Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains

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The human pathogenic fungus *Candida albicans* has traditionally been classified as a diploid, asexual organism. However, mating-competent forms of the organism were recently described that produced tetraploid mating products. In principle, the *C. albicans* life cycle could be completed via a sexual process, via a parasexual mechanism, or by both mechanisms. Here we describe conditions in which growth of a tetraploid strain of *C. albicans* on *Saccharomyces cerevisiae* ‘pre-sporulation’ medium induced efficient, random chromosome loss in the tetraploid. The products of chromosome loss were often strains that were diploid, or very close to diploid, in DNA content. If they inherited the appropriate *MTL* (mating-type like) loci, these diploid products were themselves mating competent. Thus, an efficient parasexual cycle can be performed in *C. albicans*, one that leads to the reassortment of genetic material in this organism. We show that this parasexual cycle—consisting of mating followed by chromosome loss—can be used in the laboratory for simple genetic manipulations in *C. albicans*.

Keywords: chromosome loss/mating/meiosis/parasexual/tetraploid

Introduction

Although found as a commensal in the digestive tract, *Candida albicans* is also the most common human fungal pathogen, causing both mucosal and systemic infections, particularly in immunocompromised people (Edmond *et al.*, 1999). *Candida albicans* exists naturally as a diploid yeast and until recently was thought to be asexual, as no direct observations of mating or meiosis had been reported. The lack of evidence for sexual reproduction was surprising since DNA-based taxonomies indicated that *C. albicans* was closely related to sexually reproducing yeast species such as *Saccharomyces cerevisiae*. Moreover, a number of genes in *C. albicans* were identified whose homologs function in mating and meiosis in *S. cerevisiae*; some of these *C. albicans* genes (e.g. *STE20* and *DMC1*) have even been shown to complement their *S. cerevisiae* counterparts (Diener and Fink, 1996; Leberer *et al.*, 1996; Tzung *et al.*, 2001). Population studies suggested that while *C. albicans* was largely clonal, implying that recombination in the wild occurred rarely,

there was evidence for some form of genetic exchange in natural populations (Graser *et al.*, 1996; Tibayrenc, 1997).

Evidence for a sexual cycle in *C. albicans* came from the description of mating-type like (*MTL*) loci in this organism (Hull and Johnson, 1999). The *MTL* loci of *C. albicans* are similar to the mating-type (*MAT*) loci of *S. cerevisiae* in two respects. First, they encode three closely related transcriptional regulators (called *MATa1*, *MAT α 1* and *MAT α 2* in *S. cerevisiae*), and secondly, the loci are heterozygous in standard diploid laboratory strains with the *MATa1*-like genes on one chromosome and *MAT α 1*- and *MAT α 2*-like genes on the other chromosome. It was subsequently premised that *C. albicans* might be made to mate by genetic modification of the wild-type *a*/ α strain to create both *a*- and α -type strains. These derivatives were indeed found to mate, albeit at low efficiency, either in a mammalian host (Hull *et al.*, 2000) or *in vitro* (Magee and Magee, 2000).

More recently, it has been shown that mating in *C. albicans* is directly linked to white–opaque switching (Miller and Johnson, 2002). Certain strains of *C. albicans* were known to undergo a white–opaque phase transition; white cells appeared round and formed dome-shaped colonies on agar, while opaque cells were more elongated and formed flatter colonies on agar (Soll, 1997). The ability to switch phases was found to be controlled by the transcriptional regulatory proteins encoded by the *MTL* loci. Furthermore, opaque cells were shown to mate $\sim 10^6$ times more efficiently than white-phase cells, explaining the very low mating efficiency observed in the original mating experiments, which had utilized white-phase cells. Thus, mating-competent cells must first switch from white to opaque to mate efficiently; the fact that the *MTL* locus controls both processes ensures that white–opaque switching is only carried out by mating-competent cells. Consistent with this idea, switching-competent clinical isolates of *C. albicans* were found to be either ‘*a*’ or ‘ α ’ strains (Lockhart *et al.*, 2002).

Although mating has now been amply documented for *C. albicans*, no study has reported whether the tetraploid products formed through mating can be induced to return to the diploid state. This process could, in principle, occur via a sexual cycle (meiotic divisions) or via a parasexual cycle (reductional mitotic divisions) in *C. albicans* (see Figure 1). In this study, we constructed a tetraploid strain of *C. albicans* containing several convenient genetic markers, and tested whether meiosis or chromosome loss could be induced under a number of different growth conditions. Although we did not observe meiosis, we did observe efficient and cooperative chromosome loss under certain conditions. Chromosome loss was particularly efficient in tetraploid strains that were grown at 37°C on a *S. cerevisiae* ‘pre-sporulation’ (pre-spo) medium, a medium that contains 1% yeast extract, 0.8% peptone and 10%

Fig. 1. Putative sexual cycle of *C. albicans*. Mating between *a* and α opaque cells produces an *a/α* tetraploid cell. A reduction in ploidy back to the diploid state, which could in principle occur by random chromosome loss or by meiosis, is the subject of this paper.

glucose. The resultant progeny showed a range of ploidy, although approximately one-third of the cells had reduced their DNA content to (within experimental error) that of a control diploid strain. While chromosome reduction appears cooperative (i.e. the loss of one pair of chromosomes predisposes the strain to lose other pairs), it is not as concerted as typical fungal meioses. We show that induced chromosome loss in the tetraploid can be used to complete a parasexual genetic cycle, allowing a series of simple genetic manipulations to be performed with *C. albicans*. These include: (i) establishing linkage relationships between genes; (ii) carrying out backcrosses to establish that a phenotype arises from a single mutation; and (iii) constructing recombinant diploid strains that contain genetic information derived from two different diploid parents.

Results

Marking a tetraploid strain of *C. albicans* to follow chromosome loss

Previous studies have indicated that tetraploid strains of *C. albicans* might be unstable. For example, heat shock treatment reduced the chromosome number in tetraploid cells formed by protoplast fusion of two diploid strains (Hilton *et al.*, 1985). Another study surmised that a tetraploid strain, constructed by mating two diploids, underwent chromosome loss to form a strain that was tri-allelic at the *MTL* locus (Hull *et al.*, 2000). To test for growth conditions that could specifically induce chromosome loss or reduction in the tetraploid, a tetraploid strain was created (RBY18) in which chromosome loss could be easily monitored. The tetraploid strain was created by mating *a* and α cell types, and appeared to have undergone karyogamy based on DAPI staining (data not shown). The strain was constructed to be heterozygous for the *URA3* and *GAL1* genes. Both *URA3* and *GAL1* genes are counter-selectable: strains containing wild-type *URA3* are unable to grow on medium containing 5-fluoroorotic acid (5-FOA) (Boeke *et al.*, 1984), while strains containing the *GAL1* gene are unable to grow on medium containing 2-deoxygalactose (2-DOG) as the carbon source (Platt, 1984). The tetraploid strain used in the present study contained one wild-type copy of *URA3* and two copies of the wild-type *GAL1* allele (see Figure 2A and B), and therefore is unable to grow on either 5-FOA- or

Fig. 2. Test of stability of a tetraploid strain grown on different media. (A and B) The tetraploid strain used in this study (RBY18) is heterozygous at the *GAL1*, *ADE2* and *MTL* loci on chromosomes 1, 3 and 5, respectively. (C) The tetraploid strain was grown for 8 days on different media at 22, 30 or 37°C, respectively. Cells were pooled from the different media and plated on FOA/DOG medium and YEPD. The number of cells that could grow on the FOA/DOG plates relative to YEPD was calculated (number of colonies per 10⁵ cells). (D) The tetraploid strain was grown on YEPD or pre-spo medium for 8 days at 37°C. Following incubation, cells were recovered and tested for growth on several test media. The percentage of cells that could grow on the test plates (or gave red colonies indicating Ade- colonies) relative to YEPD was calculated.

2-DOG-containing media. However, derivatives of this strain can grow on medium containing both 5-FOA and 2-DOG if they have lost both copies of the *GAL1* gene and the single *URA3* gene. The *GAL1* and *URA3* genes are located on chromosomes 1 and 3, respectively (Figure 2A and B). Note that *URA3* is present at the *ADE2* locus as a *HisG-URA3-HisG* allele, so that *URA3* can be lost by intramolecular recombination in addition to chromosome loss. Experiments using a *ura3/URA3*; *GAL1/GAL1* diploid strain confirmed that <1 in 100 000 colonies grew on medium containing 5-FOA and 2-DOG, demonstrating a very low background growth. Thus, efficient chromosome loss in a tetraploid would be expected to produce derivatives resistant to both 5-FOA and 2-DOG at frequencies well above the background level.

The starting tetraploid strain was also heterozygous at two additional alleles. First, the *ADE2* locus contained two wild-type and two mutant *ADE2* alleles. Strains from which both wild-type copies of *ADE2* were lost grew as characteristic red colonies, whereas all other configurations of the *ADE2* locus gave rise to white colonies. In addition, the *MTL* locus on chromosome 5 was represented

Fig. 3. FACS analysis of tetraploid strains that had been exposed to pre-spo medium. Single colonies were selected and grown up in liquid YEPD medium for FACS analysis, as described in Materials and methods. The x-axis of each graph (Sytox) represents a linear scale of fluorescence, and the y-axis (Counts) represents a linear scale of cell number. (A) Comparison of a control diploid strain (CAF2-1; left trace) with the starting tetraploid strain (RBY18; right trace). Control diploid and tetraploid traces are also included for reference in (B–F) and (H–K). (B–F) Five independent colonies (PS1–PS5) derived from the tetraploid strain RBY18 after incubation on pre-spo medium; all five colonies were di-allelic at *MTL*. (G) Comparison of the FACS profiles of four different laboratory diploid strains, CAF2-1, CHY257, CHY439 and CHY477 (Miller and Johnson, 2002). (H and I) Two colonies derived from the tetraploid strain after growth on pre-spo medium; these were chosen for FACS analysis because they were tri-allelic at the *MTL* locus. (J and K) Two colonies that were still tetra-allelic at *MTL* after incubation on pre-spo medium.

by four different *MTL* alleles: *a*, *alpha*, *al* deletion and *alpha1/alpha2* deletion. All four of the *MTL* alleles were easily distinguishable using whole-cell PCR and primer pairs specific to each *MTL* allele.

Growth on pre-spo medium induces allele loss in a tetraploid strain

To test for chromosome loss, the marked tetraploid strain was plated on a variety of different media, including YEPD, SD, spider, spo, pre-spo and cornmeal medium, and incubated at 25, 30 or 37°C. Following incubation on solid media, colonies were pooled by scraping cells off the plate and the suspended cells were plated at different concentrations onto YEPD plates (control) or SD plates containing 5-FOA and 2-DOG. Preliminary experiments suggested that growth of the tetraploid at 37°C on a *S.cerevisiae* pre-spo medium resulted in at least 10 times as many colonies on the selective plates than any other condition tested (see Figure 2C).

Allele loss on pre-spo medium was then carefully compared with that on YEPD. Tetraploid cells were streaked on both media and allowed to grow for 14 days at 37°C. Subsequently, cells were pooled and tested for growth on several types of selective media; growth on each type of medium required the loss of a particular genetic marker. As shown in Figure 2D, the number of colonies observed on the selective plates was much greater for samples grown on pre-spo medium than on YEPD. For example, only 0.02% of cells picked from the YEPD plates were able to grow on medium containing DOG and FOA, whereas 0.6% of cells grown on pre-spo were DOG^R and FOA^R (a 30-fold difference). Similarly, whereas only 0.1% of cells grown on YEPD were Ade[–] (indicated by red colonies), 6% of cells grown on pre-spo were red (a 60-fold difference). These results suggested that allele loss (which may occur via chromosome loss, mitotic gene conversion or meiosis) was induced upon growth on pre-spo medium.

FACS analysis of progeny cells shows a reduction in ploidy

To test whether the loss of the *URA3*, *GALI*, and *ADE2* markers occurred by chromosome loss or mitotic gene conversion, we examined by FACS the ploidy of cells derived from tetraploids on pre-spo medium. Control diploid and tetraploid strains of *C.albicans* were readily distinguishable by FACS analysis, with tetraploids showing twice the DNA content of diploids (Figure 3A). Several colonies picked from pre-spo plates were also subjected to FACS analysis (Figure 3B–F). These strains, PS1–PS5, were all di-allelic at the *MTL* locus (see below). Strains PS1–PS5 all showed a greatly reduced ploidy relative to the parental tetraploid strain, with a DNA content very close to that of the diploid control (Figure 3B–F). We cannot be certain that these strains are true diploids as several appear to have a ploidy slightly higher than that of a parental diploid (Figure 3B–E). However, different laboratory diploid strains also show slight variations in DNA content as measured by FACS, suggesting that true diploid strains give a range of FACS signals (Figure 3G).

We also selected several additional pre-spo-derived colonies for FACS analysis that were tri-allelic or tetra-allelic at the *MTL* locus (see below). Significantly, the total DNA content of these strains appeared to mirror the number of *MTL* alleles they carried. For example, the FACS scans of three colonies that were tri-allelic at *MTL* showed DNA contents between that of a diploid and a

Pre-spo medium		1° screen		
		DOG ^R	Ade-	2x MTL
		5.5%	5.5%	34%
2° screen	DOG ^R	-	17.9%	
	Ade-	14.2%	-	
	2x MTL	92%	94%	

YEPD medium		1° screen		
		DOG ^R	Ade-	2x MTL
		0.3%	0.12%	< 3%
2° screen	DOG ^R	-	10.0%	
	Ade-	2.6%	-	
	2x MTL	50%	43%	

Fig. 4. Pre-spo medium induces allele loss in a tetraploid strain of *C.albicans*. (A) A detailed analysis of growth of the tetraploid strain RBY18 was grown for 8 days at 37°C on pre-spo medium and pooled colonies were subsequently tested for growth on DOG or YEPD plates. The percentage of cells that were DOG^R, Ade- or di-allelic at the *MTL* locus is indicated (1° screen). The DOG^R and Ade- colonies were further tested to determine the percentage of these cells that had lost other chromosomal markers (2° screen). (B) The tetraploid strain was grown on YEPD for 8 days at 37°C, and subsequently analyzed as in (A). The results of (A) and (B) show that allele loss is concerted.

tetraploid (examples shown in Figure 3H and I), suggesting they are trisomic for many of their chromosomes. Similarly, three colonies that were tetra-allelic at *MTL* after growth on pre-spo medium also appeared to be tetraploid or close to tetraploid in total DNA content (examples shown in Figure 3J and K). These results show that pre-spo conditions give rise to efficient chromosome loss and confirm that the allele loss observed in the previously described experiments is due to chromosome loss, and not to gene conversion or other types of allele loss.

Allele loss is concerted for all marked chromosomes

Based on the FACS analyses described above, chromosome loss in the tetraploids appeared to occur by an imprecise mechanism, i.e. it produced both diploid and triploid products. We addressed this point in more detail by directly measuring the rate at which genetically marked chromosomes were lost. We first used PCR to examine loss of alleles at the *MTL* locus. The tetraploid contains four unique alleles at the *MTL* locus (wt *a1*, wt *alpha1/alpha2*, *Δa1* and *Δalpha1/alpha2*) and thus it is possible to follow the fate of each allele following exposure of the tetraploid to pre-spo medium. Figure 4 shows an analysis of tetraploid colonies plated on either pre-spo or YEPD plates. In this experiment, we again observed that a significantly higher fraction of cells grown on pre-spo were Ade- or DOG^R compared with cells grown on YEPD (18-fold more colonies were DOG^R and 46-fold more colonies were Ade-). In addition, analysis of the *MTL*

locus in 32 colonies picked at random from YEPD revealed that 31 of the 32 colonies were still tetra-allelic at *MTL* and one was tri-allelic, consistent with relatively stable propagation of the tetraploid on this medium. In comparison, analysis of 32 colonies randomly picked from pre-spo plates indicated that 35% (14/40) of the colonies were di-allelic at *MTL*, 35% were tri-allelic (14/40) and 30% were tetra-allelic (12/40). The simplest interpretation of these results is that approximately one-third of the cells grown on pre-spo medium lost two copies of chromosome 5 (the chromosome containing *MTL*), one-third lost one copy of chromosome 5 and one-third retained the parental configuration of all four copies of chromosome 5. The fact that one-third of the products contained three copies of chromosome 5 suggested, like the FACS analysis, that chromosome loss was occurring by an imprecise mechanism and was therefore unlikely to be a result of a true meiosis.

In order to compare the rates of loss of different chromosomes, we calculated the fraction of the population that had lost chromosome 1 (by monitoring *GAL1*), chromosome 3 (by monitoring *ADE2*) and chromosome 5 (by monitoring *MTL*). Note that if all of the cells underwent random chromosome loss to form diploids, we would expect that ~17% of the cells would be Ade- and 17% of the cells would be DOG^R. This is because one in six of the progeny (17%) would be *ade2/ade2*, whereas five out of six progeny would contain at least one wild-type copy of *ADE2*. A similar argument applies for *GAL1*. We observed that 5.5% of the colonies derived from pre-spo medium were Ade-, 5.5% were DOG^R and 35% were di-allelic at *MTL* (Figure 4A). Thus, in this experiment, we can estimate that one-third of the tetraploid cells exposed to pre-spo medium lost two copies of chromosome 1, one-third lost two copies of chromosome 3 and one-third lost two copies of chromosome 5. These results indicate that chromosomes 1, 3 and 5 were lost at a similar rate upon exposure of the tetraploid to pre-spo medium.

We also determined whether the alleles at the *MTL* locus segregated randomly, indicative of random loss of chromosome 5, by examining the different di-allelic combinations at *MTL*. All six possible combinations of *MTL* were obtained (see Figure 5), indicating that non-parental configurations of the chromosomes are generated by chromosome loss on pre-spo medium. In addition, the distribution of the six possible *MTL* alleles was analyzed in 259 di-allelic colonies. The number of colonies containing *Δa/Δa*, *WTa/Δa*, *WTa/Δa*, *Δa/WTa*, *WTa/Δa* and *WTa/WTa* alleles was 34 (13%), 29 (11%), 60 (23%), 34 (13%), 51 (20%) and 51 (20%), respectively. The relatively even distribution of *MTL* alleles strongly suggests that loss of chromosome 5 from tetraploids is random; in particular, the parental combinations are broken up and redistributed.

To determine whether chromosome loss was concerted, we monitored strains that had lost one chromosomal marker for the loss of other chromosomal markers. We picked the Ade- and DOG^R colonies derived from pre-spo and YEPD, and examined the other markers in these colonies. After exposing tetraploids to pre-spo medium, we found that 14.2% of the DOG^R colonies were Ade- and 92% were di-allelic at *MTL* (see Figure 4A). Similarly, of the Ade- colonies, we found that 17.9% were DOG^R and 94% were di-allelic at *MTL*. These proportions are

Fig. 5. Analysis of the *MTL* locus in cells grown on pre-spo medium. The configuration of the *MTL* was checked by PCR after tetraploid cells had been exposed to pre-spo medium at 37°C for 8 days. (A) Schematic representation of the PCR products used to identify the different *MTL* alleles. Black bands represent the position of the relevant PCR products, while gray bands represent non-specific products amplified in the PCR reaction. Each reaction contains three sets of primers and, in this ideal diagram, all four *MTL* alleles (*a*, *α*, *Δa*, *Δα*) are present in the strain. (B) PCR products from six different progeny colonies are shown, together with a tetraploid control. All six possible *MTL* configurations were observed, indicating the random nature of the chromosome loss.

significantly higher than those for loss of a single marker (5.5% Ade⁻, 5.5% DOG^R, 34% di-allelic at *MTL*), and are near the theoretical limits of fully concerted chromosome loss. Although the overall numbers were much lower, concerted loss was also observed with colonies from YEPD plates. Hence, while only 0.3% of the total colonies were DOG^R and 0.12% were Ade⁻, 2.6% of DOG^R colonies were Ade⁻ and 10% of Ade⁻ colonies were DOG^R (see Figure 4B). In addition, 50% of DOG^R colonies and 43% of Ade⁻ colonies were di-allelic at *MTL*, whereas <3% (0/32) of the total colonies were di-allelic at *MTL*. Taken together, these results indicate that the efficient chromosome loss observed on pre-spo (and even the low frequency loss observed on YEPD) is concerted, i.e. cells that have lost two copies of one chromosome are much more likely to have lost copies of other chromosomes.

Time course of chromosome loss

Chromosome loss on pre-spo and YEPD media at 37°C was followed over the course of 8 days. Cells were taken from pre-spo or YEPD plates and tested for the fraction of colonies able to grow on 5-FOA or 2-DOG plates and for Ade⁻ colonies on YEPD plates. As shown in Figure 6A, significant numbers of resistant colonies began to appear on FOA and DOG plates after 2 days exposure to pre-spo medium (~5% of viable colonies). The number of FOA^R, DOG^R and Ade⁻ colonies from pre-spo plates continued to increase over time so that by 8 days 15% of the pre-spo colonies were DOG^R and 18% were Ade⁻. Note that in this

Fig. 6. Time course of chromosome loss and viability. (A) The tetraploid strain, RBY18, was grown on pre-spo (solid lines) or YEPD (dashed lines) medium at 37°C. Cells were collected at regular intervals and plated onto FOA, DOG or YEPD plates. The frequency of allele loss is proportional to the percentage of cells that grew on FOA or DOG plates relative to YEPD control plates, and by the percentage of Ade⁻ (red) colonies on YEPD. (B) The percentage of viable cells was calculated by collecting cells, measuring the total OD₆₀₀ and then plating a fixed number of cells onto YEPD plates.

experiment, chromosome loss after 8 days is close to the theoretical maximum, with most of the colonies having lost two copies of chromosomes 1 and 3. The number of colonies from pre-spo plates that grew on FOA also increased daily and, by day 8, 28% of cells were FOA^R. As there is only a single copy of the *URA3* gene in the tetraploid, the theoretical limit is 50% FOA^R colonies; thus, most of these cells have also undergone very efficient chromosome loss. In contrast to pre-spo plates, tetraploids exposed to YEPD gave rise to very few colonies that were FOA (0.7%) or DOG (0.04%) resistant, even after 8 days of growth (Figure 6A).

Growth of tetraploid strains on pre-spo medium is accompanied by cell death

To quantitate the number of viable cells for the time-course experiment described above, cells were collected from pre-spo and YEPD plates at various time points, the OD₆₀₀ was measured, and equal numbers of cells were plated on YEPD. The number of viable cells obtained from pre-spo plates fell precipitously so that after 2 days <1% of cells gave rise to viable colonies on YEPD (see Figure 6B). The number of viable colonies from pre-spo remained low until days 5–8, when the number of cells began to increase (viability increased to 26% by day 8). In contrast, the number of viable cells obtained from YEPD remained high

and fell only at the end of the time course (Figure 6B). Examination of the cells from pre-spo plates by microscopy showed that a number of the cells appeared to have lysed (data not shown). Cells taken from YEPD plates did not exhibit cell lysis. There are two reasonable explanations for this phenomenon. (i) The chromosome loss induced by pre-spo medium could result in high lethality if recessive lethal mutations were present on some chromosomes, i.e. only certain combinations of chromosomes could be lost and still give rise to viable cells. However, the experiments described above indicate random chromosome loss of chromosomes 1, 3 and 5, suggesting that no recessive lethal mutations reside on these chromosomes. (ii) The pre-spo medium could induce stress that causes both chromosome loss and lethality, but the two are not directly linked.

Diploids do not undergo large-scale chromosome loss when grown on pre-spo

Given the efficiency with which pre-spo medium induced chromosome loss from tetraploid strains of *C.albicans*, we also tested whether these same conditions induced chromosome loss in diploid strains. Diploid strains that were heterozygous for the *GAL1* gene or *URA3* gene were streaked onto pre-spo medium and grown at 37°C for 9 days. After 9 days on pre-spo, only a very low fraction of the colonies were able to grow on DOG (0.08%) or FOA (0.03%), compared with the results with the tetraploid strain (5.5% were DOG^R or FOA^R; Figure 4A). In addition, when the *MTL* configuration of 16 of the DOG^R and FOA^R colonies was tested, all 16 were found to have the parental configuration at *MTL* (i.e. they were di-allelic). These results show that, in contrast to the tetraploid strain, the diploid strain does not readily undergo loss of chromosome 5. Since loss of one copy of chromosome 5 is not lethal in *C.albicans* (as shown by Janbon *et al.*, 1998), this result suggests that pre-spo conditions do not induce chromosome loss in a diploid. The low fraction of DOG^R and FOA^R colonies may represent mutations or gene conversions of the wild-type *GAL1* or *URA3* genes when grown on pre-spo medium.

The analysis of chromosome loss from tetraploids, described earlier, also supports the idea that chromosomes are not readily lost from diploids. Examination of the *MTL* locus in a large number of tetraploids exposed to pre-spo media showed that while many had lost two alleles of *MTL*, only very few (2/261) had lost more than two alleles at *MTL*. These results suggest that the diploid state is much more stable than the tetraploid on pre-spo medium and may constitute the 'ground' state of ploidy on this medium.

Growth on sorbose medium also induces chromosome loss in the tetraploid

Diploid strains grown on sorbose medium have previously been shown to undergo loss of one copy of chromosome 5, the chromosome containing the *MTL* locus (Janbon *et al.*, 1998; Magee and Magee, 2000). It is thought that an inhibitor of the sorbose utilization gene (*SOU1*) resides on chromosome 5, and that loss of one copy of the inhibitor gene allows *C.albicans* to metabolize sorbose. Based on these results, we expected that growth of a tetraploid strain on sorbose media might also lead to loss of copies of

chromosome 5. To test the effect of sorbose on tetraploids, we grew the tetraploid strain RBY18 on sorbose medium for 5–7 days at 37°C. Similar to diploid strains, many of the tetraploid cells failed to grow on sorbose, and survivors began to appear by day 3. After 5–7 days, cells were collected from sorbose plates and tested for Ade⁻ and DOG^R phenotypes and *MTL* configuration. Eighty-four percent of cells were di-allelic at *MTL*, as expected if copies of chromosome 5 are being lost (Figure 7A). Unexpectedly, a high fraction of the cells had also lost copies of chromosomes 1 and 3, as evidenced by the significant number of DOG^R (11.6%) and Ade⁻ (6.9%) colonies, respectively (Figure 7A). The analysis of these data suggests that ~70% of the cells had lost two copies of chromosome 1 and 41% of cells had lost two copies of chromosome 3. Testing of the DOG^R colonies revealed that 2.5% of them were Ade⁻ and 78% of them were di-allelic at *MTL* (Figure 7A). Similarly, of the Ade⁻ colonies, 20% were DOG^R and 67% of them were di-allelic at *MTL*. The tetraploid strain therefore appears to have undergone concerted chromosome loss of at least three different chromosomes on sorbose medium.

To confirm that the ploidy of the tetraploid is reduced upon growth on sorbose, we carried out FACS analysis on four of the sorbose-derived strains (Figure 7B). Three of the four showed a ploidy close to that of a diploid control, whereas one showed a ploidy intermediate between a diploid and tetraploid. In agreement with the genetic analysis, the FACS results suggest that the tetraploid has undergone extensive chromosome loss on sorbose medium, not only of chromosome 5, but of most of the other chromosomes as well. This is the same behavior as was observed for growth of tetraploids on pre-spo medium, with one significant difference. Whereas growth of the tetraploid on pre-spo medium very rarely gave rise to colonies with only one copy of chromosome 5 (<1%), a significant fraction of the colonies from sorbose contained only one copy of chromosome 5 (11.6%). This observation is consistent with previous results showing that specific loss of chromosome 5 gives cells a growth advantage on sorbose (Janbon *et al.*, 1998).

Progeny from pre-spo media are mating competent

A major goal for pursuing chromosome loss in *C.albicans* is to be able to manipulate a genetic cycle in the organism. In order to show that mating followed by chromosome loss constitutes a true cycle, we set out to demonstrate that two diploid strains can be mated, the resulting tetraploid can be induced to produce diploid progeny, and the diploid progeny can in turn be mated to complete the cycle. For this experiment, we first mated an *a* and an *α* strain (see Materials and methods). The resulting tetraploid was grown on pre-spo medium to induce chromosome loss, and Ade⁻ colonies were isolated, as these had probably undergone significant chromosome loss. These colonies were then analyzed by PCR to determine their *MTL* configuration. Potential mating strains, i.e. those that contained a copy of either the wild-type *MTL_a* or *MTL_α* locus, were then mated against control diploid strains (Ura⁻) of the opposite mating type. The tetraploid mating products were selected on Ura⁻ Ade⁻ plates. In every case, the pre-spo-derived *a* and *α* diploids proved to be mating

Fig. 7. Growth on sorbose medium induces chromosome loss from tetraploids. The tetraploid strain RBY18 was grown on sorbose medium at 37°C for 7 days. (A) Chromosome loss was monitored by calculating the percentage of cells that were *DOG^R*, *Ade⁻* or di-allelic at *MTL* (1° screen). *DOG^R* colonies were also tested to see what percentage were *Ade⁻* or di-allelic at *MTL*, and *Ade⁻* colonies similarly tested to find the percentage that were *DOG^R* or di-allelic at *MTL* (2° screen). (B) Four colonies picked from the sorbose medium and determined to be di-allelic at *MTL* were grown up in YEPD and analyzed by FACS. In each graph, a control diploid strain, the parent tetraploid strain and the test colony are included (see Figure 3 for details).

competent and gave rise to new tetraploid colonies. Thus, mating of diploid strains, chromosome loss on pre-spo medium and re-mating constitutes a true parasexual cycle.

Fig. 8. Chromosome loss in a tetraploid can be used to link a phenotype with a genotype. A tetraploid strain that is heterozygous for the *NRG1* gene (*nrg1/nrg1/NRG1/NRG1*) was constructed by mating two *Δnrg1/NRG1* strains, and then exposed to pre-spo medium to induce chromosome loss. (A) Both smooth and wrinkled colonies were obtained following chromosome loss on pre-spo. A control *nrg1/nrg1* diploid strain is shown with its characteristic wrinkled appearance. Colony PCR was carried out on smooth colonies (B) or wrinkly colonies (C) generated from the tetraploid exposed to pre-spo medium in order to detect the presence or absence of the *NRG1* ORF. Only smooth colonies showed the presence of the *NRG1* gene. This analysis shows that the wrinkled appearance is caused by the *NRG1* deletion (or a mutation closely linked to it). (Upper PCR bands are background bands from the PCR reaction.)

Use of a *C.albicans* parasexual cycle to link phenotype to genotype

Two experiments were carried out to demonstrate that mating followed by chromosome loss could be used to carry out useful genetic manipulations. In the first experiment, we showed that a phenotype could be unambiguously linked to a gene deletion created in the laboratory. A *Δnrg1/Δnrg1* strain of *C.albicans* is characterized by being highly filamentous and forming characteristic wrinkled colonies on YEPD (see Figure 8A; Braun *et al.*, 2001; Murad *et al.*, 2001). Although the *nrg1* deletion was created in the laboratory, it is formally possible that the phenotype also involves additional unlinked mutations that were inadvertently introduced into the strain during construction. To show that the phenotype of this strain is caused by the *nrg1* deletion, we crossed two *nrg1/NRG1* heterozygous strains to generate a *nrg1/nrg1/NRG1/NRG1* tetraploid strain. The tetraploid

strain was then incubated on pre-spo medium for 10 days to induce chromosome loss, and cells were plated on to YEPD or DOG plates. Colonies that were wrinkled or smooth in appearance were chosen from both YEPD and DOG plates and re-streaked to confirm their phenotype (Figure 8A). PCR was then carried out on these colonies to determine whether the presence or absence of the intact *NRG1* gene correlated with the smooth or wrinkled appearance of the colonies. Strikingly, 38/38 smooth colonies showed the presence of the *NRG1* gene, while 29/30 wrinkled colonies lacked the *NRG1* gene (see Figure 8B and C). The one wrinkled colony that tested positively for the *NRG1* ORF by PCR (Figure 8C, lane 2) was re-streaked for single colonies and re-checked by PCR. Derivative colonies lacked the *NRG1* ORF, suggesting that the original colony was a mixture of *nrg1*⁻ and *NRG1*⁺ cells (data not shown). These results show that mating followed by chromosome loss on pre-spo can be used to link a phenotype with a specific mutation. This manipulation is especially important in *C.albicans*, as chromosome rearrangements are known to be common in this organism, and may occur during the routine construction of deletion strains.

This experiment also shows that new diploid strains can be constructed that combine mutations from two diploid parents. We believe that, in many cases, this method of constructing strains with mutations in multiple genes will prove simpler than the current approach of creating the deletions sequentially in a single isolate. In addition, the ability to obtain homozygous mutants from heterozygotes via a parasexual cycle (as demonstrated above) could be particularly useful in determining whether a gene has an essential function in *C.albicans*. Finally, as shown above, this approach can also be used to circumvent the sometimes difficult task of deleting the second allele of a gene in generating a homozygous deletion strain.

Use of a *C.albicans* parasexual cycle for determining genetic linkage

We also demonstrated that mating followed by chromosome loss could be used to determine genetic linkage. From unpublished studies on the *HOP1* gene, we surmised that it resided on chromosome 1 together with the *GAL1* gene (R.J.Bennett and A.D.Johnson, unpublished observations). To test this idea rigorously, we performed the genetic crosses shown in Figure 9A. A *gal1/gal1/hop1/hop1* strain was crossed with a *GAL1/GAL1/HOP1/HOP1* strain, and the reciprocal cross, a *gal1/gal1/HOP1/HOP1* strain crossed with a *GAL1/GAL1/hop1/hop1* strain, was also performed. The tetraploid strains were grown on pre-spo medium to induce chromosome loss. If the *GAL1* and *HOP1* genes were linked, then they should co-segregate. Thus, in the first cross, all *gal1*⁻ strains would also be expected to be *hop1*⁻, while in the second cross all *gal1*⁻ strains would be expected to be *HOP1*⁺ (barring recombination events). After incubation of the tetraploids on pre-spo plates, colonies lacking *GAL1* were selected for on DOG media and tested for the presence of the wild-type *HOP1* gene or the $\Delta hop1$ allele by PCR (Figure 9B and C). The results showed that 20/20 of the DOG-resistant colonies from the first cross contained only the $\Delta hop1$ allele, while 20/20 DOG-resistant colonies from the second cross contained only the wild-type *HOP1* allele

(Figure 9C). The *GAL1* and *HOP1* genes are therefore genetically linked; *GAL1* has been shown to reside on chromosome 1 (Magee *et al.*, 1988), and we conclude that *HOP1* also lies on this chromosome.

Discussion

The lack of a sexual cycle in *C.albicans* has severely limited genetic analysis of this organism. In this study we provide evidence for a chromosome-loss pathway which, when combined with mating, completes a parasexual cycle for *C.albicans* that can be readily carried out in the laboratory. In particular, we show that the ploidy of a tetraploid strain of *C.albicans* (produced by the mating of two diploid strains) can be reduced by simple laboratory manipulations (growth on *S.cerevisiae* 'pre-sporulation' medium at 37°C) to that of a diploid. Although we do not understand its detailed mechanism, this ploidy decrease occurs through random chromosome loss that is concerted; that is, loss of one or more chromosomes seems to predispose cells to lose others, with the diploid state being the final product.

In this paper, we also show that concerted chromosome loss combined with mating allows for several important genetic manipulations to be performed in *C.albicans*. These include: (i) confirming that a phenotype is a consequence of a specific genotype; (ii) genetic linkage experiments; and (iii) construction of new strains. The ability to carry out these manipulations should greatly improve the ease with which *C.albicans* can be studied. For example, the ability to link a mutation with a certain phenotype (see Figure 8) is particularly important in this organism where chromosome rearrangements can occur at a high frequency (for a review, see Rustchenko and Sherman, 2003). Currently, gene 'add-back' experiments are typically carried out in which the gene of interest is re-introduced into the homozygous mutant. These experiments can be difficult to interpret, however, with multiple isolates containing the re-introduced gene often showing different degrees of complementation. The procedure described here eliminates this problem by relying on chromosome segregation to create a large collection of strains with defined sets of alleles. It is then possible to unambiguously link a phenotype with a mutation. Genetic linkage experiments (see Figure 9) should allow both confirmation of the physical map of *C.albicans* that is being generated from the sequencing of the genome (<http://sequence-www.stanford.edu/group/candida>) and the identification of regions of the genome that are 'hot' or 'cold' for mitotic recombination. Finally, the ability to construct new strains by a parasexual cycle will be of benefit as multiple gene knockouts in *C.albicans* are often laborious and time consuming. Establishing whether a gene is essential should be straightforward since mating of heterozygous strains followed by chromosome loss could reveal whether the homozygous mutant strain is viable. For these applications, 'a' and 'α' mating strains could be generated from existing mutant strains (which are predominantly 'a/α') by inducing the loss of chromosome 5 in diploid strains, as described by Magee and Magee (2000).

We have described new conditions for inducing particularly efficient chromosome loss from *C.albicans* tetraploids, and shown how chromosome loss can be

Fig. 9. Chromosome loss induced by pre-spo medium can be used to demonstrate genetic linkage. (A) Two mating experiments were carried out in which a *gal1 hop1* strain was crossed with a *GAL1 HOP1* strain (cross 1), or a *gal1 HOP1* strain was crossed with a *GAL1 hop1* strain (cross 2). The resultant tetraploid strains were incubated on pre-spo medium at 37°C to induce chromosome loss. If the *GAL1* and *HOP1* genes are linked, all the *DOG*^R (i.e. *gal1*⁻) progeny from cross 1 will lack the *HOP1* gene, while all the *DOG*^R progeny from cross 2 will contain the wild-type *HOP1* gene. (B) Schematic of the PCR used to test whether a colony contains the wild-type *HOP1* gene or the *hop1* deletion. (C) Colony PCR was performed on 14 *DOG*^R progeny from cross 1 (lanes 1–14) and 14 *DOG*^R progeny from cross 2 (lanes 15–28). All PCR reactions contained primer sets to detect both the deleted and wild-type *HOP1* alleles. The results show that *HOP1* is genetically linked to *GAL1*.

combined with mating to form a robust parasexual cycle that can be carried out in the laboratory. However, chromosome loss itself has been observed previously. In several classical experiments, diploid cells of *C.albicans* were spheroplasted and fused to generate cells that were tetraploid following nuclear fusion. It was shown that the DNA content in these tetraploid cells could be reduced by chromosome loss induced by heat-shocking the cells at 50°C for 1–2 min (Hilton *et al.*, 1985). This treatment created some cells with a diploid, or close to diploid, DNA content. In these pioneering studies, Hilton *et al.* were able to map *ADE1*, *ARG1* and *MET1* to a single chromosome through linkage analysis. In another study, tetraploid cells heterozygous for a partially dominant selectable marker were constructed by protoplast fusion. Following selection for drug resistance, 70% of the colonies picked were found to have lost chromosomes, with about half of these cells close to a diploid level (Poulter and Hanrahan, 1983). These results indicate that heat shock or drug selection can induce mitotic instability and chromosome loss, with diploid cells a significant product. Finally, growth of diploid strains of *C.albicans* on sorbose medium at 37°C has previously been shown to result in the loss of one copy of chromosome 5, the chromosome containing the *MTL*

locus (Janbon *et al.*, 1998, 1999). The majority of diploid cells die when grown on sorbose medium, but the survivors are often monosomic for chromosome 5. It has been proposed that the level of expression of the *SOU1* gene, which is essential for L-sorbose assimilation in *C.albicans*, is regulated by the copy number of chromosome 5 (Janbon *et al.*, 1998). We show in this paper that sorbose can, like pre-spo medium, induce efficient chromosome loss from a tetraploid strain. However, unlike the diploid, all chromosomes appear to be lost at random from the tetraploid, so that overall ploidy is reduced to close to that of a diploid strain. These results suggest the possibility that chromosomes other than chromosome 5 are also lost during growth of the diploid on sorbose media, but that this effect is masked either by reduplication of the missing chromosome or by cell death caused by exposure of recessive lethal alleles in the genome. In support of this idea, ~10% of diploid strains grown on sorbose were found to have alterations in chromosomes other than chromosome 5, suggesting a general chromosomal instability on sorbose medium (Janbon *et al.*, 1999).

All of the conditions found to induce chromosome loss in *C.albicans* also induce stress in the organism, as

indicated by cell death of a significant fraction of the population. For example, in this paper we show that growth of the tetraploid strain on pre-spo medium resulted in a majority of the cells dying after 3 days at 37°C (Figure 6B). The number of viable cells then began to accumulate and most of these appeared to have lost at least one chromosome (after 8 days, 70% were either di- or trisomic for chromosome 5). Similarly, heat-shock treatment of tetraploids resulted in 10% viability, and growth of a diploid strain on sorbose medium for 3 days resulted in <1% viability of the original population (Hilton *et al.*, 1985; Janbon *et al.*, 1998). Unlike the case with sorbose medium and heat shock, it is not known why cell death and chromosome instability should occur on pre-spo medium, especially as its recipe is close to that of YEPD (see Materials and methods). Perhaps chromosome loss provides a tetraploid cell with some growth advantage on pre-spo medium, although, if so, the nature of this advantage is not known. Regardless of the mechanism, it appears that the cell may increase chromosome instability and missegregation in order to survive stress conditions (Janbon *et al.*, 1999).

The present study leaves open the possibility that *C.albicans* is able to undergo meiosis. However, none of the conditions tested seemed to induce meiosis in our tetraploid strain, including conditions known to induce sporulation in *S.cerevisiae*. In addition, the reduction in ploidy on pre-spo medium was not blocked by deletion of the putative meiosis genes *SPO11*, *DMC1(DLH1)* or *HOP1* (R.J.Bennett and A.D.Johnson, unpublished observations). If meiosis does occur in *C.albicans*, it may occur by a different signaling pathway, perhaps requiring a stimulus from its mammalian host. Curiously, while homologs of several *S.cerevisiae* genes required for meiosis are present in the *C.albicans* genome, including *SPO11*, *DMC1* and *NDT80* (Diener and Fink, 1996; Tzung *et al.*, 2001), a number of conserved meiotic genes are conspicuous by their absence. These include *IME1*, *ZIP2* and *SPO13*, which are strictly required for meiosis in *S.cerevisiae* (Tzung *et al.*, 2001). In any case, the question of whether *C.albicans* can undergo meiosis has not been resolved.

Studies of *C.albicans* suggest that while populations are largely clonal, there is evidence for some mechanism of genetic exchange in natural populations (Graser *et al.*, 1996; Tibayrenc, 1997). Although the present study does not rule out a meiotic phase in *C.albicans*, we demonstrate that mating and chromosome loss can constitute an alternative mechanism for genetic exchange. We speculate that chromosome loss could be induced *in vivo* when *C.albicans* is stressed by environmental conditions inside its mammalian host. Thus, chromosome loss may have evolved to provide *C.albicans* with an alternative pathway to meiosis and lead to the rearrangement of genetic material in this organism.

Materials and methods

Strains

All strains were derived from strain CA14 (Δ ura3::imm434/ Δ ura3::imm434; Fonzi and Irwin, 1993). Both copies of *GAL1* were sequentially disrupted by means of a *hisG-URA3-hisG* cassette in the strain CHY444 (a derivative of CA14 in which the *MTL alpha1* and

alpha2 genes have been deleted; Hull *et al.*, 2000) to produce the strain RBY16. SD plates containing 5-FOA and uridine were used to counterselect against the *URA3* marker (Fonzi and Irwin, 1993). RBY16 was then mated (mating conditions described below) to CHY477, in which the *MTLa1* and *ADE2* genes had been deleted. The resulting tetraploid, RBY18, was used to assay for chromosome loss. The diploid strains used to test for chromosome loss were CAF2-1 (Fonzi and Irwin, 1993) and RBY09 (*ura3/ura3 gal1::HisG-URA3-HisG/GAL1 MTLa/mla1::HisG mla2::HisG*).

In addition, strain RBY110 (*ura3/ura3, hop1::HisG/hop1::HisG, gal1::HisG/gal1::HisG, MTLa/mla1::HisG mla2::HisG*) was mated with CHY477 (Miller and Johnson, 2002) to give RBY595. RBY16 was mated with RBY113 (*ura3/ura3 hop1::HisG-URA3-HisG/hop1::HisG, ade2::HisG/ade2::HisG, mla1::HisG/MTLa*) to give RBY593. RBY51 (*ura3/ura3 nrg1::HisG/NRG1, MTLa/mla1::HisG mla2::HisG*) was mated with RBY54 (*ura3/ura3 nrg1::HisG-URA3-HisG/NRG1 ade2::HisG/ade2::HisG, mla1::HisG/MTLa*) to give RBY540.

Chromosome loss experiment

To test for chromosome loss, the tetraploid strain RBY18 or diploid strains CAF2-1/RBY09 were streaked onto different media and incubated at 25, 30 or 37°C for 5–10 days. Following incubation, cells were picked, resuspended in H₂O and the OD₆₀₀ measured. Cells were then plated on test conditions, i.e. YEPD containing uridine and adenine, 2-DOG plates, 5-FOA plates or 2-DOG/5-FOA plates. While the exact degree of chromosome loss varied significantly from experiment to experiment, large-scale random chromosome loss was observed in six independent experiments with cells grown on pre-spo medium.

The tetraploid strain was also grown on sorbose medium (Janbon *et al.*, 1998) at 37°C for 5–7 days. Resulting colonies were analyzed as above. Chromosome loss on sorbose medium was observed in three independent experiments.

FACS analyses

FACS analyses of *C.albicans* strains were carried out by growing logarithmic cultures of cells to OD₆₀₀ ~2.0. Cells were collected by centrifugation from 0.5 ml cultures and prepared for FACS analysis as described previously (Hull *et al.*, 2000).

Media, mating conditions and MTL PCR assay

For details, see Supplementary data available at *The EMBO Journal* Online.

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