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Isolation of conditional mutations in genes essential for viability of *Cryptococcus neoformans*

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Abstract

Discovering the genes underlying fundamental processes that enable cells to live and reproduce is a technical challenge, because loss of gene function in mutants results in organisms that cannot survive. This study describes a forward genetics method to identify essential genes in fungi, based on the propensity for *Agrobacterium tumefaciens* to insert T-DNA molecules into the promoters or 5´ untranslated regions of genes and by placing a conditional promoter within the T-DNA. Insertions of the promoter of the *GAL7* gene were made in the human pathogen *Cryptococcus neoformans*. Nine strains of 960 T-DNA insertional mutants screened grew on media containing galactose, but had impaired growth on media containing glucose, which suppresses expression from *GAL7*. T-DNA insertions were found in the homologs of *IDI1*, *MRPL37*, *NOC3*, *NOP56*, *PRE3* and *RPL17*, all of which are essential in ascomycete yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Altering the carbon source in the medium provided a system to identify phenotypes in response to stress agents. The *pre3* proteosome subunit mutant was further characterized. The T-DNA insertion and phenotype co-segregate in progeny from a cross, and the growth defect is complemented by the reintroduction of the wild type gene into the insertional mutant. A deletion allele was generated in a diploid strain, this heterozygous strain was sporulated, and analysis of the progeny provided additional genetic evidence that *PRE3* is essential. The experimental design is applicable to other fungi and has other forward genetic applications such as to isolate over-expression suppressors or enhance the production of traits of interest.

Keywords Antifungal drug target, Essential gene, Inducible promoter, Proteosome

Introduction

All antifungal drugs used in medical mycology target molecules that are required for fungal viability; however, the identification and characterization of genes that encode essential functions is difficult. The following study provides a new insertional mutagenesis method to identify essential genes in the human pathogen *Cryptococcus neoformans*. While the focus of this research is on potential targets for drug development in human pathogens, the same insertional mutagenesis approach would also be applicable to investigations of other aspects of fungal biology.

The limited numbers of antimicrobial agents and narrowing pipeline to develop new ones are well-recognized problems across the fields of microbial pathogenesis. These are challenges faced in medical mycology, where antifungal drug resistance is also a major global problem (Calderone et al. 2014; Denning and Bromley 2015). The underlying reasons for this are multifactorial. However, antifungal agents are rarely sought in programs aiming to develop antimicrobials and just one new class of antifungal, the echinocandins, has been released in decades. Current interests therefore have focused on agents already approved for other uses now tested for their antifungal properties (Krysan 2015; Roemer and Krysan 2014). Nevertheless, suitable drug targets are needed, that is proteins or metabolites that are crucial for microbe viability.

The *Cryptococcus neoformans* species complex is a monophyletic group of related fungal species, currently with seven proposed species (Hagen et al. 2015), all of which are capable of causing diseases in the human population, in native wild life, and in domestic pets. An initiate estimate made by the United States Centers for Disease Control and Prevention was

~600,000 deaths occurred each year from this disease (Park et al. 2009). A revised calculation was presented at the 9th International Conference on Cryptococcus and Cryptococcosis in 2014, of about half that number (Idnurm and Lin 2015; Park et al. 2014). Thus, cryptococcosis continues to rank as a major causes of mortality attributed to infectious diseases.

Beyond the interest as a major global pathogen, the *Cryptococcus* genus is a member of the Basidiomycota phylum of fungi. The species complex has become one of the few models for molecular biology research in the basidiomycetes. This phylum diverged from the Ascomycota, a group carrying most of the known human pathogenic fungi, somewhere ~800-1600 million years ago (Berbee and Taylor 2010; Taylor and Berbee 2006). Hence, analysis of genes that may be suitable as broad-spectrum targets for new antimicrobial drugs should take into consideration *Cryptococcus* species and other basidiomycetes.

We recently described a forward genetics method to identify essential genes in *C. neoformans* (Ianiri and Idnurm 2015). Transfer-DNA (T-DNA) molecules from *Agrobacterium tumefaciens* were inserted randomly into the genome of a diploid strain of *C. neoformans*. These heterozygous strains were induced to undergo meiosis and sporulation, and the spores analyzed to infer insertions in essential genes by the absence of viable progeny carrying the drug resistance marker within the T-DNA. However, there were several limitations and drawbacks with the method. These included T-DNA insertions that triggered chromosomal translocations and the tendency for T-DNAs to insert between genes and into regions that may be the promoters or 5´ untranslated regions (UTR) of genes, thereby making it necessary to use other methods to test which of the genes on either side of the translocation or insertion is essential for fungal viability. In addition, primary screening required a

substantial amount of labor and media. Thus in the following sections, we describe a new method to identify genes that are essential for viability in *C. neoformans*.

Materials and methods

Construction of shuttle vectors for transformation of multiple species

Two plasmids were created to transform multiple species: the bacteria *Escherichia coli* and *Agrobacterium tumefaciens* and the fungi *Saccharomyces cerevisiae* and *Cryptococcus neoformans* (Fig. 1). The oligonucleotide primer sequences used in amplification during plasmid construction, and strain characterization, are provided in table 1.

Plasmid pGI3 was generated by digesting the *A. tumefaciens* binary vector pPZP-201BK (Covert et al. 2001) with ScaI restriction enzyme, whose site is outside but close to the right border, and by recombining into it the *S. cerevisiae* 2 μ circle and *URA3* gene amplified from plasmid pRS426 (Sikorski and Hieter 1989) using primers ALID2018-ALID2019 (these two primers have chimerical regions with the pPZP-201BK to allow recombination in *S. cerevisiae*). Transformation was carried out with the lithium acetate and polyethylene glycol method (Ito et al. 1983), using equimolar amounts of the PCR product and the linearized plasmid pPZP-201BK, in *S. cerevisiae* strain FY834 [*MAT*α; *his3*; *ura3*; *leu2*; *lys2*; *trp1* (Winston et al. 1995)]. Selection of *S. cerevisiae* transformants was on yeast nitrogen base (YNB) minimal medium without uracil, supplemented with histidine, leucine, lysine and tryptophan to compensate for these other auxotrophies in strain FY834. DNA from one transformant of *S. cerevisiae* was extracted and transformed by heat shock into *E. coli*, with selection on kanamycin (50 μg/ml). Plasmids were prepared from *E. coli* and inserts

sequenced. The resulting plasmid pGI3 contains pPZP-201BK and regions for replication and selection in *S. cerevisiae* (Fig. 1A).

The promoter of the *C. neoformans GAL7* gene, which encodes galactose-1-phosphate uridyl transferase, and the *NAT* cassette, which confers resistance to nourseothricin when transformed into *C. neoformans*, were recombined into plasmid pGI3 to generate the plasmid pGI7, as follows. The *GAL7* promoter was amplified from *C. neoformans* strain KN99α with primers GI114-GI115 (Fig. 1B), which are based on primers used previously to amplify this region (Ruff et al. 2009); primer GI114 has a chimerical region for recombination into plasmid pGI3 and GI115 a chimerical region for recombination with the *ACT1* promoter of the *NAT* cassette. Similarly, the *NAT* cassette (p*ACT1::NAT::*t*TRP1*) was amplified from plasmid pPZP-NATcc (Walton et al. 2005) with primers ai290 and ALID2147, of which ALID2147 has a chimerical region for recombination into plasmid pGI3. PCRs were performed using a High Fidelity Taq polymerase. *S. cerevisiae* strain FY834 was transformed with equimolar amounts of the PCR products (*GAL7* promoter and *NAT*) and the plasmid pGI3 linearized with BamHI and KpnI. Transformants were subject to colony PCR to identify positive clones. DNA was extracted from one strain (Pitkin et al. 1996) and the DNA used to electroporate strain EHA105 of *A. tumefaciens*; transformants were selected on lysogeny broth medium containing 50 µg/ml kanamycin. Plasmids were isolated from *A. tumefaciens* transformants, and the inserts in one plasmid (pGI7) were sequenced to confirm the correct assembly of the PCR fragments within the T-DNA.

***Agrobacterium tumefaciens*-mediated transformation**

A. tumefaciens-mediated transformation (*At*MT) was performed using a standard protocol (Bundock et al. 1995; Idnurm et al. 2004), except that galactose replaced glucose in all media types. That is, the induction medium contained galactose rather than glucose (at 0.9 g/L), and growth of *C. neoformans* transformants after co-cultivation with the *Agrobacterium* was on yeast extract + peptone + 2% galactose (YPG), supplemented with nourseothricin (100 µg/ml) to select for fungal transformants and cefotaxime (100 µg/ml) or tetracycline (15 µg/ml) to counter select the *Agrobacterium*.

Strain KN99α (Nielsen et al. 2003) of *C. neoformans sensu stricto*, i.e. *C. neoformans* var. *grubii* or serotype A, was used as the recipient for transformation of T-DNAs from *A. tumefaciens* strain EHA105 with the pGI7 plasmid.

Identification of mutants with impaired growth and T-DNA insertions in putative essential genes

960 transformants were arranged into ten 96 well plates containing 100 µl of liquid YPG and cultured overnight. 2.5 µl from each culture were spotted onto four agar medium plates: YPD, YPG and YNB + glucose and YNB + galactose. Strains selected from this primary screen as unable to grow on repressive media containing glucose were tested using ten-fold serial dilutions.

Genomic DNA was extracted from fungal strains using a CTAB extraction buffer (Pitkin et al. 1996). Inverse PCR was performed to identify the T-DNA insertion sites. Briefly, genomic DNA was digested with BglII or KpnI restriction enzymes, re-ligated with T4 DNA ligase, and the ligation used as the template for PCR using primers GI120-ai077 with ExTaq

polymerase (Takara Bio, Japan). Inverse PCR products were sequenced, and the sequences used to search with BLAST the *C. neoformans* strain H99 genome at the Broad Institute (Janbon et al. 2014). *C. neoformans* genes were used to search the Saccharomyces Genome Database for the *S. cerevisiae* orthologs.

Analysis of strain 3A11 and the *PRE3* gene

Strain 3A11 was crossed with strain KN99**a**, which is congenic with the *MAT***a** mating type (Nielsen et al. 2003), on Murashige & Skoog 4% agar medium in the dark at 22°C (Xue et al. 2007). Basidiospores were isolated using an Olympus microscope with an attached micromanipulation stage and plated onto YPG medium.

A wild type copy of *PRE3* was amplified with high fidelity Q5 DNA polymerase (New England BioLabs, Ipswich, MA) with primers MAI0170-MAI0171 and cloned into plasmid pPZP-NEO11, linearized with EcoRV-HindIII, using Gibson assembly (New England BioLabs). A clone without errors was identified by sequencing, and the plasmid was electroporated into *A. tumefaciens* strain EHA105 and selected on lysogeny broth + kanamycin media. The *A. tumefaciens* strain was used to transform strain 3A11 as described above. Transformed strains were selected on YPG + G418 (100 µg/ml) + cefotaxime (200 µg/ml).

One copy of *PRE3* was deleted by homologous recombination in the diploid strain AI187 [*MAT***a**/*MAT*α *ade2*/*ADE2 ura5*/*URA5*; (Idnurm 2010)]. The 5´ side of the gene was amplified with primers MAI0172-MAI0173 and 3´ side amplified with primers MAI0174-MAI0175. The *NAT* cassette was amplified with primers ai006-ai290. The three fragments

were joined together into the EcoRV-HindIII site of plasmid pPZP-201BK using Gibson assembly. The gene replacement construct was amplified using primers MAI00172-MAI0175. The DNA was precipitated onto gold beads and transformed into strain AI187 using a PDS/He-1000 biolistic apparatus [Bio-Rad, Hercules, CA (Toffaletti et al. 1993)] and plated onto YPD with nourseothricin (100 µg/ml). Mutant strains were identified by PCR, and then inoculated on Murashige & Skoog medium and incubated in the dark at 22°C to induce meiosis and the production of basidiospores. Basidiospores were micromanipulated onto YPD medium, and allowed to germinate at 30°C. Colonies were phenotyped for genetic markers (*ADE2*, *MAT*, *URA5*) on YNB + adenine and YNB + uracil media, and by crossing to *MAT***a** and *MAT*α parents on V8 juice agar and/or by PCRs specific for each mating type.

Phenotype characterization of T-DNA insertional mutants

Strains were cultured overnight in YPG medium, ten-fold serially diluted, and plated onto yeast extract peptone with 2% galactose or 0.5% galactose + 2% raffinose as carbon sources. One set of plates was incubated at 37°C and another exposed to UV light (120 Jm^{-2}). Seven plates contained stress agents: 0.04 % methyl methanesulfonate (MMS) (Sigma Aldrich, 129925), 0.06 % ethidium bromide solution (EtBr) (Sigma Aldrich, E1510), 0.25 mM menadione (Sigma Aldrich, M5625), 0.25 mM paraquat dichloride hydrate (paraquat) (Sigma Aldrich, 36541), 0.4 mM Luperox TBH70X, 0.4 mM *tert*-butyl hydroperoxide solution (tBOOH) (Sigma Aldrich, 458139), 5 mM hydrogen peroxide solution (H_2O_2) (Sigma Aldrich, 216763) and 0.01 mM cadmium sulfate ($CdSO_4$) (Sigma Aldrich, 481882). Plates were incubated at 28°C for 2 days.

Results

Development of shuttle plasmids

A method for altering the transcription of genes adjacent to the insertion of T-DNA molecules was sought for *C. neoformans* to identify genes that are essential for viability. The strategy was to insert the *GAL7* promoter as close as possible to one border of the T-DNA. The right border almost always inserts three bases, so the *GAL7* promoter was fused adjacent to this border. Plasmid pGI7 was generated that can replicate in *E. coli*, *A. tumefaciens*, *S. cerevisiae* and transform *C. neoformans* (Fig. 1).

This new plasmid pGI7 was used to transform T-DNA molecules into *C. neoformans*, with the transformants cultured on media containing galactose to allow expression of genes adjacent to the insertion event. In the primary screen using transformants arrayed in ten 96-well plates, 17 strains grew poorly on YPD. The same result was obtained on YNB with glucose as the sole carbon source, although the results on this medium were not as clear. Growth of the strains was examined using more accurate serial dilutions, confirmed that nine strains from the screen (0.9%) have reduced growth on YPD compared to YPG (Fig. 2).

Identification of genes mutated in T-DNA insertional mutants

Inverse PCR was performed to amplify the regions flanking the insertion sites of the T-DNA in the *C. neoformans* genome. Sequences were compared to the H99 genome database that features transcriptome information on the genes (Janbon et al. 2014). The positions of the insertions and associated sequences are in figure 3. The genes responsible for the inviable phenotype in two strains 5E12 and 5H11 could not be identified, due to obtaining PCR

reactions only with the native *GAL7* gene. Alternative methods to inverse PCR, or potentially genome sequencing that can be used to identify T-DNA junctions (Chambers et al. 2014; Esher et al. 2015), could potentially identify these insertion events and genes affected. One strain (4E3) inserted into a section of the genome with a complex prediction of genes, including a non-coding RNA molecule. In five strains the *GAL7* promoter inserted at the start of genes whose orthologs are essential in *S. cerevisiae* (Dowell et al. 2010; Giaever et al. 2002). The functions of the genes, *IDI1*, *NOC3*, *NOP56*, *PRE3* and *RPL17*, as characterized in *S. cerevisiae* are provided in table 2. The sixth insertion was into the *MRPL37* homolog, whose mutation blocks respiration in *S. cerevisiae*; *C. neoformans* requires respiration for survival. The homologs of five genes are also essential for viability in a second ascomycete yeast, *Schizosaccharomyces pombe* (Houchens et al. 2008; Kim et al. 2010). One gene, *RPL17*, is not essential in *S. pombe*. However, it is duplicated in both *S. cerevisiae* and *S. pombe*; in *S. cerevisiae RPL17A* is essential while *RPL17B* is not and in *S. pombe* both genes can be deleted individually, suggesting redundancy in function in fission yeast (Kim et al. 2010). The *RPL17* homolog was identified as essential in *A. fumigatus* (Firon et al. 2003). In addition to the model yeasts, the *NOC3* homolog is has been reported as essential in *Candida albicans* and *Aspergillus fumigatus* (Becker et al. 2010; Hu et al. 2007).

The T-DNA inserted before or close to the start of the *C. neoformans* gene. Two strains are associated with chromosomal rearrangements (3F5 and 8A11), because the sequences from each side of the T-DNA after inverse PCR map to different chromosomes. 8A11 has the insertion within the second exon of the *RPL17* gene, which is predicted to remove the first 31 amino acids of the Rpl17 protein. Two strains have insertions in promoter regions and three within the 5´ UTRs (Table 2, Fig. 3). The close proximity to the start of the genes is consistent with these insertions affecting their normal transcriptional regulation.

Additional evidence that *PRE3* is essential in *C. neoformans*

One strain and its mutated gene were examined in more detail. Three experiments provide additional evidence that the T-DNA inserted into the regulatory region of an essential gene. Strain 3A11 has the T-DNA inserted in the *PRE3* gene. Pre3 is an essential subunit of the 20S proteasome. The protein is a peptidyl-glutamyl peptide hydrolyzing enzyme, although this activity is dispensable for cell survival (Enenkel et al. 1994; Heinemeyer et al. 1997).

First, strain 3A11 was crossed to strain KN99**a**, which is congenic to the wild type KN99α but of the opposite *MAT***a** mating type. Basidiospores were micromanipulated and germinated on YP galactose. 24 colonies were obtained, and their growth tested on media with glucose and nourseothricin. Perfect co-segregation between nourseothricin resistance and the reduced growth on glucose was observed. As an independent genetic marker, the mating type of each progeny was determined; the mating type **a** or α properties encoded by this locus segregate independently of the growth and drug resistance properties, indicating that the progeny from the cross reflect recombination (Fig. 4).

Second, the wild type copy of *PRE3* was amplified, cloned adjacent to a marker for G418 resistance, and strain 3A11 transformed by *At*MT with this construct. After co-culture, *C. neoformans* transformants were selected on YP galactose + G418. The transformants were tested for growth on YPG and YPD. Growth was restored to wild type in the complemented strain on YPD (Fig. 5).

Third, a large deletion of *PRE3* was made in the diploid strain AI187 by replacing the open reading frame via homologous recombination, with a cassette conferring nourseothricin resistance (Fig. 6A, B). The heterozygote strain *pre3::NAT*/*PRE3* was plated on Murashige & Skoog medium to induce meiosis and sporulation. Germination of the basidiospores to form colonies was low (10 from 52 basidiospores plated). However, a similar number (13/52) of basidiospores germinated and produced two to four cells before arresting growth (Fig. 6C). Of the ten colonies obtained, only one was nourseothricin resistant, and subsequent PCR analysis indicated it was heterozygous *pre3::NAT*/*PRE3*. Phenotypes controlled by three markers that are on separate chromosomes in strain AI187 (*MAT***a**/*MAT*α; *ade2*/*ADE2*; *ura5*/*URA5*) were tested, showing independent segregation of the mating type (*MAT*), *ADE2* and *URA5* loci (Fig. 6D). The lack of nourseothricin-resistant progeny is indicative that the deletion allele of *PRE3* is deleterious for cell survival.

***In vitro* stress sensitivity phenotypes are associated with mutations of essential genes**

One disadvantage of a complete gene deletion is that this results in an inviable strain. The inducible promoter system has the potential to be manipulated to provide some level of growth, and strains can then be tested for phenotypes that can provide additional information about gene function. Hence, the strains were cultured on plates containing 2 % galactose or 0.5 % galactose + 2 % raffinose and a selection of stress agents or conditions (Fig. 7). On 2 % galactose, all strains except 4C9 showed a minor decrease in growth at 37ºC and this phenotype was augmented when grown on 0.5 % galactose 2 % raffinose medium. Decreasing the amount of galactose had little to no effect on growth without stress, however, greatly enhanced growth phenotypes under stress conditions. This suggests that specific genes also play roles in protecting against specific stresses.

Discussion

Essential genes in *Cryptococcus neoformans* have been identified by a number of approaches. One approach employed a diploid strain, with both targeted and insertional mutagenesis into the strain to mutate one copy of the gene, and those strains were then sporulated and the essentiality of the inserted DNA inferred by the inability to recover those haploid progeny (Ianiri and Idnurm 2015). Targeted mutagenesis is achieved by biolistic transformation; the disadvantage of targeted mutagenesis is that it requires knowledge of the genes to be chosen and since limited information is available for essential genes in basidiomycetes, the choice has to be made based on essential genes known in ascomycetes. Unless aiming to delete all the genes, which is an ongoing project in a haploid strain background of *C. neoformans* (Homer et al. 2016; Liu et al. 2008), this is a restriction in the discovery of novel genes as it effectively focuses on *C. neoformans* genes conserved across fungi. To overcome this issue and to be able to identify on a large scale essential genes of *C. neoformans*, random insertional mutagenesis can be performed using either *Agrobacterium*-mediated transformation (*At*MT) or biolistic delivery of the DNA (Ianiri and Idnurm 2015). However, the use of random insertional mutagenesis in the diploid strain has a number of drawbacks, and hence was a motivation to develop new approaches.

Insertional mutagenesis using *At*MT or biolistic delivery of exogenous DNA is effective for gene function discovery in haploid isolates of *C. neoformans*. To use a haploid strain of *C. neoformans* for mutant generation and to overcome issues related with the use of a diploid strain, for essential genes identification we generated a vector for *At*MT with the inducible *GAL7* promoter. The approach was to generate *At*MT strains in a haploid background of *C.*

neoformans, arrange the strains in 96-well plates, and spot them on inducible (+ galactose) and repressive (+ glucose) media. Strains only able to grow under inducible conditions should have an insertion in the promoter or 5´ UTR regions of genes essential for growth. Further evidence for essentiality can be obtained by Mendelian genetic analysis, by showing co-segregation of the T-DNA with the growth defect and absence of the T-DNA with wild type growth, as illustrated for strain 3A11 (Fig. 6). The Murashige & Skoog medium used for crossing *C. neoformans* supports the growth of the strains without the need to add galactose.

The *GAL7* genes and its promoters have been in use in *Cryptococcus* species for over two decades (Wickes and Edman 1995). The promoter is able to drive expression of adjacent genes, being repressed by glucose and induced by galactose (Baker and Lodge 2012; Ruff et al. 2009). Placing copies of the *IPC1* and *TOP1* genes, encoding inositol-phosphoryl ceramide synthase and topoisomerase, under control of the *GAL7* promoter was used to infer that these are essential for viability (Del Poeta et al. 1999; Luberto et al. 2001). An alternative promoter could be the *CTR4* promoter, which is subject to regulation by the presence or absence of copper ions in the media (Ory et al. 2004).

This approach of construct design and subsequent use as an insertional mutagenesis tool is applicable to other fungal species. The requirement is to produce two DNA fragments; one with a promoter element that enables gene regulation and the other a selectable marker. These can be joined using *in vivo* recombination in *S. cerevisiae* into plasmid pGI3. The regulated promoter could be from one of the genes for galactose utilization, as these are present in many fungi and over long evolutionary time (Okuda et al. 2014), or another source, such as the Tet-Off system (Lai et al. 2016). *S. cerevisiae* transformants are then screened by

PCR, and positive clones are subject to plasmid extraction that are electroporated in an *A. tumefaciens* strain that can be used for *At*MT. This avoids cloning procedures that rely on restriction enzyme sites and can also eliminate steps involving replication in *E. coli*.

A number of advantages are worth highlighting, listed as follows and with the focus on *C. neoformans*. (i) T-DNA insertions that are unlinked to the mutant phenotype are highly unlikely. In *C. neoformans* unlinked mutations occur in about a third to one half of mutants isolated after *Agrobacterium*-mediated transformation (Walton et al. 2005). (ii) The method ameliorates the problem of chromosomal rearrangements that hamper genetic analysis. T-DNA mutagenesis can cause chromosomal rearrangements, yielding genetic segregation results that suggest insertions are into essential genes but are not, plus add substantial challenges in interpreting all the rearrangements to the genome (Ianiri and Idnurm 2015). In addition only one side of T-DNA insertion, i.e. the region next to the *GAL7* promoter, needs to be sought to identify the gene of interest. (iii) In *C. neoformans*, unlike in other fungi, *Agrobacterium* cannot be used to target constructs to their native locus. Hence, there is no skew within the transformant population for insertions into the native *GAL7* locus. (iv) *Agrobacterium* has a tendency to insert the T-DNA into intergenic regions (Walton et al. 2005), that would include promoters or 5´ UTRs and the locations in which placement of a new promoter would work. (v) There is generally precise integration of the T-DNA at the right border of three nucleotides from that border sequence, in contrast to the more variable left border (Michielse et al. 2005). (vi) It is possible to find phenotypes associated with impairing these genes beyond an inviable state, by manipulating the carbon sources.

While there are many advantages to this type of screen, of the seven insertion sites identified, six were in genes known to be essential in other fungi, while the seventh (strain 4E3) was in

an area of gene complexity making it not possible to establish clearly which gene was affected. At least three caveats are worth raising about the ability to identify all essential genes in *C. neoformans* using this approach. (i) Overexpression of certain proteins or misregulated expression could have a deleterious effect on the cell, e.g. by the production of inadvertent artificial "toxic" products or altering the cell cycle. Hence, some essential genes may not be possible to be detected. However, these genes may still be sought, e.g. an alternative screen would be to plate transformants onto YPD and then screen for strains with growth defects on YPG. (ii) Insertions into genes influencing galactose utilization may appear as essential. These genes can be eliminated as essential by creating an independent deletion allele, such as was performed for *PRE3*. (iii) The T-DNA insertions into regulatory regions cannot distinguish between "very sick" and "essential" mutants, because there is some growth of the strains on YPG. In *C. neoformans* the question of essentiality can be further addressed by making a gene deletion allele in a diploid strain and sporulation.

In summary, we report here a method that can be used to find essential genes by the insertion of a conditional promoter into the regulatory regions of those genes. This same type of approach could be extended to other fungi or to other phenotypes whereby the differential regulation of gene expression can provide new information about gene functions.

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Table 1 – Oligonucleotide primers used in this study. Lowercase bases in ALID2018-ALID2019 indicate the chimerical regions for recombination in pPZP-201BK. Lowercase bases in GI114 indicate the region for recombination in pGI3, with the right border in bold and underlined the three bases that usually integrate in the genome; lowercase bases in GI115 are the reverse complement of ai290 used to amplify *NAT*; lowercase bases in ALID2147 indicate the region for recombination into pGI3. Lowercase bases in MAI0170 and MAI0171 are for recombination into pPZP-NEO11, and in MAI0172 and MAI0175 into pPZP-201BK.

Name	**Sequence (5´- 3´)**	**Purpose**
ALID2018	GCTGCACTGAACGTCAGAAGgaaaagtgccacctgaacga	pGI3 generation
ALID2019	CCATTTGTATGTGCATGCCAggtaataactgatataatta	pGI3 generation
GI114	tcttag**gtttacccgccaatatatcctg<u>tca</u>**TCTCAGGAGAGAATTGAGTGC	p*GAL7* amplification for pGI7 construction
GI115	gcttctgtgagtcctcccGATCAGGCGCTGGCTGTGAGTTG	p*GAL7* amplification for pGI7 construction
ai290	GGGAGGACTCACATAAGC	*NAT* amplification for pGI7 construction
ALID2147	tgattacgaattcttaattaagatatcgagGAAGAGATGTAGAAACGA	*NAT* amplification for pGI7 construction
GI120	CGTCATGTTGTCAATTGAGC	Inverse PCR to identify T-DNA
ai077	AGAGGCGGTTTGCGTATTGG	

		insertion sites.
MAI0170	taaaacgacggccagtgccaGCTGTCCATACATCACAGAC	Amplify wild type copy of *PRE3* for complementation
MAI0171	ggcgaattcttaattaagatGGAGTTTAGGTGTTACAAGG	
MAI0172	aacagctatgacatgattacgTCGGTAGGCAGTATGAGG	Amplify 5´ side of *PRE3* for gene replacement
MAI0173	GCTTATGTGAGTCCTCCCCGCACCAATGACAACACC	
MAI0174	CTCGTTTCTACATCTCTTGGTTTTGGGAAGGCAAGG	Amplify 3´ side of *PRE3* for gene replacement
MAI0175	gtaaaacgacggccagtgccaTGATTTCTGCTGCCTCAACG	
ai006	GAAGAGATGTAGAAACGAG	Amplify *NAT* cassette with ai290
ai037	ATGGCGGCCGCCACTCTTGAC	Confirm *PRE3* deletion on the 5´ side
MAI0184	GGTGATGACGGAGATACGAG	
ai270	ACTTCTCGCAAAGTGAATCC	Confirm *PRE3* deletion on the 3´ side
MAI0185	CTTCTCCTTTCTTGCTTCAC	
ai144	GAAAAGGTTACGACAGAGG	Amplify *MAT*α
ai145	TGGGAAGAACTATCAGAGG	
ai150	ACGCAGTGTCAACTGGTC	Amplify *MAT***a**
ai151	CGCACCTTTTCTGTAGGG	

Table 2 – Summary of insertional mutants and the roles of the essential genes.

Strain name	**Gene affected**	**H99 gene designation**	**Position in gene**	**Protein encoded by gene**	**References**
1A12	*NOC3*	CNAG_04298	Promoter	Nucleolar factor required for rRNA maturation and DNA replication.	(Milkereit et al. 2001; Zhang et al. 2002)
3A11	*PRE3*	CNAG_03816	5´ UTR	Proteosome subunit.	(Enenkel et al. 1994)
3F5	*MRPL37*	CNAG_04664	Promoter	Protein in the mitochondrial ribosome large subunit	(Hanlon et al. 2004; Merz and Westermann 2009)
4C9	*IDI1*	CNAG_00265	5´ UTR	Isopentenyl diphosphate:dimethylallyl diphosphate isomerase, required for isoprenoid biosynthesis.	(Mayer et al. 1992)
8A11	*RPL17*	CNAG_06447	Exon 2	Ribosomal protein L17 of the 60S subunit.	(Gamalinda et al. 2013)
8D9	*NOP56*	CNAG_02209	5´ UTR	Nucleolar factor required for rRNA maturation.	(Gautier et al. 1997)

Fig. 1 Construction of two plasmids for transformation of multiple microbes. **A.** Plasmid or DNA maps and the construction process. In step 1, the *S. cerevisiae* 2 micron circle and *URA3* gene were amplified from pRS426 with primers ALID2018-ALID2019 and cloned into the ScaI site of pPZP-201BK, to form plasmid pGI3. In step 2, the *NAT* cassette was amplified from pPZP-NATcc with primers ai290-ALID2147 and the *GAL7* promoter from *C. neoformans* genomic DNA with primers GI114-GI115, and cloned into pGI3 to form pGI7. AmpR and KanR refer to genes conferring resistance to ampicillin and kanamycin in bacteria. For simplicity, replication components in *E. coli* and *A. tumefaciens* are omitted. Tick marks indicate 100 bp intervals. **B.** Sequence of the GI114-GI115 amplicon, including the 1,020 bp immediately upstream of the *GAL7* open reading frame. Sequences corresponding to primers GI114 and GI115 are underlined. The right border sequence in the T-DNA is in bold, with the three nucleotides that usually integrate into the fungal genome in the box with the grey highlight.

Fig. 2 Insertional mutants of *C. neoformans* with impaired growth in the presence of glucose. Nine strains were identified from 960 screened transformed with T-DNAs. 10-fold serial dilutions were plated onto yeast extract peptone (YP) galactose and YP glucose and cultured for 2 d.

Fig. 3 Insertion sites of the T-DNA from pGI7 in the *C. neoformans* strains. **A.** DNA sequences of the wild type (WT) region and around the T-DNA (boxed). Nucleotides deleted during the insertion are in bold in the wild type sequence. The left and right border sequences are in bold in the mutants. **B.** Diagram of the insertion sites relative to essential

genes. The T-DNA insertion sites are black lines perpendicular to and above the sequence. White boxes indicate 5´ UTRs, dark indicates coding regions, and grey indicates introns.

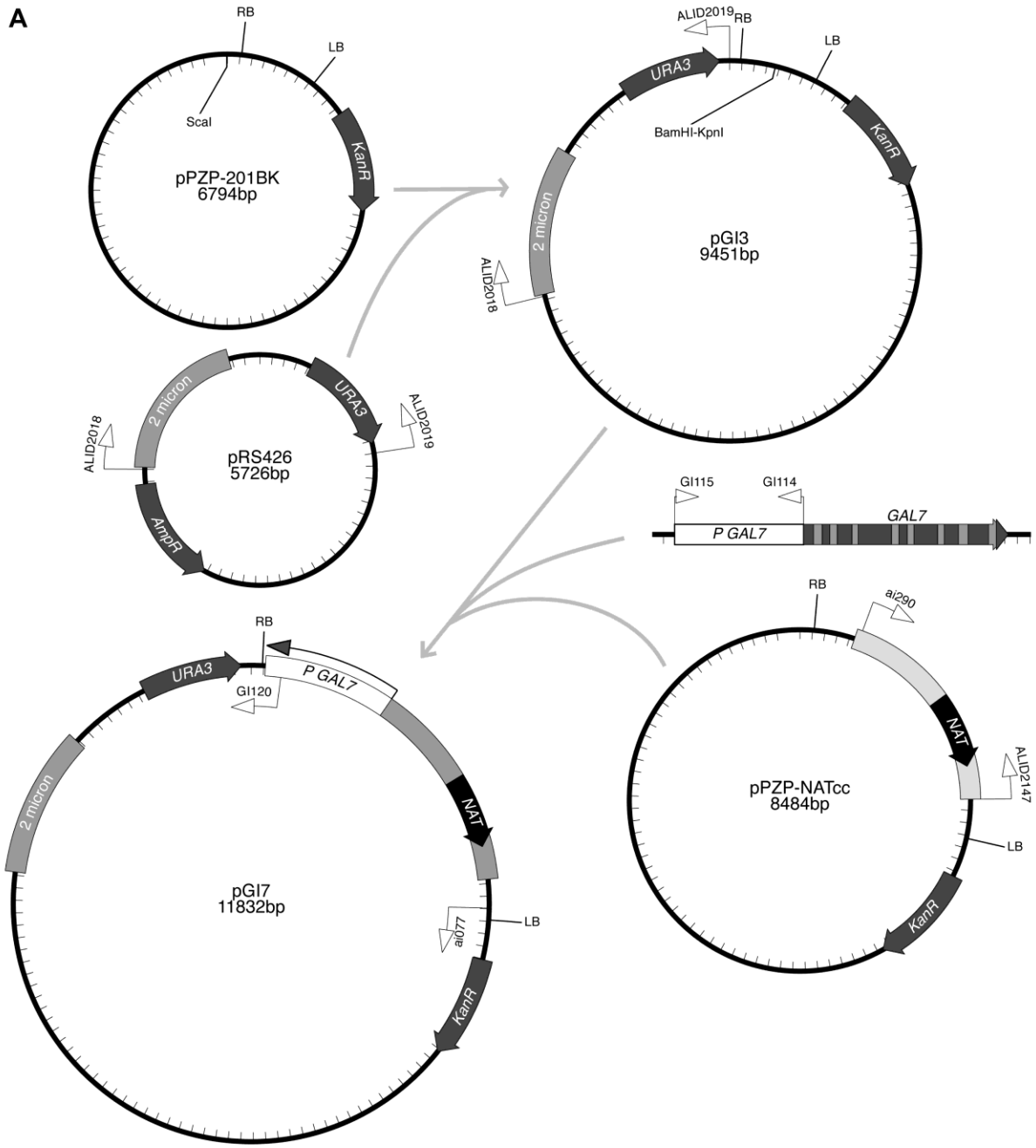
Fig. 4 The T-DNA insertion segregates with reduced growth on glucose in progeny from a genetic cross between strain 3A11 (*MAT*α) and wild type KN99**a**. The three strains at the top are wild type KN99α, 3A11 and KN99**a**. They and the progeny were plated on three media and cultured 2 days. The diagram indicates mating types of the progeny obtained from the cross: grey circles *MAT***a** and white circles *MAT*α.

Fig. 5 The wild type copy of *PRE3* complements the strain 3A11 (*pre3* mutant) growth defect on glucose. 10-fold serial dilutions of strains were grown on YP galactose and YP glucose (wild type KN99α, 3A11 *pre3::T-DNA* and *pre3::T-DNA+PRE3*).

Fig. 6 Independent confirmation that *PRE3* is essential in *C. neoformans* by targeted gene replacement into the diploid AI187 strain, sporulation and analysis of progeny. **A.** Diagram of the wild type and gene replacement alleles, with the positions of primers marked with arrows. **B.** PCR confirmation of correct replacement of *PRE3*. Faint bands in the wild type are non-specific amplification. The sizes are the 1 kb+ ladder (Invitrogen). **C.** Microcolony arrested at a four-cell stage after basidiospore germination on YP glucose agar. **D.** Segregation data of colonies after sporulation of the heterozygous *pre3*/*PRE3* strain; the *pre3*/*PRE3* strain is in the bottom right corner. Mating types are grey for *MAT***a** and white for *MAT*α.

Fig. 7 Growth defect phenotypes are associated with mutations in specific essential genes. Overnight cultures of strains grown in the presence of galactose were 10-fold serially diluted

and dotted onto plates containing stresses and different carbon sources (G = 2% galactose and GR = 0.5% galactose + 2% raffinose). After two days growth the plates were photographed. Concentrations or treatments are: no stress (28°C), 37°C, UV (120 J m^{-2}), 5 mM hydrogen peroxide solution (H_2O_2), 0.25 mM paraquat dichloride hydrate (paraquat), 0.25 mM menadione, 0.4 mM Luperox TBH70X, 0.4 mM *tert*-butyl hydroperoxide solution (tBOOH), 0.04 % methyl methanesulfonate (MMS), 0.06 % ethidium bromide (EtBr) and 0.01 mM cadmium sulfate ($CdSO_4$).



B

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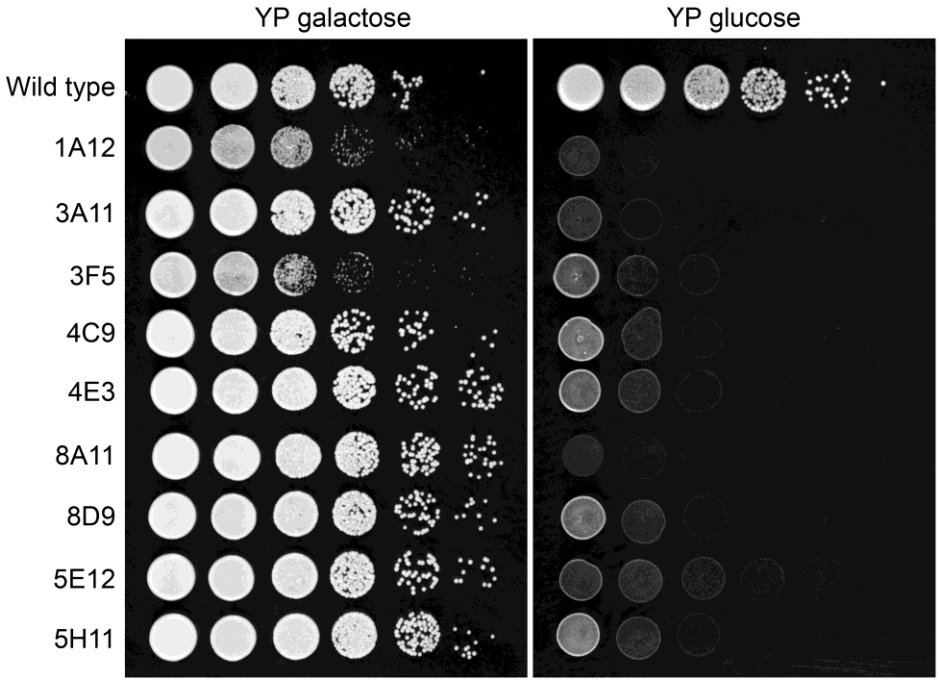

A

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WT   GGCGTTTACCACGTGGTGGGCGCCTACTTCGACCACCACCTCCGCTTGCGTTGGCGCTGCTCGCACTACTACAAGCATGCACGCAAGCGTTCGCGTTTCA
3A11 GGCGTTTACCACGTGGTGGGAGGATATATTGTGGTGTAAAC                T-DNA                TGAACGCAAGCGTTCGCGTTTCA

WT   GTGTAAGGTGGAGGTTGGGC chr 5         /         chr 10 TGCTGCTGGCCTCCGTTTGT
3F5  GTGTAAGGTGGAGGTTGGGCCAGGATATATTGTGGTGTAAAC T-DNA TGATGCTGGCCTCCGTTTGT

WT   TCCTATCATCTTCTTGCCAG--------TGTGTCCTGGCCTT--------TCAACATGTCGACAACTGCTGT
4C9  TCCTATCATCTTCTTGCCAGGATATATTGTGGTGTAAAC T-DNA TGAATCAACATGTCGACAACTGCTGT

WT   CGGCCATCCTTTCTTTCTCT---------TTCTCTACTA-----------GGGCCCGGCTCGATCTTCCT
4E3  CGGCCATCCTTTCTTTCTCTATTGCGGACGTTTTTAATGT T-DNA TGAGGGCCCGGCTCGATCTTCCT

WT   TTCGCCGAGAAGCATGTCAT chr 8         /         chr 13 CTGCCCACATCGCCGCGAG
8A11 TTCGCCGAGAAGCATGTCATCAGGATATATTGTGGTGTAAAC T-DNA TGACTGCCCACATCGCCGCGAG

WT   ATCCTTCGTTCTTGGGTACA--------------AGGA-----------GCGCAATCTCTCTCTAAACAA
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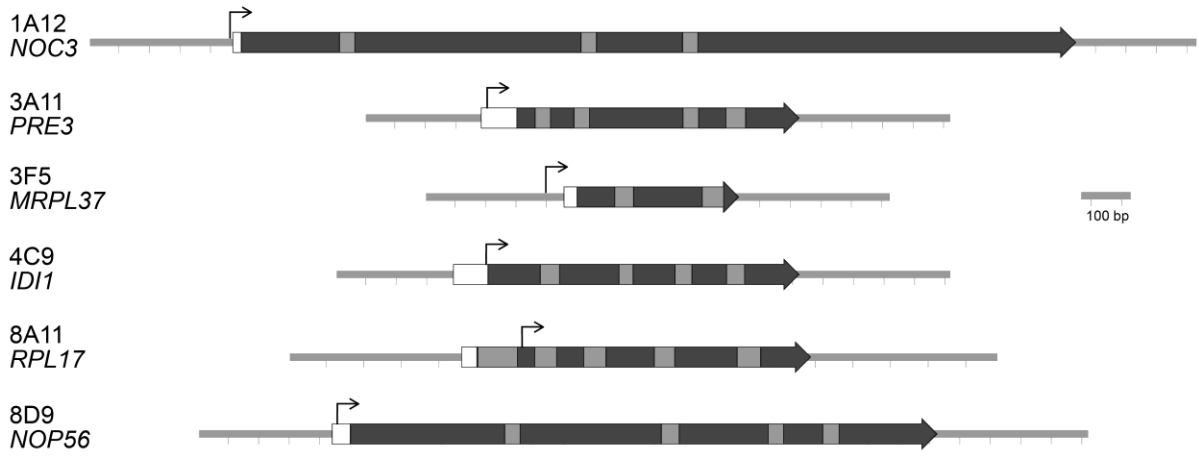
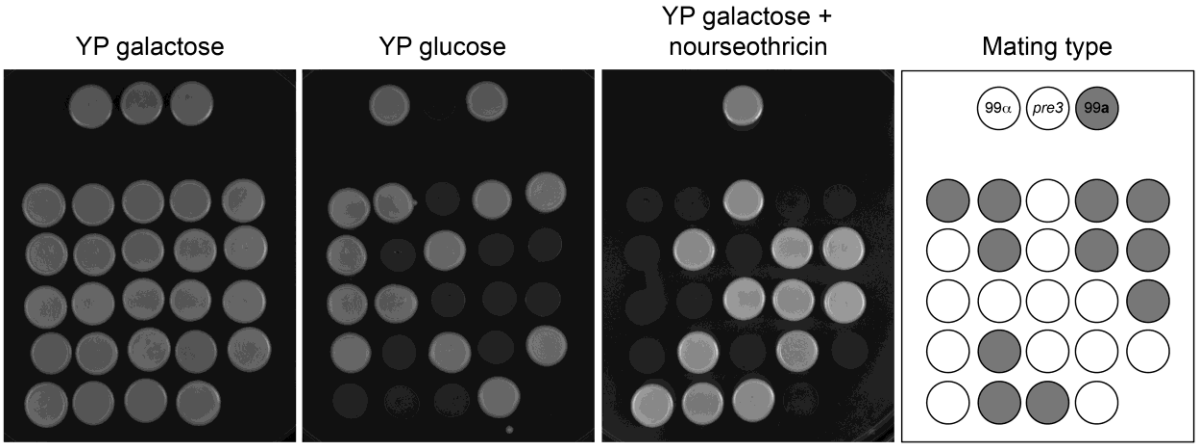
B

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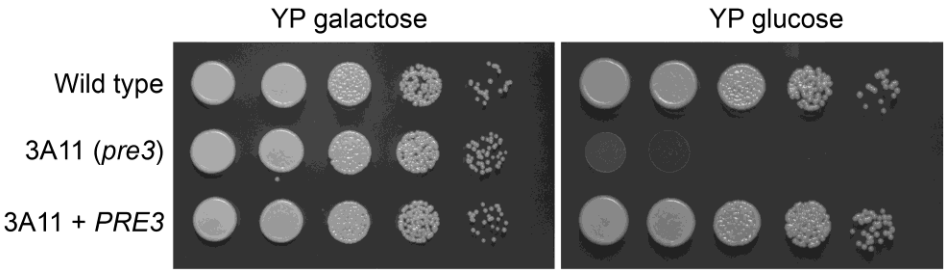
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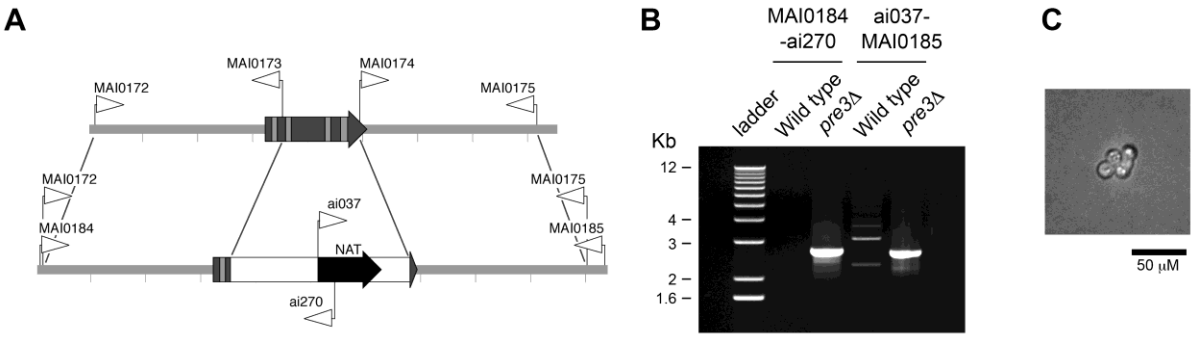
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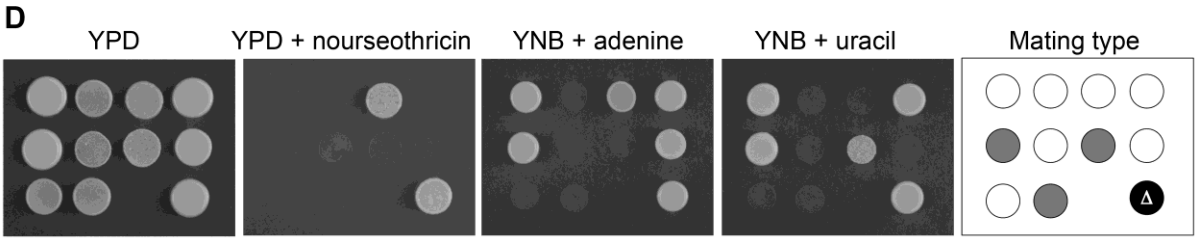
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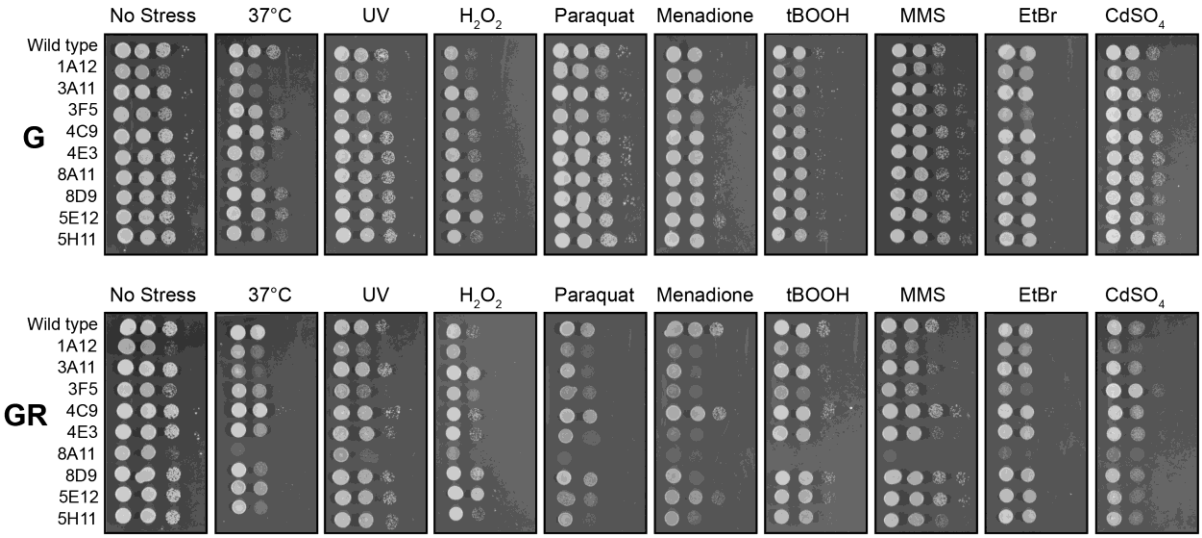
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