Genetics of *Candida albicans*, a Diploid Human Fungal Pathogen

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Abstract

Candida albicans is a species of fungus that typically resides in the gastrointestinal tracts of humans and other warm-blooded animals. It is also the most common human fungal pathogen, causing a variety of skin and soft tissue infections in healthy people and more virulent invasive and disseminated diseases in patients with compromised immune systems. How this microorganism manages to persist in healthy hosts but also to cause a spectrum of disease states in the immunocompromised host are questions of significant biological interest as well as major clinical and economic importance. In this review, we describe recent developments in population genetics, the mating process, and gene disruption technology that are providing much needed experimental insights into the biology of *C. albicans*.

INTRODUCTION

Parasexual cycle: cycle of mating followed by random chromosome loss; compare with the traditional sexual cycle of mating followed by meiosis

Commensal: organism that resides in a host without causing disease

MLST: multilocus sequence typing

Clade: highly related group of organisms

First described in 1839, the ascomycete now known as *Candida albicans* is a normal resident of the gastrointestinal tract of humans and other warm-blooded animals (56). It is also the most common human fungal pathogen. Although typically asymptomatic, *C. albicans* can proliferate even in healthy people to cause circumscribed infections of the skin, nails, and mucous membranes. In patients with deficient immune systems—because of inherited disease, malignancy, concurrent infection, or medical intervention, this same yeast can behave as an aggressive pathogen, attacking virtually any organ system, and leading to death in as many as 50% of cases of bloodstream infection (19, 25, 81).

Despite its medical importance and inherent interest to biologists, progress in understanding *C. albicans* has been relatively slow compared to some other microorganisms. In part, this situation has arisen from the difficulties of using genetics as an experimental approach to study *C. albicans*. Because it is diploid and lacks a complete sexual cycle, conventional genetic analysis is simply not possible. Despite these obstacles, researchers in the *C. albicans* field have, over the past 20 years, developed alternative strategies that have enabled many genetic approaches, including large-scale genetic screens.

In this review, we consider two broad aspects of *C. albicans* genetics: first, as a part of the natural life cycle of the organism and, second, as a research tool. We begin by summarizing population studies that reveal a largely clonal structure for the species. Next, we present experimental evidence indicating that *C. albicans*—once thought to be asexual—has a parasexual cycle where mating is followed by concerted chromosome loss. We then discuss studies showing that chromosome loss and other forms of genetic instability are important features of this organism. In the second part of the review, we discuss *C. albicans* genetics as a research approach, emphasizing methodological advances that have facilitated the first forward genetic screens in this diploid organism.

GENETICS AND THE LIFE CYCLE OF *C. ALBICANS*

Population Studies of *C. albicans*

Unlike the majority of fungi, including most other human fungal pathogens, *C. albicans* is not commonly isolated from environmental sources such as decaying vegetative matter or soil. Instead, the organism appears to propagate mainly as a commensal of numerous warm-blooded animals (14, 56). Population genetic studies of *C. albicans* have therefore relied primarily on analysis of clinical isolates obtained from hospitals. Several groups have developed methods for "typing" these strains to infer their degree of genetic relatedness. These methods [reviewed in (9, 60)] range from multilocus enzyme electrophoresis (MLEE) to DNA-based comparisons, including DNA fingerprinting (commonly using the moderately repetitive DNA sequence, Ca3), restriction length polymorphism, ABC typing (based on the number and size of rRNA internally transcribed spacer regions), and multilocus sequence typing (MLST, based on DNA sequences of sets of housekeeping genes). The DNA fingerprinting and MLST methods have converged on a set of five major clades that account for the majority of tested isolates (**Figure 1**) (57, 73). Clade 1 is the largest group of related strains, as determined by both methodologies, and includes the clinical isolate SC5314, whose genome was the first to be sequenced (32); most experimental research in *C. albicans* is based on isolates from this clade. More recently, the genome of a member of Clade 6 (57) has also been sequenced (**http://www.broad.mit.edu/annotation/genome/candida_albicans/Home.html**); this strain, WO-1, was the first strain demonstrated to undergo the white to opaque phenotypic switch (72).

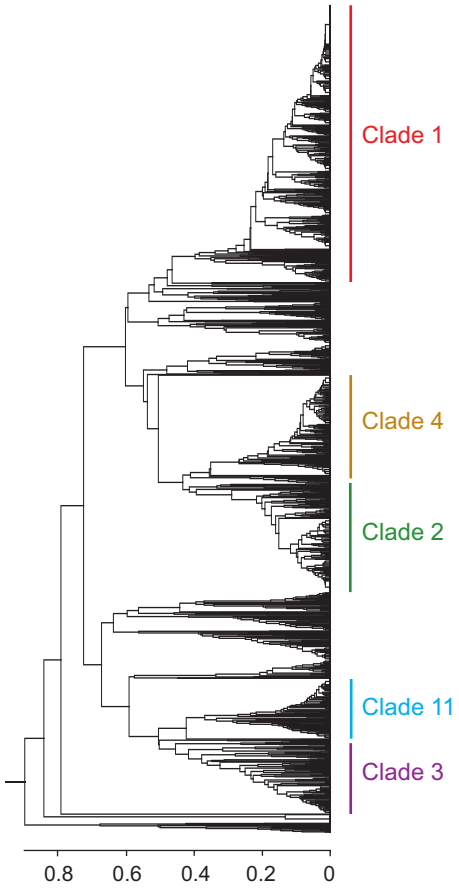

Figure 1

Population structure of *C. albicans*. UPGMA (unweighted pair group method with arithmetic mean) dendrogram drawn from MLST data for 1433 separate isolates of *C. albicans*, based on p-distance analysis. The positions marked with thick vertical lines indicate the five main clades, which together account for 74.9% of the 1433 strains analyzed. (Figure kindly provided by Frank Odds.)

Strain-typing analysis has shown that *C. albicans* propagates primarily in a clonal manner, such that essentially "pure" isolates of distinct clades can be cultured from patients in a single hospital or geographic area (2, 23, 62, 76, 87). Efforts to discover associations between specific clades and clinically important phenotypes have identified a clear association between Clade 1 and resistance to the antifungal drug, flucytosine (61, 76). Putative associations with other variables such as geographic location or anatomic site of infection are less well established or less consistent among various studies [e.g., (73) versus (76)], perhaps because of differences in composition of the strain collections. These questions should be resolved as data accumulate for larger numbers of strains.

SNP: single nucleotide polymorphism

Despite the existence of genetically distinct clades, recent MLST analysis of approximately 1400 isolates of *C. albicans* indicates that recombination between DNA haplotypes is more common than previously suspected (57). In this analysis, 170 single nucleotide polymorphisms (SNPs) were identified among 7 housekeeping genes. When phylogenetic trees were created for each of the seven genes, no significant correspondence between any of the trees was observed, implying that the genes had evolved independently rather than congruently, as one would expect for a strictly clonal mode of evolution. It is possible that *C. albicans* undergoes sexual reproduction; however, other mechanisms could also account for the observed patterns of SNPs (57).

How can one rationalize a significant rate of recombination in *C. albicans* with the maintenance of independently evolving clades? Insight into this apparent paradox is provided by studies that examine patients with serial *C. albicans* infections. Whether the infections are superficial, such as recurrent vaginitis in healthy women (42, 48, 79) and thrush in AIDS patients (5, 47, 68), or successive superficial and deep infections of normally sterile sites (58), the vast majority of infections in a given patient will result from a single strain, with few examples of strain replacement. Thus, most adult humans may be colonized with just a single strain of *C. albicans*. Should an individual host develop an immune deficiency because of inherited disease, malignancy, infection (such as AIDS), or therapeutic intervention, the same strain of *Candida* that previously existed as a commensal now acts as an opportunistic pathogen to cause virulent disease. Although mutation and

recombination may create variation among cells from the starting strain, as discussed above, there appear to be few opportunities for genetically diverse strains (i.e., those from different clades) to encounter each other and exchange genetic material. Whether *C. albicans* reservoirs other than the adult human host allow for greater mingling of genetically distant strains remains an open question.

Mating and the Parasexual Cycle in *C. albicans*

For more than a century, *C. albicans* was thought to be "imperfect" or asexual, based largely on the inability to identify mating forms in the laboratory. This idea was put into question with the recognition that a region of DNA, the *M*ating *T*ype-*L*ike Locus or *MTL*, strongly resembles the well-characterized *MAT* locus of the budding yeast, *Saccharomyces cerevisiae* (28). The *MAT* locus in *S. cerevisiae* exists as either of two alleles, **a** and α, that determine the mating type of haploid cells through expression of distinct sets of transcriptional regulators [reviewed in (7, 44)]. **a** type cells, which possess the *MAT***a** allele, can mate only with α type cells, which possess the *MAT*α allele, and vice versa (**Figure 2*a***). The product of mating is a diploid cell that is heterozygous at the *MAT* locus and cannot mate. Instead, under

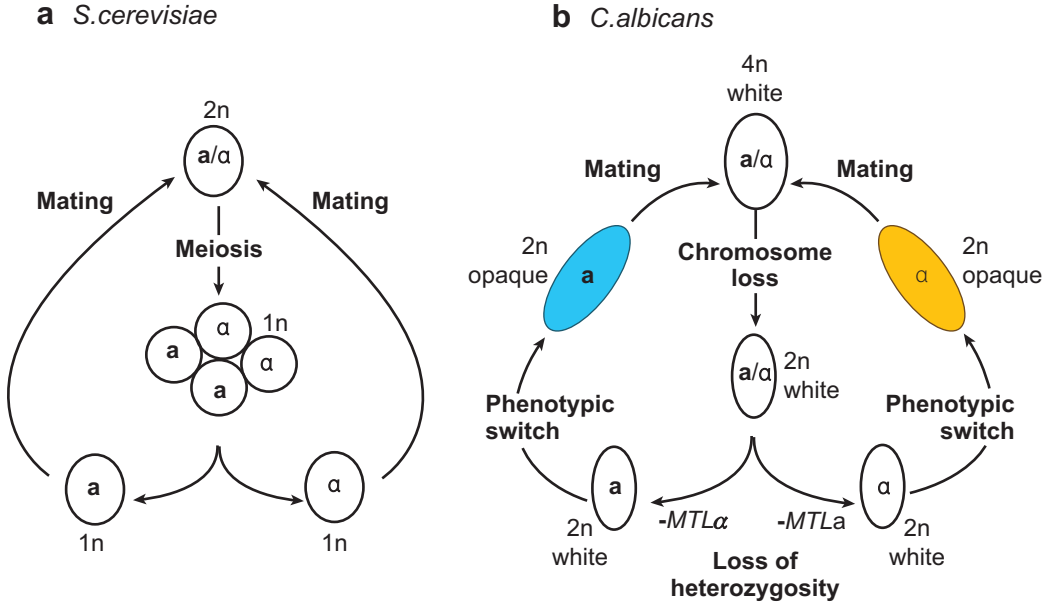

Figure 2

Sexual and parasexual cycles of *S. cerevisiae* and *C. albicans*. (*a*) Sexual cycle of *S. cerevisiae*: Haploid cells (1n) exist in either of two mating types, a or α, depending on the genes present at *MAT*. Mating between **a** and α cells results in **a** diploid cell (2n). Diploid cells can persist and grow indefinitely until, under stringent environmental conditions, they undergo meiosis and sporulation to regenerate haploid cells. (*b*) Parasexual cycle of *C. albicans*: Diploid cells (2n) of *C. albicans* are typically heterozygous at *MTL*. On certain laboratory media such as sorbose, laboratory strains undergo loss of one copy of Chromosome 5, thereby eliminating either the **a** or α allele, to create α or **a** diploid strains, respectively. At **a** frequency of approximately 10^{-4}, **a** and α strains undergo an epigenetic switch from the white to opaque phase, a transition involving hundreds of changes at the transcriptional level and a dramatic change in cell morphology. Diploid **a** and α opaque cells mate efficiently to create tetraploid (4n) **a**/**a**/α/α cells. Tetraploid cells can grow stably or, under stressful environmental conditions (e.g., PRE-SPO laboratory medium), undergo concerted chromosome loss to return to diploidy (2N).

appropriate environmental conditions, diploid cells undergo meiosis, thereby recreating haploid cells of **a** and α mating types.

Subsequent work from two laboratories has shown that, if *C. albicans* strains are engineered to express only one allele of *MTL* (either through targeted gene disruption of the opposite allele or though selection for loss one copy of Chromosome 5, on which *MTL* resides), then the engineered **a** and α strains can mate at a low frequency, both on laboratory medium and in a mouse model, to produce tetraploid cells (**Figure 2*b***) (29, 45). Analysis of *MTL* among more than 300 clinical isolates, representing the 5 major clades, has revealed that 3%–7% of natural isolates are homozygous at this locus (39, 41).

A final requirement for mating in *C. albicans* involves an epigenetic switch between white and opaque cell types. First described by Slutsky et al. (72), the white-opaque transition was initially characterized by morphological differences that arise occasionally among select *C. albicans* strains; "white" cells are oval in shape and give rise to shiny, domed colonies on solid laboratory media, whereas "opaque" cells are elongated and give rise to duller, flatter colonies. Expression analysis of the two morphological types has shown that this phase transition involves coordinated changes in expression of more than 400 genes (37, 77). Only strains that are homozygous or hemizygous at *MTL* (i.e., **a** and α cells) can undergo the white-opaque switch; furthermore, only opaque cells mate at high efficiency (49). *C. albicans* thus preserves the overall logic of the *MAT*-controlled mating system in *S. cerevisiae* but with two added complications: the need for diploid cells (*a*) to undergo loss of heterozygosity at *MTL* and (*b*) to convert from the white to the opaque physiological phase. In retrospect, the historical failure to observe mating probably results from both of these features: Most laboratory strains are heterozygous at *MTL* and therefore incapable of mating and white-opaque switching.

Whereas diploid cells in *S. cerevisiae* complete the sexual mating cycle by undergoing meiosis (**Figure 2*a***), there have been no descriptions of a similar process in *C. albicans*. Failure to observe a process, of course, does not rule it out. We do know that *C. albicans* has an alternative to meiosis. Growth of tetraploid *C. albicans* cells on certain laboratory media induces a concerted loss of chromosomes, during which a significant fraction of cells return to the diploid state, thus completing a parasexual cycle (6). On a practical note, researchers could theoretically exploit this parasexual cycle for purposes such as strain construction and linkage analysis (see below); in reality, however, such strategies are probably too cumbersome to be generally useful.

White-opaque switching: epigenetic alteration between two distinctive cell types in *C. albicans*

The purpose of mating in most organisms is assumed to be the generation of genetic diversity. Given that very little genetic exchange is evident among clades of *C. albicans*, the role of its elaborate parasexual cycle remains mysterious. One suggestion is that the purpose of mating is to produce limited diversity within an individual host (31). Because diploid strains contain a high degree of heterozygosity between chromosome pairs in *C. albicans* (26, 32, 88), mating and random chromosome loss between **a** and α variants of the same strain could result in a reasonable amount of diversity. Although such variants might not survive long outside the host, they could serve unique functions within specific niches of the host to maintain a chronic infection. Another possibility is that mating serves a specific role in host-pathogen interactions that is unrelated to the generation of genetic diversity. For example, Magee & Magee have suggested that the role of mating is to regulate white-opaque switching, providing populations of cells (**a** and α) that can switch as well as populations (**a**/α) that are blocked for switching (46).

Genetic Instability in *C. albicans*

The *C. albicans* genome is remarkably plastic. The parasexual cycle described above requires two gross genetic changes: loss of heterozygosity at *MTL* to generate **a** and α diploid strains, and concerted loss of chromosomes to

convert a tetraploid product of mating back to diploidy. Rustchenko-Bulgac et al. have published a series of studies documenting frequent, spontaneous chromosome alterations among laboratory and clinical isolates of *C. albicans*. In one study, morphological mutants associated with chromosome alterations detectable on pulse-field gels occur spontaneously at a frequency of 1.4% (67); in another study, the authors associate certain chromosomal alterations with gain and loss of carbon assimilation functions (66). In the case of sorbose utilization, the mechanism of altered carbon usage is understood. Growth of *C. albicans* on sorbose selects for loss of one copy of Chromosome 5, because that chromosome contains a negative regulator of the *SOU1* gene that is required for growth on sorbose (24, 30). Another type of chromosomal alteration was described by Selmecki et al., who found that reversible formation of an isochromosome, consisting of two left arms of Chromosome 5 flanking a common centromere, is associated with high-level resistance to the important azole class of antifungals (69). Chromosome instability may not be unique to *C. albicans*; for example, expression profiling of 300 deletion strains of *S. cerevisiae* revealed aneuploidy of whole chromosomes or chromosome segments in 8% of these laboratory strains (27). A diploid fungus, particularly one that has not been severely domesticated, may exhibit even greater tolerance for such events.

GENETICS AS A RESEARCH TOOL IN *C. ALBICANS*

Traditional genetic studies utilize random mutagens—such as chemical mutagens, UV light, or transposable elements—to introduce changes throughout the genome. Such mutagenesis schemes can be comprehensive in scope (in the limit, approaching all the genes in the genome) and can give rise to a variety of mutant types (including loss of function, gain of function, and conditional). Analysis of mutants that alter gene function rather than destroying it can be especially helpful in cases in which a gene is essential, a gene encodes a multifunctional protein (where specific mutations may unlink one function from another), and a gene participates in a redundant pathway (where a dominant gain-of-function mutation may best reveal a role in the process of interest). Nevertheless, even in organisms such as *S. cerevisiae* that are amenable to classical mutagenesis, large collections of gene deletion mutants have proven highly useful (22). Targeted gene disruption results in a highly compact mutant library, since each gene in it is represented by only one mutant. Furthermore, if a mutant is found to have a phenotype of interest, the identity of the disrupted gene is immediately available.

The obligate diploid genome of *C. albicans* has been a significant barrier to both classical and targeted genetic approaches, because recessive mutations must be introduced twice to produce an observable phenotype. Until recently, genetics in this organism was limited to studies of small sets of deletion mutants generally involving genes already suspected to affect the process of interest. In the past several years, however, technological innovations have permitted construction of large numbers of different kinds of mutants for use in forward, non-"candidate-based" genetic screens.

Techniques for Gene Disruption in *C. albicans*

The ability to selectively knock out genes in *C. albicans* was a major milestone as, for the first time, investigators could assess the contributions of individual genes to many of its important behaviors, including pathogenesis. As developed by Fonzi & Irwin (21), based on similar techniques in *S. cerevisiae* (1), the "Ura Blaster" method of gene disruption utilizes homologous recombination to replace the chromosomal copy of a target gene with a linear fragment of exogenous DNA whose ends are homologous to DNA flanking the target gene (**Figure 3**). Rare gene replacement events are selected by plating transformants on medium lacking uridine, since *URA3*

(encoding the uracil pathway biosynthetic enzyme, orotidine-5′-phosphate decarboxylase) is included in the disruption construct. Furthermore, because *URA3* can be counterselected on medium containing 5-fluoroorotic acid [5-FOA; (1, 8)], it is possible to "recycle" the selectable marker for use in disrupting the second allele of the target gene (**Figure 3**).

The "Ura Blaster" method was quickly embraced by the *C. albicans* scientific community, and dozens of knockout mutants were successfully created to address key questions such as which genes are required for virulence. Several groups developed variations of the basic protocol, including substitution of PCR for cloning to create the gene disruption construct (18, 83, 84). Although much has been learned from mutant strains constructed by these strategies, there were early indications that such strains are not always optimal for studying important phenotypes of *C. albicans*, such as virulence in a mouse model, morphogenesis, and adhesion. The problem arises because a number of these processes require high levels of *URA3* expression (4, 10, 16, 33, 75), and expression of this gene can vary greatly depending on its chromosomal context (10, 16, 38, 70). Recent studies—including those by the originator of the Ura Blaster technique (70)—have documented apparent defects in virulence, morphogenesis, and adhesion that result not from disruption of a particular target gene but from poor expression of *URA3*; as many as one third of published knockout mutants may be affected by this problem (10). Moreover, the 5-FOA used for *URA3* "recycling" is now known to be a significant mutagen that can cause major chromosomal abnormalities in addition to the intended one (80). These potential complications notwithstanding, many of the early conclusions based on mutants constructed using this technique have subsequently been verified by other methods.

There are several current strategies for ensuring adequate *URA3* expression in deletion mutants. One solution is to integrate the marker at a standard locus, where gene expression should be more consistent (51, 75).

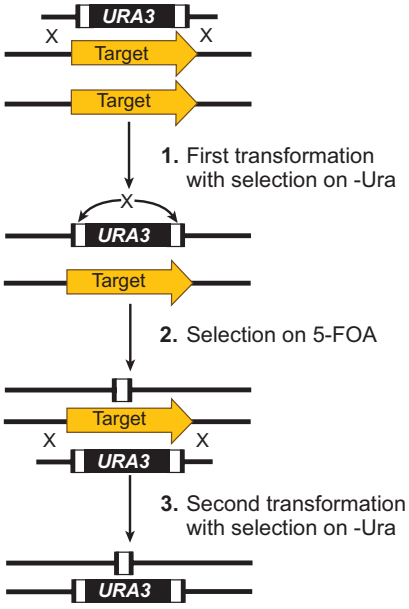

Figure 3

"URA Blaster" method of targeted gene disruption in *C. albicans*. This method results in homozygous gene disruption after two rounds of transformation (21). The steps are as follows: 1. Transformation of a strain of *C. albicans* that is auxotrophic for uracil (*ura3/ura3*) with the gene disruption fragment. This linear piece of DNA has 5′ and 3′ ends that are homologous to sequences that flank the target gene. It also contains a wild-type version of the *URA3* gene from *C. albicans*, flanked by direct repeats of the hisG sequence from *Salmonella typhimurium*. In a minority of transformants, recombination will occur between the disruption construct and sequences flanking the target gene (X's in the figure); these are selected on medium lacking uridine (-Ura). 2. Strains that have undergone disruption of the first allele of the target gene are passaged on medium containing 5-FOA, a chemical that selects against the wild-type copy of *URA3*. Strains that have deleted *URA3* via intramolecular recombination between the hisG flanks will grow on this medium. 3. Transformation with the disruption fragment is repeated, this time to disrupt the second copy of the target gene.

Another solution is to employ alternative auxotrophic markers that do not affect phenotypes such as virulence (55). In addition, dominant selectable markers conferring resistance to the antibiotics mycophenolic acid (74, 86)

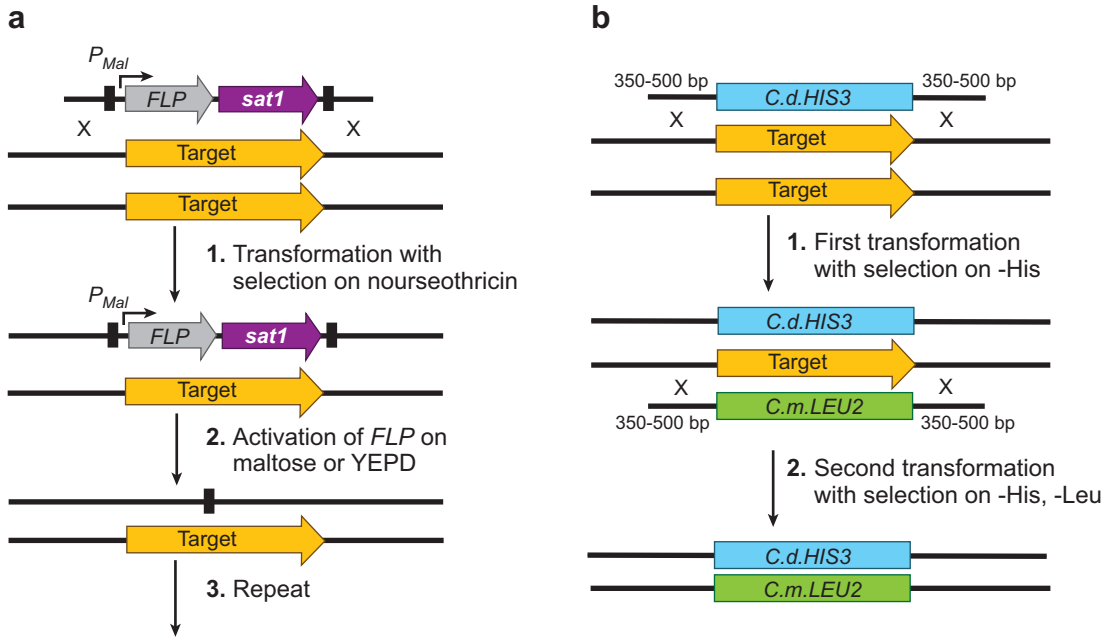

Figure 4

Tools for gene disruption in *C. albicans*. (*a*) The *SAT1* Flipper system (63) consists of a cassette containing the *SAT1* gene (which confers resistance to the antibiotic nourseothricin); the *FLP* gene, under regulation of the maltose promoter; and two *FLP* recombination sites (*solid black boxes*). Targeted gene disruption plasmids are created by subcloning sequences upstream and downstream of the target gene onto either side of the *SAT1-FLP* cassette. The linearized insert is transformed into *C. albicans*, with selection on medium containing nourseothricin. The *SAT1-FLP* region is then liberated by transferring the transformants to medium containing maltose (or YEPD, since the promoter is leaky), to activate expression of Flp recombinase. To delete the second allele of the target gene, the process is repeated. Although this method requires cloning and is therefore somewhat more laborious than PCR-based methods, it has the advantage of being applicable to most strains of *C. albicans*, including clinical isolates that lack auxotrophic markers. (*b*) In the Noble and Johnson gene disruption method (55), a linear gene disruption fragment with relatively long regions of homology (~350–500 bp) to sequences flanking the target gene is created using a fusion PCR technique. Homozygous gene disruption is accomplished in two steps: 1. Transformation of the first gene disruption fragment, marked by the *HIS1* gene of *C. dubliniensis*, with selection on -His medium, to disrupt the first allele of the target gene. 2. Transformation of the second gene disruption fragment, marked by the *LEU2* gene of *C. maltosa*, with selection on -His, -Leu medium, to disrupt the second allele of the target gene.

and nourseothricin (63, 65, 71) have been modified for use in *C. albicans*.

Recent technological innovations that have streamlined mutant construction in *C. albicans* include "flipper" systems that allow for recycling of markers without the requirement for mutagens such as 5-FOA (**Figure 4*a***) (34, 50, 63). Regulatable markers including the *MAL2* promoter (3, 13), *MET3* promoter (15), and tetracycline-repressible or -inducible promoters (52, 59) allow for conditional expression of *C. albicans* genes, which is obviously necessary in the case of essential genes. The *UAU1* cassette (discussed below) enables disruption of two alleles of a target gene after a single transformation step (20). Finally, we have developed a method that combines several techniques to improve the efficiency of gene disruption approximately 20-fold relative to previous techniques (**Figure 4*b***) (55). The method utilizes fusion PCR to create gene disruption fragments

with relatively long (e.g., 350–500 base pairs) stretches of homology to regions that flank the target gene, without the need for cloning. The gene disruptions are marked with *HIS1*, *LEU2*, and *ARG4* genes amplified from *Candida maltosa* or *Candida dubliniensis*, which are functional in *C. albicans* but sufficiently heterologous in sequence that they do not readily recombine with genomic DNA from *C. albicans*. Because deletion of these amino acid biosynthetic genes has minimal effect on virulence, knockout mutants constructed with this method are suitable for virulence analysis in the mouse.

Genetic Screens in *C. albicans*

The availability of a fully sequenced genome (12, 32), new selectable markers, tools for regulating gene expression, and more varied and efficient gene disruption techniques has inspired several groups to create collections of mutants of *C. albicans* for use in forward genetic screens. Davis et al. combined transposon mutagenesis with the *UAU1* method of gene disruption (20) to create a library of homozygous transposon insertion mutants (17). The *UAU1* selectable marker consists of a *URA3* gene of *C. albicans* that is interrupted by the *ARG4* gene (**Figure 5**) (20). Use of the *UAU1* marker in a standard gene disruption cassette allows for construction of homozygous gene disruptants after a single round of transformation. This is possible because, after disruption of the first allele of a target gene, a low frequency of mitotic recombination will result in homozygosis of this mutation. If subsequent intramolecular recombination occurs at one of the *UAU1* markers, deleting *ARG4* and thereby reconstituting *URA3*, the mutant will be prototrophic for both uracil and arginine and can be selected on medium lacking these nutrients. Unlike standard gene disruption methods, however, the *UAU1* technique does not result in precise deletion of a target gene. The mitotic recombination event used to replace the second allele is not restricted in scope and, in principle, could result in homozygosis of the entire chromosome distal to the recombination site. Because *C. albicans* has a high degree of heterozygosity between chromosomes (32, 82), mutants made with this technique will likely have numerous genetic changes in addition to disruption of the target gene.

Haploinsufficiency: production of an observable phenotype by loss of function of a single copy of a gene in a diploid organism

Using a *UAU1*-marked Tn7 transposon, Davis et al. performed random mutagenesis of *C. albicans* genomic DNA in vitro. After sequencing DNA from independent integrants to identify those predicted to disrupt gene expression, they created a library of 217 homozygous transposon mutants (**Figure 6*a***) (17). A screen for mutants defective in filamentous growth at alkaline pH led to the identification of three genes important for this process: Two of these were previously known to participate in filamentation and/or a previously described pH response signaling pathway, and *MDS3* was identified as a novel gene likely required for a second, parallel pH-signaling pathway. This library of transposon mutants has since been used successfully in screens for factors that affect formation of chlamydospores (53) and biofilms (64). Nobile et al. have subsequently utilized the *UAU1*-transposition method to create a collection of 83 mutants affecting putative transcription factors (54). Their screen for transcription factors that affect biofilm formation identified *BCR1* as a gene that regulates expression of a number of cell-surface proteins in *C. albicans*, including adhesins.

Uhl et al. (**Figure 6*b***) (78) utilized Tn7 transposition on a larger scale to create a library of strains of *C. albicans* with heterozygous transposon insertions; that is, each mutant has one normal haploid set of chromosomes and a second set that is disrupted by a transposon. Because they were interested in the yeast-to-hyphal morphological transition—a process strongly implicated in virulence [reviewed in (36)] and known to be particularly sensitive to gene dosage [e.g., (11, 35, 40, 43)], the authors were able to perform their screen based on haploinsufficiency phenotypes. After examining 18,000 independent

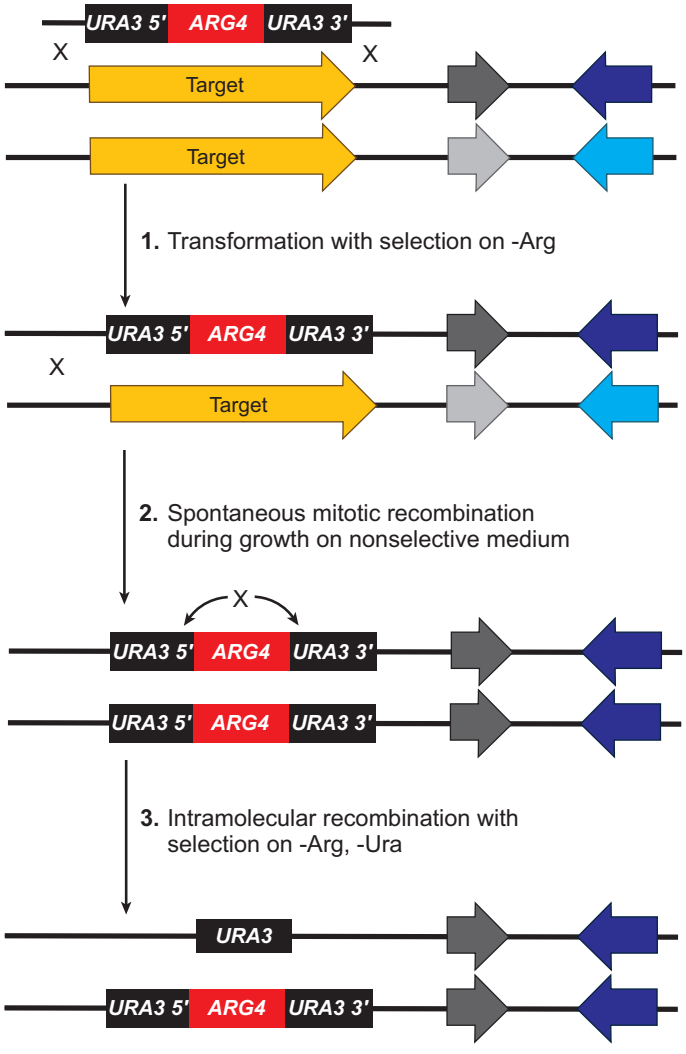

Figure 5

"UAU" method of gene disruption in *C. albicans*. This method results in homozygous gene disruption after a single round of transformation (20). 1. Transformation of a strain of *C. albicans* that is auxotrophic for both uracil (*ura3/ura3*) and arginine (*arg4/arg4*) with the gene disruption fragment. This linear piece of DNA has ends that are homologous to sequences that flank the target gene. It also contains a wild-type copy of the *ARG4* gene, flanked by 5′ and 3′ portions of the *URA3* gene. Transformants that have undergone recombination (X's in the figure) between the disruption fragment and the target gene will be prototrophic for arginine and are selected on medium lacking this nutrient. 2. Arg^+ candidates are allowed to grow on nonselective medium. A minority of cells will undergo mitotic recombination with homozygosis of the gene disruption (as well as linked DNA, such as gene variants depicted in *blue*, *black*, and *gray*). After homozygosis, some strains will undergo intragenic recombination between overlapping sequences in the *URA3* gene fragments, thereby deleting one copy of *ARG4* and reconstituting a functional *URA3* gene. 3. Arg^+Ura^+ strains are selected on -Arg,-Ura dropout medium. Homozygous knockout strains will contain disruptions of both alleles of the target gene. Alternatively, some strains (not depicted) will have both gene disruptions as well as an intact copy of the target gene (present on a duplicated chromosome or translocated chromosome fragment); only this latter type of strain will occur in the case of essential target genes.

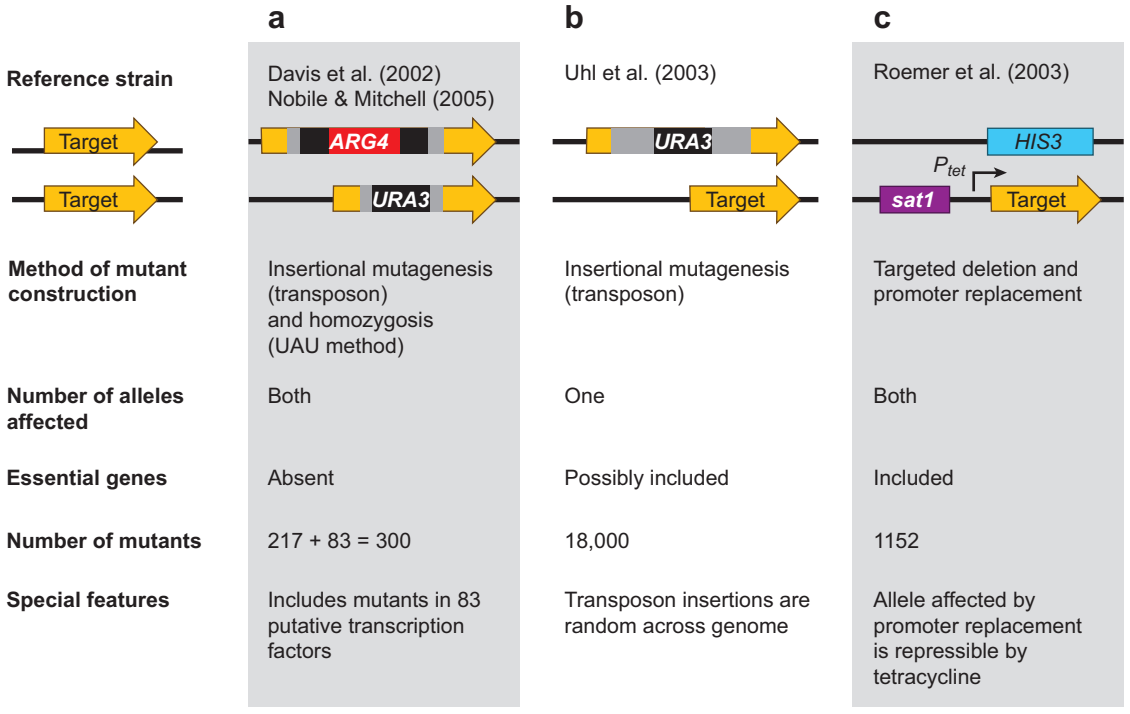

Figure 6

A comparison of mutant collections of *C. albicans*. The major features of the three largest collections of knockout mutants of *C. albicans* are presented.

heterozygous mutants for aberrations in *C. albicans* colony morphology (a function of the yeast:hypha ratio), they identified mutants affecting expression of 146 genes, whose identity was determined by sequencing genomic DNA that flanked the transposon. Several of these genes corresponded to factors previously implicated in the yeast-to-hyphal transition, but the majority were novel. Although *URA3* was used as the selectable marker for mutant construction, all of the mutants with morphogenesis defects retained their aberrant phenotypes when retested on medium supplemented with uridine; the authors speculate that *URA3* expression in these mutants is shielded from chromosome position effects by a relatively long stretch of flanking transposon sequence (several kilobases on either side). This collection of random heterozygous mutants is large; however, the nature of the library limits the types of screens for which this collection will be useful to those in which haploinsufficiency phenotypes are prominent.

The GRACE collection of conditional *C. albicans* mutants was created to identify essential genes for use as antifungal drug targets (**Figure 6*c***) (65). The collection consists of strains of *C. albicans* in which one allele of the target gene is replaced by the auxotrophic marker *HIS3*, and the second allele has undergone promoter replacement with a tetracycline-repressible promoter. The promoter-replacement strategy allows for targeting of essential genes, since such mutants will survive on medium lacking tetracycline. Among the 1152 genes targeted, 567 were determined to be essential on laboratory medium. Several of these mutants also demonstrated tetracycline-dependent defects in virulence in a mouse model. Despite the great potential utility of this large collection

of conditional mutants, the identities of most of the disrupted genes were not revealed, and the strains have not been made freely available to the research community.

The mutant collections described above have different strengths and weaknesses and different potential applications. Mutants made with the *UAU1*-transposon system have already been used successfully in several published screens. Uhl et al.'s collection of 18,000 heterozygous transposon mutants is comprehensive in scope but is useful primarily in screens for phenotypes that are highly sensitive to gene dosage. Mutants with regulatable promoters such as the GRACE collection can be used to study essential genes. Mutants made with auxotrophic markers other than *URA3* may be particularly suitable for virulence studies. The willingness of so many groups to undertake the arduous project of large-scale mutant construction in a diploid organism underscores a common belief in the power of the genetic approach. We can anticipate genome-wide collections of *C. albicans* mutants, similar to those available for *S. cerevisiae* (22, 85), for performing classical forward genetic screens in this important human pathogen.

A Few Words About Genetic Linkage

In all of the approaches described above, it is essential to know whether mutation of the target gene is responsible for the phenotype observed in the mutant strain. In particular, it is crucial to confirm that the phenotype is not caused by (or even dependent on) a second, cryptic mutation elsewhere in the genome. In *C. albicans*, this issue has traditionally been addressed by testing whether addition of a wild-type gene to the mutant restores the wild-type phenotype (**Figure 7*a***). Because there are presently no stably propagating, low-copy-number plasmids in *C. albicans*, wild-type genes are introduced by integrating them into the genome. As has been amply demonstrated for *URA3*, however, introduction of a gene even to a specific locus can result in variable amounts of gene expression and a corresponding range of phenotypes. Investigators typically choose several "add back" strains whose phenotypes resemble that of the wild-type strain and conclude that complementation has occurred. Although the logic is somewhat circular, the test does provide some assurance that the phenotype of interest is dependent on the gene in question. The test does not, however, rule out the possibility that other, unlinked mutations are also needed for the full mutant phenotype.

A different approach involves constructing and testing several independent isolates containing what should be the same mutation (**Figure 7*b***). If all of the isolates share the same phenotype, then that phenotype likely results from the common mutation. A caveat of this reasoning is that changes closely linked to the common mutation (such as neighboring genes whose expression is altered by disruption of a target gene) might contribute to the mutant phenotype and be present in each of the isolates. Further, it may be impractical to test numerous independent mutants with complicated or expensive assays such as mouse infections.

A third, rarely used test of linkage takes advantage of the parasexual cycle in *C. albicans* (**Figure 7*c***). Using appropriate markers, one can perform crosses between a mutant of one mating type and wild-type *Candida* of the opposite mating type. After growing the resultant tetraploid strain on medium that induces chromosome loss, one can examine the diploid "progeny." If 100% of diploid cells with the phenotype of interest also have the mutation in question, then the two are closely linked. Difficulties of this method include the need to create mating-competent strains with appropriate complementary markers and the possibility of changes in expression of closely linked genes, as described above.

At present, there is no perfect method for proving that a given mutation is the cause of a phenotype in *C. albicans*. All three methods discussed above have advantages and disadvantages, and it makes the most sense to

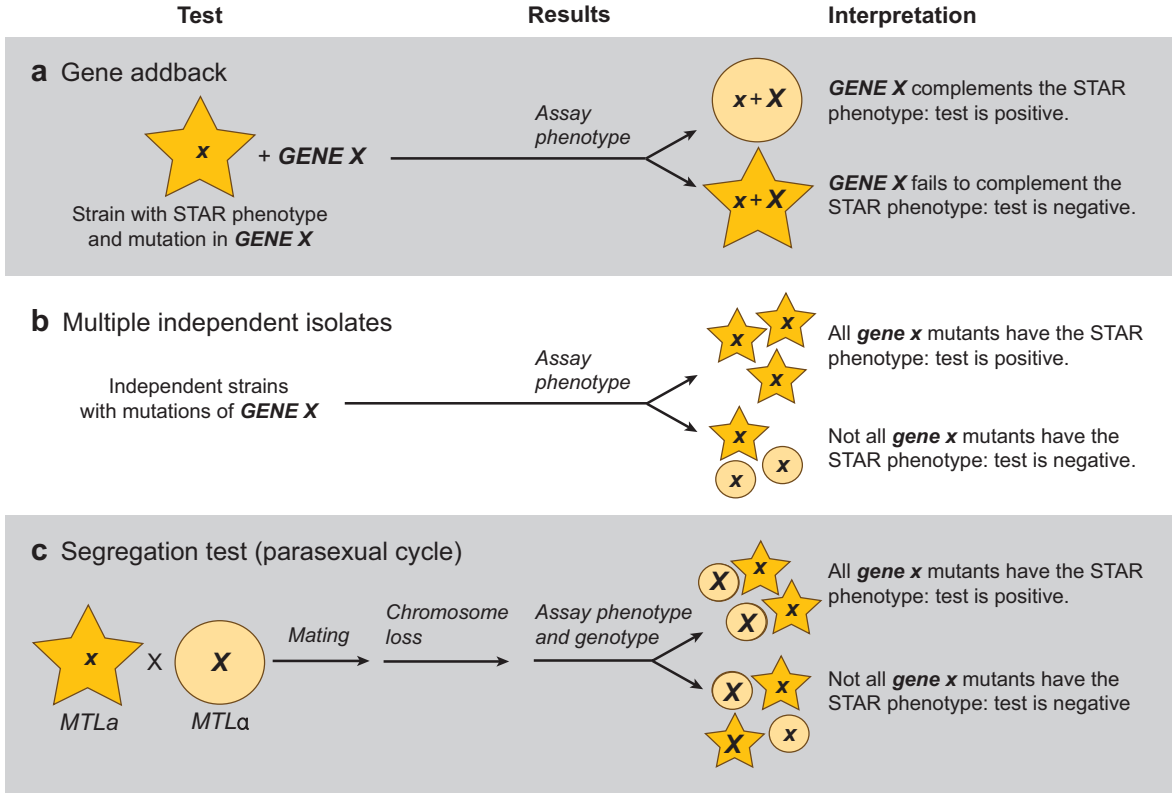

Figure 7

Methods of linking a genotype to a phenotype. Presented are three methods of testing the hypothetical association between mutation of *GENE X* and the Star cell-shape phenotype. (*a*) Gene addback test: Here, a wild-type copy of *GENE X* is introduced into the Star-shaped mutant. If wild-type cell shape is restored, then wild-type cell shape must be dependent on *GENE X*. (*b*) Multiple independent isolates: After generating many independent isolates of the *gene x* mutant, one assays their cell shape. If every *gene x* mutant is Star-shaped, then the engineered mutation is most likely the cause of the phenotype. (*c*) Parasexual cycle: A strain that is mutant for *GENE X* is mated to a wild-type tester strain that has the opposite mating type and a complementary auxotrophic marker. Tetraploid cells are selected and then induced to undergo chromosome loss. The diploid progeny are evaluated both for genotype (*GENE X* versus *gene x*) and phenotype (Round versus Star). If all *gene x* mutants have the Star shape, then the phenotype is tightly linked to *GENE X*.

selectively apply them based on the context of the biological question.

CONCLUSION

Despite the prominence of *Candida albicans* as a ubiquitous human commensal, as a cause of nonlife-threatening skin, nail, and mucous membrane infections, and as a cause of severe disease among the immunocompromised, we have relatively little understanding of its virulence mechanisms and few tools for clinical diagnosis and treatment. Nevertheless, technological and methodological advances in the past 20 years as well as a fully sequenced genome have enabled increasingly sophisticated experiments. We now have a good sense of the population structure of this organism and a detailed understanding, at least in vitro, of its unusual parasexual cycle. Several groups have contributed mutant collections useful for forward genetic screens, and many factors

important for phenotypes such as morphogenesis and biofilm formation have already been identified. There are no theoretical barriers to the use of sophisticated, genome-wide forward genetic screens to understand the basic biology of this important organism.

SUMMARY POINTS

1. Strain typing studies have converged on five major clades of *C. albicans* that appear to propagate in a clonal manner. Recent analysis of 1400 clinical isolates has revealed evidence for frequent recombination between closely related strains; such genetic exchange may be restricted to those variants of a single strain that encounter one another in a given host.
2. Whether *C. albicans* can undergo meiosis remains an open question. In any case, there is clear evidence for a parasexual cycle whereby diploid cells mate to form tetraploid cells, and tetraploid cells lose chromosomes in a random but concerted manner to return to the diploid state.
3. Chromosome loss is one of several types of chromosome alterations observed in *C. albicans*.
4. Multiple techniques and selectable markers are now available for constructing mutants of *C. albicans*. Several laboratories have utilized these methods to conduct forward genetic screens in this diploid organism.

FUTURE ISSUES

1. Do different *C. albicans* clades have specialized properties? Do they cause different disease syndromes? Are they geographically distributed?
2. What is the role of mating and the parasexual cycle in the natural life cycle of *C. albicans*? Are they important for causing disease?
3. Which genes in *C. albicans* are important for the commensal state, and which mediate virulence? Are they the same?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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