Homozygosity at the *Candida albicans MTL* locus associated with azole resistance

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Antifungal drug resistance in the pathogenic fungus *Candida albicans* is a serious threat to the growing population of immunocompromised patients. This study describes a significant correlation between loss of heterozygosity at the *C. albicans* mating-type-like (*MTL*) locus and resistance to azole antifungals. A pool of 96 clinical isolates consisting of 50 azole-resistant or susceptible dose-dependent isolates and 46 azole-susceptible isolates was screened by PCR for the presence of *MTLa1* and *MTLα1*. These genes were used as markers for the *MTLa* and MTLα loci. Both loci were present in 84 of the isolates. Six isolates failed to amplify *MTLa1* and six failed to amplify *MTLα1*. Further PCR analysis demonstrated that loss of the *MTLa1* and *MTLα1* genes corresponded to loss of all of the loci-specific genes, resulting in homozygosity at the *MTL* locus. Southern analysis and single nucleotide polymorphism (SNP) analysis were used to determine that this loss of heterogeneity was due to replacement of one of the *MTL* loci with a duplicate of the other locus resulting in two homozygous copies of the *MTL* locus. Of the 12 homozygous isolates, one isolate was sensitive to azole drugs. Statistical analysis of the data demonstrates a strong correlation between homozygosity at the *MTL* locus and azole resistance ($P<0{\cdot}003$). In a set of serial isolates, an increase in azole resistance correlated with the loss of heterozygosity at the *MTL* locus, lending further strength to the correlation. Gene disruptions of the *MTL* loci were found to have no effect on azole susceptibility.

Keywords: pathogenic fungi, mating locus, drug resistance

INTRODUCTION

Candida albicans is a pathogenic fungus responsible for frequent, recurrent oral and vaginal candidiasis in AIDS patients, and systemic infections in transplant patients (Odds, 1988). *C. albicans* is also the fourth most common nosocomial bloodstream isolate (Pfaller *et al.*, 1998). Untreated, systemic candidiasis has a mortality rate of ~40% (Wright & Wenzel, 1997). Treatment options are limited to the toxic but fungicidal polyene drugs and the fungistatic azole drugs. Prophylactic use of the azole drugs, combined with their fungistatic nature, has resulted in widespread azole resistance (Georgopapadakou & Walsh, 1996; Law *et al.*, 1994).

Abbreviations: CHEF, contour-clamped homogeneous electric field; chr, chromosome; EtBr, ethidium bromide; SNP, single nucleotide polymorphism.

A number of mechanisms have been correlated with azole resistance, including mutation and overexpression of the gene encoding the azole target enzyme, *ERG11*, and overexpression of the genes encoding the efflux pumps *MDR1*, *CDR1* and *CDR2* (White *et al.*, 1998). However, there remains a significant portion of resistant clinical isolates in which none of the resistance mechanisms previously described can be identified (Stevens & White, 2000).

The *C. albicans* mating-type-like (*MTL*) loci are homologous to the *Saccharomyces cerevisiae* mating type (*MAT*) loci. The *MAT* loci contain the transcriptional regulators *MATa1*, *MATα1*, and *MATα2* that determine the mating type (*MATa* or *MATα*) of haploid *S. cerevisiae* strains (Johnson, 1995; Herskowitz *et al.*, 1997). In haploid *MATa* strains, the *MATa1* gene product has no known function. In haploid *MATα* strains, Matα1p acts as an activator of the α mating-type

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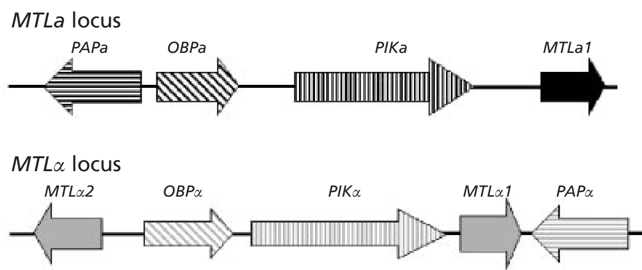

Fig. 1. Map of the *C. albicans* mating-type-like (*MTL*) loci. Genes specific to the *MTL* locus include those encoding poly(A) polymerase (*PAP*), oxysterol-binding protein (*OBP*), and phosphoinositol kinase (*PIK*), and the mating-type homologues *MTLa1*, *MTLα1* and *MTLα2*. The *a* and α versions of *PAP*, *PIK* and *OBP* have approximately 60 % homology to each other. The *MTL* genes have no significant homology to each other.

specific genes and Matα2p inhibits mating-type *a* specific genes. In diploid *S. cerevisiae*, both the *MATa* and *MATα* loci are present. In these diploid strains Mata1p and Matα2p form a heterodimer that acts to repress haploid-specific genes.

The *C. albicans MTL* loci contain genes homologous to *MATa1*, *MATα1* and *MATα2* in the same genomic arrangement as the *S. cerevisiae* genes (Hull & Johnson, 1999). However, the *C. albicans* loci also contain genes unique to fungal mating loci, including genes encoding an oxysterol-binding protein (*OBP*), a poly(A) polymerase (*PAP*), and a phosphoinositol kinase (*PIK*) (Fig. 1). A copy of each of these three genes is present in both the *MTLa* locus and the *MTLα* locus. The *a* and α copies of each of the three genes have only 60 % identity to each other, suggesting that homologous recombination within the *MTL* loci is not common, unlike the rest of the genome (Hull & Johnson, 1999). The *MTL* loci span approximately 9 kb and are flanked on both sides by identical sequence, suggesting that the flanking regions undergo homologous recombination like the rest of the genome (Hull & Johnson, 1999). Most wild-type *C. albicans* strains are diploid and have both the *MTLa* and *MTLα* loci (Magee & Magee, 2000).

The gene encoding the target enzyme for azole drugs, *ERG11*, is located on chromosome (chr) 5, the same chromosome as the *MTL* loci. Previous work has demonstrated that gene conversion or mitotic recombination associated with the *ERG11* gene is associated with azole resistance (White, 1997a). Therefore, it was of interest to determine if these gene conversions or mitotic recombinations might have affected the *MTL* loci.

The current study describes a screen of clinical isolates to determine their genotype at the *MTL* locus. That screen demonstrated a significant correlation between homozygosity at the *C. albicans MTL* locus and resistance to the antifungal fluconazole. Southern analysis was used to confirm the PCR results and to demonstrate the presence of duplicate copies of the remaining *MTL* locus in the *MTL* homozygous strains.

METHODS

Strains and growth of cultures. Fifty of the clinical isolates were sent to M.A.P. from locations around the USA for further characterization. Thirty-six of the clinical isolates were sent to D.A.S. from California locations (Stevens & White, 2000). Ten isolates were sent to T.C.W. from California locations. Isolates were plated on ChromAgar (DRG International) to characterize them as *C. albicans*. The gene disruptions were provided by Christina Hull (Duke University, Durham, NC, USA) and were constructed in the laboratory of Alexander Johnson (University of California, San Francisco) (Hull *et al.*, 2000). The FH serial isolates used in this study were described previously (Marr *et al.*, 1997, 1998). Laboratory strains 3153A (ATCC 28367, American Type Culture Collection), SC5314 (Fonzi & Irwin, 1993) and CAI4 (Fonzi & Irwin, 1993) were used as controls. The isolates were stored as frozen stocks kept at −70 °C in YEPD (10 g yeast extract, 20 g peptone, 20 g glucose per litre) with 10 % (v/v) glycerol. The isolates were struck from frozen stocks onto YEPD plates and grown at 30 °C overnight. Plates were kept at 4 °C and subcultured from single colonies every 2 weeks. Single-colony inocula were grown in YEPD liquid medium for DNA and CHEF gel block preparation.

Susceptibility testing. Fluconazole MICs were determined by broth microdilution or macrodilution using the standardized NCCLS protocol (National Committee for Clinical Laboratory Standards, 1997) as described previously (Stevens & White, 2000). Confirmation of the MICs for the *MTL* homozygous strains was done using E-test strips according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

PCR analysis. Oligonucleotides used for PCR in this study are listed in Table 1. PCR was performed in 50 μl reactions containing: 1 × PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 8·3), 2·5 mM $MgCl_2$, 200 μM dNTPs, 100 ng template DNA ml^{-1}, 20 μM each primer, and 5 U *Taq* polymerase. PCR was performed under the following conditions in a PTC-100 programmable thermal controller (MJ Research): denaturing at 94 °C for 10 min followed by 30 cycles of 1 min denaturing at 94 °C, 2 min annealing at 55 °C, and 3 min elongation at 72 °C, followed by a final extension step at 72 °C for 10 min. PCR fragments were visualized by electrophoresis on a 1·2 % agarose gel in 1 × TBE (0·089 M Tris/borate, 0·089 M boric acid, 2 mM EDTA) at 100 V for 1 h and stained with ethidium bromide (EtBr).

Statistical analysis. Freely available on-line software was used to calculate a *P*-value using Fisher's exact test (Uitenbroek, 1997).

CHEF (contour-clamped homogeneous electric field) gels. Gel blocks for CHEF gels were made using the following method (adapted from the method posted at http://alces.med.umn.edu/candida/methods.html). From overnight cultures, 2×10^8 cells were removed and washed twice in 50 mM EDTA pH 7·5. Cells were resuspended in 200 μl EDTA pH 7·5 and treated with 15 μl Zymolyase solution (50 %, v/v, glycerol, 2·5 mg Zymolyase ml^{-1}, 10 mM sodium phosphate pH 7·5). An equal volume of 1·5 % low-melting-point agarose in 1 × TAE (4 mM Tris/acetate, 1 mM EDTA pH 8) was added and the mixture poured into moulds. Blocks were allowed to cool at 4 °C for 30 min and were then added to a solution of

Table 1. Oligonucleotides used for PCR

Gene or locus	Reference*	Location†	Size (bases)	Sequence (5′–3′)
MTLa1	1	9783–10318	535	1. TAAGAATGAAGACAACGAGG 2. CGTGTTTTTCTGCTATCAATTCC
MTLα1	1	7999–8421	423	1. TACATTCTGGTCGCGATGCTC 2. GTAATCCAAAGCCTCGCATAA
MTLα2	1	1939–2553	615	1. ATGAATTCACATCTGGAGGC 2. CTGTTAATAGCAAAGCAGCC
OBPa	1	3976–5517	1551	1. AATTGCTGGTCGCTGATCG 2. ATTATTCCCAATGTGTGCCAAC
OBPα	1	3207–4498	1291	1. AATTTATCCAGCGAACATGCAC 2. CTTCTGTCCTGGAACAATCGG
PAPa	1	1674–3680	2007	1. AATCAAGCATACGGTGTTACAC 2. CCTCATGTCGCCAACCACAG
PAPα	1	8394–10205	1812	1. GACTGGTTATGCGAGGCTTTG 2. AGATCCTGGACCATAGACTCC
PDE1‡	2	800–1151	352	1. TAATATGCTAGGTGGGGGTTCCTT 2. GCCCAATATGCCTAGTTTCAAAATC
ARG4‡	2	1698–1893	196	1. TCACGGCAATTCTTGAACGAG 2. GCTAAAGCACCAGATCCTAATGGAG
C2F7‡	2	1–282	282	1. GTTTGATCTGGAACGATCTC 2. AGAAACCAACCAGCGTCTTC
ERG11‡	3	133–682	549	1. AGATCATAACTCAATATG 2. ACCCCATGAGTTTTTCTTT
ERG11 term§	3, 4	1495, 525	585	1. GGGAAAGTTTCTAAAGGGG 2. GAAGTAACGCTCATATG
MTLa-ERG11 -MTLα	5		150	1. TCGCAAGCAGGAAGAAGAAGAAGCTTTCATTTTTCCC-ACAGTAAG TACCG GGTGGTCAACATACTTCTGC 2. CTATTTCCAATCCGGAACACCAACGAGAACTATCATC-TAAATTAGCGGAT ACCTAAATGTAACAAGAACC

* 1, Hull & Johnson (1999); 2, Cowen *et al.* (1999); 3, Lai & Kirsch (1989); 4, White (1997b); 5, this study.

† Locations are based on the published sequence as referenced in the previous column.

‡ Fragments used for SNP analysis. *PDE1* digested with *Tru*9I, *ARG4* with *Hae*III, *C2F7* with *Dde*I, *ERG11* with *Hin*dIII.

§ SNPs in the *ERG11* terminator region detected by sequence analysis.

0·5 M EDTA, 10 mM Tris/HCl pH 7·5, and incubated overnight at 30 °C. Blocks were then washed three times in 0·5 M EDTA pH 9·5, treated with 2 ml of a lysis solution (2·5 mg proteinase K ml^{-1}, 1 % *N*-lauroylsarcosine, 0·5 M EDTA, 10 mM Tris/HCl pH 9·5) and incubated overnight at 50 °C. Blocks were washed three times with 50 mM EDTA pH 7·5 and stored for up to 3 months at 4 °C in 50 mM EDTA pH 7·5.

CHEF electrophoresis was done using a Bio-Rad CHEF Mapper with the following conditions: 0·8 % agarose gel run at 14 °C at 3 V cm^{-1} with a 106 degree angle in 1 × TAE. Running time was 50 h with a 2–8 min linear ramp. CHEF gels were stained with EtBr and photographed using an EagleEye II Still Video System (Stratagene). Band intensities were quantified using ImageQuant software (Molecular Dynamics).

Southern blots. CHEF gels were treated with 0·1 M HCl for 15 min followed by three washes in water. Southern blots were then performed using standard techniques (Sambrook *et al.*, 1989). Probes for labelling Southern blots were made using Prime-a-gene kits (Promega) or kinase labelling (Sambrook *et al.*, 1989). PCR products amplified from CAI4 DNA using the primers listed in Table 1 were used as probes for *MTLa1*, *MTLα1*, *OBPα*, *OBPα* and *MTLα2*. The triple probe (*MTLa1-ERG11-MTLα1*) was constructed by using two oligonucleotides containing 50 bp tails homologous to one of the *MTL* genes and 20 bp heads homologous to a region of the *ERG11* gene to amplify a 150 bp double-stranded fragment that contained 50 bp of *MTLa1*, *ERG11* and *MTLα1*. This fragment was radiolabelled and used to probe for those genes. Southern blots were scanned using a STORM phosphoimager (Molecular Dynamics) and quantified using ImageQuant software according to the manufacturer's instructions.

Single nucleotide polymorphism (SNP) analysis. PCR fragments from chr 5, chr 6 and chr 7 (*PDE1*, *ARG4* and *C2F7* from Table 1) were amplified from the *MTL* homozygous strains as previously described (Cowen *et al.*, 1999). These fragments were then digested with the appropriate restriction enzyme (Table 1) in 40 μl reactions containing 1 × reaction buffer, 10 μg PCR DNA, and 1 μl restriction enzyme incubated overnight at 37 °C. Digestion products were then electrophoresed in 1·5 % agarose in 1 × TBE at 100 V for 1 h with EtBr.

The terminator region of *ERG11* on chr 5 was amplified using the primers listed in Table 1 and the conditions used to amplify the SNP fragments. This PCR fragment was sequenced (Marr *et al.*, 1998) using the same primers as used for PCR and screened for heterozygous polymorphisms visible as over-

lapping peaks on the sequencing read. Heterozygous polymorphisms were confirmed from the sequence of the complementary strand of the PCR fragment.

RESULTS

PCR screen of the *MTL* loci of clinical isolates of *C. albicans*

Ninety-six clinical isolates were selected for analysis; fifty of these isolates were resistant or susceptible dose-dependent to fluconazole with a MIC greater than 8 μg ml^{-1}. Oligonucleotides were designed to specifically amplify the three *MTL* genes: *MTLa1*, *MTLα1* and *MTLα2* (Table 1). PCR was performed with the *MTLa1*- and *MTLα1*-specific oligonucleotides on genomic DNA from each of the isolates. PCR fragments from *MTLa1* and *MTLα1* were generated from 84 of the 96 isolates screened. Genomic DNA from six of the isolates did not amplify *MTLa1*; DNA from six unrelated isolates did not amplify *MTLα1* (Fig. 2). Of these 12 isolates homozygous at the *MTL* locus (*MTL*hom) only one was a susceptible isolate (Table 2). These PCR results were

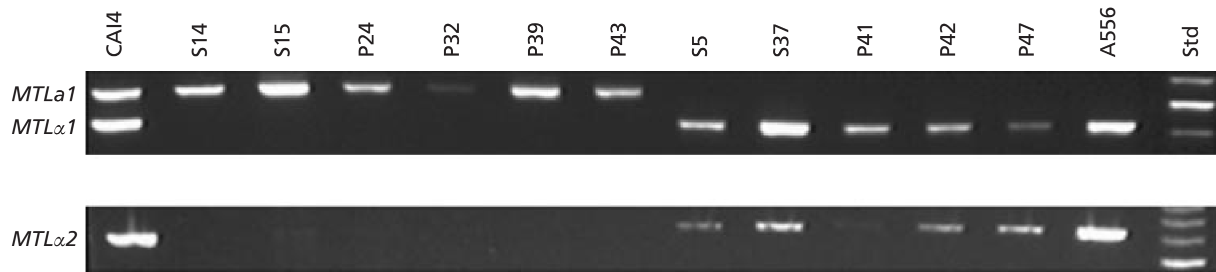

Fig. 2. PCR screen of *MTL* genotype in clinical isolates. DNA fragments from within the *MTLa1*, *MTLα1* and *MTLα2* genes were amplified using genomic DNA from 96 clinical isolates and visualized on EtBr-stained agarose gels. Reactions where one of the two PCR fragments failed to amplify were repeated. CAI4 was used as a *MTLa/MTLα* control strain. Twelve isolates failed to amplify one of the two fragments. Strains in which the *MTLα1* fragment failed to amplify correspondingly failed to amplify a fragment from *MTLα2.* Std, size standards.

Table 2. MTL genotype, fluconazole MIC and SNP analysis of heterozygosity for selected strains

Strain	*MTL* genotype*	Fluconazole MIC†	Chromosome(s) with heterozygous SNPs‡
S14	*a*	>64	5
S15	*a*	>64	7
P24	*a*	0·038	7
P32	*a*	>64	–
P39	*a*	>64	7
P43	*a*	>64	5§
S5	α	>64	5
S37	α	16	5§, 6
P41	α	>64	5
P42	α	16	7
P47	α	>64	–
A556	α	>64	7
S1	*a*/α	0·5	5
S2	*a*/α	32	5
#1	*a*/α	0·125	5
CAI4	*a*/α	2	5
3153A	*a*/α	0·125	–
FH 1	*a*/α	4	5
FH 5	α	>64	5

*Determined by PCR amplification of the *MTLa1* and *MTLα1* genes.

†MIC determined by Etest.

‡Chromosomes 5, 6, and 7 were screened for the presence of heterozygous SNPs (Cowen *et al*., 1999).

§SNPs detected by sequencing of *ERG11* terminator region.

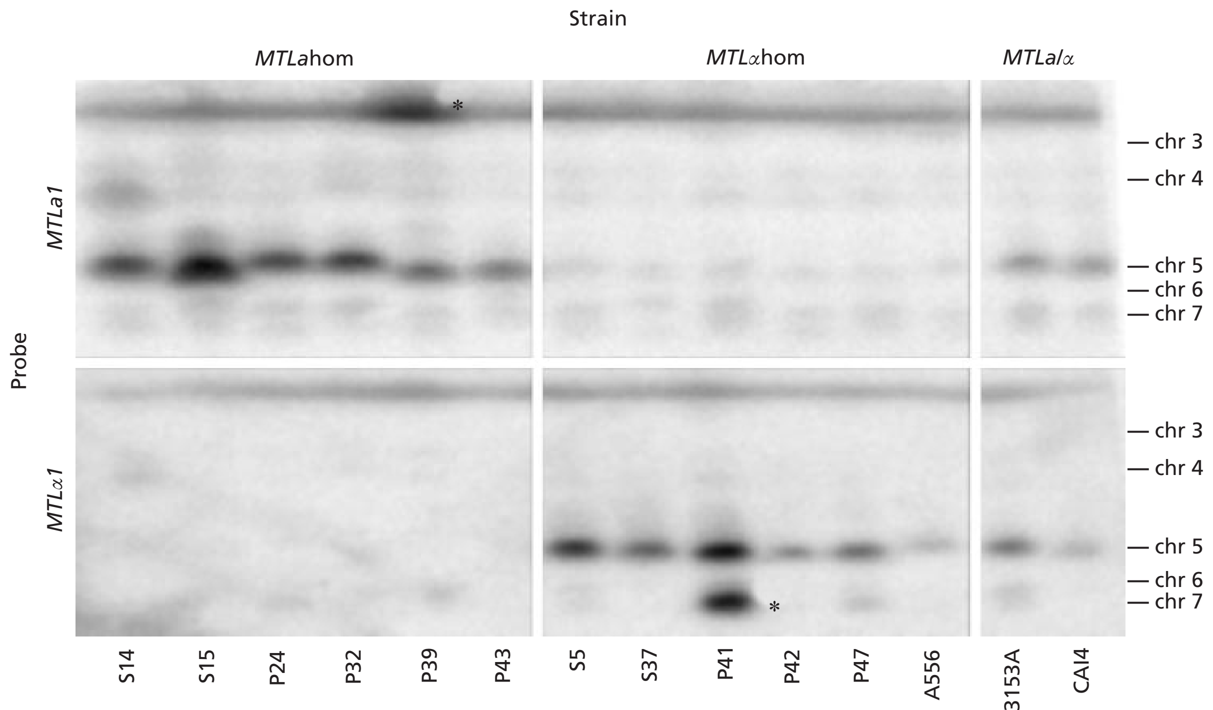

Fig. 3. Southern blot analysis using radiolabelled *MTLa1* and *MTLα1*. Genomic DNA from the *MTL*hom isolates and two control strains (3153A and CAI4) was separated into chromosomal bands by CHEF electrophoresis and blotted onto nitrocellulose paper. The blot was then hybridized to radiolabelled PCR fragments of *MTLa1* and *MTLα1* and analysed on a phosphoimager. The *MTLa1* and *MTLα1* probes selectively hybridized to chr 5 of the *MTLa*hom and *MTLα*hom respectively, and both probes hybridized to chr 5 of the control strains. Asterisks denote possible extra copies of the *MTLa1* and *MTLα1* genes on chromosomes other than chr 5.

confirmed by hybridization of *MTL*-specific probes to Southern blots of CHEF gels, which resolve the chromosomes as distinct bands on the gel (Fig. 3). Based on hybridization, strains P39 and P41 appear to have extra copies of *MTLa1* and *MTLα1* on other chromosomes. The presence of only one of the two *MTL* genes in these 12 isolates, as determined by PCR and Southern blot hybridization, suggests that these strains are homozygous at the *MTL* locus.

In order to determine the extent of the loss of heterogeneity, the presence of *MTLα2*, *OBPa*, *OBPα*, *PAPa* and *PAPα* was determined by PCR in the 12 *MTL*hom strains. The six strains missing *MTLα1* (*MTLa*hom) were found to also lack *MTLα2*. In addition, the *OBPα* and *PAPα* genes were not present in these *MTLa*hom strains. Conversely, the *OBPa* and *PAPa* genes were not present in the *MTLα*hom strains (data not shown). Neither *OBPa* nor *OBPα* could be amplified from strain P32, which is *MTLa*hom. However, a probe specific for *OBPa* hybridized to genomic DNA from strain P32 in a Southern blot (data not shown). Neither *PAPa* nor *PAPα* could be amplified from strain P41, which is *MTLα*hom. However, a probe specific for *PAPα* hybridized to genomic DNA from strain P41 (data not shown). The fact that the loci-specific *OBP*, *PAP* and *MTL* genes cosegregate in these 12 *MTL*hom strains indicates that

Table 3. Statistical analysis of the relationship between *MTL* genotype and resistance

The *P* value calculated using Fisher's exact test (Uitenbroek, 1997) was 0·003.

	***MTLa/α*†**	***MTLa* or MTLα†**	**Total**
Sensitive*	45	1	46
Resistant*	39	11	50
Total	84	12	96

* Sensitive, fluconazole MIC <8 μg ml^{-1}; resistant, fluconazole MIC $\geqslant 8$ μg ml^{-1}.

† *MTL* genotype determined by PCR amplification.

the loss of heterozygosity extends through the entire 9 kb *MTL* locus in these strains.

Statistical analysis of the 96 strains (Table 3) indicates a strong correlation between homozygosity at the *MTL* locus and azole resistance (Fisher exact, $P<0{\cdot}0029$) (Uitenbroek, 1997). The equal distribution of *MTL*hom isolates, with six missing *MTLa1* and six missing

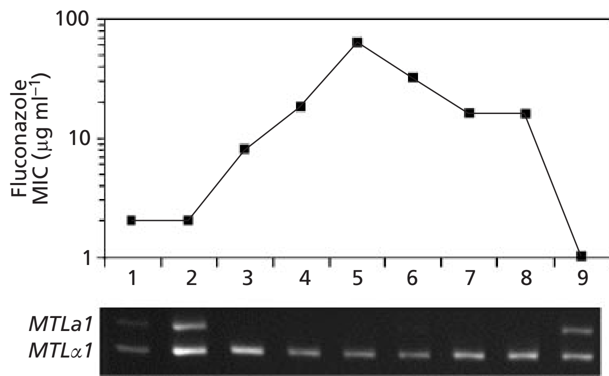

Fig. 4. *MTL* genotype and fluconazole susceptibility of a serial set of transiently resistant clinical isolates (FH1 to FH9). Fluconazole MICs for the series of nine isolates were determined using the NCCLS macrodilution method. PCR was used to amplify fragments from *MTLa1* and *MTLα1,* which were visualized on EtBr-stained agarose gels. *MTLa1* failed to amplify in FH3 to FH8, corresponding to an increase in the fluconazole MIC. The final isolate in the series is sensitive and amplified both *MTLa1* and *MTLα1*.

MTLα1, suggests that homozygosity at either locus is associated with resistance.

Loss of *MTL* heterozygosity in a set of serial isolates

This laboratory has previously studied several matched sets of susceptible and resistant clinical isolates (Marr *et al*., 1997; White, 1997a, b; White *et al*., 1997), including sets from AIDS patients and sets from bone marrow transplant patients. Each set of isolates develops azole resistance from susceptibility during the course of the series. Two of these sets have an increase in the expression of the *CDR1*-encoded ATP-binding cassette pump correlated with the reduced susceptibility to fluconazole. One of these sets sequentially gained all of the known correlates of resistance, including *CDR1* overexpression. These four sets were assessed for the presence of the *MTL* loci using the methods described above. In three of these sets, every isolate was heterozygous at the *MTL* locus (data not shown).

However, in one of these series (FH) a switch to a homozygous *MTLα* locus corresponded to the first significant increase in fluconazole resistance (Fig. 4). This increase had previously been correlated with an increase in the expression of *CDR1* (Marr *et al*., 1997). The final isolate in this series is sensitive to fluconazole and is also heterozygous at the *MTL* locus (Fig. 4) (Marr *et al*., 1997). It is possible to induce azole resistance *in vitro* in the first sensitive isolate of this series by serially transferring the isolate in the presence of fluconazole (Marr *et al*., 1998). Several of the resulting resistant isolates, induced to resistance *in vitro*, are also homozygous *MTLα* (data not shown). This correlation provides further evidence of a link between loss of *MTL* heterogeneity and azole resistance.

Polyploidy of chromosome 5 in *MTL*hom strains

Homozygosity at the *MTL* locus could be the result of shedding one copy of chr 5, which carries the *MTL* loci. The chromosomes of the 12 strains were analysed using CHEF electrophoresis to determine how many copies of chr 5 are present. The chromosomes of the *MTL*hom isolates were separated on a CHEF gel, then stained with ethidium bromide, visualized using a digital camera (Fig. 5a) and quantified. For each strain, the intensities of chr 5 and chr 6 were normalized to the intensity of chr 4 (Fig. 5b). Since chr 5 and chr 6 are smaller than chr 4, it is expected that the staining of chr 5 and chr 6 with EtBr will be slightly reduced compared with chr 4. With the exception of strains P39 and S14, the intensities of the chr 5 and chr 6 bands were as intense as the chr 4 band. Chr 5 and 6 of strain P39 stained with half the intensity of chr 4, suggesting that there are either additional copies of chr 4 in this strain or there are only single copies of chr 5 and 6. Chr 6 in strain S14 stained with half the intensity of chr 4 and chr 5, indicating that S14 has only one copy of chr 6. These twofold differences were reproduced in three independent experiments. The equal intensity of the chr 4, chr 5 and chr 6 bands indicates that there are roughly equivalent amounts of these chromosomes for most strains. Thus the *MTL*hom strains are not aneuploid for chr 5.

SNP analysis of *MTL*hom strains

Based on the CHEF chromosome quantification, it is possible that the *MTL*hom strains are euploid and haploid, rather than diploid. Although there is some evidence to indicate the existence of meiosis in *C. albicans*, haploid strains of *C. albicans* have not been previously described (Lott & Effat, 2001). However, the *MTL* locus may provide a new marker for detecting haploid strains. To assess the ploidy of the *MTL*hom strains, we screened for heterozygous single nucleotide polymorphisms (SNPs).

In these analyses, a PCR fragment is amplified and digested with appropriate restriction enzymes. One of the two alternative sequences at these SNP locations contains a restriction site, the other sequence does not. This allows for rapid screening of sequence differences without direct sequencing. Short stretches of DNA from genes on chr 5 (*ERG11*, *PDE1*), chr 6 (locus C2F7), and chr 7 (*ARG4*) containing known SNPs (Cowen *et al*., 1999) were analysed in this way. Heterozygosity was detected by the presence of both digested and undigested bands, and would indicate the presence of at least two copies of the chromosome carrying the SNP. Homozygosity could result from either homozygous multiple copies or a single copy of the SNP. In 8 of the 12 *MTL*hom isolates at least one heterozygous SNP was found (Table 2). Three of these heterozygous SNPs were on chr 5.

In addition to PCR, sequencing and Southern analysis were used to detect heterozygous SNPs. Sequencing of the noncoding region between *ERG11* and *THR1*

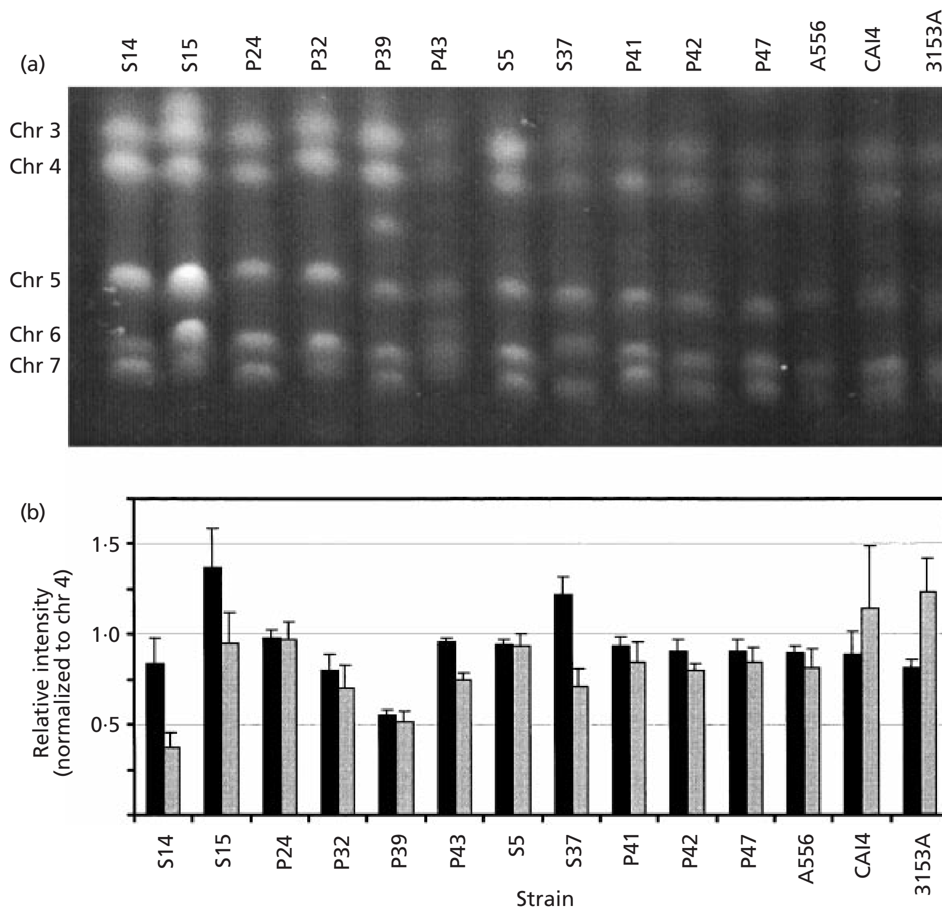

Fig. 5. Quantification of chromosomal bands from an EtBr-stained CHEF gel. (a) Genomic DNA was separated into chromosomal bands using CHEF electrophoresis. (b) A digital image of the EtBr-stained gel was quantified using ImageQuant software. The intensity of chr 5 (dark bars) and chr 6 (light bars) was normalized to the intensity of chr 4 from the same strain. Bars show the mean of three experiments with standard deviation. Chrs 5 and 6 stained with slightly less intensity compared to chr 4 for most of the isolates analysed. Isolate S14 appears to have a single copy of chr 6. Both chr 5 and chr 6 stained with half the intensity of chr 4 in isolate P39.

revealed heterozygosity on chr 5 for two additional isolates. The heterozygosity of the *ERG11* bands in Fig. 6(a) is another form of SNP analysis demonstrating that three of the 12 *MTL*hom strains are diploid at the *ERG11* locus. The heterozygosity of the *ERG11* bands in Fig. 6(a) was only seen in strains already known to have other heterogeneous SNPs on chr 5. A total of 5 of the 12 *MTL*hom strains have heterogeneity of chr 5, and 10 of the 12 strains have some known heterogeneity. This suggests that most of these *MTL*hom strains are not haploid and provides further evidence that at least five of these strains carry two copies of chr 5.

Two copies of the *MTL* locus are present in *MTL*hom strains

Another possible mechanism to explain *MTL* homozygosity is deletion of one *MTL* locus and subsequent joining of sequences flanking the *MTL* locus. Southern analysis was used to detect the copy number of the remaining *MTL* locus in the *MTL*hom strains. The relative intensities of gene-specific fragments, detected as bands on Southern blots probed with locus-specific probes, were used to determine the number of *MTL* loci present in the *MTL*hom strains. Normally, three separate probes would be used for each gene (*MTLa1*, *MTLα1* and a control such as *ERG11*). However, multiple probes would introduce variability due to differences in specific activity and binding efficiency of the probes. To reduce this variability a 150 bp oligonucleotide consisting of 50 bp pieces from *MTLa1*, *MTLα1* and *ERG11* was constructed using PCR (Table 1). This oligonucleotide was radiolabelled and used to probe a Southern blot of *Hae*III-digested genomic DNA from the 12 *MTL*hom strains and 6 *MTL* heterozygous (*MTL*het) controls (Fig. 6a).

The *MTLa1* and *MTLα1* bands were easily identified by their absence in the *MTL*hom strains. The two different higher molecular mass bands are differently sized restriction fragments of *ERG11* caused by disruption of

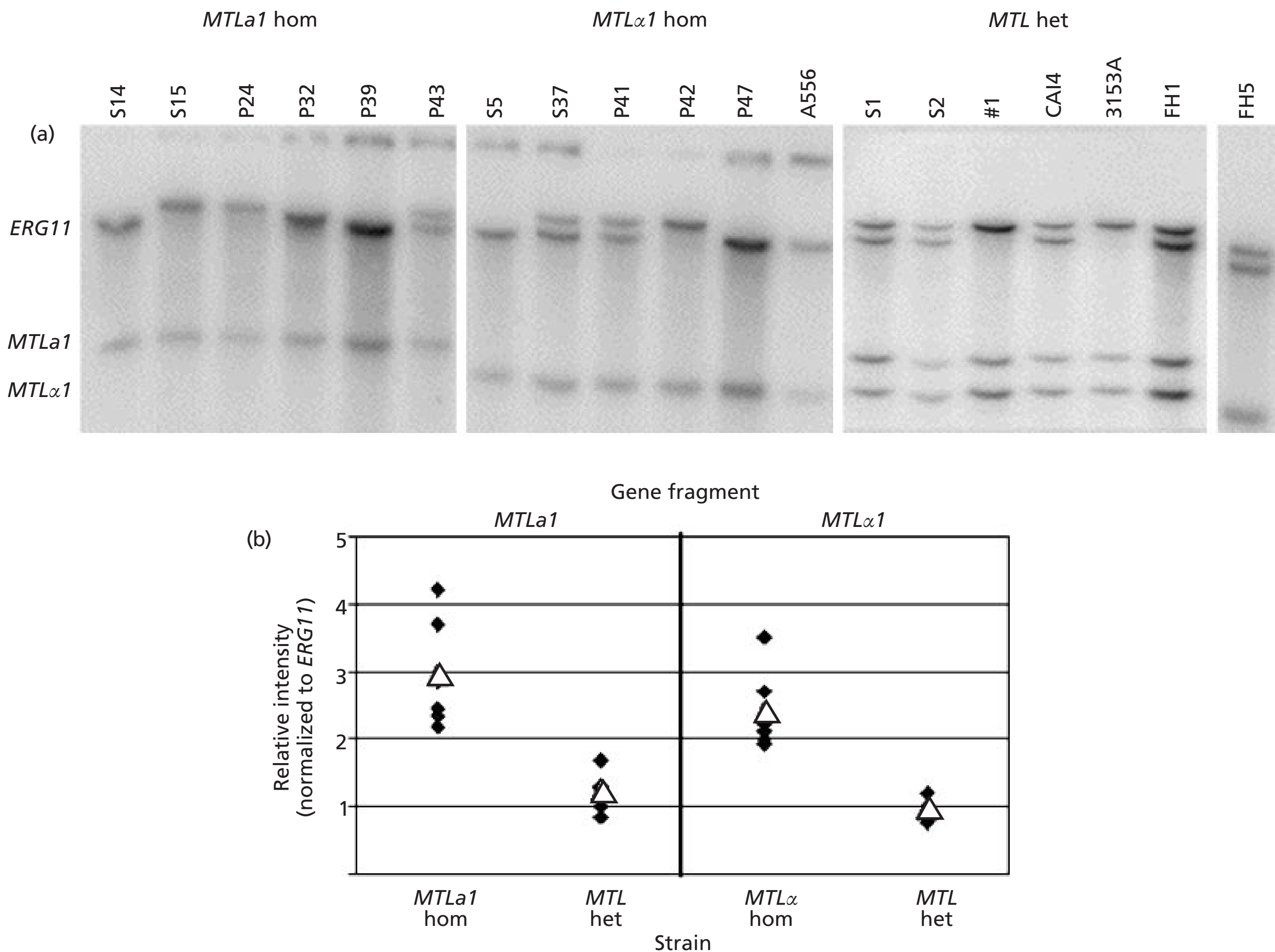

Fig. 6. (a) Southern blot of *Hae*III-digested genomic DNA probed with *MTLa1-Erg11-MTLα1* triple probe. A probe containing 50 bp of *MTLa1*, *ERG11* and *MTLα1* was constructed using PCR and radiolabelled. This triple probe was hybridized to a Southern blot of *Hae*III-digested genomic DNA. The probe hybridized to two *ERG11* bands caused by restriction fragment polymorphism. Both *ERG11* bands were present in FH1 and FH5. The *MTLa*hom, *MTLα*hom and FH5 sections are from three separate blots. (b) The intensity of these bands was quantified and the *MTLa1* and *MTLα1* bands were normalized to *ERG11* for each isolate. Each diamond represents the mean intensity of a single isolate from three experiments. The triangles indicate the mean intensity of that group of isolates. On average, the *MTL*hom strains appear to carry at least two copies of either the *MTLa* or the *MTLα* locus.

a *Hae*III restriction site. All bands were of the expected size predicted by *Hae*III restriction maps of the published sequences (Table 1). The intensity of the *MTL* bands on three separate Northern blots was quantified and normalized to the total intensity of the *ERG11* band(s). The scale was then adjusted so that the relative intensity of the CAI4 band was set to one. Although there was some variation around the mean, the relative intensities of the *MTLa1* and *MTLα1* bands of the *MTL*hom isolates were approximately twice the intensity of those bands in the control *MTL*het strains (Fig. 6b). This indicates that there are two copies of the *MTLa* or *MTLα* locus in the *MTL*hom strains, suggesting that the *MTL*hom strains are the result of gene conversion or mitotic recombination that replaced one of the *MTL* loci with a duplicate of the other. The presence of two *ERG11* restriction fragments in some *MTL*hom strains, including isolates FH1 and FH5 from a serial set of isolates, suggests that the hypothetical gene conversion or mitotic recombination did not involve the *ERG11* gene.

Fluconazole susceptibility is unaltered in *MTL* locus disruptions

It is possible that the *MTL* genes themselves have an effect on azole drug resistance. Gene disruptions of *MTLa1*, *MTLα1* and *MTLα2* have been previously described (Hull *et al*., 2000). In these gene disruptions *URA3* was inserted using homologous recombination to disrupt the gene or locus of interest in the *ura3* auxotrophic strain CAI4. The susceptibility of these isolates to fluconazole was measured using Etest strips (according to the manufacturer's specifications) and by

Table 4. Fluconazole MICs and genotypes of strains with gene disruptions

Strain	Genotype*	Fluconazole MIC†
CAI4	$\frac{MTLa}{MTL\alpha}\,\frac{ura3}{ura3}$	1·00
CHY 247	$\frac{mtla1::hisG\text{-}URA3\text{-}hisG}{MTL\alpha}\,\frac{ura3}{ura3}$	0·25
CHY 257	$\frac{mtla1::hisG}{MTL\alpha}\,\frac{ura3}{ura3}$	0·25
CHY 477	$\frac{mtla1::hisG}{MTL\alpha}\,\frac{ura3}{ura3}\,\frac{ade2::hisG\text{-}URA3\text{-}hisG}{ade2::hisG}$	0·25
CHY 420	$\frac{mtl\alpha1::hisG\text{-}URA3\text{-}hisG\ mtl\alpha2::hisG}{MTLa}\,\frac{ura3}{ura3}$	0·25
CHY 439	$\frac{mtl\alpha1::hisG\ mtl\alpha2::hisG}{MTLa}\,\frac{ura3}{ura3}$	0·50
CHY 484	$\frac{mtla\ locus::hisG}{MTL\alpha}\,\frac{ura3}{ura3}\,\frac{ade2::hisG\text{-}URA3\text{-}hisG}{ade2::hisG}$	0·25
CHY 486	$\frac{mtl\alpha\ locus::hisG}{MTLa}\,\frac{ura3}{ura3}\,\frac{ade2::hisG\text{-}URA3\text{-}hisG}{ade2::hisG}$	0·25

* All *ura3* mutants are *ura3::imm434*. † MIC determined by NCCLS.

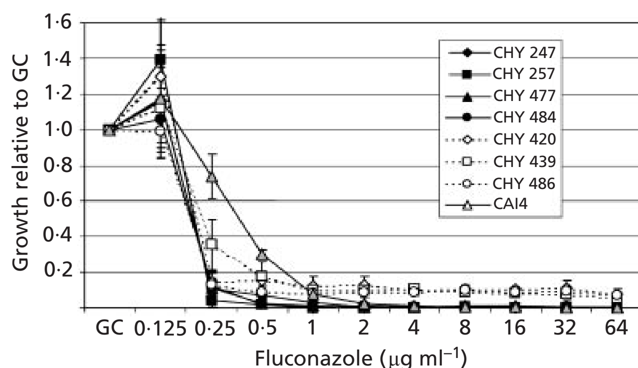

Fig. 7. Susceptibility of *MTL* gene disruptions. The fluconazole MIC of strains with disruptions of the *MTLa1* and *MTLα1* genes and the *MTLa* and *MTLα* loci (see Table 4 for genotypes) were measured using the NCCLS M27A protocol. Although the disrupted strains tended to be more sensitive than the parent strain CAI4, the differences were not significant based on NCCLS guidelines. All strains lacking the *MTLα1* gene were capable of limited growth up to the highest concentration of fluconazole tested. GC, no-drug control.

the standard NCCLS microbroth method (National Committee for Clinical Laboratory Standards, 1997). The *URA3*-disrupted strains and *ura3* auxotrophic revertants of those strains displayed no change in susceptibility compared to the parental and wild-type strains when tested on Etest strips (Table 4). These results were verified using the NCCLS microdilution assay. However, in the NCCLS assays a subtle trend was visible (Fig. 7). All of the strains in which *MTLα1* and *MTLα2* had been deleted were able to grow a small amount, even at the highest levels of fluconazole. This sort of residual growth has previously been documented as a problem in determining macrodilution MIC end-points, although the levels seen for the *MTLα1* strains would not alter the MIC of the strains.

DISCUSSION

Drug resistance in pathogenic fungi is a serious threat to the growing number of immunocompromised patients. It is critical to understand how these opportunistic pathogens are able to circumvent current therapies in order to design effective new therapies and treatments. Previous work has shown that mutation or overexpression of the enzyme targeted by azole drugs, Erg11p, and overexpression of the membrane-bound pumps encoded by *CDR1*, *CDR2* and *MDR1* are correlated with increased resistance to azoles (White, 1997a). These mechanisms are present in only a portion of the azole-resistant isolates of *C. albicans* (Stevens & White, 2000). In this study we have described a novel correlation between homozygosity at the *C. albicans MTL* loci and resistance to azole drugs.

The PCR screen of 96 clinical isolates clearly showed a relationship between *MTL* homozygosity and azole resistance (Table 3). Although the statistical relationship between drug resistance and *MTL* homozygosity is

significant, the presence of the sensitive *MTL*hom isolate P24 suggests that *MTL* homozygosity alone is not sufficient for resistance. This conclusion was further supported by the unchanged susceptibility of the *MTL* gene disruptions (Fig. 7). These sensitive *MTL*hom strains may be independent of resistance, or missing functional genes necessary for *MTL*-related resistance, or are starting to develop resistance.

The results of the PCR screen were confirmed by additional PCR screens of other genes in the *MTL* loci and by the hybridization of radiolabelled *MTLa1* and *MTLα1* genes to CHEF Southern blots (Fig. 3). The *MTLa1* and *MTLα1* probes hybridized to chromosomes other than chr 5 in some of the *MTL*hom isolates. These extra copies were present in only a few of the *MTL*hom isolates and are therefore unlikely to be related to increased azole resistance in these isolates.

Further evidence of a correlation between *MTL* homozygosity and azole resistance comes from the corresponding increase in azole resistance seen after loss of one of the *MTL* loci in the FH series of isolates (Fig. 4). The final isolate was taken from post-mortem lung tissue, and thus was likely protected from fluconazole selection pressure that appears to have caused the other isolates to develop resistance, and lose the *MTLa* locus (Marr *et al.*, 1997). The gain of resistance in this series of isolates has been previously correlated with upregulation of *CDR1*. However in three of the five *MTL*hom isolates in which *CDR1* levels have been analysed no upregulation of *CDR1* is seen (data not shown). In fact, no known resistance mechanism is present in all of the five resistant *MTL*hom isolates for which we have data.

The FH series of isolates has also been shown to have a heterogeneous resistance (HetR) phenotype (Marr *et al.*, 2001). A fraction of c.f.u. from HetR strains are able to grow on plates containing fluconazole, but the resulting colonies retain both azole susceptibility and the HetR phenotype. Preliminary experiments on FH1 colonies from azole-containing plates show no loss of heterozygosity at the MTL locus.

The multiple copies of chr 5 indicated by the presence of heterozygous SNPs on that chromosome (Table 2) suggest that the loss of MTL homozyosity is not due to a loss of one copy of that chromosome. Furthermore, duplicate copies of the *MTL* locus (Fig. 6b), and heterozygous SNPs on chr 5 (Table 2) suggest that the absence of one of the *MTL* loci is not due to deletion or chromosome loss and duplication. Given that the SNPs from chr 5 are in non-coding regions, and thus have no selection advantage, it is unlikely that these polymorphisms arose in the short time between a theoretical loss and subsequent duplication of chr 5. Homozygosity at the *MTL* locus could also be achieved through a gene conversion or homologous recombination that replaced the *MTLa* locus with a second copy of the *MTLα* locus or the reverse. Although this loss of heterogzygosity extends through the *MTL* locus, the presence of heterozygous restriction fragments in *ERG11* in the FH series both before and after heterozygosity was lost at the *MTL* locus (Fig. 6a) suggests that this region does not include *ERG11*. Mapping the size of the region of lost heterozygosity will distinguish between those two possibilities and will limit the list of genes potentially responsible for this correlation.

Changes in chromosome copy number have been linked to various morphological mutants, growth in L-sorbose and alterations in carbon and nitrogen utilization (Janbon *et al.*, 1998, 1999; Rustchenko *et al.*, 1994). Changes in copy number of chr 4 (monosomy) and chr 3 (trisomy) have been associated with azole resistance (Perepnikhatka *et al.*, 1999). However, changes in copy number of chr 5 have not previously been associated with drug resistance. This study has focused on the *MTL* locus on chr 5, which remains disomic in these isolates. Therefore, the changes observed at the *MTL* locus are unlikely to be related to the changes in chromosome number observed above.

Our results suggest a number of genes that could be responsible for a correlation between *MTL* homozygosity and azole resistance. These possibilities can be split into two categories. The first category consists of genes that cause slight resistance when mutated in one allele and much greater resistance when the mutation is in both alleles. Selective pressure would then favour replacement of the wild-type allele with a mutant allele in a population of *C. albicans* grown in the presence of azole drugs. Any gene within the region of lost heterozygosity that is present in two copies could potentially fall into this category. *ERG11* is the obvious example of this sort of selection, as it is the only gene related to azole resistance that lies on chr 5, and previous experiments have shown that gene conversion does occur following a point mutation in one allele of *ERG11* (White, 1997b; Franz *et al.*, 1998). However, the presence of heterozygous SNPs in the *ERG11* coding region and terminator in 5 of the 12 *MTL*hom suggests that there has been no recent loss of heterozygosity in *ERG11* in these isolates and therefore that *ERG11* is not the gene responsible for the correlation between *MTL* homozygosity and resistance. Other genes that could potentially fall into this category include the *OBP* and *PIK* genes in the *MTL* loci. Homologues of the *OBP* genes in particular have been shown to play a role in ergosterol homeostasis in *S. cerevisiae* (Beh *et al.*, 2001).

The other category of possibilities is a general resistance effect that results from the loss of one of the *MTL* specific genes. Of particular interest are the *MTLa1* and *MTLα2* genes. The homologues of the products of these genes in *S. cerevisiae* form a heterodimeric transcriptional regulator (Herskowitz *et al.*, 1997). It therefore follows that if a functionally similar heterodimer forms in *C. albicans*, loss of one of those genes would lead to altered levels of a number of genes, possibly including those involved in azole resistance. Although the gene disruptions of the *MTL* loci caused little change in azole susceptibility (Table 4), the laboratory strain in which the gene disruptions were performed may have a

nonfunctional copy of a gene that contributes to resistance.

Future experiments will determine which of these models is correct. These experiments include characterizing the *MTL*hom strains further and determining the mechanism by which they develop resistance – decreased import, increased export, increased resistance of the Erg11p in these isolates, or some other undescribed mechanism.

ACKNOWLEDGEMENTS

We thank Drs Raleigh Bowden and Kieren Marr (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) for the use of their clinical isolates. We thank Drs Christina Hull and Alexander Johnson for the use of their *MTL* gene disruption strains. We thank members of the White and Marr laboratories for their support and comments on this manuscript. This research was supported by NIH NIDR grants RO1 DE-11367 and RO1 DE-14161. T. C. W. is the recipient of a New Investigator Award in Molecular Pathogenic Mycology from the Burroughs Wellcome Fund.

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Received 8 October 2001; revised 7 December 2001; accepted 14 December 2001.