



METHODS

How to Use a Mutant Library to Identify Genes Required for Biofilm Formation in the Pathogenic *Candida albicans*

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SUMMARY With over 1 billion infections and the causative agents showing critical diseases such as pancreatic cancer, the study of pathogenic fungi has never been more critical. In 2017, the United States spent \$7.2 billion on fungal diseases. \$4.5 billion was allocated to 75,055 hospitalizations, while \$2.6 billion went to 8,993,230 outpatient visits. For *Candida* infections specifically, the cost was \$1.4 billion. Currently, there are few classes of antifungals available, and resistance is growing. The identification of genes required for biofilm formation is essential for new antifungal development. This review details how to identify, verify, and characterize defective biofilm formation mutants in *C. albicans*. This includes how to run an *in vitro* biofilm formation assay, how to create clean deletions using the modified CRISPR-Cas9 system, how to assay to identify the potential causes of the defect, and how to create complementation strains to confirm the mutant defect.

INTRODUCTION

Biofilm formation on medically implanted devices is a severe and ongoing public health concern. A leading microbe that forms deleterious biofilms is the fungus *Candida albicans*. In 2017, the United States spent \$7.2 billion on fungal diseases with \$4.5 billion allocated to 75,055 hospitalizations and \$2.6 billion went to 8,993,230 outpatient visits [1]. Each year the U.S. invests \$2.6 billion in antifungal therapy, yet these antifungals are not always effective [2]. *C. albicans* is an opportunistic pathogen that accounts for 75% of *Candida* infections in humans [3]. While *C. albicans* can cause urinary tract infection, genital yeast infection, and oral thrush, it is also the third most prevalent secondary infection in hospitals, making it common and dangerous [4]. When systemic, *C. albicans* has a 30%-40% mortality rate [3]. This high mortality rate is partly due to the opportunistic properties *C. albicans* has and the ability to form biofilms on surfaces such as a venous catheter, subcutaneous catheter material, and on the mucosal surfaces of oral tissues [4].

The leading cause of systemic infection is the ability of the organism to form a biofilm. A biofilm is a microbial community that forms on surfaces consisting of an extracellular matrix with interspersed yeast and hyphal cell layers [4]. Biofilms form in a sequence that begins with yeast cells adhering to a substrate; yeast cells then spread across the adhered substrate and elongate. During maturation, fungal cells proliferate, initiating the change from yeast to a filamentous cell; once a sufficient amount of hyphae and/or pseudo-hyphae are grown, an extracellular matrix forms and the biofilm has reached maturation [4]. After maturation, any cells that have not adhered are then dispersed into the environment. It is important to note that these steps may happen concurrently in an *in vivo* model [4].

Biofilms are difficult to treat as they are encased in an extracellular matrix, making it difficult for host immune cells and antifungals to penetrate [4, 5]. When formed on artificial devices, the host immune system does not detect biofilm's initial formation, resulting in rapid

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proliferation with little to no resistance. Due to the difficulty in treating biofilms pharmacologically, the current successful treatment is to remove and replace the compromised medical implant [4]. This process creates unnecessary stress on the body; therefore, it cannot be considered an effective treatment or cure. Successive repetition of this process could be very detrimental to individuals who are immunocompromised, in poor health, or elderly.

Given the severity of this situation, our lab aims to find cell wall genes required for biofilm formation and pathogenicity, providing gene targets for pharmacological discovery that target biofilm formation. Components of the cell wall are desirable for pharmacological study as they have few homologous genes in humans, and thus the chance of host toxicity is significantly reduced [6]. Our laboratory uses a mutant library of cell wall genes that are then screened for biofilm formation. Cell wall genes have a direct role in the adherence of *C. albicans* to both the substrate and in cell-cell interactions [4]. Many of the cell wall genes required for prosperous and stable biofilm formation are also required to grow and develop hyphae. In theory, targeting these genes would significantly reduce or hinder the formation of biofilms.

In our laboratory, *in vitro* biofilm assays are performed using medical-grade catheter material to create an environment that encourages yeast cells to proliferate, adhere, and create biofilms *in vitro* in an environment that best mimics the *in vivo* environment. We also work with the control *BCR1*, which has a well-established and clear defect in biofilm formation and adherence – but does not have a filamentation defect. To better understand these cell wall genes' function, the laboratory assays the mutants' ability to grow in different stresses, including osmotic stress, cell wall integrity, hypoxic conditions, and pathogenicity in a model host. In addition, to better understand the biofilm defects, density experiments are performed.

This article describes how a laboratory can 1. use a mutant library to screen for a biofilm defect in hypoxic and osmotic conditions using an established biofilm formation assay, 2. Create clean deletions of mutant genes that have a biofilm defect using a modified CRISPR-Cas9 system, 3. Assay the extent of cell wall stress via plate assays, 4. Determine any cellular morphological changes via microscopy, and 5. How to create mutant complementation strain to confirm mutant defects.

METHODS

IN VITRO BIOFILM FORMATION USING CATHETER SQUARES

Given the significance of biofilms in the clinical setting, identifying novel drug targets is of great importance. The biofilm formation assay is an easy and affordable way to identify mutants from a yeast library, with *C. albicans* being an ideal model organism. It is the largest causative agent of fungal infections. One of the benefits of this assay is that it captures many defects that lead to biofilm formation defects, including adherence, density, hyphal growth, etc., that can be established using microscopy. Thus, many of the causes of a biofilm formation defect are captured in this one assay.

Protocol. Each assay trial requires a blank catheter square, a wild type strain in our lab, we used the *his1*^{-/-} strain *DAY286*. *DAY286* is derived from *SC5314* and was created by reintroducing the removed selection markers to create a mutant library. The mutant library was used to screen for biofilm formation defect mutant. This keeps comparison closer and should reduce any potential false-positive phenotypes. Also, we use the established biofilm formation of defective strain *CJN702*, which is a *bcr1*^{-/-} mutant strain [7]. For all other wells, insertion mutants of interest are assayed. The insertion assay described here was created to include insertion at all of the known non-essential cell wall genes at the time [5] (website www.fgsc.net). The biofilm formation assay can be broken down into four steps. Step one focuses on the preparation of the different components of the assay. Step two of the assay allows for the strain being studied to form on the catheter material and adhere to yeast cells. The third step of the assay allows hyphal growth, biofilm formation, and extracellular matrix secretion. The fourth step details how to record and interpret the results. Below, each step of the biofilm formation assay is divided into a detailed description of how to do the protocol

Equipment:

- 12 well plates (Corning CoStar Clear Multiple Well Plates 3737)
- Catheter material (Cardiovascular Instrument PR72034-06N, Bentec Medical Inc.)
- Incubator
- Tweezers
- Spectrophotometer
- Aluminum foil
- Autoclave
- Bags for hypoxic assay (BD Gaspak EZ pouch system)

Materials:

- PBS
- FBS-Fetal Bovine Serum (Sigma-Aldrich)
- Standard YPD liquid and plated agar
- Ethanol

and an instructional protocol. Figures are included to aid understanding in supplementary figures.

Biofilm part 1: Preparation of materials

Catheter square prep. Catheter squares are cut using a sharp razor to a measurement of 1.5 cm x 1.5 cm (Cardiovascular Instrument PR72034-06N, Bentec Medical Inc.). The squares are washed first in water, followed by ethanol, and allowed to air dry. Squares are placed into aluminum foil and autoclaved on the dry cycle for 60 minutes. It is important to autoclave on the dry cycle. Our lab has found it convenient and results in less contamination if packs of 12 squares are made, thus encompassing one full plate. Squares are then placed into 12 well plates, one per well, and incubated overnight in fetal bovine serum (FBS) on the bench or in the 30°C incubator. This is an essential step that is required for the assay to function properly. The catheter material is now prepared for the biofilm screening.

Protocol

- 1) Catheter sheets are cut into 1.5 cm x 1.5 cm squares.
- 2) Squares are then washed with ethanol, dried, and packaged in tinfoil in groups of 12 (number of wells per plate).
- 3) The tinfoil wrapped squares are autoclaved on the dry cycle.
- 4) The catheter squares are then placed in 2 mL of FBS and incubated for 24 hours at 30° C. (FBS will dry out and ruin the catheter material if left for too long in any condition)

Preparation of strains to be assayed. Individual mutant strains are streaked and grown on standard yeast extract peptone dextrose media (YPD) agar plate for 24-48 hours at 30°C to allow for the formation of full single colonies (Suppl. Fig. 1). After the 24-hour incubation on YPD plates, single colonies are picked and diluted into 3 ml YPD liquid media and incubated overnight, 12-24 hrs, while being shaken at 280 RPMs. As most overnight (O/N) cultures will be saturated and spectrophotometers lose accuracy after an OD₆₀₀ of 0.8, dilution of the overnight cultures will need to be made. In our laboratory, a 1:20 to 1:40 dilution of the overnight samples into 3 mL of YPD is usually sufficient. Resulting ODs are used to calculate the final amount of overnight sample to add to 2 mL YPD to give a final OD₆₀₀ of 0.2. Equation 1 is used to calculate the dilutions using a 1:20 dilution as an example.

Equation 1.

X mL of sample required for final OD₆₀₀ = $\frac{(2000 \text{ mL})(0.2 \text{ (final OD}_{600} \text{ value)})}{((\text{OD}_{600} \text{ of O/N})(20 \text{ (dilution factor))})}$

Strain preparation protocol

- 1) Streak YPD plates with the strain being tested from the working stock should be stored at -80 °C.
- 2) Place streaked plates into the incubator for 24-48 hours at 30 °C.
- 3) Following the incubation period, select a single colony, swab it from the YPD plate, and place it into a test tube containing 3 mL of liquid YPD.
- 4) Place the test tube into an incubator for 24 hours, shaking at 280 RPM at 30 °C.
- 5) After the liquid culture has grown, an OD₆₀₀ is taken to determine how much of the strain (μL) will take mixed with liquid YPD to have an 0.2 OD₆₀₀ in a solution that totals 2000 μL.

Biofilm part 2

The plate containing the FBS treated squares is then removed from the incubator and goes through two rounds of PBS washes (Suppl. Fig. 2). After the washes, each square is placed

into a clean 12 well plate that contains a combination of liquid YPD and the diluted fungal strains to be tested, totaling a volume of 2 mL (Suppl. Fig. 2). The dilution for each strain is determined by calculating the amount of strain needed to achieve an OD₆₀₀ of 0.2, as described above. The squares are then incubated while shaking at 80 RPM at 37° C for 90 minutes.

Protocol

1. Remove the catheter squares from the FBS bath and place them in a well containing 2 mL of PBS, and shaken slowly by hand to remove any impurities or debris from the surface of the material.
2. From the first PBS bath, take the squares and place them into the second PBS bath again containing 2 mL of PBS and shaking slowly by hand.
3. Following the second PBS wash, the square is placed into a well containing a combination of liquid YPD and the liquid culture of the strain being tested that equals 1.5 mL. The strain amount is determined based on the OD₆₀₀ of the strain; the target OD₆₀₀ is 0.2.
4. The plate containing the squares is then placed into the incubator shaking at 80 RPM at 37° C for 90 minutes.

Biofilm part 3

Before removing the squares from the incubator, prepare two new plates containing 2 ml of 1x PBS only in each well. Following the 90-minute incubation period, the squares are treated two different ways depending on whether they are tested aerobically or anaerobically. In both cases, the squares are washed two more times with 1x PBS and placed into new sterile wells containing 3 mL of YPD (Suppl. Fig. 3). The strains being tested aerobically are then placed into the incubator shaking at 80 RPM at 37° C for 24-72 hours. If the strains are being tested anaerobically, the plate containing the squares is placed into an airtight bag along with a GasPak pouch that removes oxygen from the sealed bag. The pouch containing the plate is then placed in an incubator shaking at 80 RPM at 37° C for 24-48 hours.

Protocol

1. Before removing the squares from the incubator, prepare two new plates containing 2 ml of 1x PBS only in each well.
2. After removing the plate from the incubator, the squares are placed into wells containing 2 mL of PBS and shaken slowly by hand to remove any impurities or debris and any cells that have not adhered to the surface of the material.
3. Remove each square, using tweezers, carefully from the first PBS bath and place them into the second PBS bath again containing 2 mL of PBS, shaking slowly by hand.
4. Repeat step 3.
5. Remove the squares from the PBS wash and place them in wells containing 3 mL of liquid YPD.
6. Place the plates in an incubator shaking at 80 RPM at 37° C for 24-72 hours. (If the strains are to be tested in anaerobic conditions, place them in an airtight bag with chemical pouches that remove the oxygen as mentioned previously)

Biofilm part 4

Plates are extracted from the incubator after 24-48 hours to examine the results of biofilm growth. Defects from the aerobic screen will show in the formation, density, and growth of the biofilm (Suppl. Fig. 4). An anaerobic screen will show defects in the biofilm density more pronouncedly (Suppl. Fig. 4). Complete biofilms will appear as an uninterrupted white layer that is adhered to the catheter material's surface (Suppl. Fig. 4). If there is no defect, the liquid YPD will also be clear with no specimen floating around in the liquid (Suppl. Fig. 4). Defects can appear in different ways and can be construed with contamination fairly easily. Common

visual defects are cells clumping together in the YPD due to improper adherence to the catheter surface or incomplete growth causing an uneven biofilm surface (Suppl. Fig. 4). Beyond defects visible to the naked eye, defects are seen using microscopy often involves growth and development defects in the hyphal cells. Contamination within wells includes bacterial growth causing the liquid to be cloudy around the catheter material, or even complete bacterial cell growth in the well (Suppl. Fig. 4). Bacterial contamination can be confirmed using microscopy.

CRISPR-Cas9 DELETION OF TARGETED MUTANTS

Once our assays have identified a biofilm formation mutant phenotype, a clean deletion is created using a modified CRISPR-Cas9 system. A promising mutant is a mutant that consecutively causes an incomplete, defective, or other biofilm formation defect that creates a biofilm-like that is seen in Supplemental Figure 4. The CRISPR-Cas9 is an adaptation of the bacterial CRISPR-Cas9 system found in bacteria used for adaptation of their “immune systems” to create immunity to viruses and plasmids [8]. CRISPR with associated protein Cas9 is a RNA programmable endonuclease that allows for site-specific manipulation of a genome through inducing double-stranded breaks in DNA [8]. The CRISPR-Cas9 system is used to cause double-stranded breaks and induce homologous recombination (HR) [9-11]

In *C. albicans* the CRISPR/Cas9 systems work using a 20 bp guide gRNA sequence to attach to the host genome. To create the guide sequence, use *pV1025* as a template to transcribe the promoter sequence of *SgRNA* with 20 bps of the targeted host gene (*YFG1*) and the termination sequence of *SNR52* [11]. The construct will recruit the CRISPR/Cas9 complex to the targeted site of DNA cleavage. After the guide sequence adheres to the correct site in the genomic DNA, the SgRNA will activate the Cas9-sequence, allowing the *SNR52* to bind and for CRISPR-Cas9 to cut the host DNA at a specified gene location, in our case *YFG1*, causing a double-stranded break in the DNA. The double-stranded break in the DNA will cause stress on the DNA and will cause the cell to unravel and search for homologous pieces of DNA to repair the break [9, 12].

A. Protocol explanation of creation of guide construct

Our laboratory uses a DNA plasmid to PCR to create a guiding construct for the CRISPR-Cas9 complex to reach the desired site of creating a double-stranded break. For this assay, three specific primers are used. Primer 1(P1) is a primer that matches the SgRNA sequence. Primer 2(P2) is annealed with *YFG1*, and it anneals to SgRNA. Primer 3(P3) anneals to *YFG1* and anneals to the termination sequences in *SNR52*. Primer 4(P4) is a primer that matches the *SNR52* sequence (Suppl. Fig. 5A). Examples of several suitable primers to aid in the construction our your own primers can be in *Huang et al. 2018* [10]. It is important to note here that primers can fail to work for various reasons, so new primers may need to be created to get the protocol to function.

As P1 anneals to the sgRNA, P2 is used to transcribe the SgRNA and *YFG1* guide sequences together. P2 contains the 20 bp that becomes the guide for the CRISPR-Cas9 complex and a small primer to the SgRNA(P1); this will anneal the SgRNA promoter with our 20 bp of the guide sequence (Suppl. Fig. 5B). This creates one construct comprised of both SgRNA and *YFG1* (Suppl. Fig. 5B).

Next, *YFG1* and *SNR52* sequences are transcribed together to form one construct. P3 is 20 bp of *YFG1* and a small primer that anneals to *SNR52* (P4). Transcribing these sequences together will allow for the amplification of *SNR52*. This creates one construct that is comprised of both *SNR52* and *YFG1*. This results in two products, 1. 20 bp of *YFG1* and SgRNA, and 2. 20 bp *YFG1* and *SNR52*. This will be the guide sequence (Suppl. Fig. 5B).

To get one complete product, both constructs must be amplified using P1 and P4. Doing so will produce 1 low yield construct. A low yield construct will be difficult to see on a gel, instead purify the product and repeat the PCR experiment to produce a high yield final product (See Suppl. Fig. 5B and 5C).

Equipment:

- PCR machine
- Gel running machine

Materials:

- Primer 1 (P1) upstream of SgRNA on guide sequence
- Primer 2 (P2) connected to YFG, oriented in the downstream direction, anneals to SNR52
- Primer 3 (P3) connected to YFG, oriented in the upstream direction, anneals to SgRNA
- Primer 4 (P4) downstream of SNR52 on guide sequence
- Primer 5 (P5) oriented in the downstream direction and connected to 80bp of the upstream genome of YFG anneals to NAT1 on the upstream end
- Primer 6 (P6) oriented in the upstream direction, connected to 80bp of the downstream genome of YFG anneals to NAT1 on the downstream end
- Primer 7 (P7) oriented in the downstream direction, annealed to the 80bp upstream on the NAT1 construct
- Primer 8 (P8) inside of the NAT1 genome oriented in an upstream direction
- Primer 9 (P9) inside of the YFG section of the YFG genome, oriented in an upstream direction
- Agarose Gel
- 5 isolates for Gel
- Takara prime star buffer
- Plasmid pV1025
- Complete nourseothricin genome
- Nourseothricin plates
- Plasmid containing CRISPR with associated Cas9 construct

Laboratory protocol creation of guide sequences

1. For PCR 1:
 - a. Combine P1 and P3 and the YFG1 and SgRNA, run in PCR machine.
2. For PCR 2:
 - a. Combine P2 and P4 and the YFG1 and SNR52, run in PCR machine.
 - i. This should produce 1 YFG1 + SgRNA product and 1 YFG + SNR52 product.
3. For PCR 3:
 - a. Combine PCR product 1 and PCR product 2, and primers, and run in PCR machine.
 - i. This will create 1 product with YFG1 in the middle, SgRNA on the upstream end, and SNR52 on the downstream end.
 - ii. This will be the low yield DNA construct.
4. For PCR 4:
 - a. Run the PCR product from PCR 3 in the PCR machine again to multiply the product.
 - i. This will create a high yield DNA product.

B. Creation of *NATI* construct

After the CRISPR/Cas9 complex has cleaved the targeted DNA, a marker gene is still required to replace the cleaved *YFG1*. The marker gene is required to confirm that the transformation and the CRISPR/Cas9 complex work properly. To create the desired construct that will result in the deletion of *YFG1*, a marker gene is flanked by 80 bp upstream and 80 bp downstream of *YFG1*, with the *YFG1* ORF being replaced with the marker gene. In our lab, the nourseothricin resistant gene *NATI* gene is used as the marker. The presence of the *NATI* allows for growth on plates containing nourseothricin. Upstream and downstream primers are created containing 80 bp of *YFG1*, upstream or downstream, respectively, and a small primer part that anneals to the *NATI* cassette (Suppl. Fig. 6). PCR reactions are run, resulting in the desired transcript creation (Suppl. Fig. 6).

Protocol creation of marker construct

1. The *NATI* construct
 - a. Combine 80 bp upstream of *YFG* with the *NATI* genome and 80 bp downstream of *YFG* with P5 and P6, run in PCR machine
 - i. This should create one complete *NAI* construct, with the entire *NATI* genome.
 - ii. We use 80 bp upstream and 80 bp downstream of *YFG* because the cut gene will begin unraveling when the double-stranded break happens. When homologous recombination happens, the cell will look for the missing gene and the missing code around it. So, adding 80 bp upstream and downstream of *YFG* to the *NATI* construct allows us the construct to be recognized by the cell as the replacement for the damaged *YFG* segment.

Protocol transformation and screening. Once the guide and marker transcript has been created, a chemical transformation is performed on strain DAY286 [13]. DAY286 is a yeast strain that is *his1-/his1-*, and is the same background as the mutant library strains. DAY286 needs to be transformed with CRISPR/Cas9, the guide, and the marker PCR products in the same transformation. This is done so that only the marker sequence will be integrated into the genome, and after one cell division, CRISPR/Cas9 and the guide sequences will be lost. This is important as it ensures that we are adding only the marker DNA, so all resulting phenotypes are due to the deletion of *YFG1*.

Transformation results. Once transformed into the yeast, CRISPR-Cas9 will be transcribed and translated, giving an active CRISPR-Cas9 construct (Suppl. Fig. 7B). Simultaneously,

the guide sequence will be transcribed; from this, the guide RNA will be created and guide CRISPR-Cas9 to the endogenous genomic *YFG1* sequence, where it will then cut the sequence and create a double-stranded break (Suppl. Fig. 7B). This will be lethal to the cell and results in the repair machinery searching for complementary DNA for repair (Suppl. Fig. 7B). The cell will identify the 80 bp upstream and the 80 bp downstream of *YFG1* associated with the *NATI* genome as its own and use it for HR. This will replace the *YFG1* gene with the *NATI* genome (Suppl. Fig. 7D). Since cells with the *NATI* genome will survive on nourseothricin, an assay using nourseothricin plates can be performed. If *C. albicans* dies, the gene of interest has not been successfully removed. As *C. albicans* is diploid, there is a chance that we will create not only a homologous product with 2 sets of *NATI* but also that we get a heterogeneous product, where only one copy of the gene will be replaced with *NATI* (Suppl. Fig. 7).

C. Analysis of successful creation of a homozygous deletion mutant

To test whether a cell product is homozygous or heterozygous for the *NATI* genome, a PCR verification test will be performed (Suppl. Fig. 8). For this test, primers 7, 8 and 9 will be used. Primer 7 is upstream of the 80 bp upstream *YFG1* on the upstream end of the *NATI* genome. P8 is a reverse primer inside the *NATI* sequence resulting in a sequence that is about 600 bp, and P9 is inside of the *YFG1* genome resulting in a sequence that is about 300 bp (Suppl. Fig. 8). This will produce two potential products: 300 bp (heterozygous) or 600 bp (homozygous)—a gel of the result on 5 isolates. The gel will show if the results are heterozygous, *NATI*, and the target gene, or homozygous, both strands of DNA contain *NATI* (Suppl. Fig. 8).

Ideally, the gels show only copies of the 600 bp primer and none of the 300 bp, which is the wild type gene. The 600 bp is associated with *NATI* being integrated, and the 300 bp is associated with *YFG1*. If the gel shows both sizes, unfortunately, a heterozygote was created. In contrast, if it shows mostly or only 600 bp, then homozygous deletion strain was successfully created. It is important to note that you may need to use more than one guide to ensure that you do not miss an endogenous copy.

Protocol to confirm deletions of *YFG1*

1. This PCR is used to check if there is a heterogeneous or homogenous gene product has been produced.
2. For this PCR, a 600nt primer will be used for the *NAT* gene, and a 300nt primer will be used for the *YFG* gene.
3. To check for the presence of *NATI*, Primer 7 and Primer 8 should be used.
4. Primer 7 will anneal to the 80bp section of the genome upstream of *YFG1*, and Primer 8 (600nt primer) will anneal inside of the *NATI* construct.
5. Primer 7 will also be used to check for *YFG1*. Again, Primer 7 will anneal to the 80bp section upstream of the *YFG1* gene. Moreover, Primer 9 (300nt primer) will anneal inside of the *YFG1* gene.
6. These constructs should be run in the PCR machine, and the products should be checked by running an agarose gel.

CELL WALL SENSITIVITY PLATE ASSAYS

Plate assays allow for multiple different phenotypes to be tested, correlated to a single mutant strain. This procedure will lay out how to screen for mutant phenotypes and how to interpret the assay results. For these assays, the Congo red plate concentration should be 200 µg/ml, and the NaCl concentration used is 1.5 M. All strains need to be grown close to saturation for these assays. Positive controls in these assays will always be the WT cells. For negative controls in these assays based on a literature review, Congo Red assays will use *SSDI*, NaCl assays will utilize *HOG1* [14, 15]. Strains are grown overnight to achieve the highest level of saturation. Each plate will have varying cell densities but will be formalized by diluting each sample to the correct OD₆₀₀.

Understanding the mutant strain is important in these assays as the cell wall is a major influencer in a cell's survival. The cell wall is used for protection, nutrient and chemical

Equipment:

- 96 Well Plate

Materials:

- Mutant of Interest
- Wild Type Strain
- Negative Control
- YPD agar Plate
- Agar Plate: Congo Red (200 µg/ml) or NaCl (1.5M)
- ddH₂O
- Yeast Extract Peptone Dextrose (YPD) Liquid Media

uptake, and even biofilm formation. Congo red assays allow us to test the cell wall's integrity; a mutation in this assay will not survive. This leaves the cell unable to repair the cell wall. NaCl assays help to demonstrate any mutation with osmolarity or ion movements in and out of the cell. This is important because chemical signaling in a cell often stimulates the regulation of many processes within a cell. This can be seen in cell wall regulation as well as biofilm formation.

Cell wall sensitivity assay procedure

1. Overnight cultures from a single colony agar plate should be produced. One wild type, one negative control (*SSDI/HOG1*), and three isolates from each mutant.
Note: The wild-type control should be of the same strain background as the negative control. All genes being assayed should be grown on YPD plates to identify any growth defects in high nutrient environments.
2. A spot from each colony is taken and put into a liquid YPD culture of 2 mL.
3. Each sample should incubate for overnight/12 hours at 30 °C, shaking at 330 RPM.
4. As done in the biofilm procedure, each liquid media culture requires dilution to OD₆₀₀ of no lower than 1.2. All samples that are to be plated must be at the same OD₆₀₀ when plating onto congo red. Note, OD₆₀₀ cannot be done on filamenting cells.
5. In a 96 well plate, add 100 ul of each sample into lane 1 (Fig 9).
6. In lanes 2-6, add 80 ul of ddH₂O. Pipette 20 ul out of lane 1 and add it to lane 2. Pipette up and down thoroughly.
7. Acquire a new pipette tip and take 20 ul from lane 2 and add it to lane 3. Pipette up and down thoroughly once again.
8. Keep repeating Step 6 to produce dilutions through lane 6, each time taking 20 ul from the previously mixed lane.
9. On selected assay plate (Congo Red or NaCl), spot 5 ul for each sample Suppl. Fig. 9. Place supplemental Figure 1 below your plates to add in the application of the sample to the plate.
10. The resulting plates should have decreasing concentrations of cells, as depicted in Supplemental Figure 8.
11. Pictures should be taken every 24 hours for 3 days.

Interpreting cell wall sensitivity results

Congo red. The function of the Congo red plates is to assay for cell wall integrity. The samples will be able to grow at varying ranges when compared to the WT strain. Such growth indicates if a sample can grow in mirror to the WT within this plate, it indicates that the mutant does not function in the cell wall integrity. *C. albicans* uses this ability to create filaments to invade host cells [16]. Plasma membrane proteins are responsible for forming hyphae and are considered a target for disrupting the spread of *C. albicans* with a host. The spread of these hyphae helps dictate the virulence of a strain. To ensure that you do not see a pleiotropic effect, it is essential to compare with the YPD plate to ensure that there is not a growth defect in the mutant under normal conditions.

When investigating the congo red plate, start with the WT strain at the 100% no dilution spot. This strain will produce cultures throughout your spot plate in all dilutions. The density will be greatest in lane 1 and the least dense in lane 6 (Suppl. Fig. 11a). The *SSDI* mutant row will produce a minimal amount of cultures in lane 1. The rest of your lanes will produce little to none in each lane after that (Suppl. Fig. 11b).

Since the isolates are the same strain, it will produce a pattern where each row will look similar. A high density of cultures in a decreasing manner, similar to the wild type, will indicate that cell wall integrity remains unaffected. The strain can be considered to have an undisturbed cell wall integrity pathway. Ultimately the mutant protein in the plasma membrane is not significant to hinder the formation of a biofilm. If the isolate produces culture spots with little density, then it can be deduced that the mutant protein is responsible for the defect in cell wall integrity.

NaCl. NaCl plates test the cell's osmolarity or the ability to maintain ion movement in and out of the cell properly. This attribute is important to the cell's uptake of nutrients and biochemicals from the environment. As each mutant can have unique growth requirements, any changes are important to note and inform how this affects sensing pathways that allow a cell to regulate pathways just at cell wall pathways.

In *C. albicans*, these pathways can stimulate a response in constructing a biofilm, growth, and/or even pathogenicity [17]. Protein kinases play a major role in stimulating this response via a chemical pathway. The PKC-MAPK pathway is responsible for most *C. albicans* cell wall regulation and is thought to be a broad pathway involving complex signaling pathways. This pathway is active when the cell wall is under stress, such as in a hypo-osmotic environment. Mutations in this pathway are sought after to stop *C. albicans*' interactions with the host body.

For your results, when observing your WT strain, you will see decreasing growth across the spot plate. The density will be the greatest in lane 1 and the weakest in lane 2 (Suppl. Fig. 12a). In the negative control, lane 1 will produce some culture and will completely disappear by lane 6. Remember that the *HOG1* is a known NaCl defect mutant (Suppl. Fig. 12b).

When dealing with your isolates, each row should produce a similar pattern. Successful growth from lane 1 to lane 6 indicates that the mutant can regulate the cell wall under osmotic stress conditions. If the isolate starts to decrease and disappears altogether, it can be deduced that the mutant has a gene that disrupts osmotic regulation, with the most probable deficiency in the PKC-MAPK pathway, as it is unable to regulate its cell wall structure. If there is colony growth present in all lanes in a decreasing manner similar to that of the wild-type, it can be assumed. Simultaneously, the mutation may affect biofilm formation. It is not related to the regulation of osmotic stress.

MICROSCOPY

Microscopy is a visual assay to understand the workings within a cell. This technique is done in multiple ways, but in our lab, we start by staining the chitin in *C. albicans*. Chitin exists in fungi as a polysaccharide chain within the cell wall. This gives the cell wall rigidity and structural support. When staining chitin, a fluorescent dye calcofluor white is used to illuminate the structures under a microscope at the same wavelength as 4',6-Diamidino-2-Phenylindole (DAPI).

In our lab, a calcofluor white stain is used and can be obtained by Sigma-Aldrich. As a note, please be careful to use proper protection as this stain is a known carcinogen. This procedure is done in two steps- Part I allows visualization of fungal growth, with part II allowing for the visualization of hyphae growth.

Microscopy procedure

Part 1: Yeast formation test

1. Strains are grown overnight in YDP liquid culture at 30°C.
2. Dilute strain to an OD₆₀₀ of 0.2 with fresh YPD liquid media and incubated at 30°C overnight to an OD₆₀₀ of 0.8
3. Specimens are visualized using a Zeiss Axio Observer Z.1 microscope, settings at 40x NA 1.4 objective. Photographs are saved on Coolsnap HQ² using the Axiovision Zeiss software.

Part 2: Hyphae growth assay

4. Using unstained overnight strains from 1 dilute to an OD₆₀₀ of 0.8 in rpmi liquid medium
Note: YPD results in autofluorescence in most fungal species.
5. Cultures are incubated at 37°C, shaking at 250 rpm for 4 hours.
6. Wash with PBS, then remove from culture.
7. Incubate for 10 minutes next with PBS + 0.1 mg/ml calcofluor white (Sigma-Aldrich).
8. After, samples are visualized with a Zeiss Axio Observer Z.1 microscope with a 63x objective. Note: 40x and 100x are also sufficient, but 63x has been shown to provide

Equipment:

- Zeiss Axio Observer Z.1 Microscope
- Coolsnap HQ² Camera
- Image J (photo processor)
- Suggested PPE: Safety Glasses with side shields, gloves, lab coat, air-purifying respirator mask

Materials:

- Culture for Staining
- Calcofluor White Stain (Sigma-Aldrich)
- YPD Liquid Media
- RPMI Media (Mammalian Cell Culture Media)
- PBS

the most consistently publishable images. The image should be processed by available software within the laboratory.

9. In our laboratory, Image J is used to process the images.

Microscopy results and interpretations. Part I of the microscopy section deals with yeast formation. In Supplemental Figure 13a, normal yeast budding is observed. Each cell has a daughter cell that then pinches off to become its own cell. In contrast, Supplemental Figure 13b demonstrates cells that lack the ability to bud completely from the mother cell. In Supplemental Figure 13c, the cells are unable to bud properly, leaving a mass of cells. This is demonstrative of the mutant phenotype caused by this gene.

Part II of microscopy allows for an evaluation of hyphae formation in a cell. Supplemental Figure 14A shows normal hyphal growth in a wild-type culture stained with calcofluor white. These hyphae are straight with plasma membranes parallel to their opposing membrane. Also, they contain a visible septum that makes the hyphae appear to look more rectangular. Supplemental Figure 14 B, C portrays abnormal hyphae formation. Hyphae are not parallel, and the septum is abnormally shaped and formed. This results in the cells and their hyphae clumped together in a manner not seen in the wild type strain. Supplemental Figure 14 B, C shows just two examples of abnormal filamentation. Other examples include pinched hyphae, lack of or incomplete filamentation, bowing or pinching at septum or the mother-daughter cell junction, and/or septum formation at the mother-daughter junction. Thus, it is always essential to run a wild-type strain at the same time under the same condition as the mutant.

COMPLEMENTATION OF THE WILD TYPE GENE IN THE MUTANT BACKGROUND

The goal of running biofilm screens is to identify genes required for biofilm formation. Biofilm formation assays are conducted to identify such genes. While a result may show a biofilm formation defect, biofilm formation's precise role is not easily determined. Occasionally the defect is due to a variable outside of the mutated gene of interest. These variables include spontaneous mutations during the growth of strain colonies before the screen is run, contamination that affects colony growth, hyphal development, adherence, and/or other variables resulting in false positives. Since false positives can occur in all strains, defects must be confirmed for a specific gene. The confirmation process is done using complementation techniques. Complementation is the process where the mutant phenotype is confirmed to have caused the defect. This is done by inserting a complete wild-type version of the gene and a genetic marker to confirm this step has taken place. The “fixed” genomic strain is assayed for its ability to form a wild-type biofilm, thus confirming that the insertion mutant is the cause of the defect.

Protocol complementation

The complementation process is done using HR to return a wild-type copy of the gene to the genome, thus nullifying the homozygous mutant. Primers are created using the sequenced genome of the strain, which will reinsert the wild type gene. Primers are used to create a construct that will insert the wild type and the *HIS1* gene into the genome to make the previously *his1*^{-/-} strain to become *HIS1*⁺, allowing the absence of histidine in the agar media to be used as a marker of successful integration when grown on agar plates containing histidine (Suppl. Fig. 15). Primers are pulled from both upstream and downstream of the desired gene creating an open reading frame (ORF) containing the gene that is being manipulated. Forward and reverse copies of the primer are made. The first primer is selected from ~1000 base pairs (bp) upstream of the insertion mutant and will be ~20 bp long. The second primer closes the ORF out and is pulled from ~300 bp downstream of the target gene and is ~80 bp long. Note: Ensure that your primers do not lie within the upstream or downstream ORFs of the neighboring genes. The primers that create the ORF allow for modifying the gene by introducing starting points for recombination with primers containing the indicator histidine. Forward and backward primers are also made that have overlapping

base pairs between histidine and the gene being modified. The overlap of the gene is ~80 bp and allows for histidine insertion during replication while restoring proper function to the mutated gene (Suppl. Fig. 15).

After the primers have been created, they can be used in a PCR reaction to create the desired construct. The primers are combined with dNTP's, buffer, host plasmids, polymerase, and water and run through the PCR machine per the manufacturer's instructions. It is important to use a high-fidelity polymerase to reduce the chance of insertion mutations (Suppl. Fig. 15). The low yield product is rerun through the PCR to recover a high yield result of the desired sequence. To isolate the products' desired sequence, perform a chemical transformation, and grown on agar plates lacking histidine. Only transformations that have successfully integrated the wild type and the *HIS1* will grow on the *HIS+* plate. The isolated desired sequence is then run through the PCR machine to replicate at high volumes creating a working strain with a desired genetic sequence. The working strain biofilm screens are run to determine if the initial insertion mutant was the cause of the defect.

Protocol complementation

1. Primer 10 selected by selecting ~20 base pairs 1000 base pairs upstream
2. Primer 11 is selected by selecting ~80 base pairs 300 base pairs downstream.
3. Primer 12, 13 should be selected from the open reading frame (ORF) between the first two primers created and should be ~80 base pairs long and have overlap with the ORF containing the *HIS1* gene inserted.
 - a. A reverse copy of these two forward primers should be made to create primers 14 and 15, completing the primer set.
4. The six primers are mixed in a solution with dNTP's, buffer, host plasmids, polymerase, and water and run through the PCR machine creating a low yield DNA
5. Low yield DNA is rerun through the PCR to create high yield DNA
6. High yield DNA is then grown on *HIS* plates to isolate DNA containing the correct genomic sequence.
7. Colonies that grow on the plate are collected and put through a transformation, where homologous recombination occurs.
8. Transformation products are then integrated with the wild type, *YFG1* gene, and *HIS1* gene.
9. After integration, the product is confirmed via PCR

Conclusions Here we have detailed how to screen a *C. albicans* mutant library, how to create a clean deletion using a modified CRISPR/Cas9 system, how to assay for cell wall integrity, how to perform microscopy to identify potential morphology defects, and how to create complementation strains to verify the mutant phenotype. These assays can be performed on any gene in *C. albicans* and can help identify potential targets for drug discovery.

TROUBLESHOOTING

Observed Problem	Explanation	Solution
Biofilm Assay		
Contamination in liquid YPD after the formation of the biofilm on catheter material.	Contamination is the biggest reason for inaccuracies in screening. This is because of the nutrient-rich media used in this assay.	<ul style="list-style-type: none"> - Autoclave reagents such as YPD & PBS after making a working stock. - Sterilize catheter material regularly, autoclave again if the problem persists. If further problems ensue, replace squares. - Work in a controlled sterilized field such as a hood instead of a Bunsen flame. - Use ethanol often when moving squares into new wells.

Cell Wall Sensitivity Assay		
Both control strains show a complete lack of colony formation or abnormal growth.	The agar plates are treated with NaCl/Congo Red; the concentration of these is pivotal. Yeast cells are sensitive to the amount present in the agar plate.	<ul style="list-style-type: none"> - Check concentrations of NaCl/Congo Red.
Colonies growing other than yeast cells.	Liquid contamination can be a common source of error.	<ul style="list-style-type: none"> - Make sure to use sterile ddH₂O - Make sure plates have nothing growing on them before use. - Also, all reagents used to make plates should be autoclaved.
CRISPR Constructs		
Failed PCR	Some primers may not always work due to the system's complexity, and to be careful with your TMs, as issues with them can cause the primers not to anneal correctly.	<ul style="list-style-type: none"> - All primers should be blasted once done to ensure that the primers do not anneal at more than one location. - If this does not work, new primers should be ordered.
Microscopy		
Cloudy image	Many issues can present themselves during microscopy. Most of the ones presented in this troubleshooting section deal with the cell. Microscopy takes practice and can be fragile.	<ul style="list-style-type: none"> - Make sure to wash cell 3x's before placing culture on the slide. YPD can distort the image of the cells.
Long appearance in the cells		<ul style="list-style-type: none"> - Cells have been grown for too long of a period. Reduce cell growth time.
Clumping		<ul style="list-style-type: none"> - Another indication of too long of cell growth, especially if cultures have been grown overnight.
Popping/Bursting		<ul style="list-style-type: none"> - Reduce the amount of calcofluor white. This stain is damaging to the cell if too much is used.
Unclear/Unfocused Images		<ul style="list-style-type: none"> - Check your exposure - Z-stack can greatly improve the clarity of your images. Check if the lab has access to this program.
Complementation		
Primer Failure	Results in no PCR product because the primers failed to replace the insertion mutant with proper genomic sequence	<ul style="list-style-type: none"> - Repeat experiment at a new integration point in the genome - Be sure to create new primers for this new integration point. - All primers should be blasted once done to ensure that the primers do not anneal at more than one location.
Failure of ORF to express	The newly inserted complement is not being expressed. This can be confirmed using rt-qPCR	<ul style="list-style-type: none"> - Restart project and create new primers

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