6-1-2004

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Molecular Microbiology (2004) **52**(5), 1451–1462 doi:10.1111/j.1365-2958.2004.04068.x

Homozygosity at the *MTL* locus in clinical strains of *Candida albicans*: karyotypic rearrangements and tetraploid formation†

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Summary

One hundred and twenty *Candida albicans* clinical isolates from the late 1980s and early 1990s were examined for homozygosity at the *MTL* locus. Of these, 108 were heterozygous (*MTL*a/*MTL*α), whereas seven were *MTL*a and five were *MTL*α. Five of the homozygous isolates were able to switch to the opaque cell morphology, while opaque cells were not detectable among the remaining seven. Nevertheless, all but one of the isolates homozygous at the *MTL* locus were shown to mate and to yield cells containing markers from both parents; the non-mater was found to have a frameshift in the *MTL*α*1* gene. In contrast to *Saccharomyces cerevisiae*, *C. albicans* homozygotes with no active *MTL* allele failed to mate rather than mating as a cells. There was no correlation between homozygosity and fluconazole resistance, mating and fluconazole resistance or switching and fluconazole resistance, in part because most of the strains were isolated before the widespread use of this antifungal agent, and only three were in fact drug resistant. Ten of the 12 homozygotes had rearranged karyotypes involving one or more homologue of chromosomes 4, 5, 6 and 7. We suggest that karyotypic rearrangement, drug resistance and homozygosity come about as the result of induction of hyper-recombination during the infection process; hence, they tend to occur together, but each is the independent result of the same event. Furthermore, as clinical strains can mate and form tetraploids, mating and marker exchange are likely to be a significant part of the life cycle of *C. albicans in vivo.*

Accepted 2 February, 2004. *For correspondence. E-mail ptm@biosci.cbs.umn.edu; Tel. (+1) 612 624 7280; Fax (+1) 612 624 0426. †This paper is dedicated to the memory of Ira Herskowitz, whose work illuminated the field of cell type determination in budding yeast. ‡Present address: Department of Pediatrics, University Heidelberg, Im Neuenheimer Feld 153, Heidelberg 69120 Germany. §These authors contributed equally to this work.

Introduction

Candida albicans has emerged as the primary fungal pathogen of medical importance. This polymorphic yeast normally exists as a harmless commensal. However, in patients immunocompromised as a result of AIDS, organ transplantation or chemotherapy, *C. albicans* can cause significant morbidity and mortality (Kullberg and Filler, 2002). Despite the increasing clinical importance of *C. albicans* and other *Candida* species, we lack a clear understanding of *Candida* pathogenesis and the aetiology of candidiasis. This results in part from the lack of one of the most efficient tools for analysis of pathogenesis, classical genetics.

Candida albicans was considered for more than 100 years to be a member of the fungi imperfecti, lacking a sexual cycle. In the early 1990s, efforts in genomics led to the formulation of a macrorestriction map that determined the sites for the restriction endonuclease *Sfi*I on all eight chromosomes (Chu *et al.*, 1993). This allowed the localization of genes to a specific region of each chromosome. Subsequently, preparation of a physical map (http://alces.med.umn.edu/candida.html) and genomic sequencing (http://www-sequence.stanford.edu/group/candida/) were undertaken.

Although a complete sexual cycle for *C. albicans* has yet to be demonstrated, one of the fruits of the effort to determine the entire genomic DNA sequence was the discovery of a gene with high homology to the *Saccharomyces cerevisiae MAT***a**1 gene. This led to the demonstration that this fungus, which is diploid as usually isolated, is heterozygous for orthologues of both *MAT***a** and *MAT*α and to the cloning and analysis of the *MTL* (mating-type-like) locus (Hull and Johnson, 1999). The *C. albicans* orthologues were called *MTL***a** and *MTL*α. Subsequently, two laboratories engineered strains that were homozygous (Magee and Magee, 2000) or hemizygous (Hull *et al.*, 2000) for one of the two mating types. In both cases, mating, leading to tetraploids, occurred. The mating was

of low frequency, a fact that was explained recently when Miller and Johnson (2002) showed that the reversible phenotypic transition from 'white' cells to 'opaque' cells was repressed by the products of the *MTL**a**1* and *MTLα2* genes and that opaque cells mate at a frequency some 10^6 greater than white cells. Recently, Bennett and Johnson (2003) have demonstrated that tetraploids grown under conditions of stress show concerted loss of chromosomes at a relatively high frequency, usually culminating in a diploid progeny. These authors argued that parasexuality may have replaced meiosis in *C. albicans*, especially as several of the genes necessary for meiosis in *S. cerevisiae* are missing in *C. albicans* (Tzung *et al*., 2001).

However, all these experiments were carried out on strains that were engineered in the laboratory. As all clinical isolates that have been examined have been found to be diploid (Olaiya and Sogin, 1979; Whelan *et al*., 1980; Riggsby *et al*., 1982), the question of whether mating occurs outside the laboratory remains an important one. A survey by Lockhart *et al*. (2002) of more than 220 clinical isolates found seven that were homozygous or hemizygous for the *MTL* locus, and all but two of these carried out the white–opaque transition. The two that did not switch had variant colony phenotypes. Only the switching phenotypes were correlated with *MTL* configuration in these strains. Further work on the homozygotes showed that mixing *MTL**a*** and *MTLα* strains gave rise to mating figures that could be identified using differentially labelled parents. However, nuclear fusion (karyogamy) was not seen in these figures, and the progeny did not contain recombinants (cells containing genetic markers from each parent) (Lockhart *et al*., 2003).

Rustad *et al*. (2002) have found that there is a high correlation of homozygosity at the *MTL* loci with resistance to the antifungal drug fluconazole. They looked at 96 strains and found that 12 were homozygous at the *MTL* locus, six for *MTL**a*** and six for *MTLα*. All but one of these were resistant to fluconazole. No test was carried out for the mating ability of these homozygotes. A broader survey carried out by Pujol *et al*. (2003) found that this correlation did not extend to strains that had not been exposed to antifungal agents and that spontaneously generated *MTL* homozygous strains did not differ from their parents in antifungal susceptibility.

In this report, we analyse 120 clinical isolates from the late 1980s and early 1990s and show that the frequency of *MTL* homozygotes is about twice as large as that in the sample of Lockhart *et al*. (2002). All but one of these homozygotes are able to mate and form recombinants. We also examine the correlation of karyotypic rearrangements with switching and antifungal resistance in these strains. Our evidence suggests that these homozygotes arise in strains that have heightened recombination potential, as evidenced by the high frequency of chromosomal rearrangements among them.

Results

Some clinical strains contain only one kind of MTL *allele*

We undertook a survey of 120 clinical isolates of *C. albicans*, some of which came from a collection made by Scherer and Stevens (1987), some of which were isolates from the NIH, and some of which were common laboratory strains. The strains were tested as described for heterozygosity at the *MTL* locus. Figure 1A shows a gel containing the polymerase chain reaction (PCR) products from strains 665, 667, 745, 774, 775, 787 and 788 (all are *MTL**a*** except for 745) and from strain CAI-4. Figure 1C shows strain WO-1 along with six strains isolated at NIH from a single patient. Strains 3207, 3208 and 3209 are heterozygous at the *MTL* locus, while strains 3210–3212 are homozygous for the α allele. Of the 120 strains that we examined, seven were *MTL**a*** (two of which were probably related) and five were *MTLα* (three of which were related). The remaining 108 strains contained both *MTL* alleles. Hence, homozygosity at the *MTL* locus occurs at a frequency around 8–10% (depending on whether you count homozygosity in the related strains as a single event) in this set of clinical isolates of *C. albicans*. Among the previously examined laboratory strains that turned out to be homozygous was 1012A (Lasker *et al*., 1989).

Our original screen was for the *MTLα1* gene; in order to be sure that we were not missing copies of this gene with polymorphisms, we also screened for the *MTLα2* gene. Figure 1B shows that the distribution of this gene is the same as that of the *α1* gene. Thus, each strain seems clearly to lack one *MTL* allele and to have at least one intact copy of the complementary one.

Strains 3207–3212 are a series of sequential isolates from an AIDS patient with recurring candidiasis. The first three isolates were heterozygous for the *MTL* locus, whereas the last three were all *MTLα*. These strains were isolated over a period of 4 years, and the interval between 3209 and 3210 was 28 months. During this interval, the isolate lacking the *MTL**a*** allele arose.

In order to determine whether this change resulted from a second strain replacing the original one, we looked at some neutral genomic markers. The restriction fragment length polymorphisms (RFLPs) are markers used by Xu *et al*. (1999) in epidemiological studies for *C. albicans*. The single nucleotide polymorphisms (SNPs) are markers developed in this laboratory. Table 1 shows that the RFLP markers were identical in strains 3207–3212, suggesting that all six strains came from the same progenitor. As found by Xu *et al*. (1999), the strains were mostly homozygous for these markers. All the SNPs that we examined

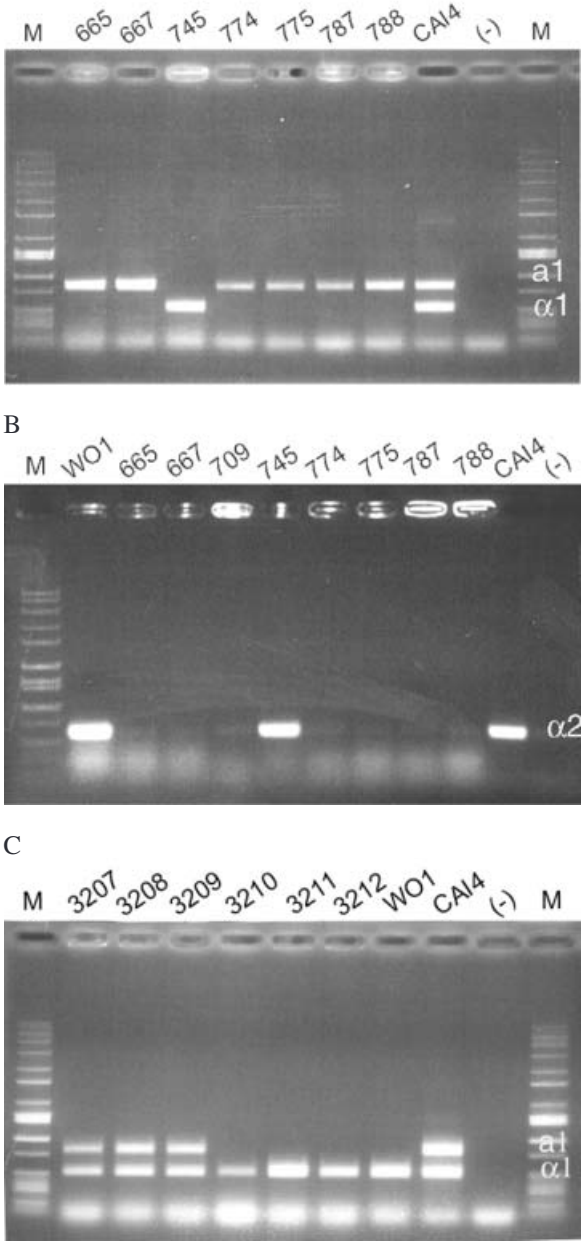


Fig. 1. PCR analysis of the *MTL* configuration of clinical isolates.
A and C. PCR of the *MTL***a**1 and α1 genes.
B. PCR of *MTL*α2.
A and B. Isolates from the collection described by Scherer and Stevens (1987).
C. Serial mouth isolates from a single patient: 3207, isolated on 6/10/93; 3208, 8/5/93; 3209, 10/18/93; 3210, 2/1/96; 3211, 6/16/97; 3212, 7/31/97. (–), no DNA control.

on chromosome 5M, the *Sfi*I fragment carrying the *MTL* locus, are in the *SNF1* gene, and they all became homozygous in strains 3210, 3211 and 3212, concomitant with the homozygosis of the *MTL* locus. *SNF1* is within 40 kb of *MTL*, so the two loci probably became homozygotic as the result of the same event. Hence, it seems most likely that, during the period between the isolation of 3209 and 3210, when the fungus existed in the commensal state, it underwent homozygosis of all or part of chromosome 5, leading to the loss of one *MTL* allele. The remaining SNPs were homozygous in all isolates, adding further evidence for the relatedness of these strains.

All but one of the clinical strains homozygous at the MTL *locus are able to mate and form recombinants*

The clinical strains are prototrophic. Most mating tests used previously for *C. albicans* use selection of prototrophs from auxotrophic parents to score for mating (Hull *et al*., 2000; Magee and Magee, 2000; Chen *et al*., 2002; Miller and Johnson, 2002). Although putative zygotic forms have been observed (Lockhart *et al*., 2003), these are difficult to identify among the usual pleiomorphic cell types and are difficult to score in a reproducible fashion. In order to determine the mating capacity of prototrophs, we used two methods. For a preliminary screen, we used the cross-streaking method described previously and looked for evidence of altered growth at the intersection. For many strains, an irregular growth pattern a bit like 'wrinkled' (Slutsky *et al*., 1985) at the intersection of the cross-streaks indicates mating. The cross-streaks of several prototrophic strains gave interesting morphology: WO-1 streaks had a special texture at the intersection with a strain of the opposite (MTL**a**) mating type, while other crosses were very sticky and difficult to replicate onto selective medium.

As the only way to confirm mating in the absence of selection was too cumbersome [colony PCR for the two *MTL* loci in isolated colonies or determining the ploidy by nuclear staining and fluorescence-activated cell sorting (FACS)], we used a set of tester strains that allows us to detect mating behaviour in prototrophs (Magee *et al*., 2002). These strains are homozygous at the *MTL* locus, *his1*/*his1 arg5*,*6*/*arg5*,*6* (hence auxotrophic for histidine and arginine), and carry the mycophenolic acid (MPA) resistance marker *IMH3*^{R} (Beckerman *et al*., 2001). Thus, mating products can be selected on minimal medium containing MPA (Magee *et al*., 2002) (Fig. 2). The homozygous clinical strains were tested against both testers. Table 2 summarizes these tests. All but strain 745 mated with the complementary mating type. Strain 745 failed to mate with either mating type, although it lacks the *MTL***a** locus and contains the *MTL*α*1* and α*2* genes (Fig. 1).

The production of MPA-resistant prototrophs as a result of mating demonstrated that the progeny contained genes from both parents, but they could be heterokaryons or products of transfer of a small number of chromosomes, as was shown to occur in parasexual crosses (Kakar and Magee, 1982; Kakar *et al*., 1983; Sarachek and Lovchik, 1989). We therefore examined them for nuclear number

Table 1. Polymorphism analysis.

Strain Locus	Size	Location	*Dde*I site	3207 RFLPs[a]	3208	3209	3210	3211	3212
F12n2	1370	ND	1370	A/A	A/A	A/A	A/A	A/A	A/A
F16n1a	1821	5I	413	A/A	A/A	A/A	A/A	A/A	A/A
F16n1b			981	A/A	A/A	A/A	A/A	A/A	A/A
E15n7	608	7G	100	A/a	A/a	A/a	A/a	A/a	A/a

Locus[b]	Size	Location	Nucleotide[c]	SNPs					
1718/2417 (SNF1)	301	5 M	89	a/g	a/g	a/g	g/g	g/g	g/g
			102	a/g	a/g	a/g	g/g	g/g	g/g
			125	a/g	a/g	a/g	a/a	a/a	a/a
			202	a/g	a/g	a/g	a/a	a/a	a/a
			216	a/g	a/g	a/g	a/a	a/a	a/a
			226	a/c	a/c	a/c	c/c	c/c	c/c
1342/2493	276	5I	172 (a/g)	g/g	g/g	g/g	g/g	g/g	g/g
			202 (a/g)	g/g	g/g	g/g	g/g	g/g	g/g
1735/2343	252	60	89 (g/t)	g/g	g/g	g/g	g/g	g/g	g/g
			190 (c/t)	t/t	t/t	t/t	t/t	t/t	t/t
1530/2473	282	7G	34 (c/t)	c/c	c/c	c/c	c/c	c/c	c/c
			183 (a/g)	g/g	g/g	g/g	g/g	g/g	g/g
2006/2431	251	4H	132 (c/g)	g/g	g/g	g/g	g/g	g/g	g/g
			195 (a/t)	a/a	a/a	a/a	a/a	a/a	a/a

a. RFLPs are from Xu *et al*. (1999). Each is a PCR fragment with a variable *Dde*I site, except that F161 has two sites.
b. SNPs from chromosome locations 5I, 6O, 7G and 4H are listed by their contigs in Assembly 6 on the Stanford Genome Technology *Candida albicans* website (http://www-sequence.stanford.edu/group/candida/). The numbers refer to the two heterozygous contigs in Assembly 6.
c. Location of polymorphism from 5′ end of forward primer.

via DAPI staining, DNA content via FACS analysis and heterozygosity at the *MTL* locus. All were mononucleate, tetraploid and heterozygous. Figure 3A shows the DAPI staining of several representative mating progeny of strains 709 and 3685, together with the parents. Although the tetraploids are larger than the parental diploids, all contain a single nucleus. Figure 3B shows FACS analysis of the mating progeny. The parents had two peaks of DNA content, G1 at 160 arbitrary units and G2 at 290 arbitrary units of fluorescence. When products of the matings were subjected to FACS analysis, the DNA content in G1 was about 350, while G2 content was in a broad peak from 580 to 640 (Fig. 3B), indicating that the DNA content of the mating products is tetraploid or close to it. Finally, Fig. 3C shows that the progeny of 10 different matings contained both *MTL***a** and *MTL*α genes. Thus, these clinical isolates were able to mate and undergo karyogamy, forming mononucleate progeny that contained markers from both parents. The range in apparent DNA content may result from chromosome loss of the sort described by Bennett and Johnson (2003). Thus, in contrast to the results of Lockhart *et al*. (2003), these matings gave rise to true tetraploids

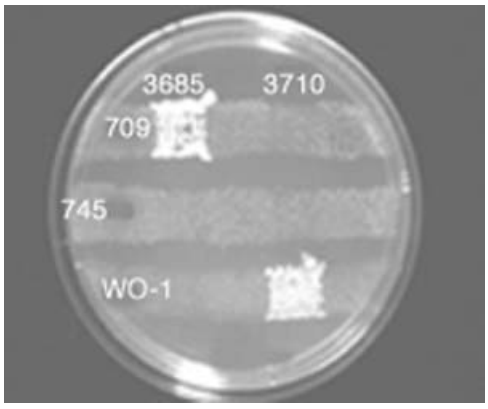


Fig. 2. Mating of prototrophs. Strains were streaked on YPD and replicated onto MPA-minimal medium as described in *Experimental procedures*. Strain 709 is *MTL***a**; WO-1 and 745 are *MTL*α. Strains 3685 (*MTL*α) and 3710 (*MTL***a**) are tester strains.

The results from the successful matings extend the results of Lockhart *et al*. (2003), as they found that none of their mating figures led to karyogamy, while all the matings reported here (except those involving 745) yielded recombinants. There are several reasons why 745 might not mate. It might have a homozygous deficiency in any of a number of genes previously shown to be required for mating (Chen *et al*., 2002; Magee *et al*., 2002), it might lack as yet uncharacterized required genes, or it might have a mutation in one or both of the *MTL*α genes. In *S. cerevisiae*, haploid strains with mutations in both *MATL*α*1* and α*2* mate as if they were *MAT***a** strains (Strathern *et al*., 1981), but 745 does not mate with *MTL*α strains. We wanted to determine whether *C. albicans* mutants behave like *S. cerevisiae*, so we prepared a strain lacking both

Table 2. Properties of clinical isolates.

Strain	*MTL*	Mate	Switch	Fluconazole resistant	Other
1012A	α	Yes	Yes	No	Small rDNA
665	**a**	Yes	Yes	No	Altered chromosome 7
667	**a**	Yes	Yes	No	
709	**a**	Yes	Yes	No	Chromosome translocation
745	α	No	No	No	Chromosome translocation
[a]774 (anal)	**a**	Yes	No	No	Extra chromosome 7
[a]775 (urine)	**a**	Yes	No	No	Extra chromosome 7
787	**a**	Yes	No	No	Extra chromosome 7
788	**a**	Yes	No	No	Extra chromosome 7
[b]3207	**a**/α	No	No	Yes	
[b]3208	**a**/α	No	No	Yes	
[b]3209	**a**/α	No	No	Yes	
[b]3210	α	Yes	Yes	Yes	Chromosome translocations
[b]3211	α	Yes	No	Yes	Chromosome translocations
[b]3212	α	Yes	No	Yes	Chromosome translocations

a. Same patient.
b. Same patient.

*MTL***a** and *MTL*α by selecting sorbose-resistant derivatives of CHY444, a strain carrying insertions in *MTLα1* and *α2* (Hull *et al*., 2000) and identifying one homozygous for the *MTL*α deletions. This strain was tested against *MTL***a** and *MTL*α strains, and it failed to mate with either. We sequenced PCR products of *MTLα1* and *α2* from 745. *MTLα2* was intact, but *MTLα1* has a single nucleotide deleted at position 20 leading to a frameshift. *MATα1* mutants do not mate in *S. cerevisiae*; thus, this mutation explains the failure of 745 to mate (Strathern *et al*., 1981).

Homozygosity at the MTL *locus sometimes but not always gives rise to opaque cells but is not correlated with fluconazole resistance*

Three phenotypes have been associated with homozygosity at the *MTL* locus: the white–opaque phenotypic transition (Miller and Johnson, 2002), fluconazole resistance (Rustad *et al*., 2002) and the ability to mate (Hull *et al*., 2000; Magee and Magee, 2000). We first surveyed our strains for switching and azole resistance. Table 2 shows that only switching was associated with *MTL* homozygosity; the association occurred frequently but not always. Using the phloxin *B* test, we found that nine of the 12 homozygous strains gave red colonies, a phenotype associated with switching. When we examined the red colonies microscopically, only five of the nine gave the typical opaque cell shape. No opaque cells were detected in the remaining homozygous *MTL* strains either by colour or upon microscopic examination after conditions designed to reveal switching. A similar phenomenon was reported by Lockhart *et al*. (2002), who found that two of their isolates showed an intermediate colour phenotype on phloxin B but did not change shape. It is, of course, possible that we failed to detect a small number of opaque cells in the cultures.

Fluconazole resistance was not a common phenotype among the *MTL* homozygotes. Only the sequential isolates from the candidiasis patient showed resistance above 1 μg ml^{-1} (Table 2). A possible reason for the divergence of our results from those of Rustad *et al*. (2002) is discussed below.

Many of the strains homozygous at the MTL *locus have karyotypic abnormalities*

A frequent finding among clinical strains is that they have non-standard karyotypes, but this has not been associated with any other properties. We have examined the karyotypes of the homozygous clinical strains. We found no differences in the larger chromosomes, R, 1, 2 and 3, under normal pulsed-field electrophoretic conditions (data not shown). [One of these strains, 1012A, has been shown to have separable homologues of chromosome R as a result of widely differing sizes of rDNA repeats (Lasker *et al*., 1989).] However, as Fig. 4 shows, 10 of the other 11 homozygous strains (all but 667) have chromosome translocations or extra chromosomal bands in the size range of chromosomes 4–7. These changes range from an extra chromosome in the chromosome 7 size range to the highly rearranged karyotypes seen in strains 709, 745, 3211 and 3212 (Fig. 4A and D). In order to determine whether the rearrangements had led to homozygosity (for example, by loss of one homologue or part of one homologue of chromosome 5), we probed the CHEF gel with *MTL***a** or α probes as well as with sequences located on the two *Sfi*I fragments 5I (480 kb) and the site of the *MTL* locus, 5M (750 kb) (Chu *et al*., 1993). The a and α probes gave the same results as the 5M probe (data not shown), except in the cases of 3111 and 3212 (see below). The results are shown in Fig. 4F and G.

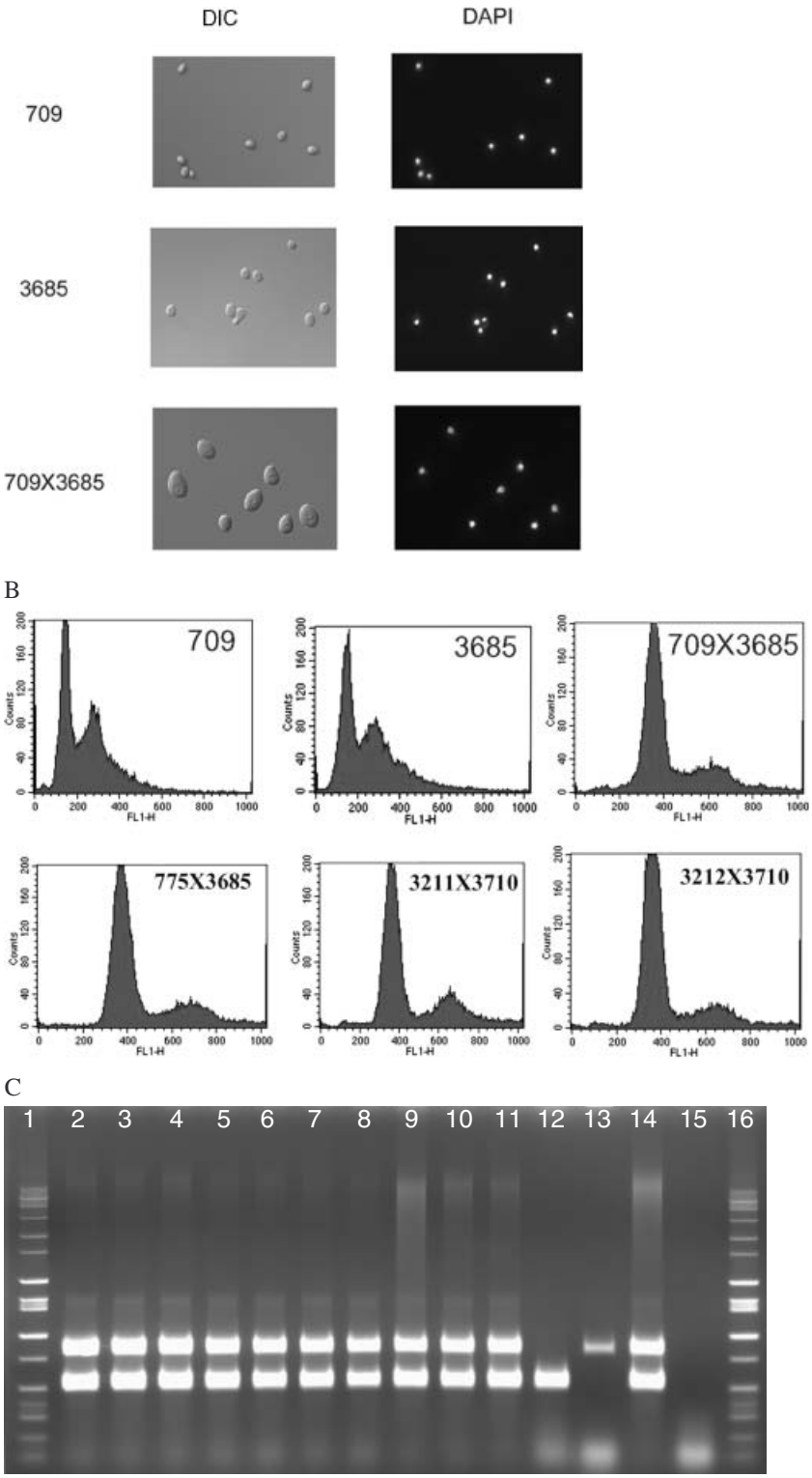


Fig. 3. The mating products are mononucleate, tetraploid and heterozygous at the *MTL* locus.
A. DAPI staining of the nuclei of 3685, 709 and their mating progeny. Left, DIC images; right, fluorescent micrographs.
B. FACS analysis of 3685, 709 and their mating progeny as well as the progeny from three other matings.
C. PCR analysis of products from matings of clinical isolates. Lane 1, marker DNA; 2, 665 × 3685; 3, 667 × 3685; 4, 709 × 3685; 5, 774 × 3685; 6, 775 × 3685; 7, 787 × 3685; 8, 788 × 3685; 9, 3210 × 3710; 10, 3211 × 3710; 11, 3212 × 3710; 12, 3685; 13, 3710; 14, SC5314; 15, no DNA control; 16, marker DNA.

Only strains 745 and 3211 lack a band in the region of chromosome 5, the site of the *MTL* locus. Strain 3211 has no band at the chromosome 5 region (1.4 Mbp) on the gel, and both the 5M and 5I probes hybridize to a band of about 930 kbp. This strain also contains a band at 960 kb that hybridizes with the *MTLα1* but not with the 5M probe, *YLR118*. Strain 3212 has a chromosome in the chromosome 5 (1.4 Mbp) range hybridizing to both the 5M and 5I probes, but it also has a 940 kb band like the 930 kb one in 3211 and an additional copy of the *MTLα1* sequence on a small band of 590 kb. Interestingly, this last band also does not hybridize with *YLR118*, which is found on a different sequence contig from *MTL* as well as on a different fosmid contig in the physical map (T. Rast, personal communication). Hence, both these isolates contain bands that include the *MTL* locus but not much of the rest of chromosome 5, and 3212, at least, must contain three copies of the *MTLα1* locus.

Although strains 3210–3212 are related and chronologically sequential, the karyotype of the last isolate, 3212, does not seem to be derived from 3211. For example, although 3210 has intact chromosomes 5 and 7, 3211 does not have an intact band at either position. However, 3212 has bands at the positions of both chromosomes. As a reversal of a chromosome rearrangement seems highly unlikely, the best explanation for the two strains is that 3212 is derived from the same progenitor as 3211 (probably 3210), not 3211 itself.

In the sequential series of strains 3207–3212, the first three heterozygous strains have standard karyotypes. However, during the period between the isolation of 3209 and 3210, homozygosis arose at the *MTL* locus. The fact that the last three isolates have divergent karyotypes but are clearly related suggests that a persisting, non-rearranged strain yielded rearranged karyotypes as the disease recurred.

Discussion

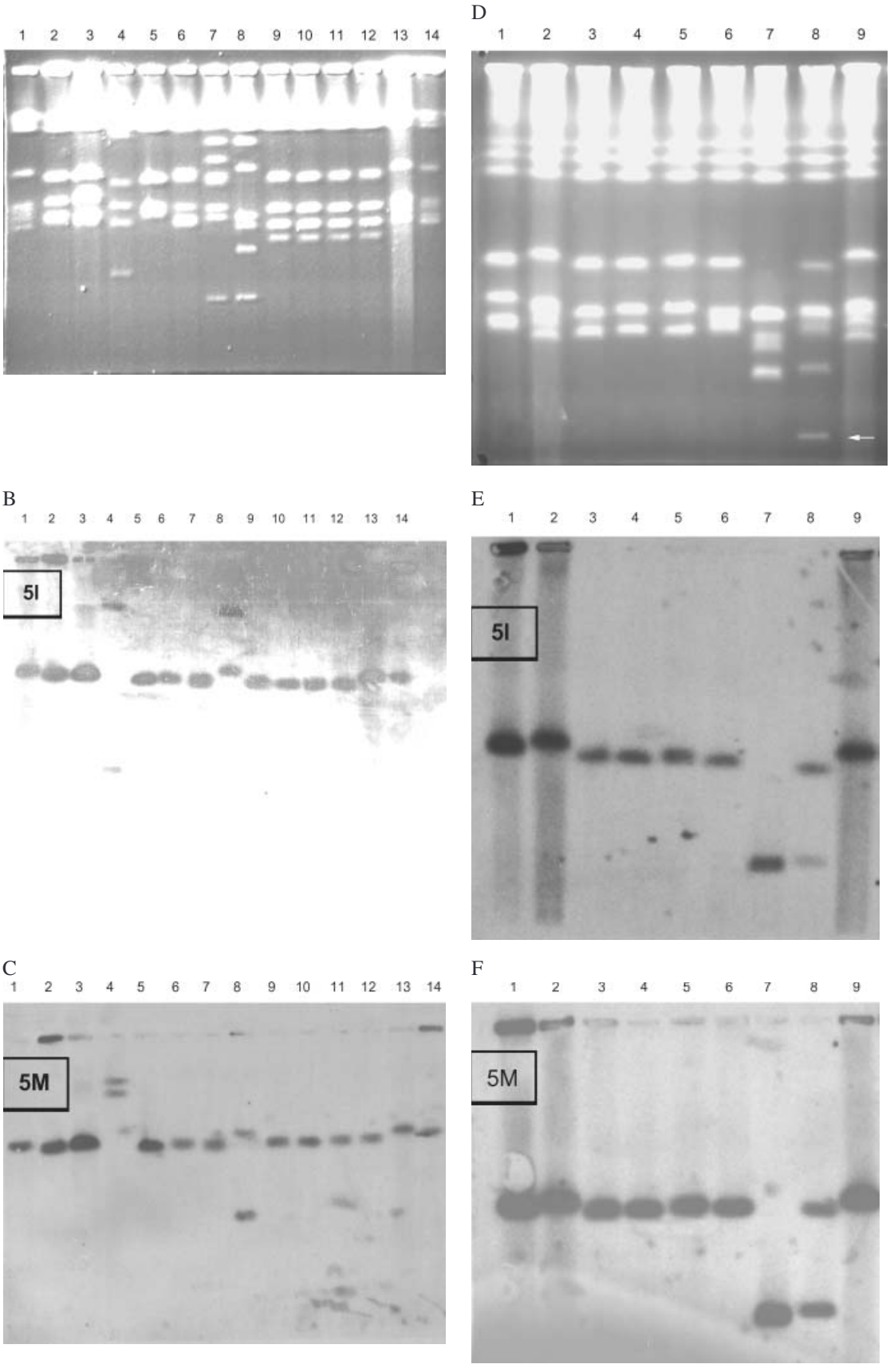
The demonstration that *C. albicans* strains engineered in the laboratory to be homozygous at the *MTL* locus can mate does not tell us whether mating is part of the wild-type life style of this pathogenic fungus. Two recent papers have shown that some clinical isolates are homozygous for one *MTL* allele or the other. Lockhart *et al*. (2002) have shown that strains homozygous or hemizygous for the *MTL* locus exist among clinical isolates. Homozygosity at this locus also exists among the clinical isolates that we have analysed, although the frequency is about twice (12/122) what Lockhart *et al*. (2002) found (7/220). Rustad *et al*. (2002) found that one of 46 azole-susceptible and 11 of 50 azole-resistant strains were *MTL***a** or *MTLα*. Lockhart *et al*. (2003) later showed that clinical isolates were able to form mating figures, but in their hands did not yield recombinants, thus leaving open the question of whether formation of recombinants could occur among clinical isolates. We found that, of the 12 clinical isolates homozygous at the *MTL* locus examined here, all but one will mate and form recombinants as judged by positive PCR analysis for *MTL***a** and *MTLα* in several mating progeny as well as by the appearance of markers from both parents. These recombinants were shown to be mononucleate and close to tetraploid in DNA content, thus ruling out heterokaryons as the mating products. This is not surprising as, in the first spheroplast fusion experiments, multinucleate syncitia were shown to reduce to mononucleate tetraploid cells at a high frequency (Sarachek *et al*., 1981).

In contrast to *Cryptococcus neoformans*, in which the *MATα* mating type is very much more common than *MAT***a**, each of the three reports on the natural distribution of the *C. albicans MTL* locus shows a relatively even balance between the two mating types. *In vivo*, therefore, mating-competent strains of both types can be found and, in principle, marker assortment can occur in human patients. However, it has not yet been demonstrated that strains of opposite mating type occur among the flora of a single individual.

The route by which strains become homozygous for the *MTL* locus *in vivo* is not clear, but the results reported here give some clues. Opaque cells were first identified in WO-1 (Slutsky *et al*., 1987), a strain which is homozygous for the *MTLα* allele (Table 3; Lockhart *et al*., 2002). This strain has undergone three chromosome translocation events, two of which involved chromosome 5, the location of the *MTL* locus. It seems possible that WO-1 went through a

Table 3. Strains used in this paper.

Strain	*MTL*	Mate	Switch	Fluconazole resistant	Other	Reference
SC5314	**a**/α	No	No	No		Gillum *et al*. (1984)
CAI-4 (*ura3*/*ura3*)	**a**/α	No	No	No		Fonzi and Irwin (1993)
WO-1	α	Yes (MPA)	Yes	No	Chromosomes 5,6 and 1,5 translocations	Slutsky *et al*. (1987)
3685 (*ura3*/*ura3*/*URA3* *his1*/*his1 arg5,6*/*arg5,6* MPA^r)	α	Yes	Yes	No	Homozygous for chromosome 5	Magee *et al*. (2002)
3710 (*ura3*/ura3/*URA3* *his1*/*his1 arg5,6*/*arg5,6* MPA^r)	**a**	Yes	No	No	Homozygous for chromosome 5	Magee *et al*. (2002)



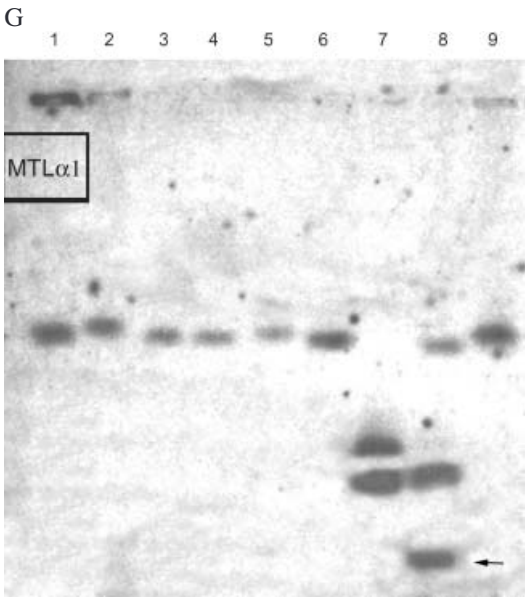


Fig. 4. Karyotypic analysis of *MTL* homozygous clinical strains. Chromosomes of the strains were separated by CHEF gel electrophoresis, transferred and probed with small DNA sequences from chromosome fragments 5M, 5I and from the gene *MTLα1*.
A. Stained CHEF gel. Lane 1, SC5314; 2, 1006; 3, 1012A; 4, WO-1; 5, 665; 6, 667; 7, 709; 8, 745; 9, 774; 10, 775; 11, 787; 12, 788; 13, SC5314; 14, CAI-4.
B. Gel in (A) Southern blotted and hybridized with a radioactive probe from chromosome *Sfi*I fragment 5I.
C. Gel in (A) Southern blotted and hybridized with a radioactive probe from chromosome *Sfi*I fragment 5M.
D. Stained CHEF gel. Lane 1, 1006; 2, SC5313; 3, 3207; 4, 3208; 5, 3209; 6, 3210; 7, 3211; 8, 3212; 9, SC5314.
E. Gel in (D) Southern blotted and hybridized with a radioactive probe from chromosome *Sfi*I fragment 5I.
F. Gel in (D) Southern blotted and hybridized with a radioactive probe from chromosome *Sfi*I fragment 5M.
G. Gel in (D) Southern blotted and hybridized with a radioactive probe from the *MTLα1* gene.
Arrows in (D) and (G) indicate the 590 kb chromosomal band.

period of hyper-recombination and that, during this period, mitotic recombination led to homozygosis of the *MTL* locus. Chromosome translocations would be another result of this process. If this were the case, WO-1 might be expected to contain fewer polymorphisms than most strains, as mitotic recombination results in homozygosity. This is a prediction that can be tested. In keeping with the idea that homozygosity and chromosome translocations might be correlated, the strains that we have examined contain a large number of karyotypic variations. In fact, only two of the homozygous strains have the standard karyotype. Lockhart *et al*. (2002) found that two out of 20 *MTL***a**/*MTL*α strains gave rise spontaneously to homozygous strains. However, the karyotypes of the original strains and the switching and non-switching derivatives were not reported.

We found no cases of azole resistance in our original group of nine homozygotes. However, among a series of isolates taken from a single patient over 4 years, azole resistance preceded mating type homozygosity, which in turn preceded chromosome rearrangement. Thus, this series indicates that, during either the commensal or the pathogenic state, there may be significant karyotypic changes, and homozygosity at the *MTL* locus is frequently among them. Our results are in agreement with those of Pujol *et al*. (2003), who found that antifungal resistance was not elevated among their *MTL* homozygotes. One possible reason for the divergence of these two findings from those of Rustad *et al*. (2002) is that most of the strains used here were isolated in the late 1980s, before the widespread use of fluconazole, while most of the strains described by Pujol *et al*. (2003) had also not been exposed to any antifungals.

Could homozygosity at the *MTL* locus be associated with the variability that would allow resistant strains to arise? The karyotypic changes that we have found suggest that homozygosity may be associated with large-scale genomic changes. The strains we studied would have had the potential variability from which resistance could be selected, but selection did not occur. As evolution of azole resistance can be studied experimentally (Cowen *et al*., 2001), it would be interesting to examine strains that are isogenic except for homo- and heterozygosity at the *MTL* loci to see whether the same population of resistant variants would arise from each.

The failure of strain 745 to mate despite the fact that it appears to be *MTL*α has been shown to result from a mutation in the *MTLα1* gene. As it has been suggested that the switching genes are repressed by the **a**1/α2 complex (Miller and Johnson, 2002), the failure of 745 to switch should not be due to its mutation in *MTLα1*. We have not, however, found conditions under which it (and several other *MTL* homozygous strains) switch to the opaque mating morphology (Miller and Johnson, 2002). Nevertheless, all strains except 745 mate. It is possible that strains 774, 775, 787, 788, 3711 and 3712 do in fact switch at a low level, but we have not been able to detect it.

We showed that a strain that is homozygous for insertions in both *MTL*α genes does not mate. This is in contrast to the case in *S. cerevisiae* (Strathern *et al*., 1981), in which such strains mate as if they were *MAT***a**. However, this strain is also homozygous for the chromosome 5 homologue that carries *MTL*α, and it may lack heretofore undetected a-specific genes on the other homologue. We tentatively conclude, however, that *C. albicans* strains lacking both *MTL* loci do not mate and thus do not exhibit the Aif (**a**-like faker) phenotype. Therefore, mating in *C. albicans* differs in this respect from that in *S. cerevisiae*. This contrast with *S. cerevisiae* is quite interesting. Use of microarrays to examine gene expression in mating type null strains of *S. cerevisiae* and *C. albicans* might show

the reasons for this divergence and provide further insight into the mating phenotype in *C. albicans*.

Our results suggest that homozygosity at the *MTL* locus is associated with karyotypic rearrangements. These rearrangements do not always lead to homozygosity; rather, they are probably also products of the process that generates homozygosity. However homozygosity occurs, mating between clinical isolates of *Candida albicans* is feasible, leads to recombinant cells and is quite likely to occur in nature. That being the case, it is important to determine whether meiosis also occurs and, if it does, whether it occurs in diploids or tetraploids.

Experimental procedures

Strains and growth conditions

The *C. albicans* strains used are shown in Tables 2 and 3. All strains were maintained in YEPD–glycerol (50%) or YEPD–DMSO (7%) suspensions at −80°C. Media used were YEPD (10 g of yeast extract, 10 g of peptone and 20 g of glucose per litre) and Min (6.7 g of yeast nitrogen base without amino acids and 20 g of glucose per litre). MinMPA contained mycophenolic acid at 10 μg ml^{-1}.

Analysis of the MTL *configuration*

The *MTL* configuration (heterozygous or homozygous) was determined by PCR using primers specific for *MTL***a1**, *MTL*α1 and *MTL*α*2*. These primers generate products of 821 bp, 515 bp and 609 bp respectively. Heterozygosity is easily demonstrated by agarose gel electrophoresis of a PCR containing both sets of primers and template DNA, as the bands are separable. Conversely, a single band indicates homozygosity or hemizygosity, and its size indicates which *MTL* allele is present. Except in strains with translocations of chromosome 5, the location of the *MTL* locus, it is very difficult to determine whether strains lacking one allele have one or two copies of the remaining allele, and PCR analyses do not distinguish between homozygosity and hemizygosity. (In translocation strains, this distinction can be made by Southern blots of karyotype separations.) For purposes of simplicity, we have referred to strains lacking either *MTL***a** or *MTL*α as homozygous. In some cases, we verified the *MTL* configuration by PCR using primers specific for the *PAP1* genes, which are also mating type specific (Hull and Johnson, 1999; data not shown). All PCR primers are listed in Table 4. Amplifications were carried out using a standard programme: 94°C for 60 s, 92°C for 40 s, 60°C for 30 s, 72°C for 60 s; repeat steps 2–4 29 times; 72°C for 10 min.

Pulse-field electrophoresis

Pulse-field electrophoresis using a CHEF DRIII (Bio-Rad) apparatus was used to analyse the karyotypes. The following conditions were used to separate the smaller chromosomes (all at 16°C): 1% agarose in 0.5× TBE, 60–120 min switch, 6 V cm^{-1}, 120°C, for 24 h, followed by 120–300 min switch at 4.5 V cm^{-1}, 120°C for 12 h. Larger chromosomes were separated in 0.6% PFGE grade agarose (Amresco III) in 0.5× TBE, 120–300 min switch, 4.5 V cm^{-1}, 120°C, for 24 h followed by 720–900 min switch, 2 V cm^{-1}, 106°C, for 12 h. DNA was transferred to Hybond-N+ (Amersham Biosciences) membranes and hybridized with ^{32}P-labelled probes. Washed filters were analysed by autoradiography (Magee and Magee, 2000).

Table 4. PCR primers.

*MTL*a2-F	CATGAATTCACATCTGGAGGCAC
*MTL*a2-R	ATAGCAAAGCAGCCAACTCAGGT
*MTL*α1-F	TTCGAGTACATTCTGGTCGCG
*MTL*α1-R	TGTAAACATCCTCAATTGTACCCGA
*MTL***a**1-F	TTGAAGCGTGAGAGGCAGGAG
*MTL***a**1-R	GTTTGGGTTCCTTCTTTCTCATTC
PAPa-R1	AAGCTGCACTTACTGTTCCGACAC
PAPa-F1	AGAATGCCTGTGATTACCCCG
*PAP*α-R1	GCATAATAGAAGAGCCGCGAGAG
*PAP*α-F1	GCAAGATTGAATATTCCTCGCGT

Determination of the switching phenotype

Phenotypic instability was observed using YEPD-phloxin B (50 μg ml^{-1}) plates. The cells were streaked onto plates, and the plates wrapped in parafilm and incubated at room temperature for 2 weeks. The appearance of both white (or pink) and red colonies was taken to indicate that the cells were unstable (switched). Cells of switching strains were examined by phase microscopy at 100× and documented with a Nikon 990 camera (data not shown).

Determination of fluconazole sensitivity

Strains were tested for fluconazole resistance by the Etest method (AB Biodisk).

Mating protocol

The strains were grown on YEPD and mated *in vitro*. Mating was assayed by streaking on YEPD, cross-replicating streaks onto YEPD plates, incubating at room temperature for 1 week and then replicating onto plates containing selective medium. Growth at the intersection of the streaks was considered positive. The tester strains, 3685 (*MTL*α) and 3710 (*MTL***a**), are His$^-$ Arg$^-$ MPA^R, and the selective medium was MinMPA. The tester strains are unable to grow on minimal medium; the prototrophs are mycophenolic acid sensitive, so only recombinants will grow.

Analysis of neutral genomic markers

Strains 3707–3712 were screened for polymorphisms using eight co-dominant DNA markers (three RFLP markers and five SNP markers). PCRs for all markers were carried out in a total volume of 25 μl with 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each dATP, dCTP, dGTP and dTTP (TaKaRa Bio), 1.0 unit of r*Taq* polymerase (TaKaRa

Bio), 2.5 μl of a 1:100 dilution of quick prep DNA and each primer at 15 mM. Amplification was performed for 34 cycles as follows: initial denaturation for 3 min at 95°C, denaturation step for 1 min at 95°C, annealing step for 30 s at 54°C, extension step for 1 min at 72°C and a final extension step for 5 min at 72°C. Three microlitres of PCR product was checked on an agarose gel (0.8% agarose in 0.5× TAE).

For RFLP analysis, 20 μl of PCR product was digested for 2 h at 37°C using conditions recommended by the enzyme supplier (New England BioLabs). Restriction fragments were resolved by gel electrophoresis on 2% MetaPhor agarose gels (Cambrex Bioscience) with 0.5× TAE buffer and visualized by ethidum bromide staining.

Sequences of SNP markers were obtained with both forward and reverse primers. Samples were prepared according to the manufacturer's instructions (Perkin-Elmer). Sequencing was carried out on an ABI 3700 capillary sequencer (Perkin-Elmer).

Nuclear staining

Ethanol-fixed cells were stained with DAPI (4′, 6′-diamidino-2-phenylindole) dissolved in water at 1 μg ml^{-1}.

FACS analysis

FACS analysis was carried out as described by Hull *et al*. (2000).

Acknowledgements

This work was supported by grants AI16567 and AI46351 and contract AI05406 from the National Institute of Allergy and Infectious Disease. P.L. was a trainee on grant T32-GM08347 from the National Institute of General Medical Sciences. We would like to acknowledge the assistance of the Flow Cytometry Core Facility of the University of Minnesota Cancer Center, a comprehensive cancer centre designated by the National Cancer Institute, supported in part by P30 CA77598.

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