its method of dissemination is still not fully understood [1].

Cn hosts a wide variety of virulence factors making it an exemplary example of a pathogen. In the environment of the host, the cells are covered with a polysaccharide capsule that allows them to escape host phagocytosis [2]. The capsule physically interferes with macrophage digestion of the organism. Additionally, the capsule produces molecules that inhibit the host's use of proinflammatory agents [2]. Host macrophages use oxidative reagents to damage the DNA of the invading pathogen [2]. *Cn*'s ability to produce melanin has been shown to prevent destruction by macrophages which allows it to bypass attack by the immune system, but it is unknown how this occurs at a molecular level. It's believed that melanin interacts with oxygen and nitrogen derived free radicals [3]. Also, *Cn* is capable of growing at 37 ºC, allowing the yeast to thrive in their human hosts.

Recently there has been a rise in the mortality rate of infected patients, especially amongst immunocompromised individuals in developing countries [4]. AIDS patients living in sub-Saharan Africa are believed to have a *Cn* infection rate between 15 to 30% [5]. There are currently few drugs on the market to combat cryptococcosis and *Cn* is capable of developing resistance to these drugs [6]. Drugs, such as amphotericin B, use

free radicals to damage the interior of the infecting organism [6]. This requires the drug to breach *Cn'*s polysaccharide capsule, which makes the drug less potent. If drugs cannot get through the protecting capsule, they cannot damage *Cn*'s membrane which would leave the organism intact and allow it to continue an infection [6]. This research could assist in understanding the relationship between pathogen and host.

Virulence factors are immensely important in *Cn*'s attack on its host, though the mechanisms behind use of these factors are not completely understood. For example, it is known that phospholipase is important in *Cn*'s ability to disseminate from the lungs but the exact mechanism *Cn* uses to accomplish this is not known [7].

Previous research in the lab led to the discovery of a transcription factor that may be involved in dissemination. By screening a knockout library, they were able to identify a knockout strain of *Cn* that could not form hyphae and also had a diminished capsule size, which is an important virulence factor (unpublished data). Further studies in mice suggested that the pathogen had reduced ability to disseminate from the lungs. It was found that mice infected intratracheally lived significantly longer than mice infected intravenously, suggesting that the knockout strain of *Cn* was unable to disseminate from the lungs (unpublished data).

This particular knockout strain has the PRO1 transcription factor deleted. Transcription factors are proteins that bind to regions of DNA that are used to regulate transcription of other genes. An organism does not need all of its genes turned on at all times as this is a waste of cellular resources, thus necessitating an on/off switch. PRO1 is also found in *Sordaria macrospora* where it is used in fruiting body (hyphal) development [8]. It is hypothesized that *Cn* similarly uses PRO1 to form hyphae, which

may be involved in how *Cn* gets out of the lungs. It is currently unknown how *Cn* moves from the lungs into the blood and this study may indicate how this mechanism works.

In order to determine if PRO1 is truly involved in dissemination, we must reconstitute the knock out strain of *Cn* with the *pro1* gene. When putting the gene back into the genome, we will also be adding an antibiotic resistance marker to the *pro1* construct so we can select for transformants, which are knock out cells that have had our PCR construct introduced into them. The antibiotic marker will allow our reconstituted strain to grow on an antibiotic enriched plate, showing us cells that have taken up the PCR construct. By placing this gene back into the knock out strain, we will be recreating the wild type strain (a knock in or reconstituted strain). The resulting reconstituted strain will then be tested to determine if the phenotype observed with the knock out strain has reverted back to the wild type phenotype. This type of experiment is referred to as a gain of function experiment, showing that placing a gene into the organism allows it to gain virulence. We are ensuring that the gene itself is responsible for creating the phenotypic differences found in the knock out strain.

RESULTS

Each of the three pieces involved in the overlap construction of *pro1* and the neomycin marker were successfully amplified using polymerase chain reaction. These pieces were observed for proper size configuration through the use of gel electrophoresis before being purified for use in PCR creation of the overlap. We observed bands at about 4, 2, and 1 kilobase pairs corresponding with the length of the left, neomycin, and right pieces of the overlap (Figure 1). Phusion DNA polymerase was used to create an overlap of the three pieces so that a construct contained untranslated regions up and downstream of the *pro1* sequence and the neomycin antibiotic resistance sequence in the middle (Figure 2). The overlap construct was then used in conjunction with a TOPO XL cloning kit to clone the created gene overlap into a commercial plasmid. Cloning the overlap allowed for quick production of the construct for purification. Five clones were created using this method, however all five had one or more base mutations rendering the clone unusable.

The construct was then biolistically transformed into the *pro1* knock out strain to create a reconstituted strain of *pro1* in *Cn*. The *Cn* cells were shot with beads incubated with the construct DNA to allow for homologous recombination of the overlap construct with chromosomal DNA. The Biology Department's gene gun was used for this biolistic transformation. These transformants are currently being screened.

DISCUSSION

Before the transcription factor PRO1 can be linked with dissemination of the pathogen *Cn* from the host's lungs into the bloodstream, the gene must be shown to play a role in changing the phenotype of the organism. The knockout strain lacking *pro1* had a phenotype with reduced virulence, lacking hyphal growth, and producing a smaller capsule than a wild type strain (Dr. McClelland, unpublished data). Reintroducing the gene into the genome and gaining functionality back will implicate the requirement of the transcription factor in dissemination and virulence.

The overlap construct was the first step in creating a reconstituted strain. The overlap construct was created through PCR and was observed through agarose gel electrophoresis. Cloning the construct allowed us to bypass continually using PCR and subsequent clean up kits. Cloning DNA constructs into bacterial plasmids has often been a tool of researchers to create a large quantity of a specific DNA fragment in a relatively short amount of time. The *pro1*/neomycin marker construct was successfully cloned into a commercial plasmid five separate times during this research project. However, each clone contained one or more base mutations which ultimately led to amino acid changes in the polypeptide. An amino acid change could possibly change the functionality of the protein and leave the construct useless, forcing us to reattempt creating a clone with the correct sequence.

The original construct was created with Fisher Taq DNA polymerase. This polymerase does not have a high fidelity rate so during PCR it will often insert a random base pair into the construct. The use of Fisher Taq to create the gene construct for cloning

and subsequent biolistic transformation likely led to the mutations observed in the sequence of each clone.

Future Directions

A reconstituted strain of *Cn* will have incorporated a lab-created construct containing both the entire *pro1* sequence, untranslated regions up and down stream of the gene, and a neomycin resistance gene. This reconstituted strain was created through biolistic transformation. This construct will also be cloned into a commercially available bacterial vector. By introducing the PCR construct into a high copy vector, copious amounts of DNA will be capable of being produced in a short amount of time. DNA sequencing will ensure this plasmid contains the proper sequence.

Further analysis of the reconstituted strain must be performed before further testing. The strain must have only one copy of the *pro1* gene. Biolistic transformation can lead to an organism having multiple copies of the same gene, leading to a phenotype different than that of a wild type strain. To ensure the strain has properly been constructed, Southern analysis will be performed on the reconstituted strain.

After the reconstituted strain has been properly constructed, it will be analyzed using RNA-Seq. RNA-Seq looks at the entire transcriptome of a cell at a given moment in time. By observing what RNAs are being produced by the knock out and wild type strains, one can identify which genes are being transcribed. Determining what genes are regulated by PRO1 could suggest a possible function of PRO1.

Knock out analysis of *pro1* in *Cn* suggests the gene is necessary for dissemination of the pathogen from the lungs of its host into the bloodstream, allowing for a more serious cryptococcal meningitis infection. PRO1 is believed to act as a transcription factor, turning on other genes to allow for a full infection. Without this molecule to turn genes on, *Cn* cannot leave the lungs and is phenotypically different than the wild type. By

creating a reconstituted strain from a knock out strain, we will be able to rescue the wild type phenotype. This correlative data will allow us to observe *pro1*'s importance in activating virulence factors. Further experiments, like RNA-Seq will provide us with more information on the role of PRO1 in gene regulation.

METHODS AND MATERIALS

Construction and amplification of an overlap of genomic DNA and marker

A sequence of DNA was created to combine the *pro1* gene and the neomycin resistance sequence into a single overlap construct. The *pro1* sequence on the left side of the construct contained 5' untranslated DNA in addition to the gene while the right piece of the overlap contained 3' untranslated DNA, so that *Cn* DNA flanked a neomycin antibiotic resistance gene. The left and right pieces of the overlap were amplified through PCR from isolated H99 DNA (see *Polymerase Chain Reactions*), with the left piece using primer #'s 1 and 3 and the right piece using primer #'s 4 and 6. The neomycin resistance marker was amplified from the pJAF plasmid (a gift from Joe Heitman, Duke University), using primer #'s 2 and 5 that also contained sequence homologous to regions amplified in the *pro1* pieces (Figure 1). A subsequent PCR was run using nested overlap primers 5' and 3' that would overlap the three individually amplified pieces (Table 1, Figure 2). The nested overlap primers were chosen from regions of DNA up and downstream of *pro1* in the untranslated regions. When used in a PCR they would create long strands of DNA that would become the overlap construct. Gel electrophoresis of the construct was used to ensure that the DNA fragment was the correct size (Figure 3). The DNA from the PCR overlap was then purified using ethanol precipitation. DNA from the PCR was incubated with 1/10 volume 3M NaOAc and 2.5 volumes of 100% ethanol for at least one hour. The precipitated pellet of DNA was then washed with 70% ethanol and resuspended in 10 µl milli Q H_2O.

Polymerase Chain Reactions

The basic PCR mixture of reagents consisted of 5x Phusion HF Buffer, 10 mM dNTPs, 10 mM 5' primer, 10 mM 3' primer, 15 ng of each DNA, Q solution , 0.5 µl Phusion DNA polymerase, and milli Q H_2O to 50 µl. The primers were specific to each reaction and were made by Fisher. The reactions were run with the following conditions in a thermocycler: 98°C for 3 minutes, 30 repeats of the following - 98°C for 30 seconds, 64°C for 30 seconds, 72°C for 7.1 minutes, then a final step at 72°C for 10 minutes, with an infinite hold time at 4°C.

Agarose gel electrophoresis

1% agarose gels were used to observe each PCR for proper DNA fragment size. Gels were constructed from 1x TAE, agarose powder, and ethidium bromide. The fragments were run with Gene Ruler ladder as a size standard. Each gel was run in 1x TAE as a buffer at 130 volts. The gels typically ran for 60 to 100 minutes. They were visualized using a gel documentation system, Gel Doc-It Imaging System.

Gel Purification

QIAGEN's QIAquick Gel Extraction Kit (Qiagen Inc.) was used to extract DNA band fragments from agarose gels. The DNA was eluted from the agarose it had been separated in so it could be used for transformations. Three volumes of Buffer QG was

added to the extracted gel bands and melted at 50°C for approximately 10 minutes. One volume of isopropanol was then added to the sample. The sample was then transferred to QIAquick spin columns and washed 2 times with Buffer QG and Buffer PE. To elute the DNA from the column, Buffer EB was added to the column and allowed to incubate at room temperature for 1 minute. The DNA was then centrifuged off the column into a microfuge tube, where it remained suspended in the elution buffer.

Biolistic Transformation of Cryptococcus neoformans

Cn strain *pro1*$^{-/-}$ (the *pro1* knock out strain) cells were incubated at 37°C, shaking at 150 rpm for 36 hours before transformation. These cells were then pelleted at 2000 rpm for 5 minutes in an ultracentrifuge and then resuspended with 1.5 ml of milli Q H_2O. Three hundred µl of resuspended cells were spread on two 1M sorbitol YPD plates then allowed to dry at 30°C. While the plates dried, the DNA was prepped for transformation. two – three µg of overlap PCR product was combined with 4 µl of milli Q H_2O in a microfuge tube.

Ten µl of 0.8 µm-gold beads (BioWorld, Gene-bullet), 10 µl of 2.5 M $CaCl_2$, and 2 µl of 1 M spermidine-free base were combined with the DNA solution. This solution was washed multiple times with 100 % ethanol through vortexing and pelleting. The solution was then added to sterile DNA microcarrier membranes and allowed to dry to completion. The DNA was then transformed into the cells through the use of the biolistic

gene gun and the cells were allowed to recover for 3-4 hours at 30 °C. The cells were then transferred to YPD + neomycin plates to begin screening.

Screening of transformants

After transformants were obtained, PCR screens were used to ensure that they received the entire construct in the correct position in the genome. Five PCR screens were run using primers obtained from Fisher Scientific. The first screen used a primer that was 300 base pairs upstream of construct on the 5' side and the 3' primer was placed in the middle of *pro1* and should have resulted in a DNA fragment 2,744 base pairs in length. The second PCR reaction used the same 5' 300 bp out of construct primer as the first while the 3' primer was located in the middle of the noursethricin (NAT) antibiotic resistance marker that should have been replaced by homologous recombination of the *pro1* construct. This reaction should not yield any results, but would give a band at 2486 bp if the transformation was unsuccessful. The third reaction was the inverse of the second PCR screen, with a primer 300 bp downstream of the construct on the 3' side and the 5' primer located in the middle of the NAT antibiotic resistance gene. This reaction also should yield no results if the transformation was successful but would give a band at 2761 bp if unsuccessful. The fourth PCR reaction used the 300 bp 3' out of construct primer and a 5' primer that was located in the middle of *pro1*, and should yield a band at 2,744 bp. The final PCR used both the 3' and 5' 300 bp out of construct primers and would yield a band at 8150 bp (Table 2).

Cloning into a Bacterial Vector

The PCR overlap was cloned into a commercial bacterial vector using the Invitrogen TOPO XL cloning kit. The construct was incubated with dATPs and taq polymerase at 72°C for 15 minutes to allow additional adenine bases to attach to the 5' and 3' ends of the construct. The TOPO XL vector has thymine bases at the ends of the multiple cloning region to allow for a construct to integrate into the plasmid. After incubating the PCR construct with dATPs, the plasmid was introduced into the mixture and allowed to incubate 30 minutes at room temperature. The mixture was added to TOPO 10 chemically competent *Escherichia coli*, then heat shocked at 42°C and allowed to recover with S.O.C. media. These cells were then plated on LB + kanamycin plates for 24 hours before they were screened by PCR and sequencing for inclusion of the *pro1* and neomycin construct. Five constructs were successfully created, however all five had point mutations in the insert (see Discussion).

Appendix A

Table 1. PCR Primers

Primers	***Sequence***
Primer #1	CGATGGCTCGAACCTACCT
Primer #2	TGACAGCGCTAATCTTGCAC
Primer #3	GTGCAAGATTAGCGCTGTCA
Primer #4	ATTGGCATCCAGAGGTTGTC
Primer #5	GACAACCTCTGGATGCCAAT
Primer #6	CCATGACGGAGCCATAAGTT
Nested Overlap 5’	CCGGCCAAGAATGAAACTC
Nested Overlap 3’	CACAGATATGCTGCCAGAGC

Each primer was designed through an online tool, Primer3. They were used in conjuction with PCR to amplify selected regions of DNA.

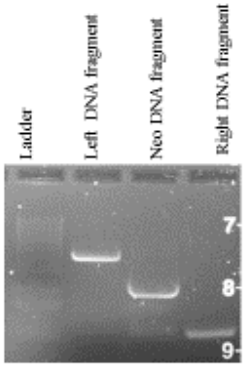

Figure 1

PCRs of the three DNA fragments. 1% Agarose gel showing the left (4,127 base pairs), neomycin (2,000 base pairs), and right (1,062 base pairs) PCR reactions which would be overlapped into a *pro1*/neomycin construct, fluorescing with ethidium bromide. Gel was run for approximately 90 minutes at 130 volts.

Table 2. PCR primers for transformant screen

Primers	***Sequence***
5’ 300 bp out of construct	ATTCACCCACGCTACAGGTC
3’ 300 bp out of construct	CGTCTCGAGGAGCTGTACCT
3’ mid *pro1*	CTTCTGGATGATACAGTGCGAGAGGGGCGAC
3’ mid NAT	AACTCGCCCAACATGTCTATCG
5’ mid NAT	CGTCTTCACCTGCATCTGATT
5’ mid *pro1*	GTCGCCCCTCTCGCACTGTATCATCCAGAAG

Table detailing primers used to screen transformants for the overlap construct through polymerase chain reaction. Primers were designed using Primer3 and ordered through Fisher Scientific.

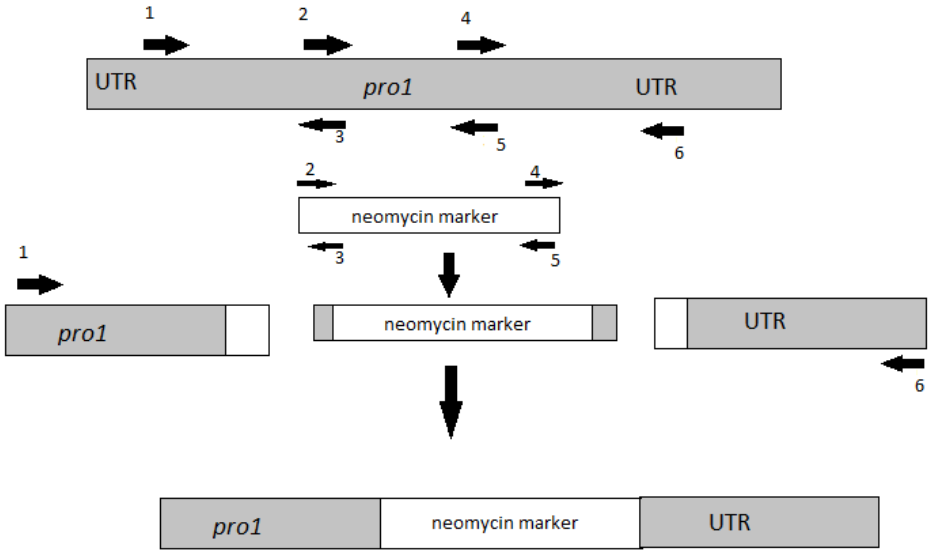

Figure 2

Purification of *pro1* and neomycin PCR pieces for overlap construct. Figure showing the use of primers to purify the left and right pieces of the overlap construct and the neomycin resistance marker. Each number corresponds with a primer found in Table 1.

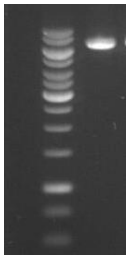

Figure 3

PCR of overlap construct. Agarose gel showing the ~ 7,000 base pair overlap construct, fluorescing with ethidium bromide, ran for approximately 90 minutes at 130 volts. The bands in the ladder correspond to the following sizes: 10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.7 and 0.5 kbp from top to bottom.

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