Sex has no detectable net benefits for

Candida albicans

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ABSTRACT

Like many other important opportunistic human fungal pathogens, for more than a century *Candida albicans* was thought to be strictly asexual until a parasexual cycle was recently discovered in the laboratory. It is uncertain, however, whether sex is still a viable reproductive strategy for *C. albicans*. In this study I tested whether or not mating enhanced survival of parental genes in this yeast, by mating 10 clinical isolates and testing recombinants' fitness.

Clinical isolates of *C.albicans* usually are diploid, carrying both the *MTL***a** and *MTL*α mating type alleles, each on a different copy of chromosome 5. These strains are apparently incapable of meiosis and cannot mate with each other, because the a1-α2 heterodimer suppresses mating. Through selection on sorbose-containing agar I induced loss of *MTL*-heterozygosity and generated 5 *MTL***a** and 5 *MTL*α derivatives of clinical isolates. Existing mating techniques involve the use of auxotrophic markers, requiring time-consuming sequential disruption of two copies of biosynthetic genes if wild-type clinical isolates are to be mated. Furthermore, auxotrophy affects the virulence of a strain, and this can potentially interfere with comparing the fitness of recombinants with that of their parents. I therefore developed a method for mating clinical isolates marked with two drug resistance markers, the mycophenolic acid (MPA) resistance-conferring allele of *IMH3* and the nourseothricin (NAT) resistance gene *CaNAT1*, allowing selection of recombinants on the basis of resistance to both agents. I marked all *MTL***a** strains

with the MPA resistance gene and all *MTL*α strains with the NAT resistance gene. This allowed 25 combinations for mating. Recombinants were obtained from 15 combinations of 9 strains. It was found that not all *C. albicans* clinical isolates could mate.

Using growth rate as the criterion, I tested the fitness of clinical isolates, *MTL*-homozygous derivatives with and without resistance markers and recombinants during adaptation to a novel environment (YPD medium), maximizing the potential benefits of sex. After computationally correcting for the impact of experimental manipulations, I calculated the net benefit of sex as the difference in the number of offspring from two cells that become mating competent and engage in sex compared to the offspring they could have produced by continued clonal reproduction. My results indicated that, as a rule, engaging in sex reduces the chances of survival of *C. albicans'* genes, in part because *MTL* homozygosis significantly reduced growth rates. Through fitness increase after recombination, sex may eventually confer a net benefit for some strain combinations in the laboratory, but this probably occurs too late to prevent elimination of recombinants by competition and genetic drift in nature. Sex in *C. albicans* therefore diminished parents' chances to pass on their genes to future generations. These findings have a significant impact on the assessment of the role of sex in *C. albicans* and other "asexual" human fungal pathogens. Recent loss of the function of sex and incomplete decay of the sex machinery are the most likely explanation of *C. albicans'*s residual ability to mate, and one that also needs to be considered in other fungal pathogens.

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ABBREVIATIONS

Amp	Ampicillin
aa	amino acid
APS	ammonium persulfate
AWCGS	Alan Wilson Centre Genome services
bp	base pair
^{0}C	degree Celsius
C. albicans	*Candida albicans*
CAIP	Calf intestinal alkaline-phosphate
cDNA	Complementary DNA
Chr (chr)	Chromosome
cm	Centimetre
CSPD	Chloro-5-substituted adamantyl-1,2-dioxetane phosphate
g	gram
DIG	Digoxigenin
DAPI	4', 6'-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dNTP	Deoxynucleoside triphosphate

DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
FACS	Fluorescence Activated Cell Sorting
GPG	General purpose genotype
h	hour
IPTG	Isopropyl-ϒ-D-thiogalactopyranoside
kb	kilobase
L	litre
LB	Luria-Bertaini medium
mg	milligram
μl	microlitre
M	Molar, moles per litre
ml	millilitre
MPA	mycophenolic acid
MM	Minimal medium
MTL	Mating-type like
NAT	nourseothricin
Non-GPG	non-general purpose genotype
OD	Optical Density
ORF	open reading frame
PAGE	Polyacrylamide gel electrophoresis
pBSKS(+)	pBluescript KS(+)
PCR	Polymerase chain reaction

pH	-Log[H^+]
RNase	Ribonuclease
rpm	Revolutions Per Minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
TAE	Tris/ Acetic acid /EDTA
TBE	Tris/Borate/EDTA
TNE	Tris/NaCl/EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
X-gal (BCIG)	bromo-chloro-indolyl-galactopyranoside
YPD	Yeast Extract Peptone Dextrose Medium
w/v	Weight/volume

1.0. INTRODUCTION

1.1. *Candida albicans*: The Predominant Pathogenic Fungus of Humans:

1.1.1. Fungal pathogens of humans

Some fungi can cause disease in humans. The frequency of fungal infections has increased steadily over the past 30 years, and has become a prominent problem for human health (Bouza and MuÒoz, 2008; Martin et al., 2003; Ruhnke, 2004). Two important aspects contribute to this problem. Firstly, the population of immunocompromised individuals is growing worldwide due to HIV/AIDS and immunosuppression after organ or bone marrow transplantation or cancer therapies. The opportunistic fungal infections in these patients have an increasing ability to cause mortality and morbidity, as the immune system in these patients is weakened (Groll et al., 1998; Martin et al., 2003; Pfaller and Diekema, 2007). Secondly, many antifungal drugs are limited in their use due to their toxicity or side effects (Georgopapadakou and Tkacz, 1995; Georgopapadakou, 1994). This is probably because fungi are eukaryotes and very closely related to humans. Most of the main structures of fungal and human cells are similar at the molecular level. Therefore it is hard to find a target in fungal cells that is different in human cells, and there are often side-effects to many antifungal drugs. Some of these side-effects can be life-threatening and have proven to be highly toxic to the human host (Sternberg, 1994). In addition, resistance to antifungal drugs in the clinic has also been observed over the past 20 years (Boschman et al., 1998; Denning, 1995; Ruhnke, 2006; Segal et al., 2006; Walsh et al., 2007). All of these features make fungal infections an active

research area and much attention is focused on the identification of fungal virulence factors.

To date, over 100,000 fungal species have been identified. Among these fungal species, surprisingly only a few (approximately 10 species) routinely cause disease in humans (Perfect, 2005). The most common fungal pathogens include *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, which are well characterized. Other less important or less characterized fungal pathogens are *Pneumocystis carinii*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii, Candida glabrata,* and *Aspergillus flavus* (Nielsen et al., 2007). The fungi pathogenic for humans are polyphyletic and closely related to other non-pathogenic fungi instead of forming an evolutionarily distinct monophyletic class of organisms (Bowman et al., 1996). Therefore, the features that allow a fungus to evolve to become pathogenic in humans are not clear. This has become a topic of interest for many researchers. A common feature of these fungal pathogens is that most of them appear asexual in nature, while retaining the machinery needed for sex (Nielsen et al., 2007). This observation leads to a suspicion that loss of sex could be a prerequisite to enable a fungus to become pathogenic in humans. Therefore, understanding the role of sexual recombination in the evolution of fungal pathogens may help us identify virulence factors and search for antifungal solutions.

1.1.2. *C. albicans*: the major fungal pathogen

The genus *Candida* is part of the hemiascomycetes group and contains more than 150 species (DoctorFungus.org., Retrieved on 2011-11-08; Odds, 1988). Among

them, *C. albicans* is the predominant species responsible for opportunistic infections in humans. It is a normal part of the human gut flora and under normal circumstances can live in the human gastrointestinal tract and mouth without causing harmful effects. However, if the microbial flora is disturbed, or host defenses become compromised, it can cause disease. Indeed, it causes 7% of all nosocomial infections and is best known for causing mucosal infections such as thrush or vaginitis (Khan and Gyanchandani, 1998). More importantly, it can also cause life-threatening systemic or disseminated infections affecting virtually any human tissue and organ in immunocompromised patients with associated mortalities of up to 50% (Fridkin and Jarvis, 1996; Kibbler et al., 2003; Wisplinghoff et al., 2004).

C. albicans is a diploid yeast in which no sexual forms have been observed in microscopic observations of clinical samples during more than 100 years (Odds, 1988), although mating can be induced in the laboratory (Hull et al., 2000; Magee and Magee, 2000). *C. albicans* can grow in a temperature range of 20-38^{o}C; a pH range of 2.5-7.5; and in the presence of serum. (dEnfert and Hube, 2007; Odds, 1988). Several features are believed to make it easy to adapt to environmental signals, and thus can help it colonize hosts. *C. albicans* has the ability to switch between a unicellular yeast morphology and multicellular hyphae. It is "dimorphic"- as it grows predominately as yeast cells and hyphae - or may be better described as "polymorphic" (Fig.1.1), because microscopic examinations of infected human tissues almost always show a mixture of budding yeast, pseudo-hyphae and true hyphae. It can also form chlamydospores that are large, thick-walled, cells developing at the tips of pseudo-hyphae or hyphae (Odds, 1988). In addition, *C.*

albicans is capable of undergoing phenotypic switching, a process in which different cellular phenotypes and morphologies are generated spontaneously. The switching is reversible, but switch phenotypes are inherited epigenetically (Huang et al., 2006; Soll et al., 1993). One example of the phenotypic switching is so called "white-opaque transition", in which cells can change phenotype from round, white cells to elongated, flat gray cells under certain conditions (Rikkerink et al., 1988) (see Section 1.3.3.). This transition was first discovered in the strain WO-1. White-opaque transition was discovered to be involved in the *C. albicans* mating process (Miller and Johnson, 2002). The other switching system was first reported by Slutsky and co-workers (1985), who demonstrated that the *C. albicans* strain 3153A switched colony morphologies among seven different types at a high frequency (approximately 10^{-4}) (Slutsky et al., 1985). This phenotypic switching has also been observed in other clinical isolates (Soll, 1992). The molecular mechanisms involved in these switch events are not fully understood yet. It seems that *C. albicans* uses these mechanisms to adapt to different host niches, evade the host immune system, and to make itself a highly successful commensal of humans and an opportunistic pathogen (Odds, 1988; Slutsky et al., 1985).

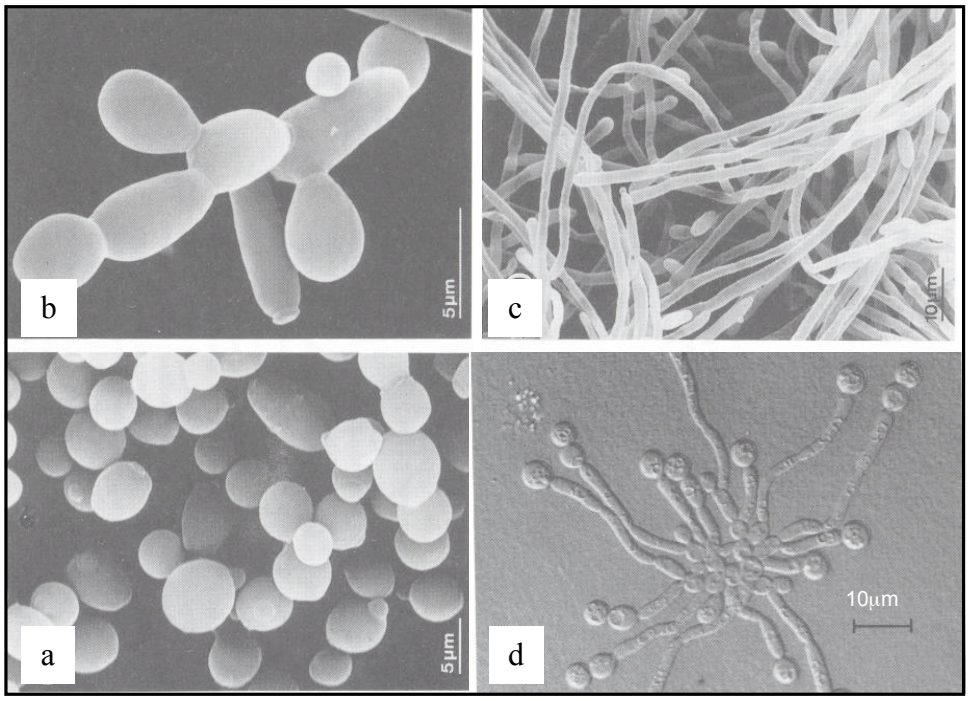


Figure 1.1. *C. albicans* is a polymorphic yeast.

Alternative morphologies of *C. albicans*. (a) Blastospores -- spherical budding cells. (b) Pseudohypha -- chains of blastospores with the obvious constrictions at septal junctions. (c) True hyphae -- with minimal or unapparent constrictions at septal junctions and only a few lateral blastospores. (d) Chamydospores -- large, thick-walled structures developing at the tips of pseudohyphae. (Source: Odds, 1988).

1.1.3. GPG strains are the most successful strains within the species *Candida albicans*

Genetic variation is one of the most interesting features in *C. albicans* and studies of the structure and dynamics of the *C. albicans* genome are important for understanding its commensal-pathogen transition (dEnfert and Hube, 2007; Wickes and Petter, 1996). It is reported that *C. albicans* shows a wide range of genetic variations that affect a broad spectrum of phenotypes (Homann et al., 2009). This feature could be an important factor in making this yeast a successful opportunistic human pathogen. It is not clear what makes the transition of *C. albicans* from a commensal to a pathogen, however, variation at the genomic level likely plays a role (Wickes and Petter, 1996).

Molecular epidemiological studies have identified a geographically widespread cluster of related *C. albicans* strains that cause disease 10-100 times more often than other groups of strains (Schmid et al., 1999) (Fig.1.2). Using Ca3 fingerprinting on a global collection of 266 disease-causing isolates, Schmid et al. (1999) identified a cluster of strains of very similar genotype, which comprised 37% of all infection-causing isolates tested. The remaining strains fell into 37 groups. In addition, in several regional studies in the US, Great Britain and New Zealand, it was found that this group of strains also are the most prevalent commensal colonizers. However, their prevalence as pathogens was often increased among candidiasis isolates, predominantly from superficial infections, compared to their frequency among commensal isolates. This indicated that this cluster has genetic

and phenotypic traits that at least contribute to the success of commensal colonization, possibly also pathogenicity. Because this cluster was highly prevalent in all patient groups and confers selective advantages over other groups of strains, Schmid and colleagues proposed that its members constitute a widely adapted general-purpose genotype (GPG) (Schmid et al., 1999).

Studies from other groups have also identified GPG strains (also called clade 1 strains) as the predominant infection-causing genotype (Botterel et al., 2001; Bougnoux et al., 2002), by using multi-locus sequence typing (MLST) and microsatellite analysis. Odds et al. (2007) undertook a large global MLST study of 1391 isolates, and confirmed that clade 1 strains are the most common globally distributed type of *C. albicans* and may have a greater tendency than other major clades for causing superficial infections, although a lower propensity for causing systemic infections (Odds et al., 2007). Also, recent studies carried out by us show that the high prevalence of GPG strains in causing disease is probably because they possess a multitude of "fitness-enhancing traits", and is not a result of genetic drift. We also showed that "the distribution of genes marked by GPG-specific mutations across functional and structural categories could identify physiological traits that are of particular importance to the success of GPG strains in their interactions with the human host" (Zhang et al., 2009). In addition, analysis of 635 European *C. albicans* candidemia isolates using a duplex PCR assay developed by us showed that GPG strains are more virulent than non-GPG strains in younger patients (Schmid et al., 2011b) .

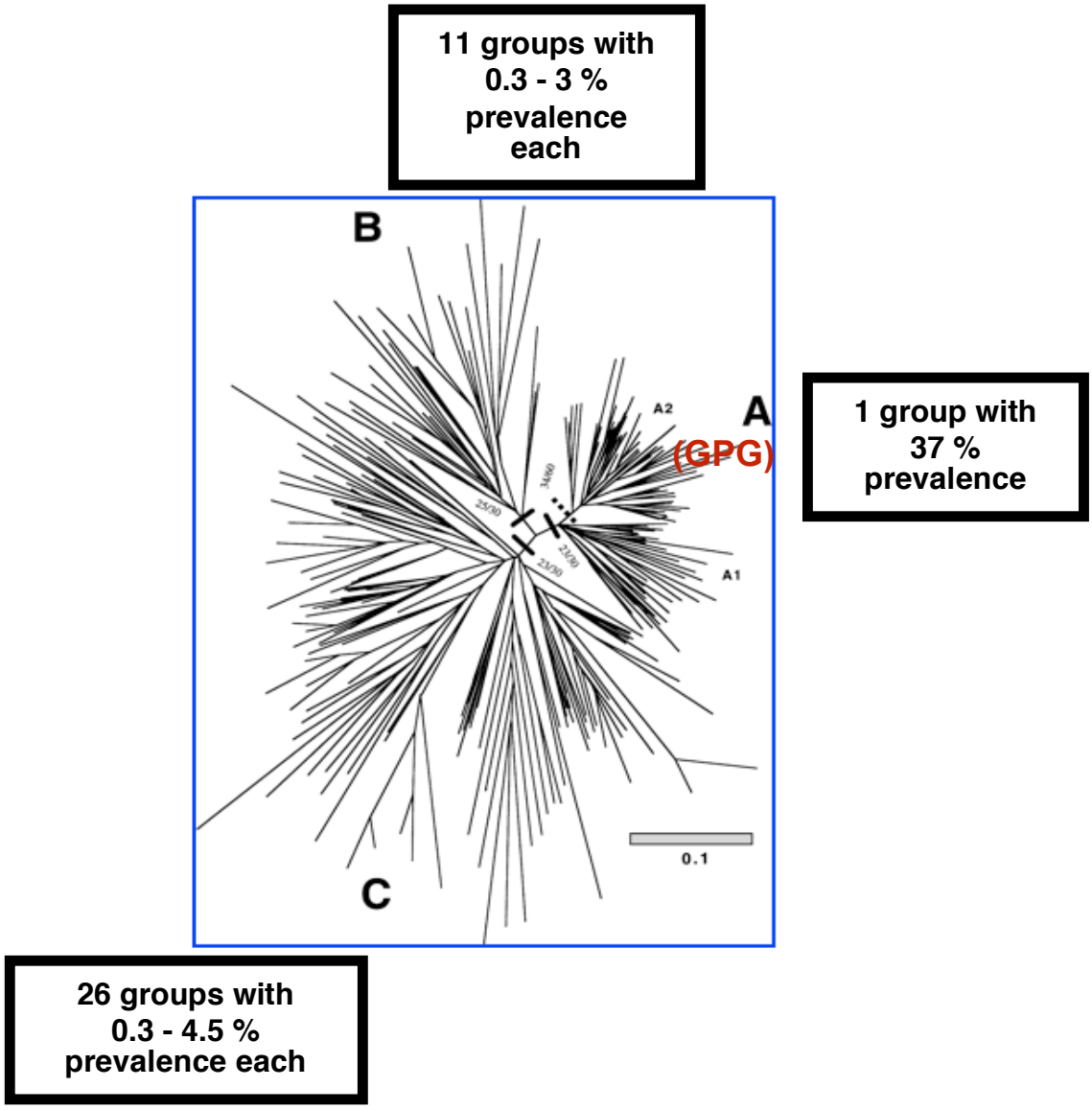


Figure 1.2. GPG strains and non-GPG strains.

Neighbour joining tree of 266 infection-causing isolates of *C. albicans,* collected from a variety of types of infections and geographical locations all over the world. The major branches A (equivalent to cluster A, or GPG), B and C are labelled. Cluster A strains are of very similar genotype, which comprised 37% of all infection-causing isolates. Using the maximum genetic distance between isolates in cluster A as a cut-off the remaining strains can be separated into 37 groups. The prevalence of each of these groups among epidemiologically unrelated isolates ranged between 0.3 and ≤ 4.5%. (Source: Schmid et al., 1999; modified)

All of these data indicate that GPG strains are the most successful *C. albicans* genotype universally, and also the most common etiological agent of human infection. Interestingly, preliminary evidence from a population study showed that GPG strains are probably more clonal than other groups of strains (Holland, 2001; Holland et al., 2002; Zhang et al., 2004), and thus may have had less sex in the past compared to other strains (Schmid et al., 2004).

1.2. The Role of Sex in the Pathogenesis of Fungal Infections:

1.2.1. Sex in Fungi

Organisms pass genes to the next generation by two major means: sexual reproduction or clonal reproduction. Sexual reproduction "generates genetically novel progeny by mating and meiotic recombination of genetically different parental nuclei". In contrast, progeny produced by clonal reproduction "have only one parent and the genome is an exact mitotic copy of the parent" (Taylor et al., 1999). Sex is proposed to have an adaptive value, because it is wide-spread and most of the species identified today have the ability to undergo sex at least at some stages of their life cycles (Otto and Lenormand, 2002). Sexual reproduction is seldom completely absent even in organisms that normally reproduce asexually, such as microbes. However, it remains unclear how sexual recombination confers sufficient benefits to outweigh its higher cost, compared to clonal reproduction (Kaltz and Bell, 2002; Nielsen et al., 2007; Otto, 2009; Otto and Lenormand, 2002).

The most obvious cost is that two parents are required for sex whereas clonal reproduction only requires one parent (Fig. 1.3). Interestingly, recombination itself, often seems to be a cost, by breaking up successful combination of alleles: "Most biologists are comfortable with the idea that sex evolved to provide variability, but mathematical models have proved that this comfort is unwarranted: sex need not be beneficial and evolution need not favour sex, even when it does increase variability and variability is beneficial" (Otto and Lenormand, 2002).

Most fungi can produce both asexual and sexual progeny to adapt to different environmental conditions. Asexual reproduction generates progeny via mitotic processes to produce daughter cells in yeasts or asexual spores such as conidia in filamentous fungi. Sexual reproduction produces progeny by nuclear fusion of haploid nuclei and subsequent spore production (Nielsen et al., 2007). It might be an advantage to maintain the ability to generate both sexual and asexual progeny. Sexual reproduction is supposed to create genetic variation, which promotes adaptation by helping beneficial mutations to spread and enables removal of bad genes. However, asexual reproduction preserves co-adapted combinations of genes that might be broken during sexual recombination. Many fungi use asexual means for reproduction when conditions favor growth and occasionally use sex in response to stressful conditions (Dyer, 1992).

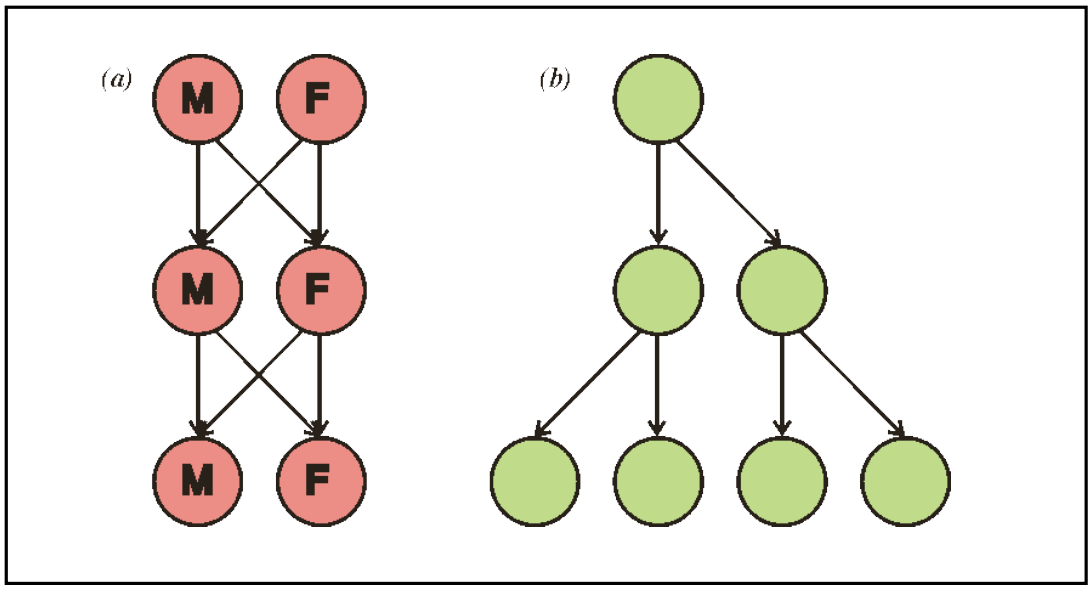


Figure 1.3. The two-fold cost of sex.

If each individual were to contribute to the same number of offspring (two), (a) the sexual population remains the same size each generation, (b) where the asexual population doubles in size each generation.
(Source: http://en.wikipedia.org/wiki/Evolution_of_sexual_reproduction).

Most fungi have genetic systems that prevent mating between genetically identical cells. Instead of the sex-determining chromosomes in plants and animals, fungi determine self-nonself interaction using so-called mating type loci. Similar to the sex chromosomes in other organisms, recombination within the mating type loci is suppressed by rearrangements and extensive sequence divergence between alleles of the mating type locus to avoid producing self-fertile, sterile, or inviable progeny (Carlile et al., 2001). There are two major mating systems in fungi. Some fungi have only two mating types. In these fungi, a single mating type locus with two alleles (**a** and α) controls cell identity by encoding transcriptional factors. This mating system is common in the ascomycetous fungi including the model yeast *S. cerevisiae* and many of the human fungal pathogens, such as *C. albicans*, *A.*

fumigatus, *C. immitis*, and *H. capsulatum*. Other fungi have two independent, unlinked mating type loci that can be multiallelic and can give rise to many different mating types. In this system, one mating locus encodes transcription factors and the other encodes pheromones and pheromone receptors. This system is common in the basidiomycetous fungi (Carlile et al., 2001; Nielsen and Heitman, 2007). Note that many fungi 'subvert' the mating type system, making genetically largely identical strains mating-compatible with each other; the best known method of "subversion" is the mating type switching in *S. cerevisiae* (see below).

1.2.2. Sex and virulence in human fungal pathogens

1.2.2.1. Many "asexual" human fungal pathogens retain their sexual machinery

Interestingly, many important human fungal pathogens appear to be asexual in nature, such as *C. albicans*, *C. glabrata*, *A. fumigatus*, *C. immitis*, and *C. posadasii* (Heitman, 2006). In addition, it seems that not only do many important human fungal pathogens tend to have clonal population structures, but strains that are more virulent or more successful within these species also are more clonal than the remainder of the species, for instance, *C. neoformans var. grubii* strains (Lengeler et al., 2000; Simwami et al., 2011) or GPG strains in *C. albicans* (Schmid et al., 2008). An explanation for these observations could be that loss of sex or severe restriction of its frequency is positively correlated with success in the human host. This led to the hypothesis that sexual reproduction would have destroyed complex combinations of genes which arose on rare occasions and allowed a small number of fungi to adapt to survival in the human, and that loss of sex is a necessary

prerequisite for a fungus to become a human pathogen, explaining why human pathogenic fungi are predominantly asexual (Whelan, 1987).

However, recent studies revealed that many "asexual" human pathogenic fungi actually retain the machinery to undergo sexual reproduction and population studies also show some evidence of recombination from these organisms. Also while mating has not been directly observed in nature, it has been observed in the laboratory under particular conditions for some of these fungi. It seems likely that while these human fungal pathogens are capable of sexual reproduction, mating occurs either rarely (or using alternative ways) or never in their natural environment (Nielsen and Heitman, 2007).

For example, a sexual cycle was observed over 30 years ago for *C. neoformans* in the laboratory (Kwon-Chung, 1975; Kwon-Chung, 1976), but never in nature. *C. neoformans* seems to prevent or limit its sexual reproduction in nature by having a nearly unisexual population, i.e. ∝ mating type strains predominate in both environmental and clinical isolates. In *var. neoformans*, **a** mating type strains account for less than 2% of the population. In *var. grubii*, the predominant pathogenic variety in this species, only 17 **a** mating type strains have been identified from >2000 isolates worldwide and 15 of them were clinical isolates from sub-Saharan Africa; thus mating could be geographically restricted as well (Litvintseva et al., 2003). *C. neoformans* can undergo a homothallic sexual reproduction (∝-∝ mating) in the laboratory, which involved only strains of one mating type (Lin et al., 2005) . It is also reported that there is some evidence for same-sex mating of *C. gattii* in nature (Fraser et al., 2005).

Another important fungal pathogen of humans, *A. fumigatus* has also been considered as an asexual organism for a long time. However, recent genome analysis has not only identified two different mating type loci but also genes involving pheromones, receptors, and components of the pheromone-signaling pathway (Paoletti et al., 2005). Although sexual reproduction was not discovered in nature, mating was observed in this fungus in the laboratory by O'Gorman et al. (2009). They discovered an extant sexual cycle in *A. fumigatus* by mating strains of opposite mating types. However, mating in the laboratory required half of a year of incubation of the mating compatible strains from a naturally occurring recombinant population in the dark on specialized medium (Heitman, 2011 ; O'Gorman et al., 2009).

In *C. albicans*, sexual forms have never been observed in clinical isolates. It has been discovered that strains can be induced to undergo mating (Hull et al., 2000; Magee and Magee, 2000) and undergo a parasexual cycle, without meiotic recombination, in the laboratory (Bennett and Johnson, 2003). To mate, strains however first need to become homozygous at the mating type locus to generate **a/a** or $\propto/\propto$ strains that are mating compatible and then need to undergo white/opaque switching to become mating competent (the details of mating in *C. albicans* will be described in section 1.3.). This would make mating very rare in *C. albicans'* natural environment, as most of the clinical isolates (90-97%) are a/α heterozygous strains and not able to mate with each other. Other than this, an alternative mating route, unisexual mating (**a-a** mating) has also been discovered recently in *C. albicans* (Alby et al., 2009), which is very similar to unisexual mating discovered previously in *C. neoformans.* It was found that alpha-pheromone produced by *MTL*α cells

promoted low frequency same-sex mating between wild-type *MTL***a** cells.

Genome sequencing reveals that all of the other common human fungal pathogens including *H. capsulatum*, *P. carinii*, *A. flavus*, *C. immitis*, and *C. posadasii* have mating-type loci, although a sexual cycle has not yet been identified in these species (Nielsen et al., 2007).

1.2.2.2. Population genetic studies reveal some evidence of recombination in many human fungal pathogens

Mating in the laboratory shows that the potential for recombination remains but may not accurately predict the impact in nature. For example, *Trypanosoma cruzi*, the agent of Chagas disease has long been known to undergo sexual recombination in the laboratory (Gaunt et al., 2003), but its natural populations are highly clonal, indicating that recombination does not contribute to its population structure (Gauthier and Tibayrenc, 2005; Tibayrenc et al., 1981; Tibayrenc et al., 1986). This implies that recombination either does not happen in its natural environment or that the recombination-generated lineages do not (usually) survive.

The biological significance of mating (out-breeding) can only be determined by population genetic methods which assess if a species contains recombinant[1] lineages. The principle of these methods is as follows: A clonal population structure means the mutations occur independently, are confined to individual lineages and

[1] Recombinants in this thesis is defined as mononuclear mating products containing genetic material from both parents.

are not exchanged between lineages. Thus only a limited number of combinations of mutations should be detected. On the other hand, a recombinant structure means that mutations can be exchanged between lineages and most possible combinations of mutations can be observed (Schmid et al., 2004; Tibayrenc, 1999; Xu and Mitchell, 2002).

Population genetic studies of many important "asexual" human fungal pathogens revealed that although their population structures are largely clonal, some evidence of recombination was observed in these organisms (Nielsen and Heitman, 2007). For example, in *C. neoformans*, population genetic studies in the early years examining strains throughout the world suggested only a very low level of recombination (Brandt et al., 1995; Xu and Mitchell, 2003; Xu et al., 2000). However, newer population genetics studies examining strains from HIV/AIDS patients in southern California strongly support the presence of ongoing, or very recent, recombination in *C. gattii* (Edmond et al., 2011). Other population studies also showed evidence of recombination in *C. neoformans* and *var. grubii* (Campbell et al., 2005; Litvintseva et al., 2003). In *C. albicans*, population genetic studies revealed some degree of genetic recombination (as will be discussed in more detail in section 1.3.6). For the *Aspergillus* species, genetic variation was detected within populations of both "asexual" pathogenic species *A. fumigatus* and *A. flavus*, although mating has never been detected in nature (Bertout et al., 2001; Paoletti et al., 2005; Pildain et al., 2004; Varga and Toth, 2003).

These population genetic studies indicate that the "asexual" human fungal pathogens may have some form of genetic exchange between individuals. However,

the recombination levels in these organisms are normally very low, probably due to the lack of a classical sexual cycle. Therefore, based on this evidence, an alternative hypothesis of reproductive biology of these organisms proposed by Neilson et al., (2007) is that: "the human pathogenic fungi have retained the ability to generate either clonal or recombining population structures in response to either constant or changing environments by preserving their ability to undergo sexual (or parasexual) reproduction but limiting the conditions under which sexual reproduction occurs in unique ways". However, indications of a low level of recombination in a population genetics analysis may not necessarily be caused by ongoing rare sex. This evidence could also be explained as an "echo" of past sex (Schmid et al., 2004), which will be mentioned in detail below.

1.3. Sex in *C. albicans*:

1.3.1. *C. albicans* was thought to be asexual

C. albicans is an ascomycetous yeast (Odds, 1988). Sexual reproduction in ascomycetes leads to the formation of readily identifiable morphological structures, the asci, which contain the ascospores, generated by sexual recombination. However, for over a hundred years, mycologists have checked large numbers of patient samples because microscopic examination is part of the diagnosis of candidosis, but never found any asci. In addition, asci have never been observed in the laboratory (Odds, 1988). Furthermore, population genetic studies revealed that the *C. albicans* population structure is predominantly clonal, with very little

evidence of recombination (Gräser et al., 1996; Tibayrenc, 1997). Based on this evidence, *C. albicans* was classified as an asexual species until recently.

1.3.2. Discovery of the mating type locus in *Candida albicans*

This opinion began to change in 1999, when Hull and Johnson identified a set of *C. albicans* genes from genome sequencing that were homologous to the *S. cerevisiae* mating-type (*MAT*) locus (Hull and Johnson, 1999). The genes identified included homologues of the key regulators of *S. cerevisiae* mating, including the transcriptional regulators a1, $\propto$1, $\propto$2, and were organized in a similar way to the *MAT* locus of *S. cerevisiae* and other fungi, suggesting that *C. albicans* has a mating type-like (*MTL*) locus (Hull and Johnson, 1999). Following this, Tzung and co-workers screened the whole *C. albicans* genome and discovered, by conducting a comparative genomic analysis between *C. albicans* and *S. cerevisiae*, genomic evidence for a complete sexual cycle in *C. albicans* (Tzung et al., 2001). They found *C. albicans* appears to have homologues of all of the genes involved in the functional pheromone response mating pathway in *S. cerevisiae*, but many genes for meiosis in *S. cerevisiae* are missing from the *C. albicans* genome. However, they found homologues of meiotic genes from other organisms in *C. albicans* genome, indicating potential alternative mechanisms of genetic exchange in *C. albicans* (Tzung et al., 2001).

The sexually reproducing model yeast *S. cerevisiae* possesses three mating type loci on chromosome 3, two of them are silent (*HMR, HML*) and one (*MAT*) is expressed (Haber, 1998). Mating is controlled by the expressed *MAT* locus. Two alleles of this

locus exist, **a** and ∝ (Fig. 1.4a). Haploid **a**-type cells only contain the *MAT***a** locus and haploid ∝-type cells contain the *MAT*∝ locus. In **a**-type cells, a single homeodomain protein **a**1 is encoded by *MAT***a** locus; and in ∝-type cells a homeodomain protein ∝2 and an alpha-domain protein ∝1 are encoded by the *MAT*∝ locus. Therefore, the three transcriptional regulators, **a**1, ∝1 and ∝2, define the mating type of *S. cerevisiae* haploid cells via a simple circuit: In haploid ∝ cells, ∝1 activates ∝-specific gene (an alpha-domain protein ∝1) while ∝2 represses **a**-specific gene (a homeodomain protein ∝2), making the cells mate as ∝ cells. In haploid **a** cells, on the other hand, only **a**-specific genes exist and can be expressed (a homeodomain protein **a**1), making the cells mate as **a** cells. These two types of cells are capable of mating with each other. The resulting diploid **a**/∝ daughter cells contain both the *MAT***a** and *MAT*∝ alleles that express both **a**1 and ∝2 proteins, which would repress many mating genes and therefore turn off the mating process while turning on meiosis and sporulation under suitable conditions (Johnson, 1995; Souza et al., 2003). Another interesting aspect of mating in *S. cerevisiae* is that the haploid cells can change mating type through gene conversion at the *MAT* locus. The two silent loci contain *MAT***a** and *MAT*∝ genes, respectively, and recombination of a DNA copy between the expressed *MAT* locus and one of the silent loci containing opposite mating type genes would change the cell mating type (Fig. 1.5) (Haber, 1998).

In the diploid yeast *C. albicans*, the two copies of the "mating-type like" (*MTL*) locus, *MTL***a** and *MTL*∝, are located on chromosome 5 (Fig. 1.4b). The *MTL***a** copy contains genes *MTL***a**1, a homologue of *S. cerevisiae MAT***a**1 (Hull and Johnson, 1999), and *MTL***a**2 which has no homologue in the *S. cerevisiae MAT***a** locus.

*MTL***a**2 encodes an HMG box protein, a transcription factor, with homologs in other fungi, such as *Neurospora crassa* (Coppin et al., 1997), *Klyveromyces lactis* (Aström et al., 2000) and *Podospora anserina* (Debuchy et al., 1993). The *MTL*∝ locus contains *MTL*∝1 and *MTL*∝2 that are homologues of *S. cerevisiae MAT*∝1 and *MaT*∝2, respectively (Hull and Johnson, 1999).

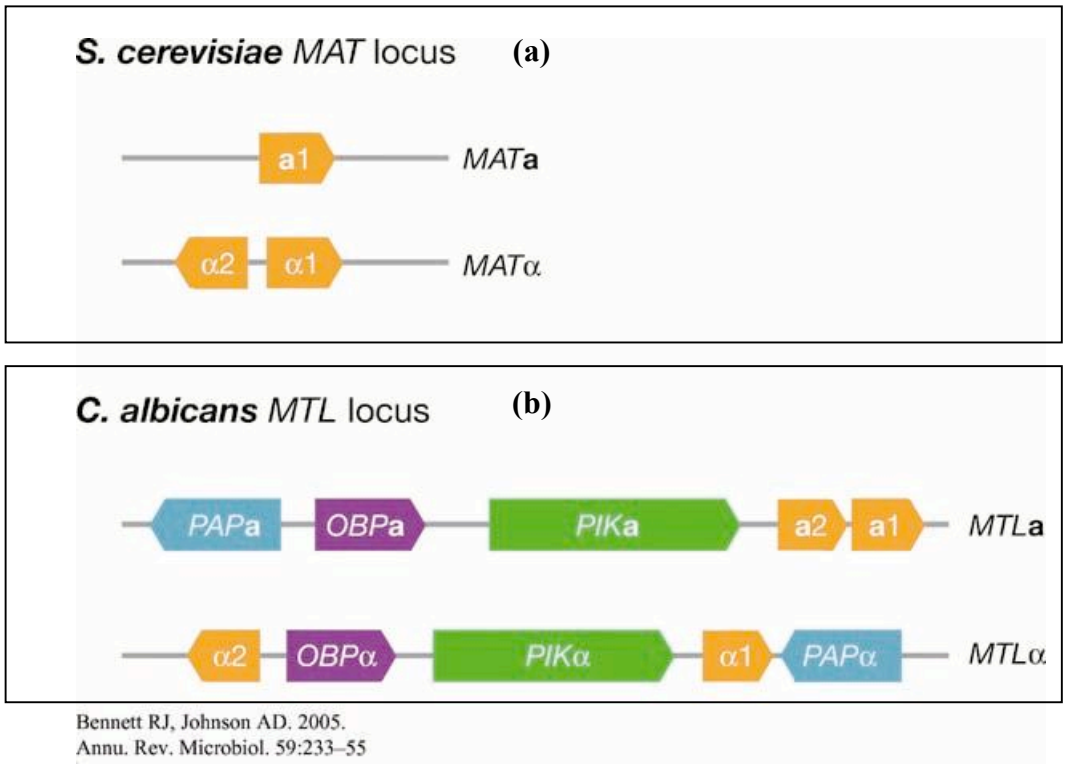


Figure 1.4. The *S. cerevisiae* and *C. albicans* mating-type loci.

(a) The *S. cerevisiae* locus has two alleles, *MAT*a and *MAT*α, which together encode three transcriptional regulators: a1, α1, and α2. (b) The *C. albicans* mating locus also contains two alleles, *MTL*a and *MTL*α. *MTL*a encodes the a1 and a2 transcriptional regulators, and *MTL*α encodes the α1 and α2 transcriptional regulators. The *C. albicans* mating locus contains three additional pairs of genes, *PAP*, *PIK*, and *OBP*, which encode a poly(A) polymerase protein, a phosphoinositol kinase protein, and an oxysterol binding protein, respectively.
(Source: Bennett and Johnson, 2005).

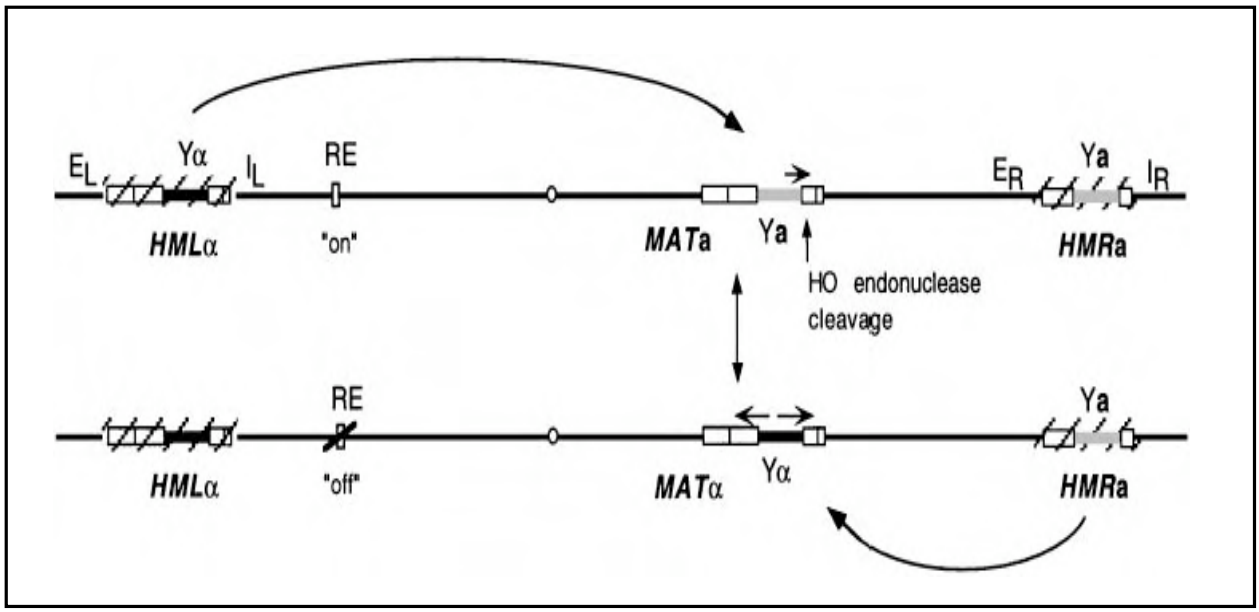


Figure 1.5. Mating-type loci of *S. cerevisiae* on chromosome III.

In addition to the expressed *MAT* locus, chromosome III harbors two unexpressed donor loci, *HML* and *HMR*. These donors are maintained in a heterochromatic structure (*diagonal lines*) enforced by two adjacent silencer sequences *HML*-E (EL) and *HML*-I (IL), and *HMR*-E (ER) and *HMR*-I (IR). When the HO endonuclease is expressed, MAT alleles can be switched by a gene conversion. (Haber, 1998).

The *MTL* locus (9kb) in *C. albicans* is much larger and more complicated than the *S. cerevisiae MAT* locus (0.7 kb). Three additional open reading frames with mating type-specific alleles (*PAP***a**, *PAP*∝**,** *PIK***a**, *PIK*∝, and *OBP***a,** *OBP*∝**)** are present in the *C. albicans MTL* locus, but not at the mating locus in *S. cerevisiae* and most other fungi (Fig.1.4b). These genes encode a poly(A) polymerase protein, a phosphoinositol kinase protein, and an oxysterol binding protein, respectively (Hull and Johnson, 1999). The nucleotide sequences of the **a** and ∝ alleles of each of the three genes are only approximately 60% identical to each other, while the similarity of the two alleles of typical *C. albicans* genes exceeds 99%. This implies that the two alleles may carry out distinct functions (Bennett and Johnson, 2005). The *MTL***a** and *MTL*∝ are each present as a single copy in the lab strain SC5314, eliminating

the possibility of mating type switching using silent mating type loci as found in *S. cerevisiae* and other fungi (Hull and Johnson, 1999). Because *C. albicans* appears to be an obligate diploid, if strains contain both *MTL***a** and *MTL*∝**,** they are not able to mate with each other, due to the expression of both **a1** and ∝**2** proteins (**a1**-∝**2** hetero-dimer**)** that, similar to *S. cerevisiae*, repress mating (Tsong et al., 2003). Indeed 90-97% of all *C. albicans* strains are **a**/∝ heterozygous at the *MTL* locus and thus not capable of mating in nature (Legrand et al., 2004; Lockhart et al., 2002; Tavanti et al., 2005).

1.3.3. *C. albicans* can mate in the laboratory

The discovery of a mating type-like locus in *C. albicans* suggested that it might be able to undergo sexual recombination. In 2000, mating was reported both *in vivo* and *in vitro* from two separate laboratories using different approaches, but both involved making **a** or ∝ derivatives from the laboratory strain SC5314 which is heterozygous (a/∝). Hull et al. (2000) demonstrated mating between *MTL***a** and *MTL*∝ strains after co-inoculation into a mouse model. The *MTL***a** derivatives had been created by deleting either the entire *MTL*∝ locus or only the ∝1 and ∝2 genes, while the *MTL***a** derivatives were created by deleting either the entire *MTL***a** locus or only the **a**1 and **a**2 gene (Hull et al., 2000). An alternative approach used in the Magees' laboratory (Magee and Magee, 2000) involved sorbose selection to obtain *MTL***a**/**a** and *MTL*∝/∝ derivatives. In *C. albicans*, one homologue of chromosome 5 which harbors the *MTL* locus is often lost during growth on medium containing sorbose as the sole carbon source (Janbon et al., 1998), and the remaining chromosome 5 is duplicated after transfer to YPD medium. Magee and Magee

(2000) converted several *MTL*a/∝ strains including SC5314 into homozygous *MTL***a**/**a** and *MTL*∝/∝ derivatives and demonstrated mating *in vitro*. In both studies, the parent strains were auxotrophic strains and the mating products were selected by the complementation of the auxotrophic markers. They were mononuclear and tetraploid, indicating nuclear fusion occurs in the matants. But no meiosis was discovered in both cases.

The mating efficiency in these experiments was extremely low. The reason for this was discovered by Miller and Johnson (2002). They found that unlike in *S. cerevisiae*, where any *MAT***a** and *MAT*∝ cells are competent to mate, *C. albicans* *MTL***a** and *MTL*∝ cells must first undergo a rare phenotypic switch to become mating competent.

The initial mating experiments had used the normal yeast phenotype *C. albicans* in which cells are ovoid-shaped. This phenotype is also referred to as "white" because the cells form off-white colored colonies, resembling those of *S. cerevisiae*. It was discovered in 1987 that some *C. albicans* strains can switch from this phenotype to another phenotype called "opaque" because of the grayish appearance of the colonies at a high frequency (10^{-2}); the cells of this phenotype are elongate and only stable at low temperature (<25^{o}C), as the opaque cells switch back to white cells at 30-37 ^{o}C (Slutsky et al., 1987). While this switch system was well studied, only very few clinical isolates seemed capable of this switch (Lockhart et al., 2002).

Miller and Johnson (2002) observed that when they made *MTL***a** and *MTL*∝ derivatives of SC5314, a strain which cannot form the opaque phenotype, the

derivatives could switch to the opaque phenotype. The ability to undergo this switch is apparently controlled by the transcriptional regulatory proteins encoded by the *MTL* locus. The two homeodomain proteins **a**1 and **∝2** work together to repress the white-opaque switch, suggesting that this switch may be involved in mating. Miller and Johnson then tested opaque cells in mating experiments and found that they are approximately 10^6 times more efficient at mating compared to white cells (Miller and Johnson, 2002). Thus the opaque cell phenotype is the mating-competent state of *C. albicans*.

Since naturally occurring isolates are diploid and apparently lost the ability to undergo meiosis, and the progenies of mating are tetraploid, if the type of mating observed in the laboratory is to occur in *C. albicans'* natural environment, it must be followed by chromosome loss. Bennett and Johnson (2003) indeed observed that on some media ("pre-sporulation" medium) rapid random chromosome loss could be induced which, while accompanied by dramatic loss of viability of the cultures, did produce diploid genotypes different from those of the parents.

In summary these experiments showed that in the laboratory *C. albicans* can produce new recombinant diploid genotypes through a paresexual cycle (Fig. 1.6).

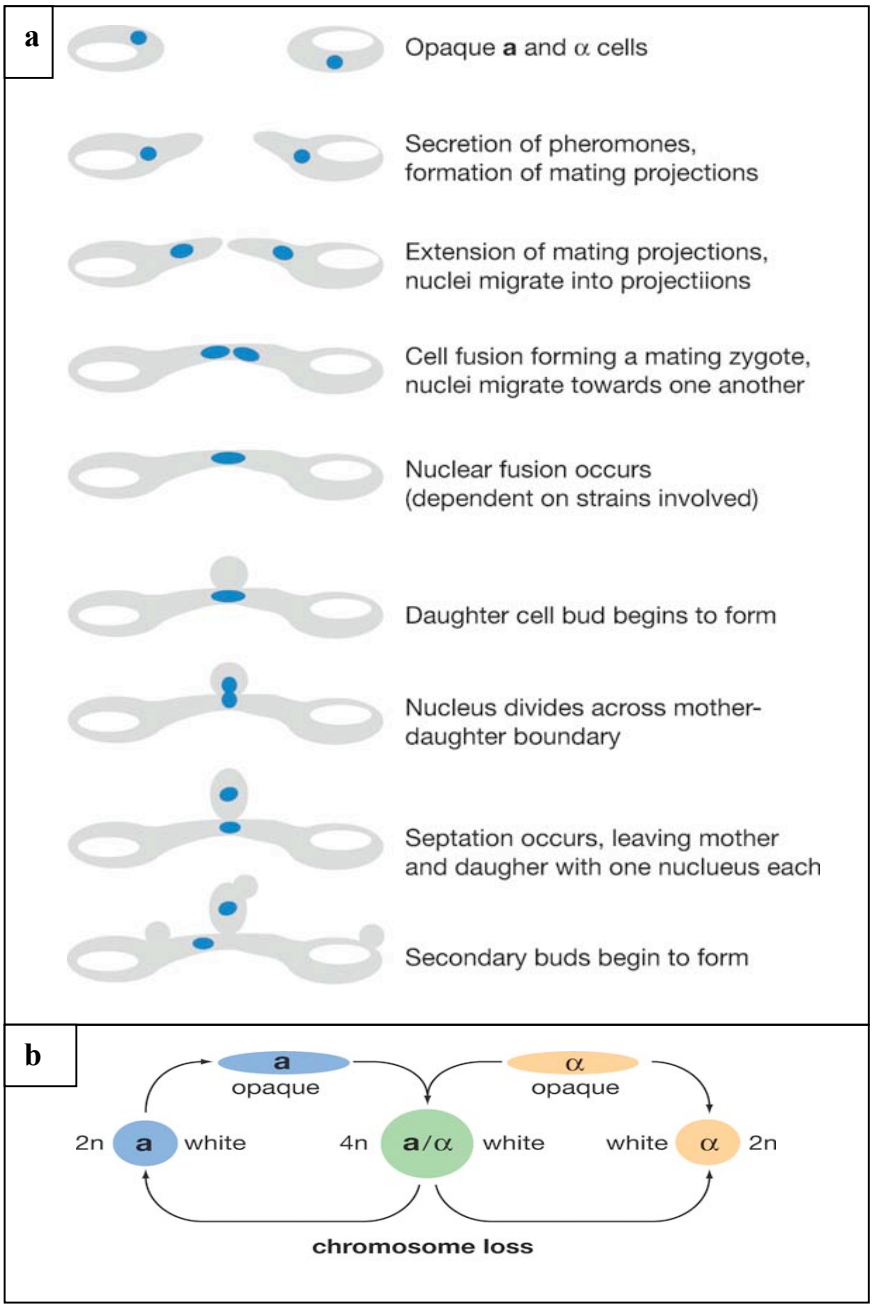


Figure 1.6. Mating in *C. albicans*.

(a) Steps in the mating of *C. albicans*. Nuclei are depicted in blue. The parental mating cells are diploid, and the mating products are mononuclear and tetraploid. (b) A parasexual cycle for *C. albicans*. Diploid a and α cells must first switch from the white phase to the opaque phase for mating. Mating of opaque cells generates a mononuclear, tetraploid a/α cell. A reduction in ploidy can be achieved by chromosome loss, regenerating a and α mating-competent progeny as part of a parasexual cycle. Note that a/α progeny (nonmating) are also formed via chromosome loss from the tetraploid (not shown). A meiotic program has not been identified in *C. albicans*. (Source: Bennett and Johnson, 2005).

1.3.4. Mating between clinical isolates

Up to this point, all evidence for *C. albicans'* ability to mate was based on mating very closely related auxotrophic relatives of the same laboratory strain, SC5314. When Lockhart et al. (2003) tried to mate opaque cells of other naturally occurring *MTL*-homozygous clinical isolates, the efficiency of zygote formation as observed microscopically appeared quite high. All of the 12 crossings tested (involving 7 strains) underwent cell fusion- but nuclear fusion and recombination was not observed either by microscopy or by genetic analysis of parental markers. The latter analysis involved the progeny of 6 individual zygotes, 12 single colonies from each zygote; i.e. the frequency of recombination in these experiments was below 1.4% of zygotes. These results were contrary to what happened in mating between the derivatives of laboratory strain SC5314, in which nuclear fusion and recombination occurred after cell fusion (Miller and Johnson, 2002). The most likely reason for the discrepancy would be that the earlier SC5314 experiments had crossed auxotrophic mutants so that only recombinants could survive, whereas the clinical isolates were prototrophs (Lockhart et al., 2003). To overcome the apparently low nuclear fusion efficiency between clinical isolates, Magee et al. (2002) constructed so-called "universal mating strains", which can mate with prototrophic clinical isolates to produce selectable genotypes (Magee et al., 2002). The universal mating strains are auxotrophic **a** or ∝ derivatives of the original lab strain SC5314 carrying a MPA (mycophenolic acid) resistance marker. When these strains mate with any other prototrophic strains and nuclear fusion occurs, the resulting matants can be selected on minimal plates containing MPA (Magee et al., 2002). Legrand and co-workers found that 11 out of 15 (73%) naturally occurring

MTL-homozygotes were capable of mating with the universal mating strains, and capable of undergoing nuclear fusion (Legrand et al., 2004). But they did not test directly whether these clinical isolates could mate with each other. In 2005, Bennett and co-workers demonstrated that nuclear fusion did occur when they tried to mate one of the pairs of *C. albicans* clinical isolates used previously by Lockhart et al. (2003), but rarely and at a low efficiency (0 - 10%) compared to mating between SC5314 derivatives (in which nuclear fusion efficiency was 93%). They found that all of six clinical isolates tested could mate (including nuclear fusion) with the *MTL* compatible SC5314 derivatives. The nuclear fusion efficiencies were intermediate (33%-93%) between those of isogenic lab strains (93%, SC5314 derivatives) and matings of clinical isolates (0-10%) and varied greatly between different pairs of strains (Bennett et al., 2005). The nuclear fusion efficiency also varied for different environmental conditions. The most efficient mating occurred on so-called "Spider medium". In addition, media containing mannitol generated more recombinants than media containing glucose (Bennett et al., 2005). However, while all six clinical isolates could mate with SC5314, Bennett and coworkers could only obtain recombinants from mating between one of two pairs of clinical isolates tested. They concluded that many clinical isolates may be defective in some aspect of nuclear fusion, and that this defect is partially rescued by mating with the more robust mating partner SC5314 (Bennett et al., 2005).

In summary, a variety of *MTL*-homozygous strains of *C. albicans* can be induced to mate in the laboratory with strain SC5314, but not all clinical isolates can mate with each other. Even the ability to mate with SC5314 does not predict the strain's ability to mate with other strains. Many clinical isolates may be genetically incompatible,

and thus unable to mate with each other. This could be a reason why mating has never been observed in nature.

1.3.5. Mating between clinical isolates is restricted in nature

Several barriers have been discovered blocking *C. albicans* ability to mate in the natural environment. First of all, most clinical strains (90-97%) are heterozygous at the *MTL* locus (Legrand et al., 2004; Lockhart et al., 2002; Tavanti et al., 2005) and can not mate with each other because *C. albicans* is a permanently diploid yeast having apparently lost the ability to undergo meiosis (Bennett and Johnson, 2003; Odds, 1988). To mate, strains need to become homozygous at the *MTL* locus to generate **a**/**a** or α/α strains that are mating compatible. However, while spontaneous *MTL*-homozygotes can arise from *MTL*-heterozygotes, which generally caused by loss of one copy of chromosome 5 homologue, harboring the *MTL* locus, followed by duplication of the retained homologue (Lockhart et al., 2002; Wu et al., 2005), it has been discovered that *MTL*-heterozygous strains (**a**/α strains) are normally more virulent than *MTL*-homozygous strains. Also, natural **a**/α strains are better at colonizing the host than their *MTL*-homozygous derivatives (Wu et al., 2007). These results suggest a competitive advantage of natural **a**/α strains over *MTL*-homozygous derivatives (Lockhart et al., 2005; Wu et al., 2007). This could be another factor restricting mating in the human host, as even if spontaneous *MTL*-homozygous strains arise in nature, they could be out-competed quickly by their *MTL*-heterozygous ancestors and not have the chance to mate.

Aside from the low percentage of *MTL*-homozygous clinical isolates, the instability

of the mating-competent opaque phenotype at 37ºC would be a major obstacle for mating in the human host. As only the opaque cells are mating competent, most clinical isolates need to undergo white/opaque switching to become mating competent. However, in most niches (e.g. human mouth and gastrointestinal tract) in *C. albicans* natural host, which is 37^{o}C, opaque cells frequently switch back to white cells, further restricting mating. A number of authors have investigated the possibility that niches exist in the human host in which the opaque phenotype may be stable and mating may be able to occur. Lachke et al. (2003) reported that opaque cells readily colonized the skin of baby mice (32^{o}C) and that mating (or at least cell fusion) could happen on mouse skin at a high frequency (up to 50%), although the fused cells rarely formed daughter cells from the conjugation bridge and it was not reported if the daughter cells exchanged genetic material between the parents (Lachke et al., 2003). However the skin is not typically heavily colonized by *C. albicans* (Odds, 1988) and therefore mating on the skin would likely be a rare event. Dumitru et al. (2007) reported that anaerobic conditions stabilize the opaque state at 37^{o}C, and mating occurs *in vitro* and *in vivo* under anaerobic conditions at 37^{o}C, such as in the gastrointestinal (GI) tract. They recovered prototrophic recombinants from the mouse GI tract that were tetraploid and mononuclear from mating between isogenic auxotrophic parent cells of *C. albicans*, although their methodology did not allow them to ascertain if mating in the GI tract occurred at a high frequency (Dumitru et al., 2007).

The existing evidence suggests that mating could occur - although probably only on very rare occasions - between *C. albicans* strains in their natural environment and could lead to new gene combinations. This raises the questions of why *C. albicans*

mating has never been observed in human hosts, why the *C. albicans* population structure is largely clonal (as will be discussed in more detail below) (Tavanti et al., 2004; Tibayrenc, 1997) and whether there is a connection between a low frequency of sex and pathogenicity (Nielsen and Heitman, 2007) .

1.3.6. Population genetic studies reveal evidence of sex -but not much

A number of *C. albicans* population genetic studies using different approaches reached a similar conclusion: that the population structure is predominantly clonal, however, some degree of recombination has been detected (Bougnoux et al., 2008; Fundyga et al., 2002; Gräser et al., 1996; Holland, 2001; Holland et al., 2002; Lachke et al., 2003; Pujol et al., 1997; Pujol et al., 1993; Tavanti et al., 2004; Tibayrenc, 1997; Xu and Mitchell, 2002; Xu et al., 1999). These studies all yielded very similar data, which were interpreted quite differently before and after the discovery that *C. albicans* could undertake sex in the laboratory. The data could suggest that *C. albicans* clinical isolates may mate and generate recombinant lineages on occasion. However it could also be explained as "an echo of sexual recombination" in *C. albicans'* evolutionary past. As mentioned in section 1.1.3, GPG strains, the most successful group of *C. albicans* strains, have a significantly more clonal population structure than other less successful strains (Holland, 2001; Holland et al., 2002), suggesting that sex may no longer be advantageous to the species. In addition, the very existence of a general-purpose genotype argues strongly against ongoing sexual recombination, since such genotypes cannot survive in the presence of recombination (Forbes et al., 1997; Lynch, 1984). Also, a recent

population study carried out by Schmid et al. suggested that the frequency of sex may have progressively declined during *C. albicans'* recent evolutionary history (Schmid et al., 2011a), supporting the idea that the very limited evidence for recombination could be largely based on past rather than on present recombination events. Although *C. albicans* still possesses limited functional sexual machinery, the sexual lineages may be inferior to clonal lineages and may not survive.

All these observations led to two major hypotheses in the reproductive biology of *C. albicans*. Hypothesis I, ongoing rare sex: Many researchers believe that *C. albicans* still uses rare or converted sex to generate new recombinant lineages to meet adaptive challenges outside the laboratory. This rarity of mating and the lack of meiosis - and thus meiotic recombination - explain the limited population genetic evidence for recombination. However, when a species abandons sex, clonal reproduction will continue to copy genetic marker distributions that were generated by sex - new mutations and genetic drift will only slowly generate evidence of clonality. Likewise mutational decay will only destroy the genes required for sex over time (Schmid et al., 2004). Therefore our research group developed an alternative hypothesis: Hypothesis II: "Echo" of past sex: While some remnants of the sexual machinery in *C. albicans* still function and sex may still happen on occasion, the recombinant lineages generated by sex are not able to compete with clonal lineages and do not survive. The limited evidence of genetic exchange in the population may be better explained as a result of recent loss of biological role of sex in *C. albicans* instead of a sign of ongoing rare sex.

1.4. Selection markers for assessing the ability of clinical isolates to mate with each other

As described above we have no clear view of the ability of clinical isolates to mate with each other. One major obstacle seems to be that frequency of recombination is very low even when two isolates are competent for mating. Thus an accurate assessment of the ability of clinical isolates to mate with each other requires a selection system for efficient recovery of rare recombinants.

1.4.1. Auxotrophic markers

Mating two strains with different auxotrophic markers provides a powerful selection system of recombinants- as prototrophic yeast. However, since the *C. albicans* life cycle does not include a haploid phase, making auxotrophic mutants requires the disruption of both copies of a biosynthetic gene, unless the isolate already has one defective allele. Except in the latter case it will be very difficult to generate these auxotrophs by mutagenesis (Whelan and Magee, 1981). The most popular tool for disrupting gene function in *C. albicans,* the Ura-blaster cassette (Fonzi and Irwin, 1993) cannot be used to generate auxotrophs from prototrophic strains, because it relies on using the *URA*3 gene in a disruption cassette to complement a *ura*3 auxotrophy –i. e. it can only be used on auxotophic strains and not on prototrophic clinical isolates. Even it were possible, mating auxotrophic parents limit research on the resulting recombinants. Auxotrophy is usually deleterious to *C. albicans* fitness in animal models (Chen et al., 2008; Lay et al., 1998; Manning et al., 1984), which

means that there will be selection for recombinants that retain those copies of chromosomes that contain the functional alleles of genes that complement the auxotrophy. The mating of diploid *C. albicans* parents results in tetraploid recombinants which then return to a diploid state by chromosome loss (Bennett and Johnson, 2003). Chromosome loss will be influenced by selection, and if selection takes place in an animal model, it will be biased towards retention of chromosomes complementing the auxotrophy. In other words, if the marked strains will be used in animal models the auxotrophy can interfere with natural selection following mating, Moreover, it is also reported that the Ura-blaster cassette reduces virulence in many strains (Lay et al., 1998), and the expression of *URA*3 was influenced by position effects. Thus introducing *URA*3 marker into parents for mating might affect downstream experiments, e.g. competition experiments between the recombinants and their parents. An alternative approach is to mark the wild type, prototrophic clinical isolates with dominant selective markers.

1.4.2. Dominant selectable markers

Although a number of selectable markers have been used in fungi, very few dominant selective markers are effective in *C. albicans*. This is because *C. albicans* is naturally resistant to most of the drugs that form the basis of selectable markers in other fungi, such as benomyl, tunicamycin, cycloheximide, and mitomycin C. Moreover, *C. albicans* translate the CTG codon as leucine instead of serine unlike most of the organisms except some other *Candida* species (Santos and Tuite, 1995). Thus genes derived from other organisms may not function in *C. albicans* due to the

presence of CTG codon or unusual codon usage (Cormack et al., 1997; Shen et al., 2005).

Recently, Basso's group has developed a synthetic hygromycin B resistance gene with optimized *C. albicans* codons, which is able to confer hygromycin B resistance when introduced into wild-type *C. albicans* strains (Basso et al., 2010). However, when the work for this thesis began, only two dominant selectable markers were available in *C. albicans*. One is the mycophenolic acid (MPA) resistance (*MPA*^{r}) marker, conferred by three point mutations in the *IMH3* gene encoding Inosine monophosphate (IMP) dehydrogenase (Beckerman et al., 2001), which is a target of MPA. This gene was cloned by Kohler et al. from a naturally MPA-resistant clinical *C. albicans* isolate (Köhler et al., 1997). It was found that a single-copy of the *IMH3* allele sufficiently conferred resistance to MPA in *C. albicans* (Beckerman et al., 2001).

The second marker is *CaNAT1* which confers resistance to nourseothricin (NAT) (Shen et al., 2005). *C. albicans* is susceptible to moderate nourseothricin concentrations (250 to 400 μg/ml). *CaNAT1* was constructed from the *Streptomyces noursei nat1* gene, which encodes nourseothricin acetyltransferase, conferring resistance to the aminoglycoside antibiotic nourseothricin. The codon usage was optimized for that of *C. albicans*. It was found that a single copy of *CaNAT1* was an efficient positive selectable marker in *C. albicans* (Shen et al., 2005).

The availability of two dominant selectable markers in *C. albicans* should make it possible to mark one clinical parent with MPA and the other one with

nourseothricin (NAT). Selection of recombinants can be accomplished in principle by plating the recovered yeasts on plates containing MPA and NAT. However, a potential problems for the MPA selection of recombinants could be that MPA selection gives a high background of false-positives (Beckerman et al., 2001). MPA does not kill susceptible cells. If selection of very rare resistant cells among a large number of susceptible cells was required (as mentioned above, nuclear fusion frequency between clinical isolates is very low), MPA selection could result in many false-positive putative recombinants.

1.5 Assessments of the benefit of sex in the laboratory

Given that the population genetics analyses do not answer the question of whether *C. albicans* still forms viable recombinant lineages, other approaches need to be explored. A direct experimental test of the biological function of sex in *C. albians* is measuring whether cells benefit from choosing sex by improving their chances of passing on their genes to future generations. This is most easily done in the laboratory, not only because of the ease of experimental manipulation but also because for clinical isolates this will mimic the situation in which sex confers the greatest benefit, adapting to a new environment (in this case laboratory culture). Accelerated adaptation to altered environments, by generation of new allele combinations from the parental genomes, is held to be one of the main benefits of sex (de Visser and Elena, 2007).

1.5.1 Sex is expected to be more beneficial if adaptation to a novel environment is required

The biological significance or benefits of sex for eukaryotic organisms has remained unknown for many years, although many theories and explanations have been proposed (Hurst and Peck, 1996; Kaltz and Bell, 2002; Michod and Levin, 1987; West et al., 1999). Sex has costs. One is that during meiotic recombination sex breaks up a co-adapted combination of genes. However, despite the biological costs associated with sexual reproduction, sexual eukaryotic organisms predominate while asexual species of multicellular eukaryotes are, in general, short lived and rare (Burt, 2000; Kaltz and Bell, 2002). This suggests that sexual eukaryotes should have advantages over asexual eukaryotes (Kondrashov, 1998; Otto and Barton, 1997). One of the most widely accepted explanations for the advantage of sexual reproduction is given by Weismann in 1889 and proposed that sex provides the variation necessary for adapting to environments. Subsequently, Fisher (1930) and Muller (1932) developed the hypothesis further by predicting that meiotic recombination could bring together advantageous mutations (Fisher, 1930; Muller, 1932) arising in different lineages, into a single individual, thus reducing the time required for combining these mutations. By using different theoretical models, researchers have expanded on the Weismann-Fisher-Muller hypothesis. The most popular theory is that "sex increases the variance and, subsequently, the mean fitness in novel environments" (Kaltz and Bell, 2002). The combination of alleles that would be best suited to the new environment are likely to be present in only a minor proportion of the population when the environment changes. This can be either because in the previous environment, the combinations of beneficial alleles

were less fit, or because if populations are finite, some combinations of beneficial alleles are probably absent by chance (Kaltz and Bell, 2002). This "negative linkage disequilibrium" should be reduced by sexual recombination. The genetic variance of the population and the general fitness of the population should be increased if sex was beneficial (Burt, 2000; Maynard Smith, 1988; Pamilo et al., 1987). When advantageous genes appear on a chromosome that also contains harmful genes, the advantageous genes may escape their surroundings and hence spread more easily due to the presence of sexual lineage (Kaltz and Bell, 2002; Maynard Smith, 1988; Otto and Barton, 1997). Therefore sexual reproduction would be more beneficial if adaptation to a novel environment is required.

1.6. Summary

C. albicans and many other major opportunistic human fungal pathogens were long considered asexual, since sexual recombination had never been observed in nature. But the discovery that *C. albicans* can be induced to mate in the laboratory (Heitman, 2010) suggests that it may occasionally reproduce sexually (Schmid et al., 2004). *C. albicans* can undergo a parasexual cycle and can be expected to do so, probably on very rare occasions, in its natural environment. The population genetics evidence does not, however, unequivocally support the hypothesis that the recombinant lineages survive. It is quite possible that *C. albicans* no longer generates viable recombinant lineages and that the evidence for recombination in population studies are based on past recombination events only. If so, sex in the laboratory (and very rare sex in the host) is still possible only because sex lost its biological function only recently and the genes involved have not yet lost all of their

functionality through mutational decay. Thus recent loss of a biological function of sex is an equally convincing explanation of the available evidence.

1.7. Aims of the research

The available evidence supports two conflicting hypotheses. Either rare sex generates viable recombinant lineages. Alternatively, sex, while still observable in the laboratory, no longer generates viable lineages in *C. albicans*.

To distinguish between these hypotheses I: (1) developed molecular tools, which allowed me to mate clinical isolates; (2) mated a selection of clinical isolates to determine the fraction of pairwise combinations of clinical isolates capable of producing recombinants; and (3) tested, using recombinants from each successful mating whether choosing sex over clonal propagation enhances the chances of clinical isolates to pass on their genes to future generations, during their adaptation to a new environment, where sex should be most likely to confer selective advantage.

2.0. MATERIALS AND METHODS

2.1. Biological materials

2.1.1. *Candida albicans* strains

C. albicans strains used for mating experiments in this study are listed in Table 2.1a. The universal tester strains were obtained from the University of Minnesota (Prof PT Magee) (Magee et al., 2002) and are listed in Table 2.1b. Representative recombinants[1] obtained from mating used for growth competition experiments are listed in Table 2.1c. Twenty-three *MTL* homozygous clinical isolates used for growth rate determinations were obtained from Brown University (Assistant Professor Richard Bennett) (Miller and Johnson, 2002; Schmid et al., 1999; Slutsky et al., 1987; Wu et al., 2007) and are listed in Table 2.2.

Table 2.1. *C. albicans* strains used in this study

(A) Clinical isolates*

Strain	Ca3 type**	Geographical origin	Clinical origin
AU7	GPGA2	New Zealand	Urine
AU90	GPG A2	New Zealand	Skin wound
HUN97	GPG A2	Britain	Blood
OD8916	GPG A2	Britain	Oral
W43	GPG A2	New Zealand	Oral
AU35	C	New Zealand	Sputum
FJ11	C	Fiji	Throat

[1] The term "recombinant" used in this study is defined as mononuclear mating products containing genetic material from both parents.markers.

RIHO11	C	United States	Blood
W17	C	New Zealand	Throat
YSU63	C	Malaysia	Urine

(B) Universal tester strains

Strain	Genotypes	Original strains
3685	*His*$^{-}$, *Arg*$^{-}$, *MPA*^{r}, *MTL*α	Derivatives of the lab strain Sc5314
3710	*His*$^{-}$, *Arg*$^{-}$, *MPA*^{r}, *MTL***a**	Derivatives of the lab strain Sc5314

(C) Recombinants[***]

AU35**a** x HUN97α.1	AU90**a** x YSU63α.3
AU35**a** x OD8916α.1	RIHO11**a** x OD8916α.1
AU35**a** x OD8916α.2	RIHO11**a** x OD8916α.2
AU35**a** x W17α.1	RIHO11**a** x OD8916α.3
AU35**a** x W17α.2	RIHO11**a** x W17α.1
AU35**a** x W17α.3	RIHO11**a** x W17α.2
AU35**a** x YSU63α.1	RIHO11**a** x W17α.3
AU35**a** x YSU63α.2	RIHO11**a** x YSU63α.1
AU35**a** x YSU63α.3	W43**a** x OD8916α.1
AU7**a** x YSU63α.1	W43**a** x OD8916α.2
AU90**a** x HUN97α.1	W43**a** x OD8916α.3
AU90**a** x HUN97α.2	W43**a** x W17α.1
AU90**a** x OD8916α.1	W43**a** x W17α.2
AU90**a** x W17α.1	W43**a** x W17α.3
AU90**a** x W17α.2	W43**a** x YSU63α.1
AU90**a** x YSU63α.1	W43**a** x YSU63α.2
AU90**a** x YSU63α.2	W43**a** x YSU63α.3

* Source: Schmid et al. (Schmid et al., 1999)

** Based on Ca3 fingerprinting; GPG = general purpose genotype, equivalent to major group A which is subdivided in to subgroups A1 and A2 (Schmid et al., 1999).

All of the recombinants are (*MPA*^{r}) (*NAT*^{r}) *MTLa**/α.

Table 2.2. Naturally occurring *MTL*-homozygous clinical isolates

Strain	Genotype	Reference
WO-1	*MTL*α/α	B. Slutsky *et al.*,1987; Miller and Johnson, 2002
L26	*MTL*a/a	Wu et al., 2007
GC75	*MTL*α/α	Wu et al., 2007
p37005	*MTL*a/a	Wu et al., 2007
19F	*MTL*α/α	Wu et al., 2007
p87	*MTL*a/a	Wu et al., 2007
12C	*MTL*a/a	Wu et al., 2007
p60002	*MTL*a/a	Wu et al., 2007
p78048	*MTL*α/α	Wu et al., 2007
p57072	*MTL*α/α	Wu et al., 2007
P94015	*MTL*a/a?	Wu et al., 2007
85/005	*MTL*a/a	Odds et al., 2007
AM2005/0377	*MTL*a/a	Odds et al., 2007
T101	*MTL*a/a	Odds et al., 2007
M97105	*MTL*a/a	Odds et al., 2007
81/139	*MTL*a/a	Odds et al., 2007

SCS103353G	*MTL*a/a	Odds et al., 2007
SCS103354N	*MTL*a/a	Odds et al., 2007
AM2003-018	*MTL*α/α	Odds et al., 2007
AM2003/0165	*MTL*α/α	Odds et al., 2007
AM2002/0087	*MTL*α/α	Odds et al., 2007
RIHO9	*MTL*a/a	Schmid et al., 1999; Odds et al., 2007
81/196	*MTL*a/a	Odds et al., 2007

2.1.2. *Escherichia coli* strains

E.coli strains used in this study are listed in Table 2.3.

Table 2.3. *E. coli* strains used in this study

Strain	Genotypes	Reference
DH5α	F^-, φ80*lacZ*, ΔM15, Δ*lacZYA-argF*, U169, *recA1*, *endA1*, *hsd17* (r_k^-, m_k^-), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*	Invitrogen
TOP10	F^-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), φ80*lacZ*, ΔM15, Δ*lacX74, deoR*, *recA1*, *araD139,* Δ(*ara-leu*)7697*, galU, galK, rpsL(*Str^R*),* *endA1, nupG*	Invitrogen

2.2. Sources of materials and equipments

Sources of materials and equipment are listed in Table 2.4.

Table 2.4. **Suppliers of Materials and Equipment**

Materials and Equipment	Suppliers
1 Kb plus DNA ladder	Invitrogen
100 bp DNA ladder	Biolabs
Agar	Oxoid Ltd
Agarose	Roche
Ammonium persulfate	Sigma
Ampicillin	GmbH
Anti-Digoxigenin-AP	Roche
Bacto®-tryptone	Difo Laboratories
Bacto®-peptone	Difo Laboratories
Boric Acid	Invitrogen
Branson 2510 Sonicator	Bransonic
Calf intestine alkaline phosphatase	Roche
CSPD	Roche
Cryovial (2ml)	Global Science
Diethylpyrocarbonate	Sigma
DIG PCR Probe Synthesis Kit	Roche
DIG Easy Hyb	Roche
DNA extraction kit	Qiagen

dNTP	Roche
DTT	Sigma
DMSO	Sigma
Expand™ long template PCR polymerase	Roche
Ethidium bromide	Sigma
Gel Doc™ 2000	Bio-Rad
Gel purification Kit	Zymo
Gel casting units	Bio-Rad/ Hoefer/ Owl
Glucose	Asia Pacific Specialty Chemical Ltd
High Pure PCR Product Purification Kit	Roche
High Pure™ plasmid isolation kit	Roche
IPTG	Global Science
Klenow fragment	Roche
KOD DNA polymerase	Novagen
LABOFUGE 400 Centrifuge	Thermo Electron Corporation
Lithium acetate	Sigma
Long ranger™	BMA
Lyticase	Sigma
Mannitol	Merck
MPA	Invitrogen
NAT	Werner BioLabs
Nutrient broth	Difco Laboratories
Nylon Membranes, positively charged	Roche
PCR clean up kit	Roche
PEG3350	Sigma

Pepsin	Sigma
pGem^{R}-T Easy Vector Systems	Promega
Phloxine B	Sigma
Proteinase K	Roche
Qiagen taq DNA polymerase	Qiagen
Ribonuclease A (RNAse A)	Sigma
Salmon sperm DNA	Invitrogen
Savant DNA 110 SpeedVac ® System	Global Market Insite, Inc
Cellulose acetate filter (0.2μm).	Toyo Roshi Kaisha Ltd
Spectrophotometer	NOVA TECH
L(-)Sorbose	Merck
Sorvall^{R} RC-5B Refrigerated Super-speed Centrifuge	DuPont company
Sytox green	Invitrogen
T4 DNA ligase	New England Biolabs
T4 DNA polymerase	New England Biolabs
T4 polynucleotide kinase	New England Biolabs
Tris Base	Invitrogen
X-ray film	Kodak
Yeast extract	Difco Laboratories
Yeast nitrogen base w/o amino acids	Difco Laboratories
Zymoclean™ Gel DNA Recovery Kit	Zymo Research

All other chemicals were of analytical grade and from Merck. Restriction enzymes and buffers were supplied by Invitrogen, Roche and Global Science.

2.3. Plasmids used in this study

The plasmids used in this study are listed in Table 2.5.

Table 2.5. Plasmids used in this study

Plasmid	Contains	Reference
p3408	*IMH3*^{r}	Beckerman *et al*., 2001
p99RLU	TRp/tetR-ScHAP4AD	Nakayama *et al*., 2000
pCAITHE5	*tetR-ScHAP4AD*	Nakayama *et al*., 2000
pJK850	*ACT1*promoter-*CaNAT*-*ACT1*terminator	Provided by P.T. Magee and B. B. Magee, Department of Genetics, Cell Biology, and Development, University of Minnesota, MN. USA
pBluescript II®KS+	*E. coli* cloning vector	Stratagene
pGemT easy	*E. coli* cloning vector	Promega

2.4. Plasmids developed during this study

The plasmids developed during this study are listed in Table 2.6.

Table 2.6. Plasmids developed during this study

Plasmid	Features
pNZ1	pBlueScriptII® KS(+) containing: the ORF encoding the tetracycline activator tetR-ScHAP4-WH11 under the control of the *CaENO1* promoter; *Amp*^{r} .
pNZ2	pBlueScriptII® KS(+) containing: *IMH3*^{r} under the control of a tetracycline-responsive promoter (pTR); *Amp*^{r} /*MPA*^{r}
pNZ3	pBlueScriptII® KS(+) containing: *IMH3*^{r} under the control of a tetracycline-responsive promoter (pTR); the ORF encoding the tetracycline activator tetR-ScHAP4-WH11 under the control of the *CaENO1* promoter; *Amp*^{r} /*MPA*^{r}
pNZ4	pBlueScriptII® KS(+) containing: *IMH3*^{r} under the control of a tetracycline-responsive promoter (pTR); the ORF encoding the tetracycline activator tetR-ScHAP4-WH11 under the control of the *CaENO1* promoter; two sequences TS1 and TS2 from a non-coding region of *C. albicans* chromosome 7; *Amp*^{r} /*MPA*^{r}
pNZ11	pBlueScriptII® KS(+) containing: a 1.9-kb *Kpn*I/*Sac*I fragment from pJK850; *Amp*^{r} /*NAT*^{r}
pNZ18	pBlueScriptII® KS(+) vector containing: a native Ca*ACT1* promoter plus the *IMH3*^{r} gene and the *IMH*3 terminator from p3408
pTS1	pGemT Easy vector containing: a 0.8-kb TS1 fragment from *C. albicans* chromosome 7; *Amp*^{r}
pTS2	pGemT Easy vector containing: a 1.1-kb TS2 fragment from *C. albicans* chromosome 7; *Amp*^{r}

2.5. Media and solutions

All media were prepared with deionized water and sterilized at 121^{o}C for 15-20 min (except where otherwise noted). To make solid medium, 1.5%-2% (w/v) agar was added to the medium.

2.5.1. Yeast media

2.5.1.1. Yeast Extract Peptone Dextrose (YPD)

YPD medium (Ausubel et al., 2006) contained 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose.

2.5.1.2. Minimal medium (MM)

MM (Chu et al., 1993) contained 0.67% (w/v) yeast nitrogen base without amino acids (aa), and 2% (w/v) glucose.

2.5.1.3. Sorbose medium

Sorbose medium (Rustchenko et al., 1994) was prepared with 2% (w/v) sorbose, 0.67% (w/v) yeast nitrogen base without aa, and was filter sterilized.

2.5.1.4. Spider medium

Spider medium (Liu et al., 1994) contained 1% (w/v) nutrient broth, 1% (w/v) mannitol, 0.4% (w/v) K_2HPO_4, 0.08% (w/v) KH_2PO_4, pH was adjusted to 7.2.

2.5.1.5. MPA plates

MPA plates (Beckerman et al., 2001) were prepared by adding MPA (Invitrogen, dissolved in ethanol) to MM with 1.5% agar to yield final concentrations ranging from 1 to 10 μg/ml. For selection of transformants or recombinants, MM containing 5 μg/ml MPA was used. The stock solution of MPA was 25 μg/ μl.

2.5.1.6. NAT plates

NAT plates (Shen et al., 2005) were prepared by adding NAT (Werner BioLabs, dissolved in deionized H_2O, filter sterilised) to YPD with 1.5% agar to yield final concentrations ranging from 100 to 400 μg/ml. For selection of transformants or recombinants, YPD containing 200 μg/ml NAT was used.

2.5.1.7. Phloxine B plates

Phloxine B plates (Lockhart et al., 2002) were prepared by adding Phloxine B (Sigma) in deionized H_2O (then filter sterilized) to 10 μg/ml in YPD with 1.5% agar.

2.5.2. Bacterial media

2.5.2.1. Luria-Bertani medium (LB)

LB medium contained 0.5 % (w/v) NaCl, 1% (w/v) tryptone and 0.5% (w/v) yeast extract; pH was adjusted to 7.0-7.5 with 1M NaOH.

2.5.3. General buffers and stock solutions

All buffers and stock solutions were prepared according to Ausubel et al. (2006) unless otherwise indicated.

2x SSC:	0.3 M NaCl, 0.03 M Sodium citrate, adjusted to pH 7.0 with NaOH.
20x SSC:	3 M NaCl, 0.3 M Sodium citrate, pH 7.0.
50x TAE (1 L):	242 g Tris Base, 100ml 0.5 M EDTA (pH8.0), 57.2 ml glacial acetic acid.
10x TBE (1 L):	108 g Tris Base, 55 g Boric Acid, 40 ml 0.5 M EDTA (pH8.0).
$CaCl_2$ solution (1 L):	6.6 g $CaCl_2$, 187 ml glycerol, 3 g PIPES, adjust pH to 7 with 8 M NaOH.

EDTA (0.5M in 1 L):	186 g NaEDTA·2 H_2O in 700 ml H_2O, adjust pH to 8.0 with 10 M NaOH (~50ml), add distilled water to 1 litre.
Gel loading buffer (10x):	20% Ficoll 400; 0.1 M disodium EDTA, pH 8; 1% SDS; 0.25% bromophenol blue; 0.25% xylene cyanol.
Glycerol solution:	60% glycerol, 0.1 M Mg_2SO_4, 0.025 M Tris, pH 8. Autoclave to sterilize.
IPTG stock solution (0.1 M):	1.2 g IPTG, add water to 50 ml final volume. Filter-sterilize and store at 4°C.
Polyacrylamide gel (8%) (5 ml):	0.8 ml long ranger™ (BMA) acrylamide-bis, 10x TBE 0.5 ml, N,N,N',N'-Tetramethylethylenediamine (TEMED) 3 µl, 10% APS, 30 µl, 3.7 ml deionized H_2O.
X-Gal (50 mg/ml):	100 mg 5-bromo-4-chloro-3-indolyl-ß-D-galactoside dissolved in 2ml N,N'-dimethyl-formamide. Store protected from light at -20°C.
RNase A solution:	10 mg/ml in H_2O. Heat to 70°C for 10 min. Store at -20°C.
10x TNE (pH 7.4, 250 ml):	3.02 g Tris base (100mM), 5 ml 0.5 M EDTA stock (10mM), 29.2 g NaCl (2 M). Adjust pH to 7.4 with HCl. Add distilled H_2O to 250ml. Autoclave. Store at 4ºC for up to 12 months.
Hoechst Dye H 33258 Stock (1 mg/ml):	Prepared by dissolving dye in distilled H_2O which has been filtered using a 0.2 µm cellulose acetate filter (Toyo Roshi Kaisha Ltd). Stored at 4°C, protected from light, for up to 6 months.
1 M sorbitol:	Prepared by dissolving 18.2 g sorbitol in distilled H_2O, adjusted to 100 ml.
10x TE (pH 7.5, pH 8.0):	100 mM Tris base, 10 mM EDTA in distilled H_2O. Adjusted pH to 7.4 or 8.5 using HCl. Autoclave. Store at room temperature for up to 12 months.
Ethidium bromide dye:	1µg/ml in 1xTBE or 1xTAE buffer.
1 M Lithium acetate (LiOAc):	Prepared by dissolving 10.2 g LiOAc in distilled H_2O, adjusted to 100 ml. Autoclave to sterilize.
TELiOAc (1 M) (Magee, 2008):	10 mM TrisHCl, pH 7.5, 1 mM EDTA, pH 7.5, 1 M Lithium acetate; prepared by combining 1 volume 10x TE (pH 7.5) and 1 volume LiOAc (1 M) with 8 volumes sterile water.

PLATE mix (Magee, 2008):	10 mM Tris HCl, pH 7.5, 1 mM EDTA, pH 7.5, 0.1 M Lithium acetate, 50% PEG3350 (Sigma). Prepared by diluting 1 volume 10x TE (pH7.5) and 1 volume 1 M LiOAc with 8 volumes 50% PEG3350.
Salmon sperm DNA (10 mg/ml):	Prepared by dissolving 0.1 g Salmon sperm DNA (Invitrogen) in 10 ml of distilled H_2O. Break DNA by sonication. Autoclave to sterilize. Aliquot and freeze at -20.
RNase A (2 mg/ml, for FACS analysis)	Prepared by dissolving 10 mg RNase A (Sigma) in 10 ml of 5x TE buffer (pH 8.0). Prepare fresh by boiling 15 min, and cooling on bench top.
Pepsin (5 mg/ml):	Prepared by dissolving 100 mg Pepsin (Sigma) in 20 ml of 55 mM HCl. Prepare freshly.
Sytox Green (1 mM):	Prepared in dimethyl sulfoxide (DMSO). Aliquot and store at -20° for up to 1 year.

2.6. Primers used in this study

Primers used in this study are listed in Table 2.7.

Table 2.7. Primers used in this study

Name	Oligonucleotide Sequence (5' to 3')
MG1pf	CCTCCCTTCTCTTAAGAG
MG1pr	AACAGGAGAGGTTAAGAG
MC0repf***	AACCATCATGACGATCACCA
MC0repr***	GATAAATCTCATCTGCAGGC
MC5pf***	AGAGTTAACTCTATCTCCTC
MC5outpr***	CTAGTTCCGATAGATACTCG
M13pf	CTCACTATAGGGCGAATTGGAG
M13pr	AATTAACCCTCACTAAAGGGAACAAAAGCTGG
Sacpf1	ACTGGAGCTCATTTTATGATGGAATGAATGGG
MTL**a**1-F*	TTGAAGCGTGAGAGGCTAGGAG
MTL**a**1-R*	GTTTGGGTTCCTTCTTTCTCATTC
MTLα*1*-F*	TTCGAGTACATTCTGGTCGCG
MTLα1-R*	TGTAAACATCCTCAATTGTACCCGA
MTLapr:	ATCAATTCCCTTTCTCTTCGATTAGG
ENOpf	GGGATCAAGATTTGTTTACAG
tetRWH11pr	CCATGGTGAGACGCGACAGA
TRpf	CTCGAGCCCGGGTGGACTTCTTCG
TRpr	CTGCAGGTCGACTTTTCTGA G

IMH3pfatg	ATGGTGTTTGAAACTTCAAAAG
IMH3pr	CTCGAGTCTAGAACTCAGTATATCTT CA
TRpIMHp	ATCTCAGAAAAGTCGACCTGCAGATGGTGT
	TTGAAACTTC AAAAG
TS1pf	GCGGCCGCCCCGGGTCCTTTCTTACTAAAATATAGA
TS1pr	GCGGCCGCGATCTTACACACACAATCAG
TS2pf	AAGCTTAGAAGGAAAGAAGGAAAGAA
TS2pr	AAGCTTCCCGGGCCTTATCATCATGATCACCA
pNZ11pf	CGAATTGGAGCTCATTTTATG
CaNATpr	GGACATGGCATAGACATATAC
pACTFpf	TGCCTCTTACCAACTATTTCA
TS1pr-p	GCGGCCGCGATCTTACACA
TS1Fpf	CACTACTACTACCTACTACTAC
TRsepr	GGTGTGGTCAATAAGAGCGA
TS2Fpr	TACCTATGCACTACTACTACTC
CaACTpr	CTAAAACATACCACCGTCCA
pENOpr	ATCGTTAGTCAACTTTTGCAAC
MCEA1outpf2***	TGGGTCATGTGGTATTGGAG
MCEA1outpr2***	ATGGCCGGATGTTTCCAGAA
SSR1-2**	CTAGTTCAGCCAAGGCTTCTTC
SSR1-3**	AGAAGAAGCCTTGGCTGAAC
tetRpf	ATGTCTAGAT TAGATAAAAG TAAA
tetRpr2	AGTCGTCATCAGTACCGGC
TS1se	CTCTCTCCTT TTGTTACGAG
CaNATpf	CTACTACTTTGGATGATACTG

* designed by Legrand and coworkers ((Legrand et al., 2004).

** designed by Zhuo Zhou (Zhou, 2011).

*** designed by N. Zhang (Zhang et al., 2003).

2.7. Microbial growth conditions

2.7.1. Storage, growth and manipulation of *C. albicans* cultures

2.7.1.1. Glycerol stocks for *Candida albicans*

For permanent storage, *C. albicans* was grown overnight at 37ºC in a 19 mm x 20 cm Erlenmeyer test tube in 2 ml YPD medium on a LAB-LINE rotary shaker (150 rpm) and 0.5 ml overnight culture was mixed with 0.5 ml glycerol solution (described in section 2.5.3.) in a 2 ml plastic cryovial (Global Science) with a screw cap. The tube was then stored at -80°C. To revive cells, a red-hot inoculating loop was lowered into the glycerol stock, and the molten drop attached to the loop was used to inoculate a YPD plate, which was then incubated at 37°C for 24 hours, and stored at 4°C for up to two months for use.

2.7.1.2. Drug susceptibility assay for clinical isolates

MPA (mycophenolic acid) susceptibility assay: MPA plates containing different concentrations of MPA (2 μg/ml, 5 μg/ml, 10 μg/ml) were made to test the resistance of the model strains to MPA. MM plates without MPA were used as controls. Approximately 300 cells (cell numbers were determined microscopically

using a counting chamber (ZINTL, Western Germany)) of each strain were spread evenly over each plate. The plates were incubated at 37^{o}C for up to 5 days.

NAT (nourseothricin) susceptibility assay: NAT plates containing different concentrations of NAT (100 μg/ml, 250 μg/ml, 400 μg/ml) were made to test the resistance of the model strains to NAT. YPD plates without NAT were used as controls. Approximately 300 cells of each strain were spread evenly over each plate. The plates were incubated at 37^{o}C for up to 2 days.

2.7.1.3. Sorbose selection of *MTL*-homozygous derivatives

MTL-homozygous derivatives of clinical isolates were generated through chromosome 5 loss by sorbose selection as described by Janbon et al. (Janbon et al., 1998). Cells of each strain were grown in 2 ml of liquid YPD medium at 37^{o}C overnight. Approximately 10^{6} cells of each strain were spread on sorbose medium agar plates (2% w/v sorbose, 0.67% w/v yeast nitrogen base w/o amino acids; sterilized by filtration, 1.5% agar). The plates were incubated at 37^{o}C for one to two weeks. Colonies were checked by colony PCR (see section 2.9.2) for the presence of *MTL***a** and *MTL*α alleles, Primer combinations used were *MTL***a**1-F/*MTL***a**1-R and *MTL*α-1F/*MTL*α1-R (Table 2.7). The *MTL***a** or *MTL*α derivatives of each strain were purified twice on sorbose plates and stored in 30% glycerol at -80 ^{o}C

2.7.1.4. Selection of spontaneously arising *MTL*-homozygotes

To obtain spontaneously arising *MTL*-homozygotes the method developed by Lockhart et al. was used (Lockhart et al., 2002), except that the phloxine B plates were made in YPD instead of Lee's medium. Approximately 4000 to 6000 *C. albicans* cells of each strain were spread on YPD agar plates containing phloxine B (5 µg/ml) at low density (50 to 150 colonies per 85-mm plate) and the plates were incubated at 25° for 2 weeks. Red sectors were checked by multiplex colony PCR (section 2.9.6.) for the presence of *MTL***a** and *MTL*α alleles. Potential *MTL*-homozygotes were streaked onto YPD plates to get single colonies and the colonies were checked again for the *MTL* alleles by PCR.

2.7.1.5. Serial transfer conditions

For serial transfer experiments, one loop of cultures was inoculated into 2ml of YPD medium from patches of each sample on YPD plates. This culture was incubated at 37°C for 24 hours to saturation. Cell numbers were estimated by measuring the absorbance, at 600 nm wavelength, of the saturated culture using a NOVA TECH spectrophotometer. This saturation culture was designated as culture at time point 0 (t0). For each sample, 10µl of the saturated culture (approximately 1.5 to 3 x 10^6 cells) were then transferred into another 2 ml YPD medium and were grown to saturation (for 24 hours) again at 37°C. Cell numbers of starting culture and saturated culture were estimated using spectrophotometer as described above. This allows 8 generations for one transfer (from 1.5 - 3 x 10^6 cells to 3-6 x 10^8 cells). The cultures were serially transferred using the same procedure until reached

100 generations of propagation.

2.7.1.6. Growth rate determination

All *C. albicans* strains were maintained on YPD agar plates at 4 °C prior to use. For growth rate determinations, a pre-culture was made by inoculating one loop-full of cells from a YPD plate patch into 2 ml of YPD broth in a 19 mm x 20 cm Kimax glass test tube which was placed in a shaking incubator at 37 °C and 150 rpm. After 16 h, 80 µl of culture was used to inoculate 40 ml of YPD broth in a 200 ml Erlenmeyer flask, which was incubated under the same conditions; medium for growth rate experiments was always prepared in the same pressure cooker loaded with the same amount of medium to avoid differences in medium between different growth rate experiments (Fig. 2.1). Starting 2 hours after inoculation, absorbance, at 600 nm wavelength, of exponentially growing cultures of optical densities between 0.05 and 0.30 was measured at 8 time-points, using a NOVA TECH spectrophotometer. The growth rate (doubling time) was determined as the slope of the line of best fit in plots of $\ln(OD_{600})$ versus time (r^2 values were > 0.99 in all cases). Growth rates reported are averages of at least two, and usually three, independent experiments.

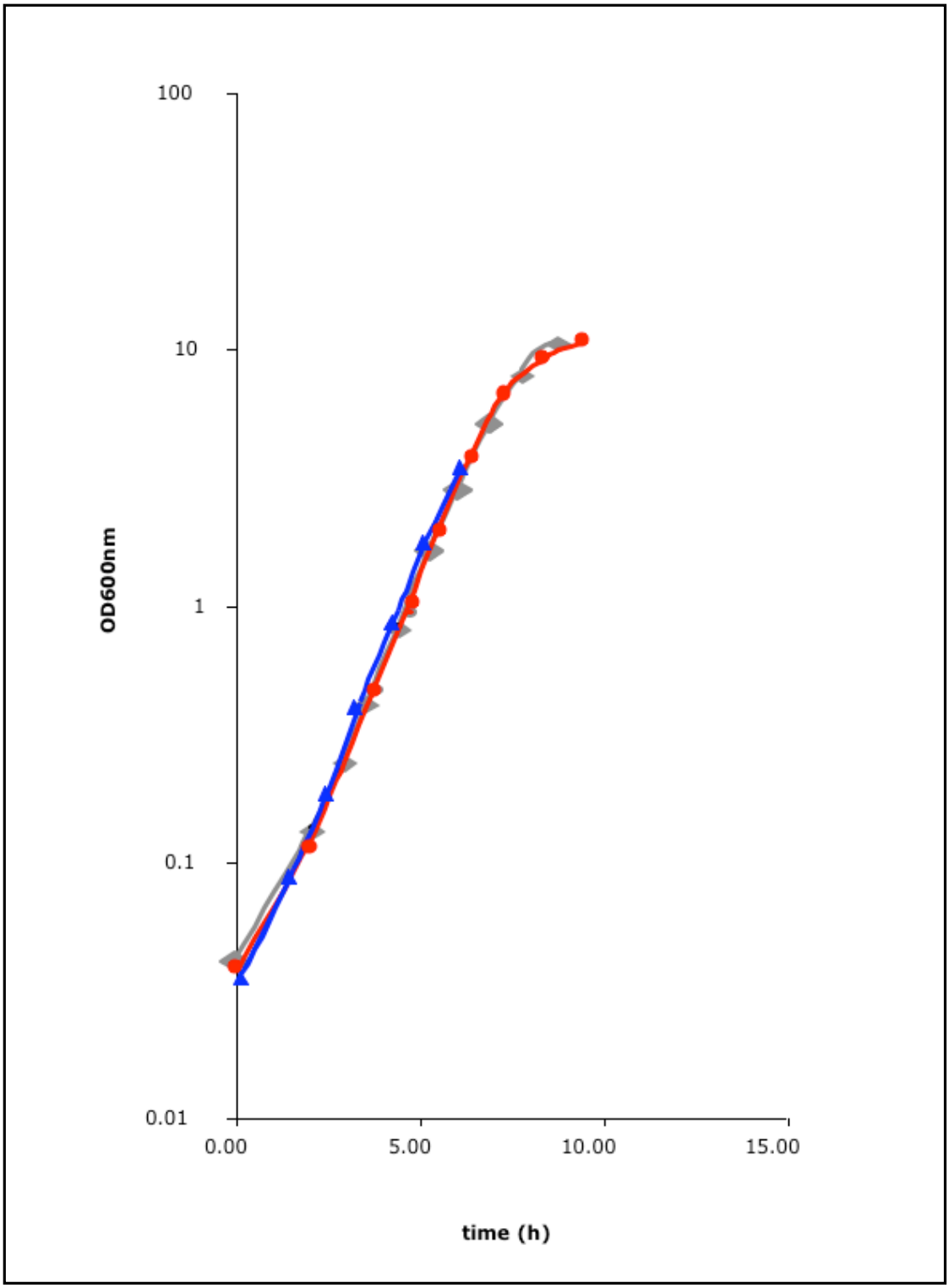


Figure 2.1. An example of multiple growth rate determinations at different times for that same strain (W43).

Grey rhombuses, red dots and blue triangles with lines represent growth experiments conducted on 03/08/09; 19/08/09 and 30/11/09, respectively. The rates calculated from the three experiments were 1.10 d/h (doublings per hour), 1.15d/h, and 1.11d/h, respectively.

2.7.1.7. Replica plating

Replica plating for transferring cells from one plate to another plate was performed using a method developed by Roberts in 1959 (Roberts, 1959). A sterilized cotton velveteen stamp was used to stamp a replica of the pattern of cells growing on one plate to one or several other plates. The apparatus consisted of a cylindrical holder for the velveteen that was just the right size to fit inside the bottom of a petri plate, and a ring of some sort to hold the velveteen in place. To replica plate, the master agar plate with the yeast on it to be replicated, was inverted over the velveteen on the holder and pressed firmly against the sterile fabric surface. Then the plate was slowly peeled off, leaving a replica or print of the cell pattern on the velveteen. These cells were transferred to one or a series of sterile agar plates by pressing each plate gently onto the velveteen and peeling it off.

2.7.2. Growth conditions for *Escherichia coli*

E. coli strains were grown on LB agar plates or in LB broth (with shaking at 200 rpm) at 37°C, overnight. For the selection of ampicillin-resistant (*Amp*^{r}) cells, ampicillin (Amp) was added to the medium to a final concentration of 100 μg/ml. For white-blue colony selection, IPTG (100 μl, 100 mM) and X-gal (20 μl, 50 mg/ml) were spread on top of pre-made LB-Amp (containing 100 μg/ml ampicillin) agar plates (30 ml) with a glass spreader. Then, the plates were dried for about 30 min. After inoculation with *E. coli* cells, the LB plates were incubated at 30°C overnight. The plates were stored at 4°C for up to two weeks. *E. coli* cultures for plasmid isolation were grown overnight in 5 ml LB broth containing Amp (100

μg/ml), at 37°C. Cultures were then stored at -80°C in 30% glycerol stock (section 2.5.3).

2.8. DNA manipulation

2.8.1. Plasmid DNA isolation

Plasmid DNA was isolated using the High Pure™ plasmid isolation kit (Roche) according to the manufacturer's instructions.

2.8.2. *C. albicans* genomic DNA isolation

C. albicans DNA was extracted from strains using a modification of the method of Scherer and Stevens (1987) (Scherer and Stevens, 1987). Cells were grown overnight at 37°C in 25ml YPD and collected from 5 ml of saturated culture (or more if not saturated) by centrifugation for 10 min at 1000 x g in tabletop centrifuge (Sigma) and suspended in 1 ml of 1 M sorbitol in an eppendorf tube. The cells were then collected by centrifugation for 5 min in an Eppendorf centrifuge at 1500 x g and the pellet was suspended in 1 ml of 1M sorbitol, 50 mM potassium phosphate, pH 7.5 with 0.1% mercaptoethanol and 100 to 200 units lyticase (Sigma). The cells were mixed vigorously on a vortex mixer immediately after addition of the lyticase solution. Cells were completely suspended so that the enzyme could get access to the wall of each cell. Cells were then incubated at 30°C to 37°C with shaking (100 rpm), and/or inverted every 5 min until all cells had been turned into spheroplasts (around 40 min to 2 hours). The spheroplasts were collected by centrifugation at

1000 x g, 2 min using a tabletop centrifuge. The supernatant was decanted and the spheroplasts were suspended in 0.5 ml of 50 mM Na-EDTA (pH 8.5) with 2 mg/ml SDS and 0.6% diethylpyrocarbonate under the fume hood while incubating at 70 to 80^{o}C for about 30 min. The tubes were flicked every 5 min or so until all spheroplasts were lysed. At the end of the 30 min period, no clumps should be apparent any more. Then 50 µl of 5 M potassium acetate were added, the solution was mixed by invertion and the tube was put on ice for 30 min or longer. The lids were kept opened and the icebox was kept under the fume hood to get rid of diethylpyrocarbonate traces that might be still present in the sample. The samples were then centrifuged for five minutes at 15000 x g and the supernatant was poured into 1 ml of ethanol in another eppendorf tube and mixed gently but thoroughly. A clump of DNA was formed from each sample and rinsed with 1 ml of 70% ethanol. The 70% ethanol was decanted and the pellet was dried in the Savant DNA 110 SpeedVac ® System (Global Market Insite, Inc) for 7 to 10 minutes. The pellet was then dissolved in 50 – 300 µl TE (pH7.4) buffer with 100 µg heat-treated RNAseA per ml. Suspension was aided by heating the sample to 65^{o}C, flicking it violently, or, as a last resort, sucking it slowly up and down with a pipette. Twice the sample volume of isopropanol was added and mixed gently but thoroughly with the supernatant. If isopropanol and supernatant were not completely mixed, or if the mixing was not carried out gently, DNA was lost. The clump of DNA was rinsed with 70% ethanol and dried in the SpeedVac as described above. The final pellet was suspended in 50 µl 1 x TE (pH 7.5) and store at 4^{o}C until use.

2.8.3. Gel purification of DNA fragments

Following electrophoresis in a TAE agarose gel (section 2.8.10), DNA bands of interests were excised from the gel and purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research) according to the manufacturer's instructions.

2.8.4. PCR product purification

PCR products were purified using High Pure PCR Product Purification Kit (Roche), according to the manufacturer's instructions with the exception that the final volume of elution buffer used was 30 μl.

2.8.5. DNA quantification

The DNA concentration of samples was determined using a TKO 100 Mini-Fluorometer (Hoefer), according to the manufacturer's instructions. The standard assay (0.1 μg/ml Hoechst Dye 33258 Stock in 1x TNE working buffer) was used for DNA samples between 10 ng/ml and 500 ng/ml. The extended range assay (1.0 μg /ml Hoechst Dye Stock in 1x TNE working buffer) was used for DNA samples between 100 ng/ml and 2000 ng/ml.

2.8.6. Restriction endonuclease digestion of DNA

Genomic DNA (1 μg) for Southern blot analysis was digested at 37°C overnight with 100 units of restriction enzyme in 1x commercial buffer provided with the enzyme and deionized water to a final volume of 20 μl. Plasmid DNA was digested for 3 h at 37°C, using 10 units of restriction enzyme/μg of DNA, 1x commercial buffer provided with the enzyme and deionized water to a final volume of 20 μl.

2.8.7. Calf intestinal alkaline-phosphate (CIAP) treatment of vectors

To prevent self-ligation of vectors cut by restriction enzymes, vectors were treated with calf intestinal alkaline-phosphatase (CIAP) by incubating 2-5 μg of digested vector with 1 unit of CIAP per μmol of DNA ends in a dephosphorylation buffer provided with the enzyme and deionized water to a final volume of 40 μl, at 37 °C for 1 h. The enzyme was then heat-inactivated at 65°C for 15 min.

2.8.8. Ligation

Ligation of fragments derived from PCR reactions into pGEM-T Easy vectors (pGEM®-T Easy Vector Systems, Promega. Refer to Appendix 10.7. for plasmid map) was performed according to the manufacturer's instructions. Ligation of DNA fragment of interest into CIAP-treated pBSKS(+)-derived vectors was performed by incubating 20 ng of vector DNA with insert DNA (1:3 molar ratio) with 1 unit of T4

DNA ligase in 1x ligation buffer supplied with the enzyme in a final volume of 20 μl at 4 ^{o}C overnight.

2.8.9. Creating blunt-ended DNA fragments

To create blunt-ended DNA for ligating to blunt-ended vectors, 5' overhangs of the DNA fragments were repaired using Klenow fragment (Roche) while 3' termini were removed with T4 DNA polymerase as described by Sambrook et al. (1989) (Sambrook et al., 1989).

2.8.10. Agarose gel electrophoresis

Agarose gels for electrophoresis were prepared in 1x TBE or 1x TAE buffer. The percentage of agarose used for each gel was determined by the sizes of the DNA fragments to be separated (Ausubel et al., 2006). Agarose was added to the buffer and heated in a microwave oven with regular mixing until dissolved. The solution was cooled to 50-55°C before being poured into a gel-casting unit. All DNA samples were mixed with 1 or 2μl of 10x gel loading buffer (according to the amount of sample used), prior to loading. For Southern blot analysis, DNA samples were separated at voltages of 30 V overnight, in a 15 x 20 cm gel (200 ml). All other DNA samples were separated at a voltage of 80-100 V, for 1-2 hours (or until the bromophenol blue dye front was three-quarters down the gel), in a 15 x 10 cm gel. After electrophoresis, gels were stained with ethidium bromide (1 μg/ml in distilled water) for 30 min, and then de-stained with water for 10 min. The DNA

fragments were visualized and photographed using a Bio-Rad Gel Doc™ 2000 system.

2.8.11. Polyacrylamide gel electrophoresis (PAGE)

A Mini Protean® 3 system (Bio-Rad) was used in all PAGE experiments. Gel plates were cleaned thoroughly with 5% SDS and their surfaces were brushed gently to remove dust or any residual gel, then rinsed with 70% ethanol and left to dry. An 8% polyacrylamide gel was prepared using the recipe as described in section 2.5.3. and used to separate DNA fragments between 100 and 500 bp. Each polyacrylamide gel was made from 5ml of polyacrylamide mixture. The gel was polymerized for 2 h between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After polymerization was complete, the combs were removed and the gels placed in the electrophoresis tank. 1x TBE buffer was poured in the space between the two sets of glass plates to cover the wells, and then samples were mixed with 1 µl of 10x gel loading buffer (the same gel loading buffer as used for agarose gels) and added to the wells. For each polyacrylamide gel, ~6 µl 100 bp DNA ladder (Biolabs) was loaded as a size marker. 1x TBE buffer was then poured into the space around the gels to half way up the container. Electrophoresis was carried out at 65 V for 2.2 hours. The polyacrylamide gel was carefully removed from the glass plate and stained with ethidium bromide and photographed as described for agarose gels (section 2.8.10).

2.8.12. Southern blotting

Genomic DNA of *C. albicans* clinical isolates was digested with restriction enzymes and separated by gel electrophoresis, stained and visualized as described in section 2.8.10. DNA fragments were transferred to positively charged nylon membranes using a modification of the method of Southern (Ausubel et al., 2006). Briefly, the gels were prepared for blotting as follows: The initial step was depurination in 0.25 M HCl for 15 min, followed by denaturation in 0.5 M NaOH plus 0.5 M NaCl for 2 x 20 min and lastly neutralization in 2M NaCl, 0.5 M TrisCl, pH 7.4 for 2 x 30 min, under gentle agitation. After neutralization, the gel was washed for 2 min in 2x SSC and assembled onto a blotting apparatus which as follows: a glass plate was placed across a glass tray containing 200 ml of 20x SSC buffer. A 3 MM paper wick soaked in 20x SSC was placed on the top of the glass, covered with plastic wrap, which was cut to have a surface area slightly smaller than the gel. The gel was placed on top of the soaked paper wick with the DNA side facing the top. A nylon membrane cut to the same size as the gel was pre-soaked in 2x SSC and then placed on top of the gel; three sheets of 3 MM paper of the same size as the gel were pre-soaked in 2x SSC and placed on top of the membrane, followed by a stack of paper towels and a 500 g weight. The blot assembly was left overnight to allow DNA to transfer from the gel to the membrane. After transfer, the membrane was washed in 2x SSC for 5 min and DNA was crosslinked to the membrane using an Ultraviolet crosslinker Cex-800 (Ultra-Lum Inc) for 5 min (120,000 IJ/cm3).

2.8.12.1. Probe labeling using Dig-labeling system

The DNA probes used in Southern blot analysis were labeled using PCR DIG Probe Synthesis Kit (Roche), and the efficiency of probe labeling was evaluated, as per the manufacturer's instructions.

2.8.12.2. Hybridization

The membranes were prehybridized in hybridization buffer, DIG Easy Hyb (Roche), for 2 hours at 42^{o}C in glass hybridization tubes (Amersham Biosciences). The labeled probes were denatured by boiling for 10 min, followed by cooling on ice for 5 min, and added to the hybridization tubes. The final concentrations of the probes were adjusted to 12-15 ng/ml according to the efficiencies of labeling determined as described in section 2.8.12.1. Hybridizations were carried out at 42°C for 16 h according to the manufacturer's instructions (Roche). Afterwards the blots were washed and probe-target hybrids detected with the Anti-Digoxigenin-AP kit (Roche) in detection buffer containing CSPD (Roche), according to the manufacturer's instructions (Roche). The blots were then exposed to X-ray film (Kodak) at 37^{o}C for 30 min. Films were developed in a 100Plus^{TM} automatic X-ray processor (All-Pro Imaging Corp.).

2.8.13. DNA sequencing

DNA sequencing was performed by the Allan Wilson Centre Genome Service (AWCGS), Massey University. Samples for sequencing were prepared as per the facility's instructions: the reaction mixture of 15 µl contained 300 ng of plasmid DNA or 2 ng/100 bp of PCR products and 3.2 pmol of each sequencing primer. The samples were sent to AWCGS and were sequenced by the dideoxynucleotide chain termination method using BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit on the capillary ABI3730 Genetic Analyzer, from Applied Biosystems Inc. Sequences. Electrophoregrams were assembled using AB DNA Sequencing Analysis Software version 5.2. (Applied Biosystems Inc.).

2.9. PCR methods

Oligonucleotide primers used in this study are listed in Table 2.7. All primers were synthesized by Invitrogen. Primers were re-suspended in sterile deionized water to a concentration of 100 pmol/µl (stock solution) and stored at -20^{o}C. Primers used in PCR reactions were diluted to a concentration of 10 pmol/µl (working solution).

2.9.1. Routine PCR

Routine polymerase chain reactions (PCRs) were performed in a final volume of 20 µl containing 1 U of Qiagen *Taq* DNA polymerase, 4 µl of Q-buffer and 1x PCR buffer supplied by the manufacturer (Qiagen), 10 pmol of each primer, 200 µM of each dNTP, and 10–100 ng DNA. The cycling conditions, varied according to

primer sets and the size of the products (Ausubel et al., 2006) and included an initial incubation for 2 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 50–60°C, and 30 s to 3 min at 72°C. All PCR protocols included a final 5 min extension step at 72°C. Reactions were carried out in an Eppendorf Mastercycler thermocycler (Eppendorf, Hamburg, Germany).

2.9.2. Colony PCR

Colony PCR was performed as described in Zhang et al. (2010). Briefly, a portion of a *C. albicans* colony or *E. coli* colony was picked with a sterile 10 µl pipette tip and mixed with 20 µl PCR reaction mixture prepared as described above; the initial step in the cycling program was altered to 5 min (for *C. albicans*) or 3 min (for *E. coli*) at 96°C.

2.9.3. High-fidelity PCR

High-fidelity enzyme KOD DNA Polymerase (Novagen) was used, according to the manufacturer's instructions, for amplifying fragments used in constructing resistance markers where sequence accuracy was crucial.

2.9.4. Recombinant PCR

Recombinant PCR was performed as described by Zarrin et al. (Zarrin et al., 2005). In short, two rounds of PCR reactions were performed (Fig. 2.2). For first-round PCR, fragment 1 and 2 were amplified separately with primers p1 and p2, and p4

and p5, respectively. Each reaction was performed in a final volume of 50 µl containing 1 U of KOD DNA polymerase (Novagen), 5 µl of 10x PCR buffer supplied by the manufacturer (Novagen), with 10 mM $MgCl_2$, 20 pmol of each primer, 200 µM of each dNTP, and 10–100 ng DNA. Hot start amplification was initiated with 2 min at 94 °C denaturation, followed by 30 amplification cycles (94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min) and a final extension cycle of 72°C for 5 min. PCR products from each reaction were gel-purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and used in a subsequent amplification. Second-round PCR was as for the first-round, except that 10 ng each of fragment 1 and fragment 2 PCR product from the first round of PCR, together with 20 pmol of primers p1 and p5, 2 pmol of primer p3 were used. PCR conditions were as for the first-round of PCR, except that the extension time at 72 °C was 2.5 min. PCR product was gel-purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research).

2.9.5. PCR screen for GPG strains

Three sets of primers (set 1: MC5pf/MC5outpr; set 2: MG1pf/MG1pr and set 3: MC0repf/MC0repr) were used for the screen. The primer sequences are listed in Table 2.7. All PCR reactions were performed as described above (section 2.9.1) except for primer set 1 and set 3, a "touchdown" protocol was used (Don et al., 1991); that is, the annealing temperature was 65 °C for cycles 1-3, 60 °C for cycles 4-6 and 55 °C for cycles 7-33.

The PCR products were then run on a 0.8%, 1% or 2% agarose / TBE gel for primer sets 1, 2, or 3, respectively, at 30 V overnight.

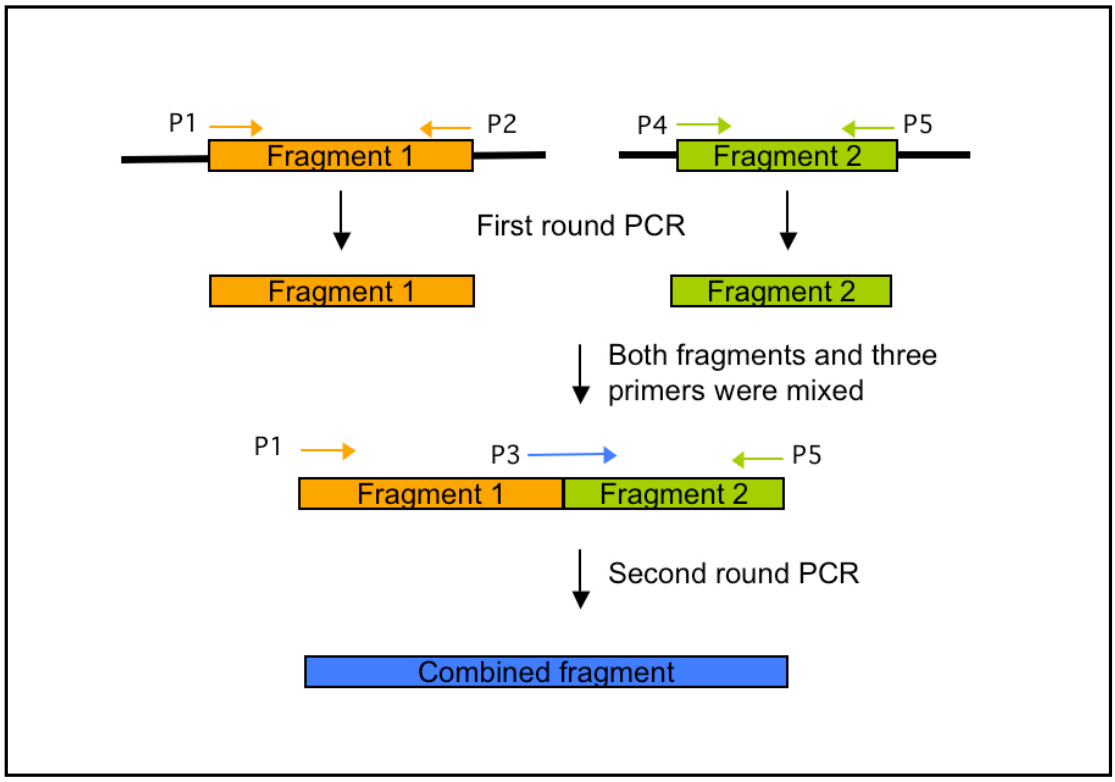


Figure 2.2. Principle of recombinant PCR used to combine two fragments.
An example of using recombinant PCR to combine two fragments from two resources.

2.9.6. Multiplex colony PCR screen for *MTL* heterozygosity

Colonies were checked by multiplex colony PCR for the presence of *MTL***a** and *MTL*α alleles. Colony PCR was performed as described in section 2.9.2, except that four primers (10 pmol each) were added in the 20 µl PCR reaction mixture. Primer combinations used were *MTL*a1-F/*MTL*a1-R and *MTL*α1-F/ *MTL*α1-R (Table 2.7) (Legrand et al., 2004).

2.10. Transformation of *E. coli* and *C. albicans*

2.10.1. *E. coli* transformation

2.10.1.1. Competent cell preparation

Competent *E. coli* cells were prepared according to the protocol of Ausubel et al. (Ausubel et al., 2006). One loop of *E. coli* DH5α cells was used to inoculated 50 ml LB medium in a 500 ml flask and the culture incubated overnight at 37°C with moderate shaking at 150 rpm on a orbital shaker (LAB-LINE). Four ml of this culture was used to inoculate 400 ml LB medium in a 2L flask and incubated, at 37°C, with moderate shaking, to an OD_{590} = 0.375. The culture was dispensed into eight 50 ml sterile polypropylene tubes, which were left on ice for 10 min. After centrifugation at 3000 x g in a Sorvall^{R} RC-5B Refrigerated Super-speed Centrifuge (GSA rotor) at 4°C for 7 min, the supernatant was discarded and each pellet suspended in 10 ml ice-cold $CaCl_2$ solution. The tubes were then centrifuged at 2500 x g (SS34 Rotor) for 5 min at 4°C, the supernatant was discarded, and the pellet resuspended in 10 ml ice-cold $CaCl_2$ solution. The tubes were left on ice for 10 min. After centrifugation at 2500 x g for 5 min at 4°C, the supernatant was discarded, and the pellet resuspended in 2 ml ice-cold $CaCl_2$ solution. The tubes were then left on ice at 4°C for 24 hours. Aliquots of 250 µl of cells were dispensed into pre-chilled sterile eppendorf tubes and stored at -80°C for up to 1 year.

2.10.1.2.Transformation

For each transformation, 100 µl of competent DH5α cells, prepared as described above, or 50 µl of commercial TOP10 competent cells (Invitrogen) was added to an eppendorf tube containing 10 µl of ligation mixture, mixed by pipetting and incubated on ice for 10 min. The solution was then heat-shocked by incubating the tube at 42°C for 2 min, and then put on ice immediately. After 2 min, 1 ml of LB medium was added and the tubes were incubated at 37°C with shaking for 1 h. After incubation, cells were plated on LB plates containing the appropriate antibiotic.

2.10.1.3. Detection of colonies with insert-containing plasmids

LB-Amp plates, with 100 µl of 100 mM IPTG and 20 µl of 50 mg /µl X-gal on the agar surface (section 2.7.2.), were dried for 30 min at 37°C. Portions (100 µl) of transformed cells (section 2.10.1.2.) were spread on the surface of each plate. The plates were then incubated at 37°C for 18 h. The presence of plasmids with inserts was indicated by white colonies growing on plates while blue colonies indicated insert-less (self-ligated or undigested) plasmids. The presence of the correct plasmid in the transformed white colonies was confirmed by amplifying the insert region with primers (M13pf/M13pr, Table 2.7, or other primer sets according to the plasmid constructs) spanning the region, using a colony PCR technique (section 2.9.2.). The size of the products were checked on 0.8 to 2% agarose gels (depending on the predicted size of the products) by loading 8 µl of each PCR product onto the gels. The rest of the PCR products (12 µl) were purified and sequenced if required.

2.10.2. *C. albicans* transformation

C. albicans cells were transformed with 1 to 3 μg of DNA with approximately 2.5 to 5 x 10^7 cells per transformation with the heat shock method and 0.1 to 1 μg DNA with approximately 1.0 to 2.0 x 10^8 cells with electroporation method.

2.10.2.1. Heat shock transformation method

C. albicans cells were transformed using a modification of the method described by Beckerman et al. (2001). Briefly, a single colony from an YPD plate was picked, used to inoculate 2ml of YPD broth in a test tube and grown at 30°C at 150 rpm in a orbital shaker (LAB-LINE). A portion of the overnight culture was used to inoculate 50 ml of YPD broth to an OD_{600} value of 0.05 and this culture was grown under the same conditions for 4 to 4.5 hours until it reached an OD between 0.5 and 1.0. The cells were pelleted in a 50 ml Falcon tube by centrifugation at 3000 x g in a HERAEUS LABOFUGE 400 Centrifuge (Thermo Electron Corporation) for 10 min, resuspended in 5 ml H_2O and re-pelleted by further centrifugation. The pellet was resuspended in 0.5 ml TELiOAc (section 2.5.3) and transferred to an eppendorf tube, re-pelleted by centrifugation at 3000 x g for 2 min using a bench-top centrifuge. Cells were then resuspened in 300 μl TELiOAc.

For transformation, 5μl of a 10 mg/ml stock of salmon sperm DNA (carrier DNA) was added to the transforming DNA (0.5 to 10 μg; cleaned prior to use with a Roche PCR clean up kit). When transforming with a PCR cassette, this was

amplified in five 100 µl reactions, products were pooled, ethanol precipitated and suspended in 40 µl of sterilized distilled water. To 0.1 ml aliquots of TELiOAc-competent cells 10 µl of DNA of interest was added. Following 30 min incubation at room temperature, 0.7 ml of PLATE mix (vortexed before use, refer to section 2.5.3) was added to each tube and mixed by pipetting up and down slowly. The solution was then incubated at room temperature overnight. After this, cells were heat shocked at 42°C for 1 hour, pelletted in a microcentrifuge at 3000 x g for 2 min and resuspended in 200 µl H_2O by gentle pipetting.

For mycophenolic acid (MPA) selection, cells were plated on MPA plates (section 2.5.1.5, with 5 µg/ml MPA) and incubated at 30°C for 3 days. For Nourseothricin (NAT) selection, cells were plated on YPD medium, incubated at 37^{o}C for 7 to 8 hours, and then replica-plated onto NAT plates (section 2.5.1.6, with 250 µg/ml NAT). The NAT plates were then incubated for 3 days at 30 0°C

2.10.2.2. Electroporation method

Transformation of *C. albicans* by electroporation was performed by adapting a method described by De Backer et al. (De Backer MD, 1999) as follows: A single colony of *C. albicans* from an agar plate that had been incubated at 30°C for 24 to 48h was used to inoculate 50 ml YPD broth and this culture was grown overnight at 30ºC with shaking (150 rpm in a orbital shaker) to an OD_{600} of 1.6 to 2.0 (approx. 0.5 to 1.0 x 10^7 cells/ml). Cells were centrifuged in a 50 ml falcon tube at 2,500 x g for 5 min, resuspended in 10 ml of TELiOAc (1M). This mixture was incubated at

30ºC with shaking (150 rpm), for 1h. Then 250 µl of 1M DTT were added and incubation continued at 30ºC for 30 min. Then 40 ml of H_2O was added and cells were spun down as above. Cells were washed in 50 ml of ice-cold water, spun again, washed in 10 ml ice-cold 1M sorbitol, and spun again for 2 min. After removal of all traces of supernatant (the tube was spun once more for 5-10 s to collect any remaining liquid), the pellet was re-suspended in 50 µl 1M sorbitol and kept on ice.

Forty microliters of this cell suspension was used for each transformation. DNA (0.5 to 10 µg; cleaned prior to use with a Roche PCR clean up kit) in a volume of 5 µl water or TE was mixed in gently with a pipette tip and the mixture was transferred to a chilled (4ºC) 0.2 cm electroporation cuvette. Electroporation was performed in a Bio-Rad "MicroPulser™" on *Fungi Sc2* setting. Immediately after the pulse, 1 ml YPD was added to the cuvette. The transformed cells were then placed in a sterile test tube and incubated with shaking for 2-3 h at 37ºC. The transformants were selected as described above in section 2.10.2.1.

2.11. Selection of model strains

The model strains were selected using a modification of the greedy best-total-coverage (greedy-BTC) method (Holland and Schmid, 2005). In order to select the most typical GPG or non-GPG strains from a collection of 266 strains which consisted of 98 putative GPG strains and 166 putative non-GPG strains (Schmid et al., 1999), a neighbour-joining tree was constructed for every possible quartet consisting of 2 putative GPG strains and 2 putative non-GPG strains. For each strain

the proportion of quartet-trees (which included that strain) that separated the two GPG strains from the two non-GPG strains was recorded. From among strains for which ≥ 70% of quartets united them with another member of their group a representative set of five was chosen using the method of Holland and Schmid (Holland and Schmid, 2005). To pick strains that were definitely members of their group (GPG or non-GPG, hence the quartet score threshold), from a range of countries, and fairly representative of the diversity of strains (hence the requirement that they be good in terms of greedy best total coverage score), many random sets of strains with quartet proportions above 0.68 were generated, and only those strain sets that were within 0.25 of the coverage score of the greedy-BTC score were kept. From these sets of strains the sets with best coverage score (13.94 for GPG set and 14.45 for nonGPG set) which included strains from different countries were selected, these are listed in Table 3.1.

2.12. Mating between *C. albicans* strains

2.12.1. Mating procedure between clinical isolates

The mating protocol was a modification of the plate mating method of Legrand and coworkers (Bennett et al., 2005). In brief, both parents were grown as broad bands across YPD plates at room temperature overnight, and replica-plated at right angles onto a single YPD or spider medium (Legrand et al., 2004) plate (Fig. 6.1). The cross-replicated plate was incubated at room temperature for seven days, at which time the potential mating products on the crossing area were ready to be selected.

2.12.2. Selection of mating products using MPA and NAT

The mating plate was replica-plated onto a YPD plus NAT (200 µg/ml) plate (NAT plate). After incubation at 30°C for 2 days, all cells from areas where mating could have occurred (the intersections of the bands of cells) were scraped off the plate with a loop, suspended in 1 ml water, and 100 µl aliquots of this suspension were plated on 10 minimal medium plates containing MPA (5 µg/ml) (MPA plate) and incubated at 30°C for 5 days. Large colonies were picked and patch-plated (from each single colony) on both MPA and NAT plates again. After incubation at 30°C for 2 days, the patches that grew on both NAT and MPA plates were tested by PCR for the presence of both *MTL* alleles. If both *MTL* alleles could be amplified, cells from the patches on the NAT plate were streaked on YPD and the PCR assay was repeated for single colonies. These precautions were necessary because MPA selection has a high false-positive background (section 5.2). Only single colonies from the final YPD plates containing both *MTL* alleles were considered as true recombinants. Clones thus verified as recombinants were stored at -80°C. In some cases PCR analysis to detect the MPA^r and NAT^r markers using primer sets TS2Fpr/pENOpr and pACTFpf/CaNATpr, respectively, was used to confirm recombinants.

2.12.3. Mating of clinical isolates with universal mating tester strains

Mating of clinical isolates with universal mating tester strains was performed according to a mating method developed by Legrand et al. (Legrand et al., 2004):

The *MTL***a** derivatives of unmarked clinical isolates were mated with the universal mating tester strain 3685 (*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL*α) and the *MTL*α derivatives were mated with the tester strain 3710 (*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL***a**) as described above and recombinants were selected as prototrophic MPA-resistant colonies on MPA plates (5 μg/ml) as described by Magee et al. (2002).

2.13. Microscopy

2.13.1. Fluorescence microscopy of DAPI-stained nuclei

Cells from patches grown on YPD plates at 37°C overnight were fixed in 70% ethanol for at least 1 h at 4°C and stained with DAPI (4', 6'-diamidino-2-phenylindole) dissolved in water (1 μg/ml) for at least 5 min. Fluorescence microscopy was performed in the Manawatu Microscopy and Imaging Centre (MMIC). Cells were visualized using a DFC320 camera and a FM Olympus BX-51 microscope using a U-MWU2 Ultraviolet excitation (wideband) filter set (excitation 330-385 nm, emission 420 nm, dichromatic mirror cut-off 400 nm) with 100 X Magna File Objective.

2.14. Fluorescence Activated Cell Sorting (FACS) analysis of DNA content

2.14.1. FACS analysis of recombinants and their parents

Cells from glycerol stocks were patch plated on YPD agar and, after overnight incubation at 30°C, the patch was used to inoculate liquid YPD medium to an initial OD_{600} of approximately 0.2. This culture was incubated at 30°C with shaking (150 rpm) for 4 to 5 h and cells were harvested when the OD_{600} was between 1 and 2. The cells were then fixed in 70% ethanol at 4°C for 1h to up to 4 days. Then the cells were collected by centrifugation at 13,000 x g in a tabletop centrifuge at room temperature for 5 min. The supernatant was carefully removed with a vacuum aspirator, leaving 5-10 μl to avoid accidentally loss of the pellet. Upon collection, cells were washed 2 times with 750 μl of 5 x TE (pH 8.0) to remove trace amounts of ethanol, which can inhibit the subsequent RNase A treatment. During the second wash cells were sonicated using a Branson 2510 Sonicator (Bransonic) for 5 min (40kHz). The pellet was resuspended in 500 μl RNase A solution and incubated for 2 hr at 37^{o}C. Cells were then pelleted again and the RNase A solution was removed by aspiration. The pellet was resuspended in 250 μl pepsin solution (5 mg/ml) and incubated at 37° for 60 min. Cells were pelleted again and the pepsin solution removed. Cells were then washed with 750 μl 50 mM TrisCl pH 7.5, 5 mM EDTA, pelleted and resuspended in 50-250 μl TrisCl pH 7.5, 5 mM EDTA. A sample of 50 μl cell suspension was then placed in a eppendorf tube containing 0.5 ml 1 μM

Sytox in 50 mM TrisCl pH 7.5, 5 mM EDTA and incubated at 4° overnight in the dark.

FACS analysis was performed on a BD FACSCalibur (BD Biosciences), using an excitation wavelength of 488 nm (15 mW argon-ion laser). Emission from Sytox green was measured using a 530/30 nm band pass filter. The sample was collected at a rate of 12 µl/min equating to a rate of >500 events per second. A minimum of 50,000 events were collected.

2.14.2. Calculation of DNA content of recombinants and their parents

DNA content of the recombinants relative to their parents were calculated as average of the median value of M1 (of the recombinant) divided by average of median value of M1 of parent1 and parent 2, and the median value of M3 (of the recombinant) divided by average of median value of M3 of parent1 and parent 2 (Refer to Fig. 7.6 and Appendix Table 10.2.).

2.15. Statistical analysis

2.15.1. General statistical analysis

The two-tailed binomial tests were to determine if two categories are equally likely to occur by chance. The null hypothesis states that the observed frequency in the sample is identical to a predetermined frequency. The two-tailed paired or unpaired Student's t test (equal variance) was used to determine if the means of two groups are statistically different from each other. The 95% confidence intervals for the median were obtained from http://www.math.unb.ca/~knight/utility/MedInt95.htm. Box and Whisker plots were generated using a template obtained from http://www.vertex42.com/ExcelTemplates/box-whisker-plot.html.

2.15.2. Randomization procedure for comparing fitness of parents and recombinants

To assess the overall fitness impact of mating, I wanted to utilize the growth rates of multiple recombinants arising from the same pair of parents where possible. To do so a randomization test was developed (by my supervisor Barbara R. Holland) to preserve the structure of the non-independence between multiple recombinants from the same successful mating. The test statistic was the sum, for all recombinants, of the difference between their fitness and the average fitness of their parents. The test statistic D was defined as the sum for all 34 recombinants (representative recombinants from all successful matings, up to 3 per mating, less if fewer

recombinants had been recovered) of the fitness of the recombinant minus the average fitness of its two parents. The null hypothesis was that there is no difference between the fitness of the recombinants and their parents (D=0). The two-sided alternative hypothesis that there was a difference ($D \neq 0$) was tested. To do so, the 43 fitness values (34 recombinants plus nine parents which produced the recombinants) were randomized 1000 times amongst both the parents and the recombinants and it was determined how often the D value of randomized sets was higher than the actual D value.

2.16. Growth simulations

2.16.1. Simulation of the number of offspring from a single cell, or pairs of cells, engaging in mating relative to the number of offspring from clonally reproducing cells

To measure the direct impact of recombination I simulated the number of offspring produced by each recombinant and compared this to the number of offspring produced by one or both of its homozygous parents if they continued clonal reproduction. For each recombination, up to three recombinants were tested, less if fewer than three recombinants had been obtained. I determined the growth rates of *MTL*-homozygous parents after they had been marked with resistance cassettes and again after they have been transferred for 100 generations in YPD medium (section 2.7.1.5.). The growth rates of recombinants were also determined initially and after 100 generations. Growth rates of recombinants were generally higher after 100

generations and, for the simulations, it was assumed that this increase was linear (total increase/(No. of hours taken for 100 generations of each recombinant) per hour). There was no significant increase in parents' growth rates and their growth rate was set as the average of initial rates and rates after 100 generations. The timescale is given in hours, which is equivalent to the time it takes the recombinant to double 100 times (i.e. to reach a cell number of 1.26765×10^{30}). For single (Fig. 8.7) or pairs (Fig. 8.8.) of cells (strains) engaging in recombination the number of their offspring was expressed as a fraction of offspring produced by the recombinants relative to offspring produced by continuous clonal propagation of each (Fig. 8.7.) or the sum (Fig. 8.8.) of drug resistance marked sorbose-derived *MTL* homozygous parent(s). The ratio of the growth rates of recombinants to the growth rates of individual (Fig. 8.7.) or the sum of pairs (Fig. 8.8.) of parent strains was calculated, assuming mating happened at time 0, i.e. mating happened in the first generation.

The mating process, during which only one new cell is produced from two parents, was assumed to take 1 generation (about 1.1 h).

2.16.2. Simulation of the number of offspring from pairs of cells losing *MTL* heterozygosity and engaging in mating relative to the number of offspring from clonally reproducing cells

The simulation was based on empirically determined growth rates measured as described in section 2.7.1.6. For the first part of the simulation I calculated the hourly increase in the number of a pair of *MTL*-homozygotes for 30 h, which is a

close approximation of growth over 30 generations; the time it would take individual homozygotes to divide 30 times ranged from 27 -34h. I then calculated, in hourly intervals, the number of offspring that the pair of their *MTL*-heterozygous ancestors would have produced by clonal reproduction and the ratio of *MTL*-homozygotes/*MTL*-heterozygous ancestors. For the simulations shown in Fig 8.15, growth rates (determined after 30 generations of serial propagation) of *MTL*-homozygous cells without resistance cassettes were used in the calculations, for Fig. 8.17. growth rates of *MTL*-homozygotes transformed with resistance cassettes (the times required for 30 doublings of individual homozygotes ranged from 28 - 48h; the higher doubling times were those of W17 transformants) were used.

I simulated the mating process as being part of the 30^{th} doubling so that after that doubling recombinants were present at a number equal to those of the faster growing *MTL*-homozygote. To generate Fig. 8.17, I then calculated how long it would take these recombinants to double in numbers 100 times, based on the actual empirically determined growth rates of recombinants, determined at t_0 and after 100 generations of serial propagation. If the growth rates were higher after 100 generations than at t_0, I assumed that growth rates increased at a constant percentage each generation to increase from the rates measured at t_0 to the rates measured after serial propagation for 100 generations. If the rates were lower after 100 generations than at t_0, I used the average growth rates of the two time points. Using these growth rates I calculated the number of recombinants and *MTL*-heterozygotes present after each hour until recombinants had doubled in number 100 times. The time it took to double 100 times differed somewhat between individual homozygotes (range 89–122 h).

In Fig. 8.15, I followed the same procedure, but instead of using the actual growth rates of recombinants, the growth rate of recombinants at a given time (t_n) was calculated as:

growth rate of marked recombinant at t_n x (average of growth rates of both marked parents at t_0 and t_{100})$^{-1}$ x average growth rate of parental homozygotes w/o resistance cassettes

The estimated time it took individual recombinants to undertake 100 doublings based on these modified rates ranged from 69-112 h; the lower times were those of W17 recombinants.

Because, as explained above, the time it took for the entire simulated 130 generations varied between pairs of isolates, I scaled the x axes of individual data sets so that all graphs were of the same length, representing 130 generations. I did this by pasting all individual graphs (generated in MSExcel) into MSPowerpoint.

2.16.3. Simulation of the number of offspring from a single cell losing *MTL*-heterozygosity and engaging in mating, relative to the number of offspring from a clonally reproducing cell

For a cell engaging in recombination, the number of its offspring was expressed as a fraction of the offspring produced by continuous clonal propagation of the *MTL*-heterozygous clinical isolate from which it had been derived. The simulation was

the same as described above (section 2.16.2) except that the progeny from one cell losing *MTL* heterozygosity for 30 generations (approximately 30 hours) and engaging in sex was compared hourly to the progeny of each of the heterozygous parents it was derived from instead of comparing it to the progeny of a pair of parents. For the simulation shown in Fig. 8.14, the impact of loss of one *MTL* allele on the growth rate for a given strain was based on the growth rate of a *MTL*-homozygous derivative without the resistance cassette (determined after 30 generations of serial propagation), for Fig. 8.16 growth rates of *MTL* homozygotes transformed with resistance cassettes were used. In Fig. 8.14 instead of using the actual growth rates of recombinants, the growth rate of recombinants at a given time (t_n) was calculated as:

growth rate of marked recombinant at t_n x (average of growth rates of the marked parent at t_0 and t_{100})$^{-1}$ x growth rate of the parental homozygote w/o resistance cassettes

3.0. SELECTING AND GENERATING PARENT STRAINS FOR MATING

3.1. Introduction

The main objective of this research was to determine if sex still generates recombinant lineages that are viable in *C. albicans'* natural environment, by mating a set of *C. albicans* clinical isolates and testing recombinants' fitness during their adaptation to a novel environment, to test directly whether engaging in sex enhances *C. albicans*'s chances of passing on its genes to future generations. Since the majority of laboratory mating experiments reported have involved derivatives of one laboratory strain (as described in section 1.3.3), a possible explanation for the fact that mating has never been observed in nature is that in the human host mating is restricted because clinical isolates are often incompatible with each other. Possibly many combinations cannot mate. The significance of sex in *C. albicans* should be best demonstrated by mating a wide variety of genetically different wild-type strains. Because different members of the *C. albicans* species could differ in their reproductive strategy, mating attempts between GPG and GPG, non-GPG and non-GPG and mating attempts between GPG and non-GPG strains may have different outcomes. It is believed that general purpose genotypes (GPG) can only exist without sex, because the co-adapted combination of genes in GPG strains would be broken up by sex, i.e. GPG strains must protect themselves by at least not mating with other groups of strains (Forbes et al., 1997; Lynch, 1984). Also, the population structure of GPG (clade 1) strains appears to be more clonal than that of the remainder of the species as described in section 1.1.3 (Forbes et al., 1997; Holland et al., 2002; Lynch, 1984; Schmid et al., 2004). Indeed, GPG strains may have barriers against mating, in particular with other strains, so that either mating could not happen, or the lineages could be non-viable.

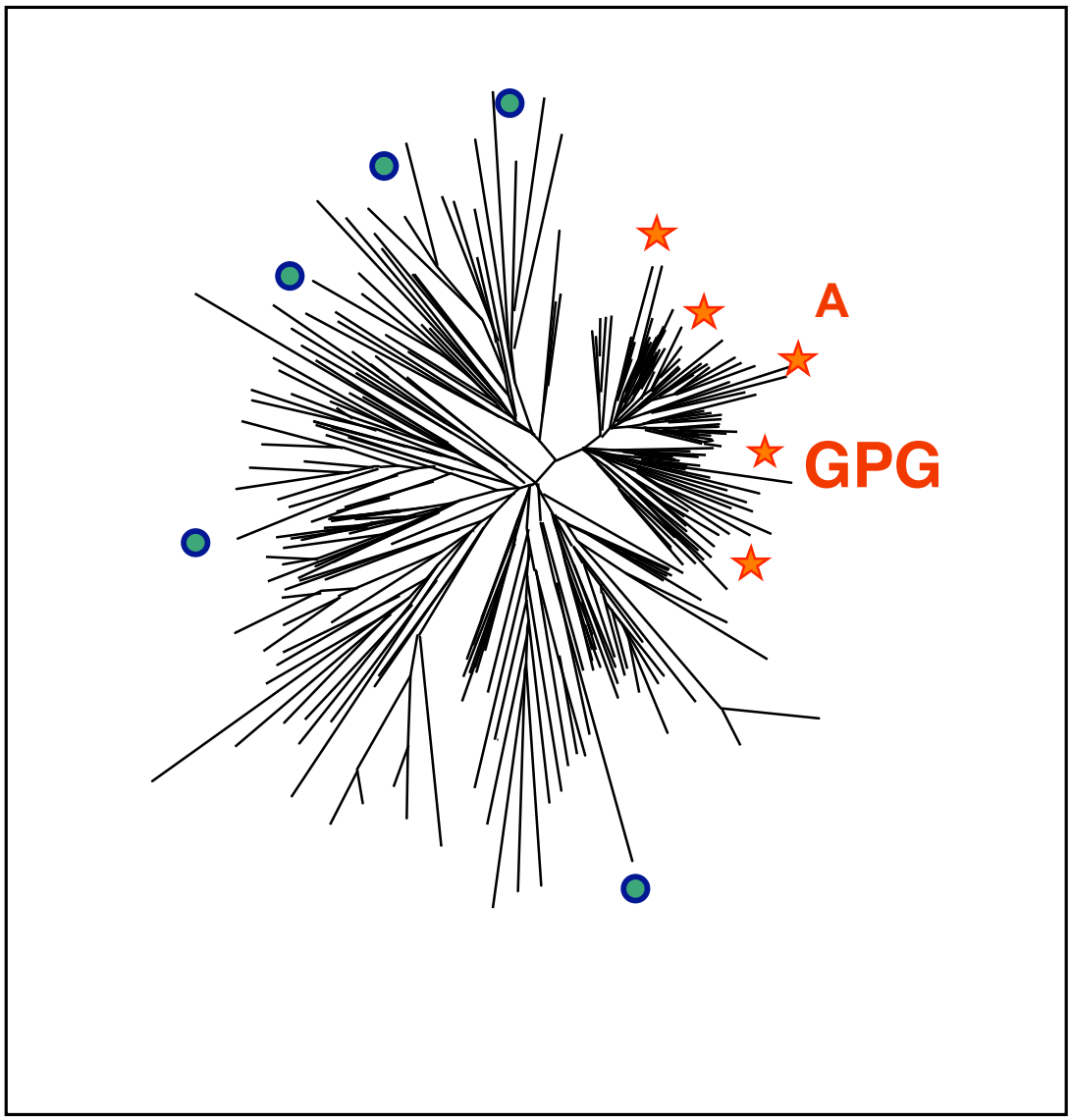


Figure 3.1. Neighbour joining tree of 266 infection-causing isolates of *C. albicans*, collected from a variety of types of infections from 11 locations worldwide, between 1984-1991 (Schmid et al., 1999).

Group A strains are equivalent to the GPG type (Schmid et al., 1999; modified). 5 GPG strains chosen for this project are marked with stars and 5 other strains with circles. Note: The symbols do not indicate any particular branches.

I therefore decided to select for my mating experiments 5 GPG strains and 5 other strains, each set chosen to optimally represent its part of the species in a global clinical isolate collection (Fig. 3.1) as the model strains for this study (Schmid et al., 1999), and to mate their *MTL***a** and *MTL*α derivatives in all 25 possible combinations. This design would make it possible to test if mating can happen within GPG or non-GPG groups as well between these two groups.

In addition, to be suitable parental strains, the ideal model strains chosen had to be (i) sensitive to mycophenolic acid (MPA) and nourseothricin (NAT), as they have to be marked with the two drug resistance genes; and (ii) able to lose either of the mating type-like loci (*MTL*) by sorbose selection (Magee and Magee, 2000) to become mating competent strains, as most of the clinical isolates of *C. albicans* are *MTL* heterozygotes and not capable of mating.

This section describes how the model strains were selected and manipulated.

3.2. Model strain selection and verification

Five GPG strains and five non-GPG strains (Table 3.1) were chosen with the help of my supervisor Barbara Holland from a collection of 266 infection causing isolates from 12 geographical regions in 6 countries which consisted of 98 putative GPG strains and 166 putative non-GPG strains (Schmid et al., 1999). None of them had been extensively subcultured after their isolation from patients and were revived from glycerol stocks for these experiments. The most representatives strains were selected using a modification of the greedy best-total-coverage (greedy-BTC)

method as described in method section 2.11. (Holland and Schmid 2005) as instead of just wanting to maximise coverage I also wanted to ensure that the strains selected came from a range of countries, and that they definitely belonged to either the GPG or non-GPG groups, i.e. they weren't marginal strains that could belong to either group. The latter was assessed by using a quartet score (Schmid et al., 1999), which is the frequency with which a strain is closest to another strain of its group in quartets containing one other strain from the same group and two strains from the other groups. Only strains with a score of ≥ 0.68 were considered for inclusion in this work (Table 3.1).

The two sets chosen were very close to optimal with a representation score within the top 10% achievable for a set of five isolates when no restrictions regarding country of origin were imposed.

To confirm if the model strains were typical GPG or non-GPG strains, PCR screens amplifying GPG specific polymorphisms (*PNG*2, *ALS*7 and MG1) were performed using three sets of primers (Table 2.7; MC0repf/MC0repr for *PNG*2 (Zhang et al., 2010), MC5pf/MC5outpr for *ALS*7 (Zhang et al., 2003) and MG1pf/MG1pr for MG1 (Zhang et al., 2009)), as described in method section 2.9.5. All of the GPG strains had the typical GPG band patterns, while non-GPG strains had other band patterns (Fig. 3.2).

Table 3.1. Model strains selection and verification

Strain name	Ca3 type*	Country of origin	Quartet score**	Clinical origin
AU7	GPG	New Zealand	0.830	Urine
AU90	GPG	New Zealand	0.867	Skin wound
HUN97	GPG	Great Britain	0.799	Bloodstream
OD8916	GPG	Great Britain	0.859	Oral
W43	GPG	New Zealand	0.863	Oral
AU35	non-GPG	New Zealand	0.744	Sputum
FJ11	non-GPG	Fiji	0.708	Throat
RIHO11	non-GPG	United States	0.681	Bloodstream
W17	non-GPG	New Zealand	0.829	Throat
YSU63	non-GPG	Malaysia	0.905	Urine

* Based on Ca3 fingerprinting (Schmid et al., 1999); GPG = general purpose genotype, equivalent to major group A which is subdivided in to subgroups A1 and A2 (Schmid et al., 1999). All of the strains were infection causing isolates. Strains were further verified if they were typical GPG or non-GPG strains by PCR using GPG specific primers MG1pf / MG1pr, MC0repf / MC0repr, MC5pf / MC5outpr (Table 2.7).

** Quartet score: shows how representative the isolates are of GPG or non-GPG groups (Schmid et al., 1999).

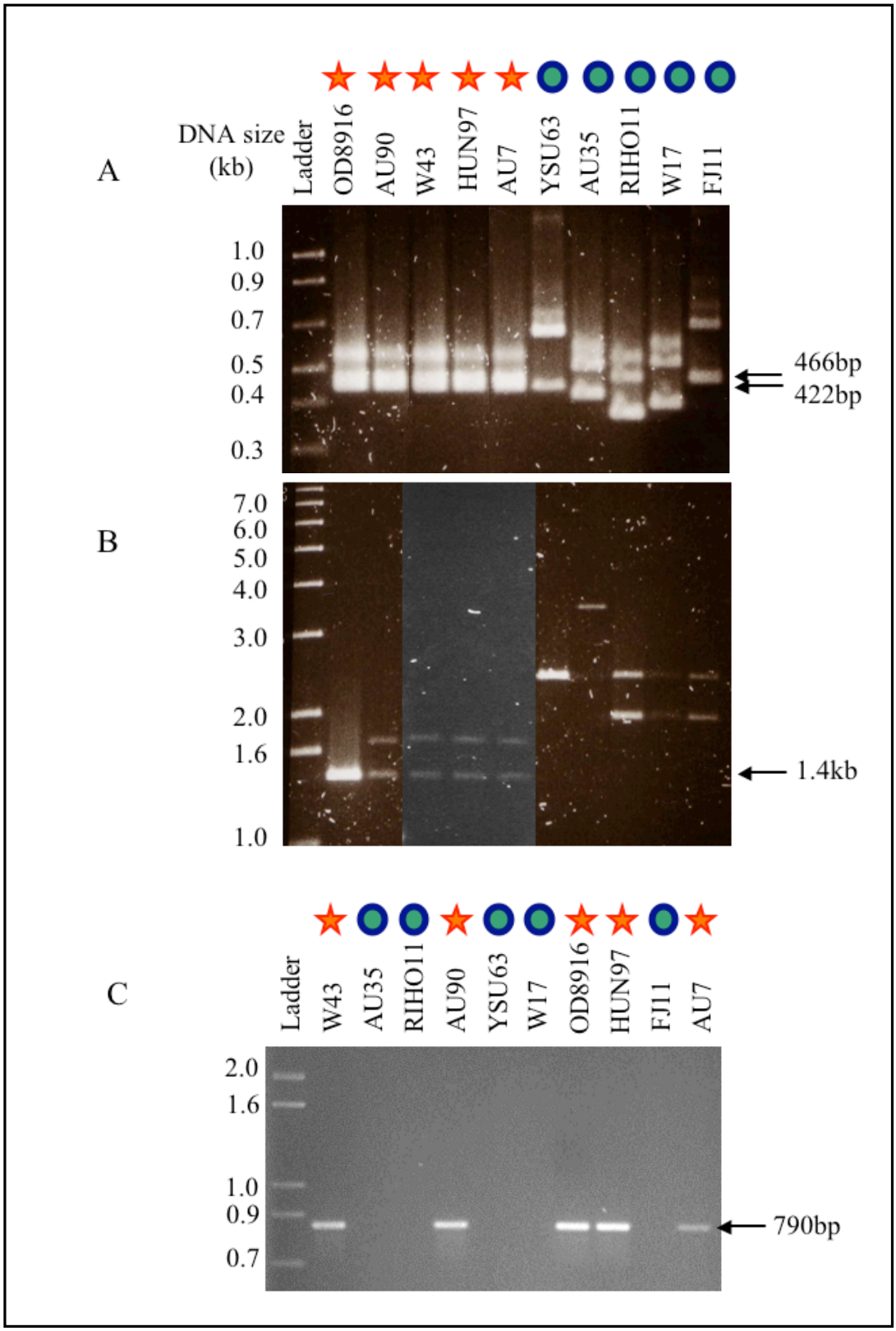


Figure 3.2. PCR screen for verification of GPG or non-GPG strains.

(A) *PNG2* screen. (B) *ALS7* screen (combine from two gels). (C) *MG1* screen. Names of the strains are shown above lanes. GPG strains are marked with stars, non-GPG strains with circles. Ladder: 1kb plus ladder; molecular weights (MW) are shown on the left. Arrows on the right mark the typical GPG products and their molecular weights.

3.3. Model strains were all susceptible to MPA and NAT

In order to select recombinants arising from mating between clinical strains, the parental strains needed to be marked with either MPA or NAT resistance genes. The suitability of each strain for mating experiments was thus tested to see if they were sensitive to MPA or NAT. Three concentrations of MPA (2 μg/ml, 5 μg/ml and 10 μg/ml) or NAT (100 μg/ml, 250 μg/ml and 400 μg/ml) were tested as described in Method section 2.7.1.2. The model strains were all sensitive to 2 μg/ml MPA or 100 μg/ml NAT (data not shown).

3.4. All model strains were *MTL*-heterozygotes

Only strains homozygous for the mating type-like locus (*MTL*) can mate (Tsong et al., 2003). I therefore tested each model strain for the presence of these alleles. All of the model strains were checked by colony PCR to see if they were *MTL* heterozygotes (section 2.9.2.) using primers MTLa1-F/MTLapr and MTLα1-F/ MTLα1-R. All strains had both the *MTL***a** and the *MTL*α locus (Fig. 3.3), as is typical for clinical isolates (Lockhart et al., 2002).

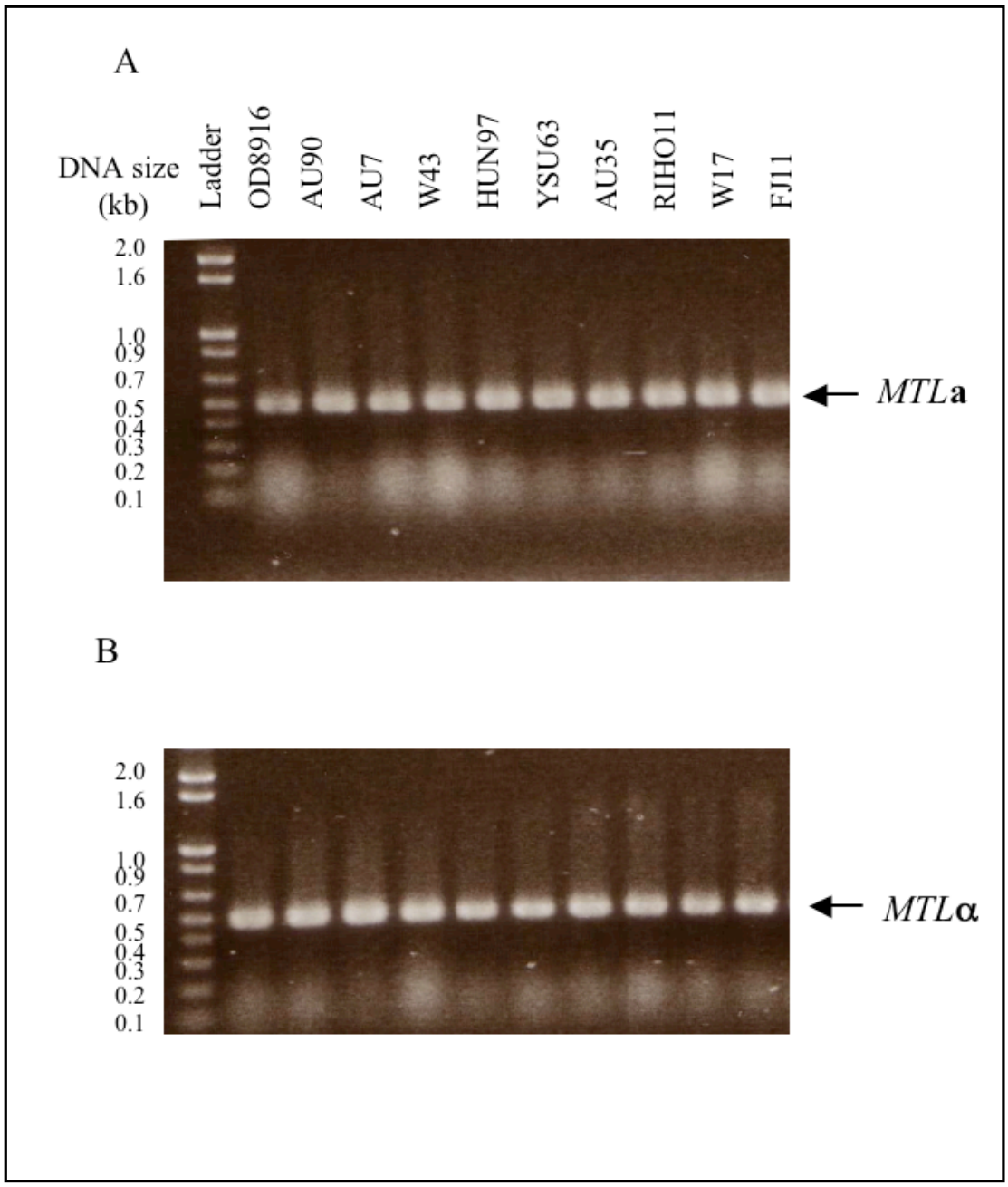


Figure 3.3. PCR screen of *MTL* alleles for the model strains (refer to Table 3.1).

All isolates had both the *MTL***a** (A) and the *MTL*α (B) locus. Names of the strains are shown above lanes**.** Ladder: 1kb plus ladder; DNA sizes are shown on the left. Arrows on the right mark the *MTL***a** and *MTL*α alleles. The primers used for the PCR screen were: MTLa1-F/MTLa1pr for *MTL***a** and MTLα1-F/ MTLα1-R for *MTL*α. Primer sequences are listed in Table 2.7.

3.5. Sorbose selection to generate *MTL* homozygotes for mating

I next assessed if the model strains could be induced to lose one *MTL* allele by losing one copy of chromosome 5, which contains the *MTL* locus, through sorbose selection - a method commonly applied to generate *MTL***a** and *MTL*α strains that are competent for mating (Magee and Magee, 2000). Each strain was plated on sorbose-containing medium and sorbose-derived single colonies were purified on YPD medium and checked by PCR for the presence of both *MTL* alleles, as described in Method section 2.7.1.3. & 2.9.5. As shown in Table 3.2, all strains could be induced to lose one mating type allele, however, it was evident that only one GPG strain (W43) and one non-GPG strain (FJ11) frequently lost either mating type locus. The remaining eight strains readily lost one mating type locus, but rarely the other (Table 3.2). Rather than trying to find both **a**/**a** and α/α derivatives for each of these strains, I was able to generate a set of 5 GPG strains and 5 non-GPG strains, with half of them being *MTL***a** (3 GPG plus 2 non-GPG strains) and half of them *MTL*α homozygotes (2 GPG plus 3 non-GPG strains). This set allowed 25 matings (Fig. 3.4) without having to find rare derivatives of strains. For strains for which I obtained both *MTL* homologues, the more common mating type was usually chosen for mating experiments. The exception was W43. Even though W43 *MTL*α derivatives arose three times more frequently than *MTL***a** derivatives I chose the *MTL***a** derivative in order to have five strains each of *MTL***a** and *MTL*α for mating (Table 3.1, Table 3.2).

Table 3.2. *MTL* status of colonies recovered from sorbose plates

Strain	Percent *MTL***a**/MTLα (number of colonies)*	Percent *MTL***a** (number of colonies)*	Percent *MTL*α (number of colonies)*
AU7	94 (45)	6 (3)	0 (0)
AU90	25 (6)	75 (18)	0 (0)
HUN97	50 (6)	0 (0)	50 (6)
OD8916	79 (19)	0 (0)	21 (5)
W43	4 (1)	25 (6)	71 (17)
AU35	71 (17)	29 (7)	0 (0)
FJ11	4 (1)	17 (4)	79 (19)
RIHO11	96 (23)	4 (1)	0 (0)
W17	42 (10)	0 (0)	58 (14)
YSU63	25 (6)	0 (0)	75 (18)

* determined by colony PCR as described in section 2.9.2.

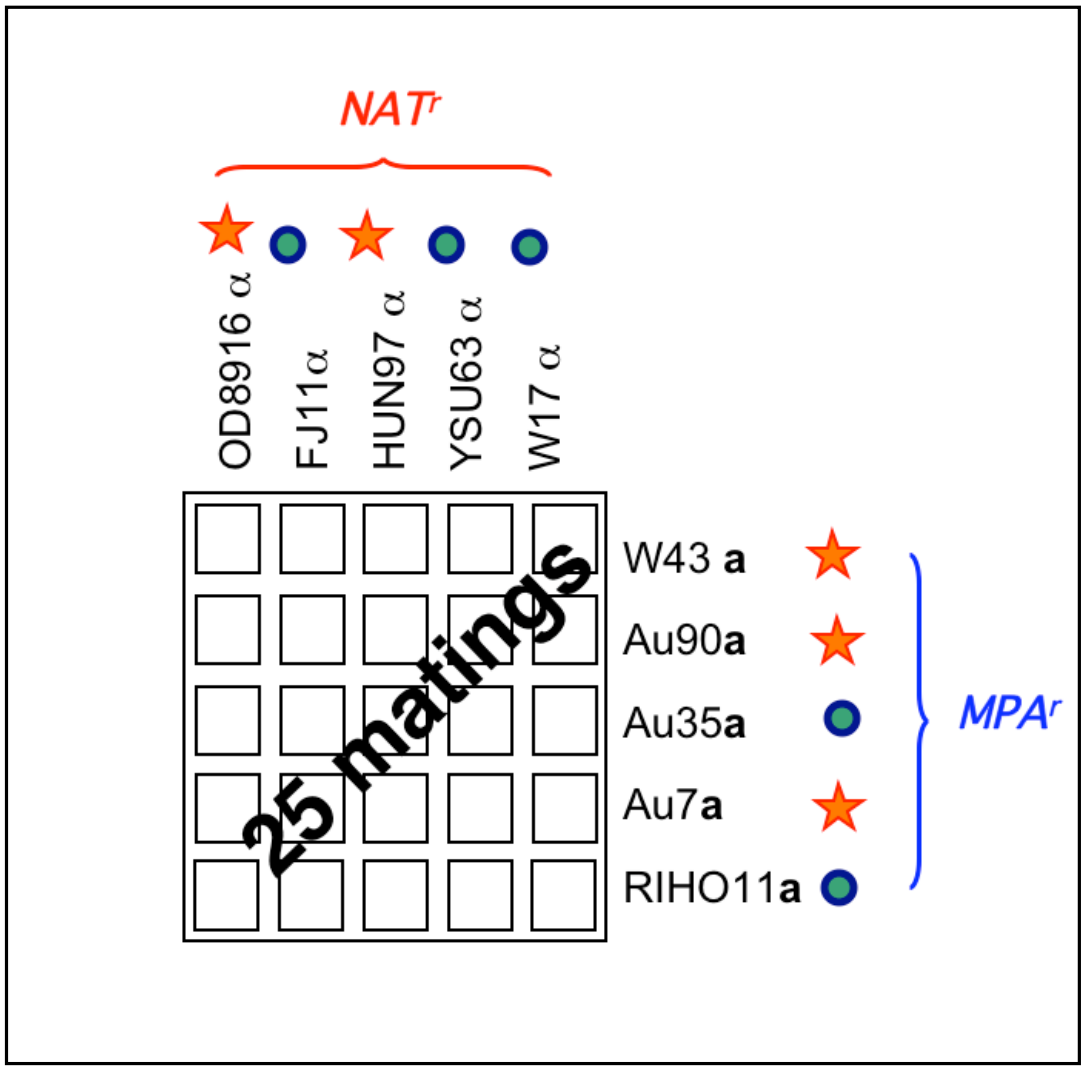


Figure 3.4. Matrix of 25 possible matings.

Five *MTL* α strains are shown in the top row, the *MTL***a** strains are on the right. GPG strains are labeled with stars non-GPG strains with circles. All of the *MTL***a** strains would be marked with the MPA resistance cassette and all of the *MTL*α strains would be marked with the NAT resistance cassette as described in section 5.2. & 5.3.

4.0. DEVELOPMENT OF MARKER CASSETTES

4.1. Introduction

As described in Introduction 1.4, to be able to efficiently select recombinants arising from the mating of clinical isolates, the parents need to be modified with selectable markers. The mating strategy used was to transform each of two parent strains with one of the two drug resistance markers currently available for *C. albicans*: an MPA resistance-conferring allele of the *C. albicans IMH3* gene (*MPA*^{r}) (Beckerman et al., 2001), or the *CaNAT* nourseothricin resistance gene (*NAT*^{r}) (Shen et al., 2005). After mating, recombinants would be selected on the basis of their resistance to both drugs (Fig. 4.1). All of the *MTL***a** strains would be marked with the *MPA*^{r} cassette and all of the *MTL*α strains would be marked with the *NAT*^{r} cassette.

Because one aspect of this project is to compare the fitness of recombinants with that of the original clinical isolates, resistance gene cassettes were designed so that their integration would not disrupt any known native genes in the parent strains. This chapter describes the construction of two resistance cassettes. A *CaNAT* resistance cassette, in pNZ11, was designed to integrate through single crossover of the intact plasmid into the *ACT1* promoter region of chromosome 1. This integration/promoter duplication event would place the *CaNAT* gene under the control of the *ACT1* promoter (Shen et al., 2005) without disrupting the native *ACT1* gene. An MPA resistance-conferring cassette, in pNZ4, was designed so that it would integrate, through double crossover, in a non-coding region of chromosome 7, as described below (Fig. 5.2, Fig. 5.4).

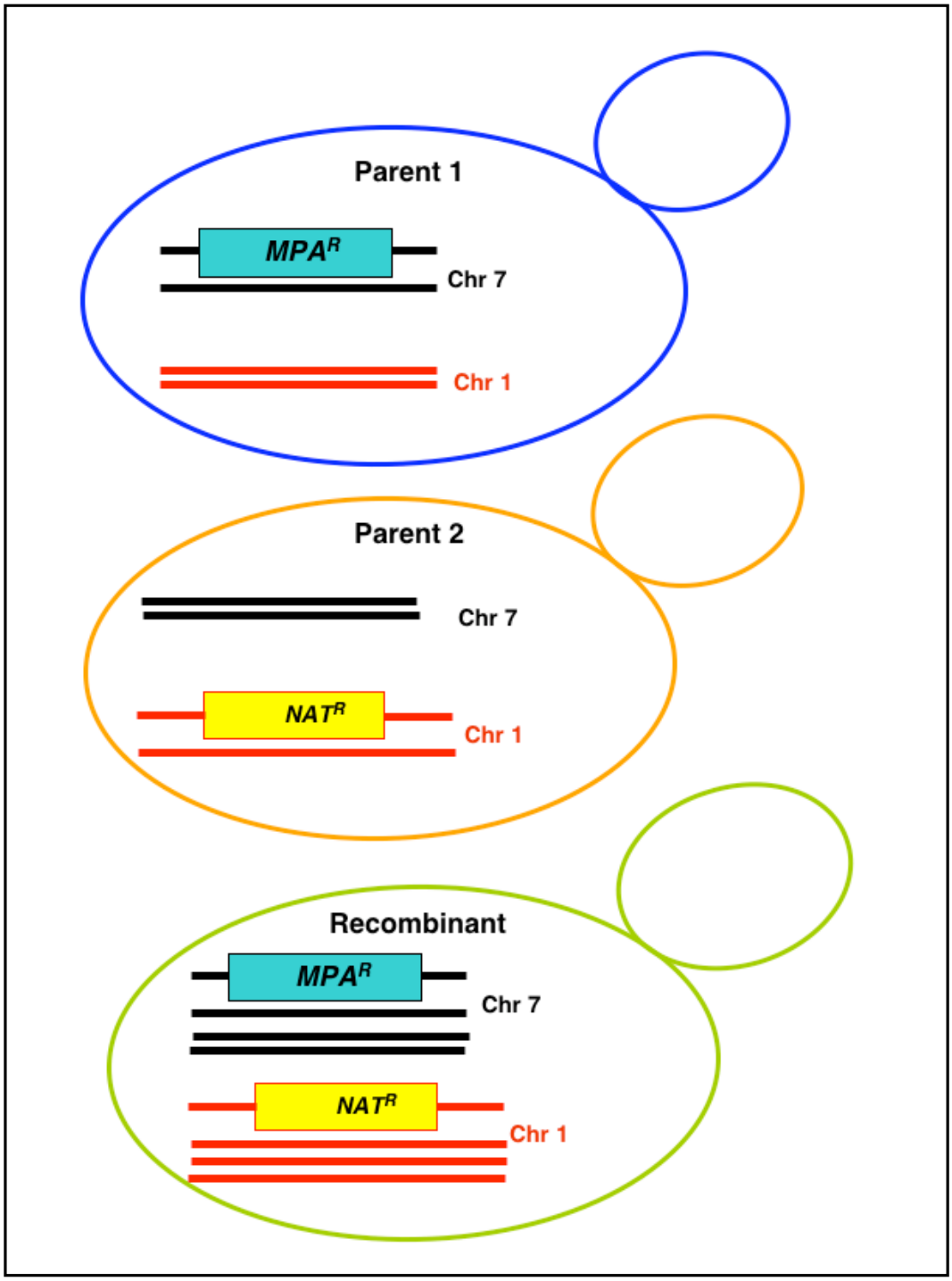


Figure 4.1. Principle of the two-marker system for marking parental strains and the generation of recombinant strains.

Parent 1 is made MPA-resistant by integrative transformation with a MPA resistance cassette (*MPA*^{r}) into chromosome 7 (chr7). Parent 2 is made NAT-resistant by integrative transformation with a NAT resistance cassette (*NAT*^{r}) into chromosome 1 (chr1) and recombinants can be selected on the basis of resistance to both drugs.

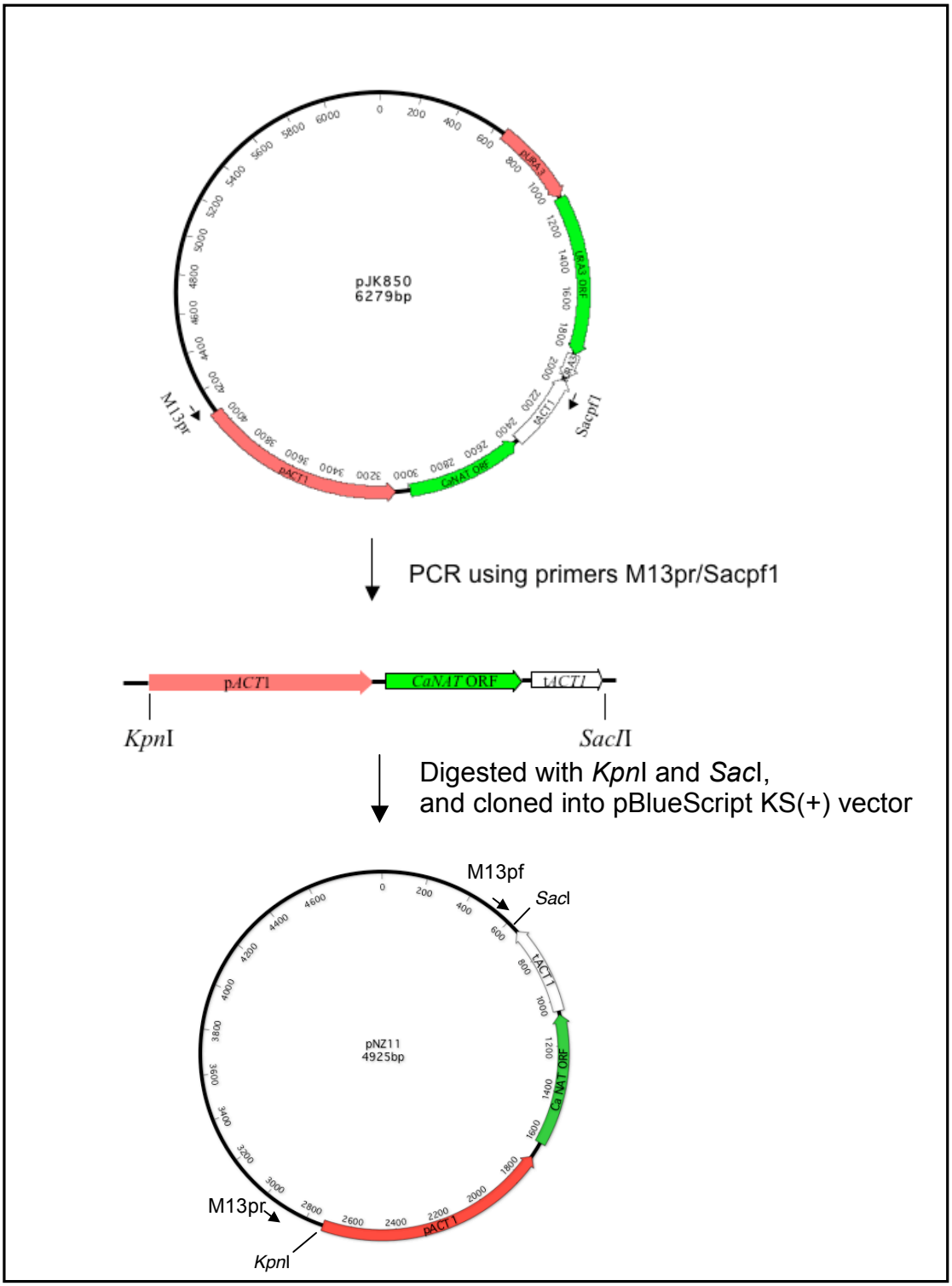


Figure 4.2. Construction of NAT resistance cassette-containing plasmid pNZ11.

Plasmid pNZ11 was constructed from plasmid pJK850, containing a *URA3* ORF with a *URA3* promoter (pURA3) and a pCaACT1+CaNAT+tCaACT1 (a NAT resistance gene (Shen et al., 2005) created for *C. albicans* under the control of a *C. albicans ACT1* promoter plus an *ACT1* terminator) cassette, in a pBSKS(+) backbone. The pCaACT1+CaNAT+tCaACT1 cassette was amplified using primers M13pr/Sacpf1, digested with *Kpn*I and *Sac*I, and cloned into the multi-cloning site of a *Kpn*I/*Sac*I-digested pBlueScript KS(+) vector. Locations of primers (arrows) and restriction sites used in cloning are labeled. A plasmid map of the backbone pBlueScript II KS (+) (pBSKS(+)) is shown in Appendix 10.2.

4.2. Construction of a NAT resistance cassette

The NAT-resistance vector pNZ11 was designed and constructed as follows (Fig 4.2.): The cassette containing a *CaNAT*^{r} (*C. albicans* nourseothricin resistance gene, Shen, 2005) flanked by a *CaACT1* (*C. albicans ACT*1 ORF) promoter and a *CaACT1* terminator (p*CaACT1*+*CaNAT*+t*CaACT1*) was amplified from plasmid pJK850 (Table 2.5, Appendix 10.1.), using KOD polymerase with primers M13pr / Sacpf1 (containing a *Sac*I cutting site in this primer), as described in section 2.9.3. The fragment was purified using PCR clean up kit (section 2.8.4.) and then double digested with restriction enzymes *Kpn*I and *Sac*I, as described in section 2.8.6. The cassette was then purified from 8% TAE agarose gel (section 2.8.3.) and inserted into K*pn*I/*Sac*I double-digested vector pBlueScript KS(+) multiple cloning site (MCS, Appendix 10.2.) by ligation (section 2.8.8.). The ligation mixture was then used to transform *E.coli* DH5α to ampicillin resistance (section 2.10.1.2.). If the ligation was successful, the transformed *E.coli* cells should form white colonies in the presence of X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside). White colonies were screened by colony PCR (section 2.9.2.) using primers M13pf / M13pr for the correct insertion of the cassette (p*CaACT1*+*CaNAT*+t*CaACT1*, Fig. 4.2), as described in 2.10.1.3. The PCR products were checked on a 0.8% agarose gel (section 2.8.10.). A white colony containing a plasmid of the correct size was picked and used to inoculate 50 ml LB+Amp (100 μg/ml) medium for plasmid extraction (section 2.8.1.). The plasmid was then sent for sequencing as described in section 2.8.13 to confirm the construct was correct. Glycerol stocks of *E. coli* containing pNZ11 were made as described in section 2.7.1.1 using a portion of this culture. Plasmid pNZ11 was stored in 1xTE buffer (section 2.5.3.) for future use.

4.3. Construction of a MPA resistance cassette

Some problems with the construction of the MPA-resistance vectors were encountered initially. Initially I wanted to use plasmid p3408 (Appendix 10.3, containing a native *IMH3* promoter, an *IMH3*^{r} gene (Inosine monophosphate dehydrogenase resistance gene) from *C. albicans* strain 1161 which confers MPA resistance and a native *IMH3* terminator (Beckerman et al., 2001). However, this plasmid did not give stable *C. albicans* transformants with the cassette integrated at the correct position (data not shown). Another plasmid pNZ18, containing a native Ca*ACT1* promoter plus the *IMH3*^{r} gene and the *IMH3* terminator from p3408, was then constructed (Appendix 10.4.). But unfortunately this vector did not give stable *C. albicans* transformants either, probably because the cassette integrated at the native *IMH3* locus instead of at the *ACT1* promoter, and looped out during growth by homologous recombination.

Stable transformants could, however, be obtained using plasmid pNZ4, containing the *IMH3*^{r} under the control of a tetracycline-responsive promoter (pTR), the ORF encoding the tetracycline activator *tetR-ScHAP4-WH11* under the control of the *CaENO1* (*C. albicans* Enolase 1) promoter and two flanking sequences targeting its integration to a noncoding region of chromosome 7 (Fig. 4.3). PNZ4 was constructed as follows: A blunt-end DNA fragment containing *pENO1* (*ENO1* promoter) plus *tetR-ScHAP4-WH11* was amplified by PCR from the plasmid pCAITHE5 (Table 2.5, Appendix 10.5.) (Nakayama et al., 2000) using KOD polymerase with primers ENOpf and tetRWH11pr (section 2.9.3.) and then cloned into *Sma*I-linearized plasmid pBSKS(+) (MCS site, Appendix 10.2.) to produce

plasmid pNZ1 (Fig. 4.3). The cloning, *E. coli* colony screening (using primers M13pf/M13pr spanning the inserts) and plasmid isolation procedures were similar to those used to generate pNZ11 described above, except that the blunt-ended *Sma*I-linearized plasmid pBSKS(+) needed to be dephosphorylated with CAIP before cloning to prevent self-ligation as described in section 2.8.7.

Recombinant PCR (section 2.9.4.) was used to generate a combined fragment (pTR+*IMH3*^{r}ORF): The tetracycline-responsive promoter (*pTR*) was PCR-amplified from plasmid p99RLU (Table 2.5, Appendix 10.6.) (Nakayama et al., 2000) with primers TRpf (p1) and TRpr (p2). The *IMH3*^{r} ORF was amplified from plasmid p3408 (Table 2.5, Appendix 10.3.) (Beckerman et al., 2001) using primers IMH3pfatg (p4) and IMH3pr (p5). Both fragments were purified using a PCR clean-up kit (section 2.8.4.) and used as templates for recombinant PCR to combine the TR promoter and the *IMH3*^{r} ORF using primers TRpf (p1), TRpIMHp (p3) and IMH3pr (p5), as described in section 2.9.4. The blunt-end recombinant PCR product was gel purified (section 2.8.3.) and cloned into *EcoR*V-linearized blunt-ended plasmid pBSKS(+) to produce plasmid pNZ2 (Fig. 4.3), as described above for pNZ1. All of the PCR reactions mentioned above used high fidelity polymerase KOD enzyme (Novagen). The primer sequences are listed in Table 2.7.

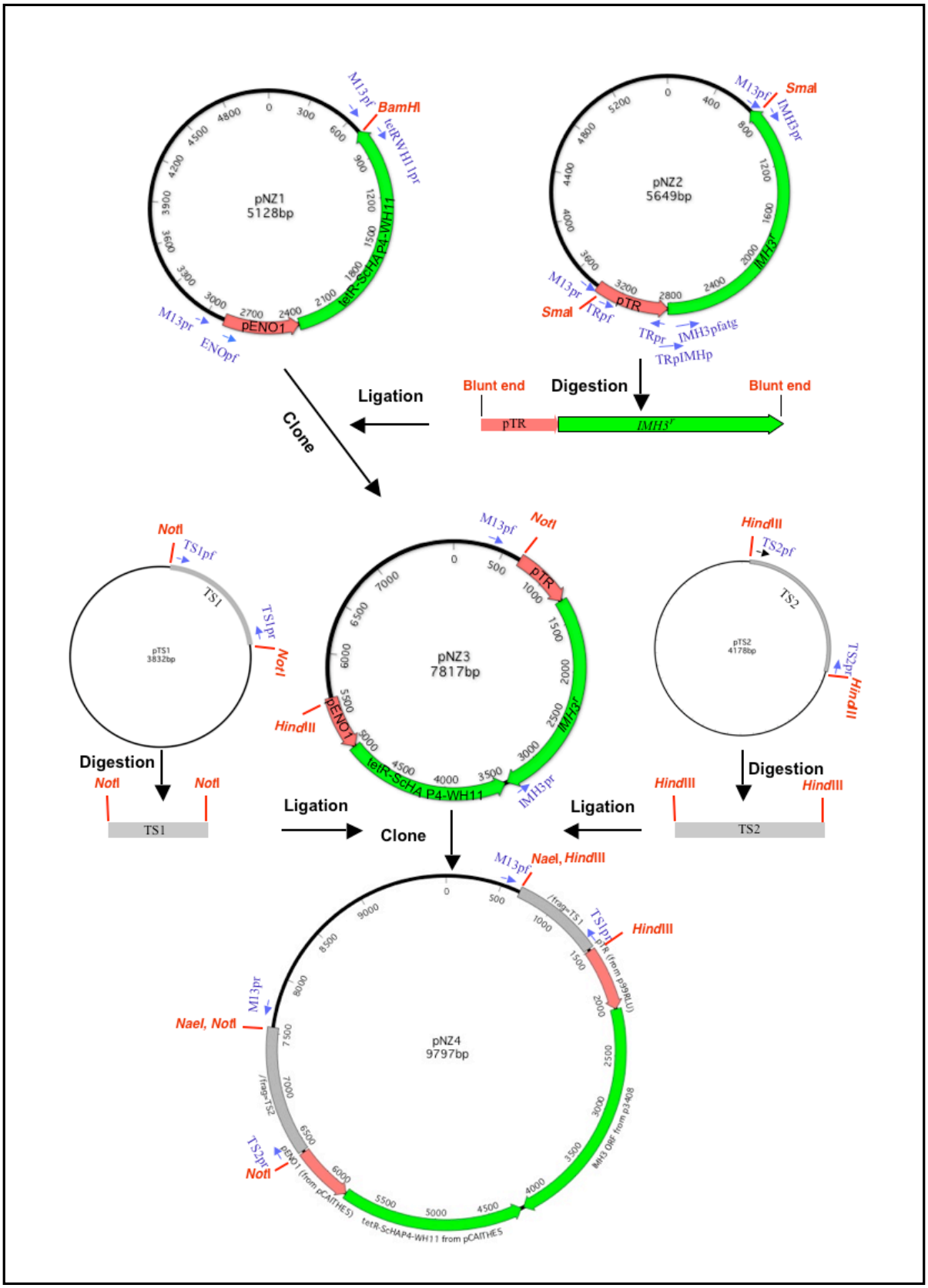

pNZ1
5128bp
M13pf
BamHI
tetRWH11pr
tetR-ScHAP4-WH11
pENO1
M13pr
ENOpr
pNZ2
5649bp
M13pf
SmaI
IMH3pr
IMH3f
M13pr
SmaI
TRpf
pTR
TRpr
IMH3pfatg
TRpIMHp
Ligation
Clone
Blunt end
Digestion
Blunt end
pTR
IMH3f
NotI
TS1pf
TS1
pTS1
3832bp
TS1pr
NotI
Digestion
NotI
NotI
TS1
pNZ3
7817bp
M13pf
NotI
pTR
IMH3f
IMH3pr
tetR-ScHA P4-WH11
pENO1
HindIII
HindIII
TS2pf
TS2
pTS2
4178bp
TS2pr
HindIII
Digestion
HindIII
HindIII
TS2
Ligation
Ligation
Clone
M13pf
NaeI, HindIII
/frag=TS1
TS1pf
HindIII
pTR (from p399BLU)
IMH3 ORF from p3408
tetR-ScHAP4-WH11 from pCAITHES
pENO1 (from pCAITHES)
TS2pf
NotI
/frag=TS2
M13pr
NaeI, NotI
pNZ4
9797bp

Figure 4.3. Construction of MPA resistance cassette-containing plasmid pNZ4.
Plasmid pNZ4 was constructed from pNZ1, pNZ2 and pNZ3 as described in the text. TS1 and TS2 are targeting sequences from *C. albicans* genome. The promoter pTR (promoter of tetracycline-responsive gene) was amplified from plasmid p99RLU (Nakayama, et al., 2000, Appendix 10.6.), while the promoter pENO1 (promoter of the enolase 1 gene) and tet-ScHAP4-WH11 (tetracycline activator ORF) were amplified from plasmid pCAITHE5 (Nakayama, et al., 2000, Appendix 10.5.). The MPA resistance gene *IMH3*^{r} was amplified from plasmid p3408 (Beckerman et al., 2001, Appendix 10.3.). Locations of primers (arrows) and restriction sites used in cloning are labeled. Plasmid map of the backbone pBlueScriptII KS (+) (pBSKS(+)) is shown in Appendix 10.2. Refer to the text (section 4.3) for the details of pNZ4 construction.

Plasmid pNZ1 was then cut with *Bam*HI and treated with Klenow enzyme to generate blunt ends (section 2.8.9.). The cassette containing *pTR* and *IMH3*^{r} was cut out from pNZ2 with *Sma*I generating a blunt-end fragment and was then cloned into dephosphorylated, blunt-ended, *BamHI*-linearized pNZ1 as described above to generate plasmid pNZ3 (Fig. 4.3). The correct insertion of the cassette was verified by PCR using primers M13pf/IMH3pr (section 2.10.1.3.).

Two targeting sequences, TS1 and TS2 (to direct the integration of the resistance cassette to a non-coding region of the *C. albicans* genome), were PCR-amplified using Taq polymerase (Qiagen) from chromosome 7 (bp 367394 to 368202 and 366038 to 367194, respectively, on chromosome 7 of strain SC5314 as per genome assembly 21; http://www.candidagenome.org/) with primers TS1pf/TS1pr and TS2pf/TS2pr (Table 2.7.), and cloned into plasmid pGemT-Easy (Appendix 10.7.) (Promega), as described by the manufacturer, to create plasmids pTS1 and pTS2 respectively (Fig. 4.3.) Fragments TS1 and TS2 were removed from pTS1 and pTS2, using *Not*I and *Hin*dIII, respectively, purified from 0.8% TAE agarose gel and cloned into *NotI* and *HindIII* -linearized pNZ3, subsequently, to obtain pNZ4. The desired cloning of TS1 and TS2 fragments in pNZ4 were verified as described

in section 2.10.1.3, using primers M13pf/TS1pr and M13pr/TS2pf, respectively, by colony PCR as described in section 2.9.2.

All constructs mentioned above were verified by DNA sequencing. Both pNZ11 and pNZ4 have the expected sequences (Appendix 10.8. and 10.9.). The sequences were submitted to GenBank and the accession numbers are FJ804172.1 and FJ804173.1.

5.0. MARKING OF PARENTAL STRAINS FOR MATING EXPERIMENTS

5.1. Introduction

As the next step towards undertaking mating experiments, the chosen *MTL* homozygotes (Table 3.2) were transformed with the resistance cassettes.

5.2. Transformation of *MTL***a** strains with the MPA resistance cassette

*MTL***a** derivatives to be used for mating were transformed with the *IMH3*^{r} cassette. Plasmid pNZ4 was isolated as described in section 2.8.1. The *IMH3*^{r} cassette (6.7 kb) was removed from pNZ4 (Fig.4.2) by *Nae*I digestion (section 2.8.6.), gel purified (section 2.8.3.), and used to transform *MTL***a** derivatives, using either the lithium acetate heat shock method (section 2.10.2.1.) (Beckerman et al., 2001) or electroporation (section 2.10.2.2.) (De Backer et al., 1999). After transformation, 100 μl aliquots of the transformation mixture were plated on each minimal plate containing MPA (5 μg/ml). Big colonies on a lawn of light growth were considered as potential transformants. These potential MPA-resistant colonies were re-streaked on minimal medium with MPA (10 μg/ml) to isolate single colonies and verify resistance, as MPA selection has a high false-positive background (Beckerman et al., 2001) (Fig. 5.1). Integration of the pNZ4 *IMH3*^{r} cassette should be driven by the terminal targeting sequences (TS1 and TS2) directing the cassette to a non-coding region on chromosome 7 by a double cross-over (Fig. 5.2). The electroporation method yielded 20 to 30 integrative transformants per μg DNA, the heat shock

method yielded 2 to 10 integrative transformants per μg DNA. The transformants were then tested for correct insertions.

The presence and correct insertion of the pNZ4 cassette was investigated by colony PCR (Fig. 5.3) with primer combinations TS1Fpf/TRsepr and TS2Fpr/pENOpr (Table 2.7, Fig. 5.2). A PCR reaction using primers TS1Fpf and TS1pr-p (Table 2.7, Fig. 5.2) was used to detect tandem integration (Fig. 5.3). These, and subsequent PCR reactions were carried out as described in section 2.9.2. Southern hybridization (which will be described in detail below) of *Eco*RI digests of transformants' genomic DNA using as a probe a 1134 bp fragment amplified from pNZ4 plasmid with primers tetRpf/tetRpr2 containing a partial tetR-SchAP4-W11 fragment, confirmed that insertion had occurred only at the intended target locus.

One transformant with correct insertion of a single copy cassette was chosen for each *MTL***a** strain for mating (Table 5.1). For strain AU35*MTL***a**, no single copy transformants were obtained after several attempts. A transformant with tandem copies at the correct position of this strain was chosen to be a parental strain (Table 5.1).

Transformants were stored at -80°C (glycerol added to overnight YPD cultures to 30% v/v) until further use as described in section 2.7.1.1.

Table 5.1. *MTL* homozygous derivatives marked with drug resistance cassettes

MTL homozygote	Genotype	Drug-resistant derivative	Genotype
AU7**a**	*MTL***a**	AU7**a**-pNZ4	*MPA*^{R}, *MTL***a**
AU35**a**	*MTL***a**	AU35**a**-pNZ4	*MPA*^{R}, *MTL***a**
AU90**a**	*MTL***a**	AU90**a**-pNZ4	*MPA*^{R}, *MTL***a**
RIHO11**a**	*MTL***a**	RIHO11**a**-pNZ4	*MPA*^{R}, *MTL***a**
W43**a**	*MTL***a**	W43**a**-pNZ4	*MPA*^{R}, *MTL***a**
FJ11α	*MTL*α	FJ11α-pNZ11	*NAT*^{R}, *MTL*α
HUN97α	*MTL*α	HUN97α-pNZ11	*NAT*^{R}, *MTL*α
OD8916α	*MTL*α	OD8916α-pNZ11	*NAT*^{R}, *MTL*α
W17α	*MTL*α	W17α-pNZ11	*NAT*^{R}, *MTL*α
		W17α-pNZ11.2*	*NAT*^{R}, *MTL*α
YSU63α	*MTL*α	YSU63α-pNZ11	*NAT*^{R}, *MTL*α

* A second transformant of W17α not used for mating (see section 8.4.3.)

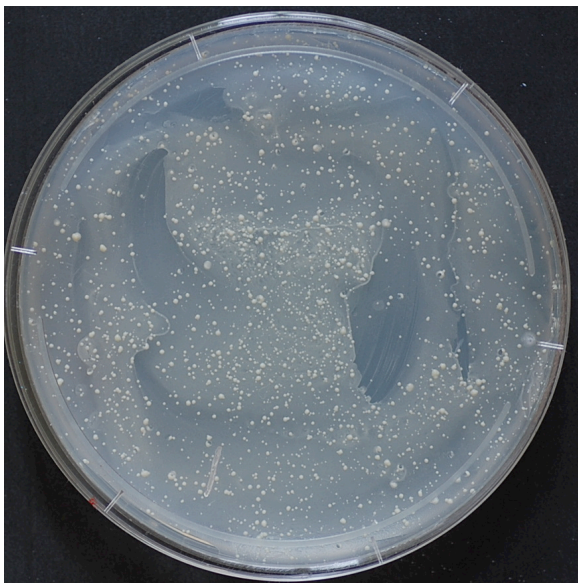


Figure 5.1. MPA selection has a high background.

Typical appearance of a MM plate containing MPA (5 µg/ml) 5 days after inoculation with 100 µl of a cell suspension of strain W43*MTL***a** transformed with the MPA resistance cassette, and incubation at 30 °C. The larger colonies were considered putative *MPA*^{r} cassette-bearing clones.

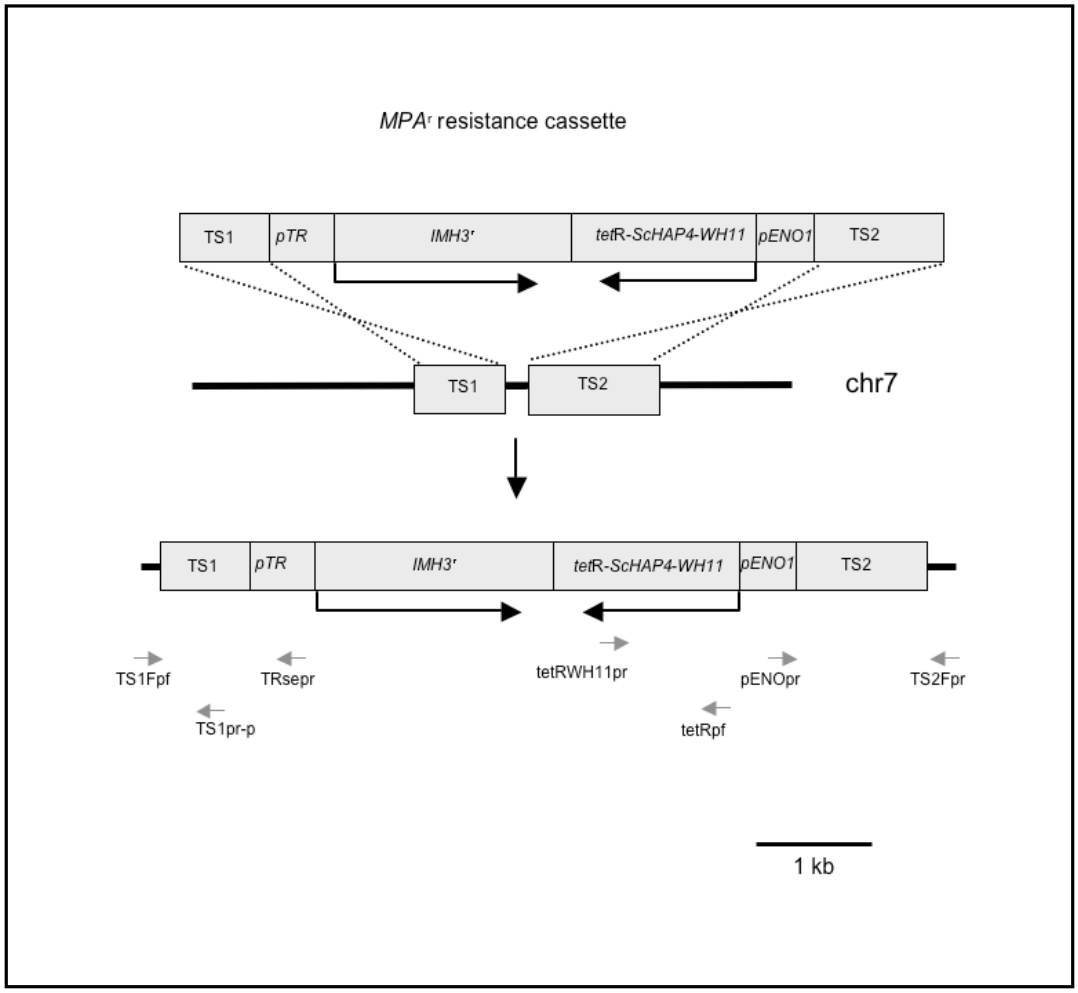


Figure 5.2. Intended integration of *MPA*^{r} resistance cassette on chromosome 7 by double cross-over.

The cassette contains two non-coding sequences, TS1 and TS2, homologous to a region of *C. albicans* chromosome 7 (chr7) (bp 367394 to 368202 for TS1 and bp 366038 to 367194 for TS2) to which integration of the cassette is targeted, the *MPA* resistance-conferring *IMH3* allele (*IMH3*^{r}) under the control of a tetracycline-responsive promoter (*pTR*), as well as the gene encoding the tetracycline transactivator (*tet*R-*ScHAP4-WH11*) under control of the *ENO1* promoter. In the absence of tetracycline, the *ENO1* promoter controlling tetracycline activator expression induces the tetracycline-responsive promoter controlling *IMH3*^{r} expression thus conferring resistance to MPA. Integration occurs by crossing over in TS1 and TS2. Primers used to confirm correct integration by PCR (Fig. 5.3) are shown as arrows.

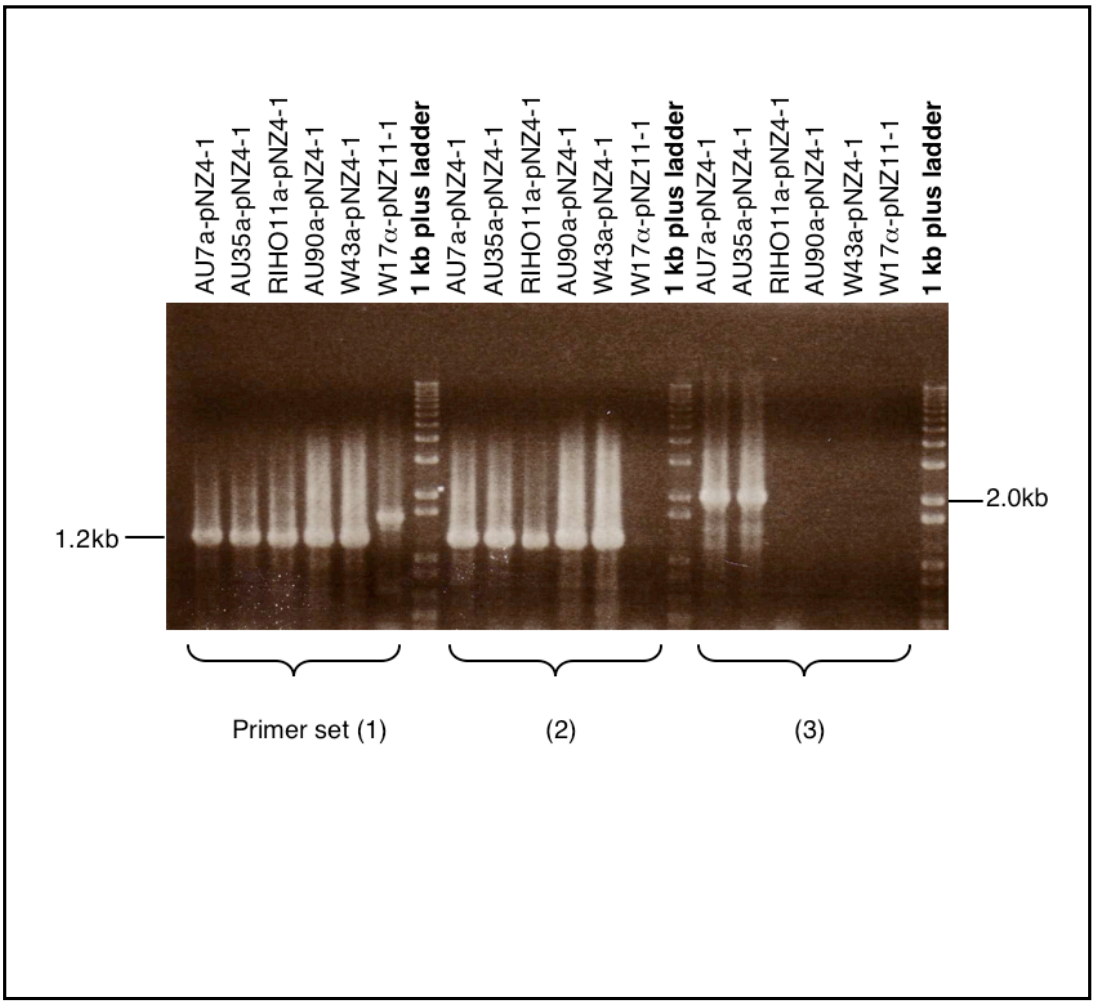


Figure 5.3. Examples of PCR screens for the correct integration of MPA resistance cassette in marked parents.

Three primer sets were used for identifying double cross-over transformants. Primer set (1), TS1Fpf/Trsepr; primer set (2), TS2Fpr/pENOpr; and primer set (3), TS1pr-p/pENOpr. Refer to Fig. 5.2 for the positions of the primers. The sequences of the primers are listed in Table 2.7. If the MPA resistance cassette had integrated correctly as a single copy, a 1.2 kb band and a 1.1 kb band would be amplified with primer set 1and 2, respectively, while no product would be amplified using primer set 3 (examples were transformants RIHO11a-pNZ4-1, AU90a-pNZ4-1 and W43a-pNZ4-1). If a 2.0kb band was amplified when using primer set 3, and a 1.2kb band and a 1.1kb band was present using primer set 1 and 2, the cassettes had integrated in tandem repeats at the correct position (examples were transformants AU7a-pNZ4-1 and AU35a-pNZ4-1). W17α-pNZ11-1 was a negative control.

5.3. Transformation of *MTLα* strains with the NAT resistance cassette

MTLα derivatives were transformed with uncut plasmid pNZ11 to obtain single cross-over integration of the whole plasmid (Fig. 5.4.), using the lithium acetate heat shock method (section 2.10.2.1.) (Beckerman et al., 2001) or electroporation (section 2.10.2.2.) (De Backer et al., 1999), as described above. After transformation, 100 μl portions of the transformation mixture were spread on YPD plates and incubated at 37°C for 7 hours, at which time a thin lawn of cells had formed. Transformants were selected by replica plating onto YPD plates containing NAT at 200 μg/ml (200 μg/ml was used because at high cell density, such as that used here, too much background growth occurred even with strains sensitive to 100 μg NAT/ml) (section 2.7.1.2.). Approximately 20 integrative transformants per μg DNA were obtained with either method. Table 5.1 lists the transformants obtained for the *MTLα* strains.

The correct integration of the *NAT*^{r} cassette in the parental strains was verified by PCR with primers M13pr/CaACTpr and pACTFpf/CaNATpr (Table 2.7; Fig. 5.4) flanking the target insertion sequence. The NAT resistance cassette contains the *ACT1* promoter and terminator, favoring double cross-over and consequential deletion of the native *ACT1* gene (Fig. 5.4b). In order to not compromise the parent strains, I decided to isolate transformants in which integration had occurred by single cross-over (Fig. 5.4a) and in which the native *ACT1* gene remained intact. In these, the cassette would integrate the *NAT*^{r} gene under control of the *ACT1*

promoter in front of an intact native *ACT1* gene (Fig. 5.4a). A PCR using primers M13pr/pNZ11pf (Table 2.7; Fig. 5.4) was used to detect tandem integration. Figure 5.5 shows typical results of the PCR screen to confirm the insertions. Southern hybridization (which will be described in detail below) of *Sal*I digests of transformants' genomic DNA using as a probe a 564 bp DNA fragment amplified from pNZ11 using primers CaNATpf/CaNATpr containing the *CaNAT* ORF, confirmed that insertion had occurred only at the intended locus. One transformant with correct insertion of a single copy cassette was chosen for each *MTL*α derivative for mating experiments (Table 5.1; Fig. 3.4). For strain FJ11*MTL*α, no single copy transformants were obtained after several attempts. A transformant with tandem copies at the correct position of this strain was chosen to be a parental strain (Table 5.1).

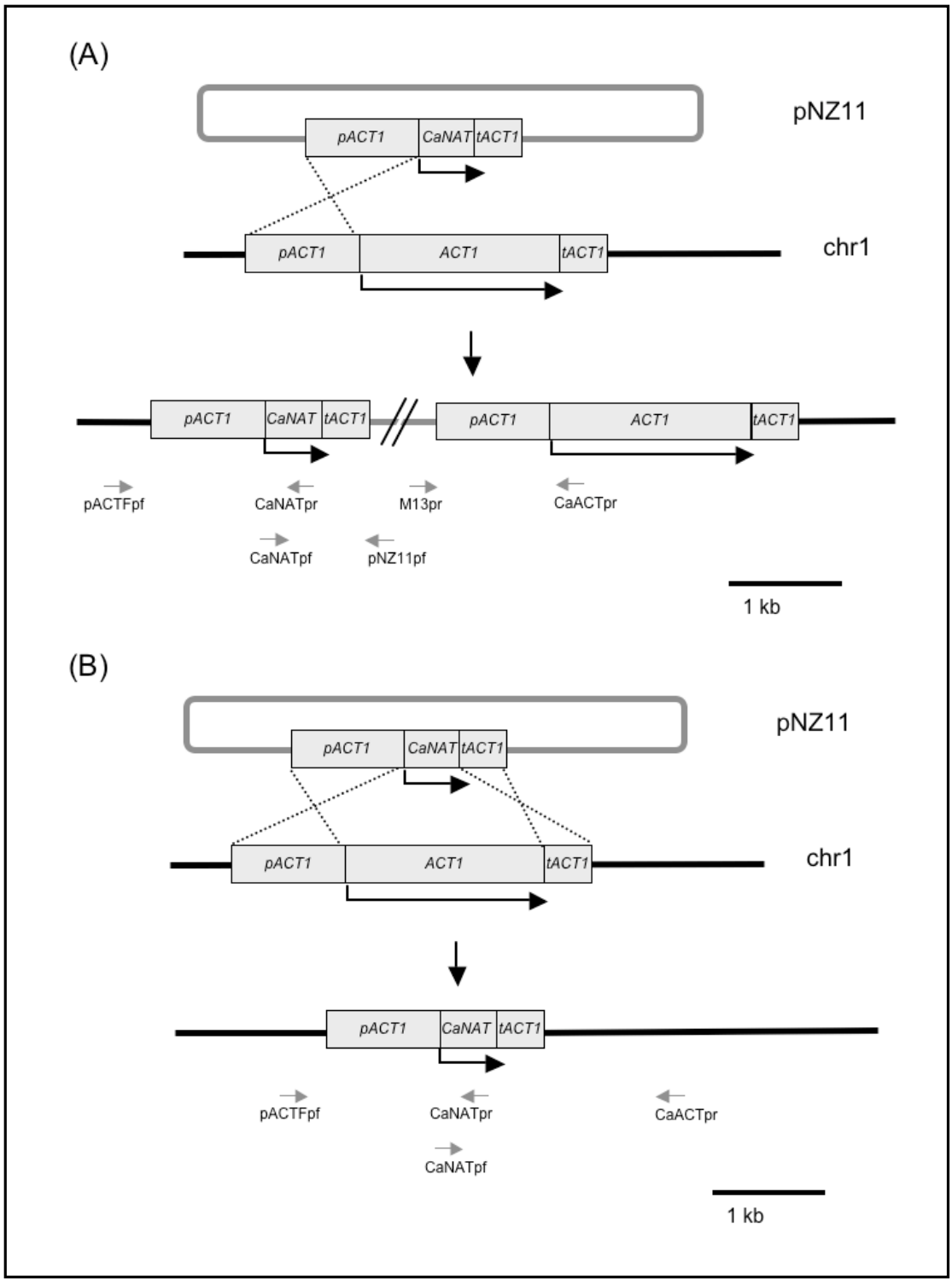


Figure 5.4. Intended integration of the NAT resistance cassette on chromosome 1 by single cross-over (A) or double cross-over integration (B).

In (A) the pNZ11 cassette, containing the *CaNAT*^{r} gene (conferring resistance to nourseothricin) under the control of the *ACT1* promoter integrates by a single cross-over, directing the whole pNZ11 plasmid to the *ACT1* locus. In (B), double cross-over results in removal of the native *ACT1* gene. Primers used for verifying correct integration by PCR (Fig. 5.5) are shown.

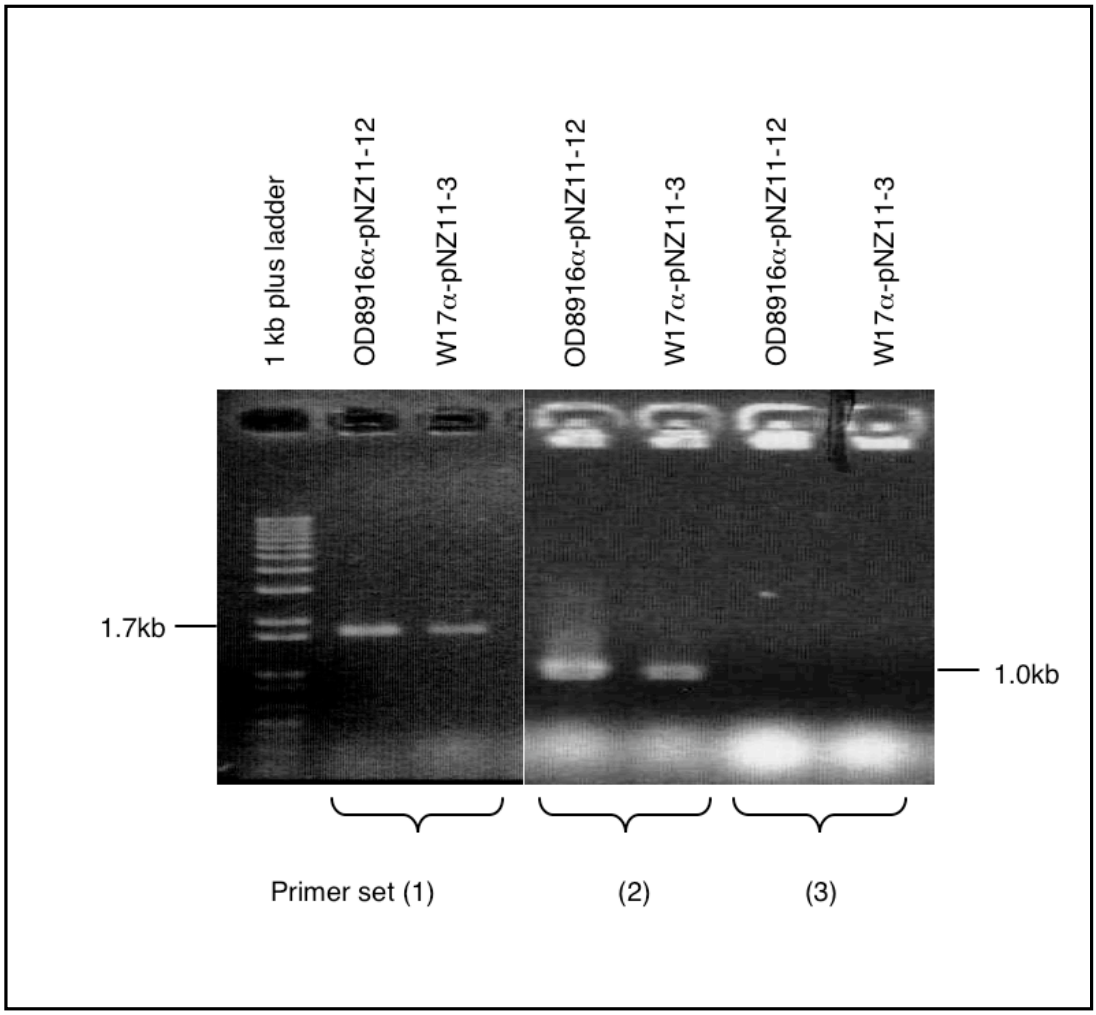


Figure 5.5. Examples of PCR screen for the correct insertion of NAT resistance cassette in marked parents (OD8916α-pNZ11 and W17α-pNZ11).

Three primer sets were used for identifying single or double cross-over transformants and tandem inserts. Primer set (1) pACTFpf/CaNATpr, (2) M13pr/CaACTpr and (3) M13pr/pNZ11pf. Refer to Fig. 5.4 for the positions of the primers. The sequences of the primers are listed in Table 2.7. Correct single-copy integration of the NAT resistance cassette results in a 1.7 kb band and a 1.0 kb band with primer set 1 and 2, respectively (marked by arrows) while no product should be amplified using primer set 3. A 3.0 kb band produced by primer set 3, plus a 1.7 kb band and a 1.0 kb band produced using primer sets 1 and 2, respectively, would indicate tandem repeats at the correct position. Upon double cross-over, no band would be detected when using primer set 2 and set 3, while a 1.7 kb band would be detected with set 1.

5.4. Marking of parental strains with both MPA and NAT resistance markers

Three parental strains (W43*MTL***a**, OD8916*MTL*α and W17*MTL*α) were double marked with both MPA and NAT resistance cassettes as control strains to investigate the possible effect of single markers and double markers on the fitness of the parents, as the recombinants would have both drug resistance markers.

The transformation procedures performed were identical to those described above, carried out sequentially. Parental strain W43*MTL***a** was first marked with an MPA resistance cassette and then marked with a NAT resistance cassette, while OD8916*MTL*α and W17*MTL*α were first marked with the NAT resistance marker and then the MPA resistance marker. The correct positions and copy numbers of the markers were verified by PCR as described above for each strain.

5.5. Verification of the position of the resistance cassettes by Southern blot analysis

To confirm, in transformants selected for further work, single copy integration of the drug resistance cassettes at the expected position in the genome and the absence of additional ectopic copies, Southern hybridization was performed for all of the 10 parental strains marked with MPA (*MTL***a**/**a** parents**)** or NAT (*MTL*α/α parents) resistance genes.

Genomic DNA was prepared from all of the MPA and NAT resistance cassette-marked parents (pNZ4 and pNZ11 transformants, respectively) (Table 5.1), and digested with restriction endonucleases *EcoR*I and *Hin*dIII for MPA transformants and *Sal*I for NAT transformants (section 2.8.6.). The DNA digests were then probed by Southern hybridization with a 1134 bp DNA fragment amplified from pNZ4 plasmid and a 564 bp DNA fragment amplified from pNZ11 plasmid using primers tetRpf/tetRpr2 and CaNATpf/CaNATpr, respectively (Fig 5.6., Fig. 5.7.) (section 2.8.12.2). *Eco*RI cuts twice within plasmid pNZ4, while *Sal*I cuts once within plasmid pNZ11 (Fig 5.6, Fig. 5.7.). For the desired transformants with a single integrated copy pNZ4, a Southern blot of an *Eco*RI digest of transformant DNA should show two bands (3.8 kb and 1.0 kb) produced by *Eco*RI cutting twice in the plasmid pNZ4 (once within the sequence homologous to probe) and in the flanking DNA of TS2. Tandem integration of the cassette would produce three bands from *EcoR*I digests (1.0 kb, 3.8kb, 5.8 kb). DNA from single-copy pNZ11 transformants should generate a single 5.0 kb band when digested with *Sal*I, which cuts once within the plasmid pNZ11, and two bands for tandem integration (4.9 kb, 5.0 kb). Ectopic integration of additional copies of pNZ4 and pNZ11 resistance cassettes should produce other bands in addition to those mentioned above.

These experiments confirmed the PCR results, i.e. single copy integration except in cases of Au35*MTL***a**-pNZ4 and FJ11α- pNZ11 (tandem integration) and absence of any additional ectopic integration events (Fig. 5.6 & Fig.5.7).

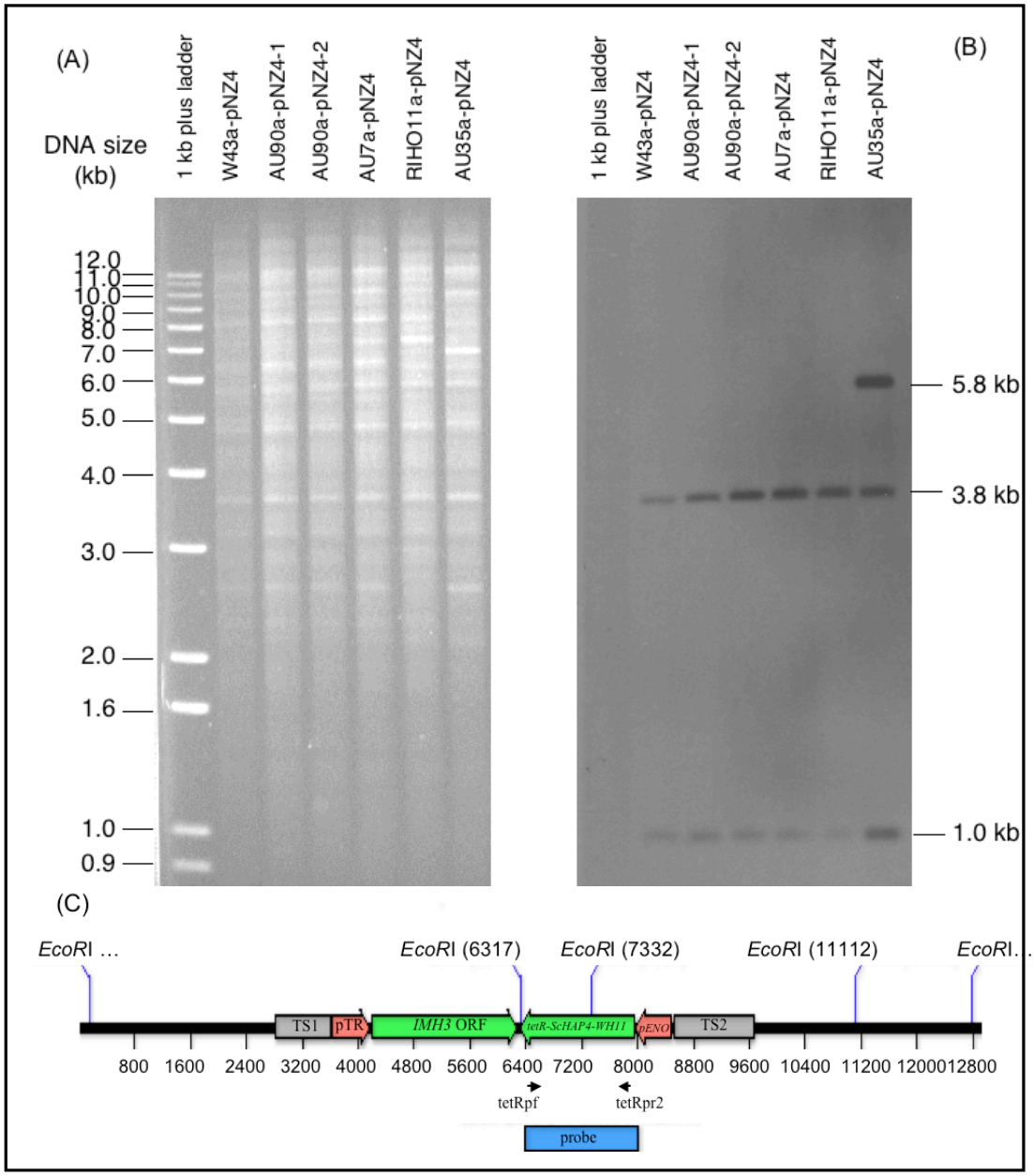


Figure 5.6. Southern blot of parental strains containing the MPA resistance marker.

Transformants with insertions at the correct genomic locus, identified by PCR, were analyzed by Southern blot. (A) DNA from six *C. albicans MTL***a**/**a** derivatives marked with MPA resistance cassette (1 µg each) was digested with *Eco*RI, separated on a 0.8% agarose gel (a 1 kb plus ladder was included on the gel as a DNA size standard - DNA sizes are indicated on the left). (B) Southern blot of the gel in (A) probed with a 1134 bp probe (see (C)) amplified from the *tetR-ScHAP4-WH11* cassette in plasmid pNZ4 using primers tetRpf/tetRWH11pr. (C) Schematic diagram showing a correctly integrated cassette, *Eco*RI sites used for verification and the probe (as a blue rectangle). A 3.8 kb and a 1.0 kb band were detected (marked by lines on right in panel B), validating that most transformants had a single copy of the pNZ4 cassette inserted at the correct position. An additional 5.8 kb band (also marked in panel B) indicated that in AU35**a** the insertion was a tandem repeat. Ectopic insertions would give extra bands, such were not detected.

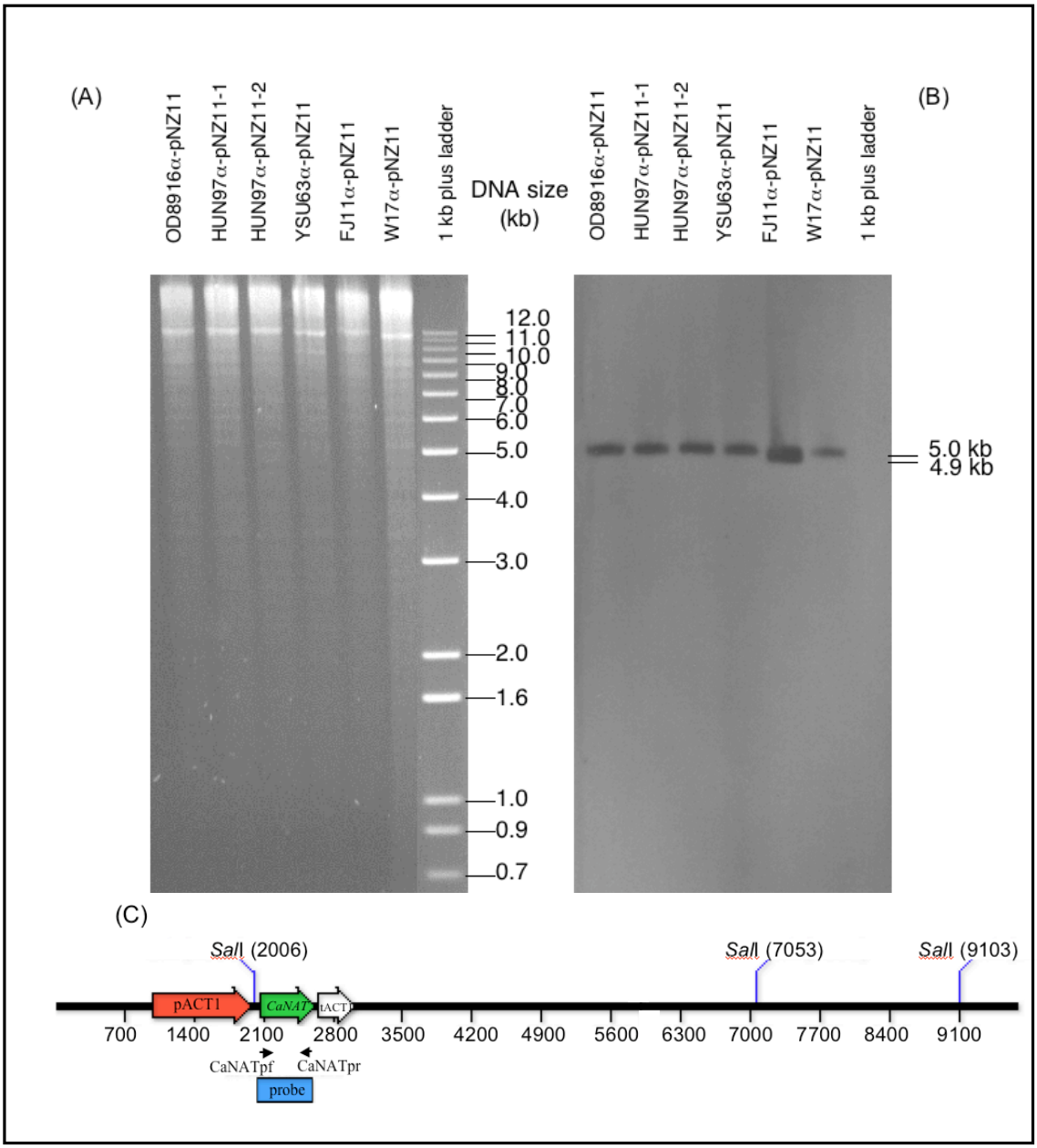


Figure 5.7. Southern blot of parental strains containing the NAT resistance marker.

Transformants with insertions at the correct genomic locus, identified by PCR, were analyzed by Southern blot. (A) DNA from six *C. albicans MTL*α/α derivatives marked with NAT resistance cassette (1 μg each) was digested with *Sal*I separated on a 0.8% agarose gel (a 1 kb plus ladder was included on the gel as a DNA size standard - DNA size are indicated on the left). (B) Southern blot of the gel in (A) probed with a 564 bp probe (see (C)) amplified from the *CaNAT* gene in plasmid pNZ11 using primers CaNATpf/CaNATpr. (C) Schematic diagram showing a correctly integrated cassette, *Sal*I sites used for verification and the probe (as a blue rectangle). A 5.0 kb band was detected (marked by lines on right in panel B), validating that the transformants had a single copy of the pNZ11 cassette inserted at the correct position. An additional 4.9 kb band (also marked in panel B) indicated that in FJ11α the insertion was a tandem repeat. Ectopic insertions would give extra bands, such were not detected.

6.0. DEVELOPMENT OF A NEW MATING PROCEDURE

6.1 Introduction

A new method was established for mating clinical strains with the new two dominant selectable marker system described above (Fig. 4.1). It was based on the existing standard white cell plate mating system (Magee and Magee, 2000).

6.2. Development of a new mating procedure for marked clinical isolates

The entire mating procedure is shown in Fig. 6.1. As in the method of Magee et al, (Magee et al., 2002; Magee and Magee, 2000) strips of parent strains were replica plated at right-angles onto either YPD medium or spider medium for mating. In the original method, involving two parents with different auxotrophies or one wild-type strain and one MPA-resistant auxotrophic strain (so-called universal mating tester strain), another round of replica plating onto plates selecting simultaneously for both markers (minimal medium or minimal medium plus MPA) can be used to directly identify mating products which arise in the area where the strips intersect (Legrand et al., 2004; Magee et al., 2002). Unfortunately I was not able to find a growth medium in which both MPA and NAT were sufficiently effective to allow one-step selection of recombinants[1]. Selection for MPA resistance is usually carried out on minimal medium (Beckerman et al., 2001) and selection for NAT resistance on YPD medium (Shen et al., 2005). Inclusion of both drugs in YPD agar did not

[1] The term "recombinant" used in this study is defined as mononuclear mating products containing genetic material from both parents.markers.

prevent growth of cells without the MPA resistance marker and inclusion of both drugs in minimal medium did not prevent growth of cells without the NAT resistance marker. The ineffectiveness of MPA in YPD is presumably caused by nucleic acids present in the medium, since MPA interferes with nucleic acid biosynthesis (Sweeney et al., 1972). NAT works by inhibiting protein synthesis (Dembitsky, 2005; Haupt et al., 1978) and may require media that promote fast growth such as YPD for effectiveness. I explored the use of a minimal medium with amino acids to promote faster growth (0.67% Yeast Nitrogen Base with Amino Acids, 2% Glucose) with both drugs (MA-NAT-MPA). This medium allowed identification of colonies with both markers on the basis of their larger size, but only when a plate was inoculated with ≤100 viable cells (Fig. 6.2), too low a number for recovery of recombinants from the patches produced by replica plating the mating plates.

I therefore developed a procedure in which recombinants were selected using a series of sequential enrichment and selection steps as described in section 2.12.1 (Fig. 6.1). First, cells from the mating plates were replica-plated onto YPD plus NAT (200 μg/ml) to reduce the frequency of MPA-resistant (NAT-sensitive) parental cells. This was followed by plating a suspension of cells, from the replicas of areas where mating may have occurred onto MPA-containing (5 μg/ml) minimal plates (I initially tried replica plating onto MPA-containing medium but this proved infeasible due to high background growth of cells without the MPA^r marker). Larger (putative MPA-resistant) colonies from these plates were then used to make patches on both MPA-containing and NAT-containing plates (Fig. 6.1) as described in section 2.12.2. When cells from a large colony grew on both media and both

MTL alleles could be PCR-amplified from these cells, the patches were considered to contain putative recombinants. The PCR screen was carried out using cells from

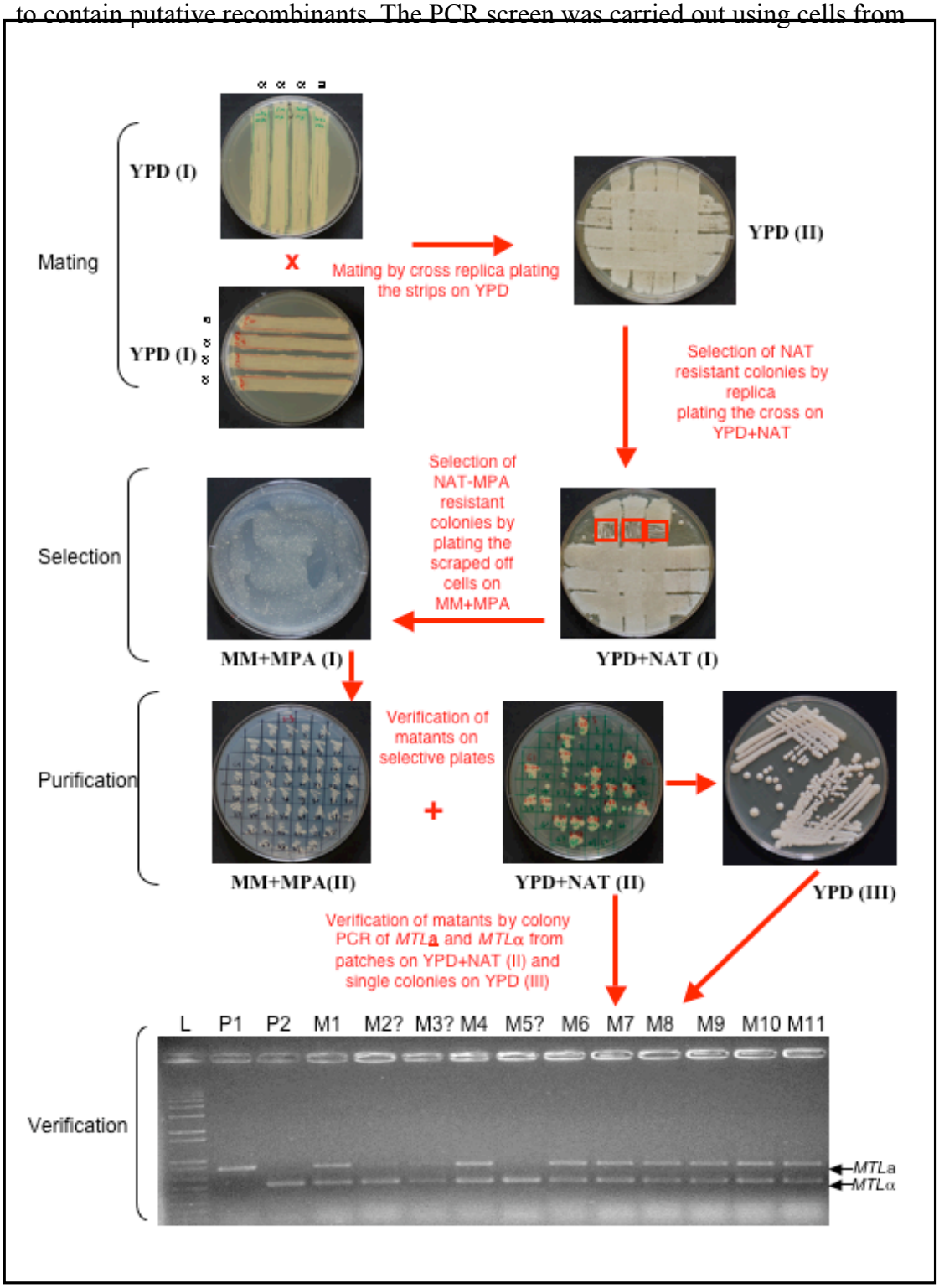


Figure 6.1. Overview of the mating procedure and selection of recombinants from three *MTL*α strains crossed with one *MTL*a strain.

The parental strains marked with MPA (**a**/**a**) and NAT (α/α) are termed as **a** and **α**, respectively, Refer to the text for more details about the mating procedure (section

6.2). YPD (I): YPD plates for incubation of the strips of parental strains; YPD (II): YPD plate for incubation of the mating crossings; MM+MPA (I): the first MM plate containing 5μg/ml MPA for the first round MPA selection; YPD+NAT (I): the first YPD plate containing 200 μg/ml NAT for the first round NAT selection; MM+MPA (II) and YPD+NAT (II): the MM plate containing 5μg/ml MPA and the YPD plate containing 200 μg/ml NAT for the second round MPA/NAT selection; YPD (III): a YPD plate for the final purification of the matants. The bottom panel shows a gel picture of detection of both *MTL* alleles using a duplex PCR as described in section 2.9.6. with primers MTLa1-F/MTLa1-R for the *MTL***a** locus; MTLα1-F/MTLα1-R for the *MTL*αα locus. For primer sequences refer to Table 2.7. Names of the strains are shown above lanes. P1: an example of the *MTL***a** parent. P2: an example of the *MTL*α parent. M1 to M11: putative matants. L: 1 kb plus ladder; arrows on the right indicate the *MTL***a** and *MTL*α alleles.

patches on YPD+NAT plates as NAT selection has much less background than MPA selection. However, I found that the PCR results could be misleading presumably because patches could still contain some parental cells. Therefore I included a final verification step: putative recombinant patches from NAT plates were streaked out to produce single colonies on YPD agar and, from these, true recombinant colonies were identified by the presence of both *MTL* alleles, as assessed by PCR.

I verified that this method can be used to recover recombinants, using several of the *MTL* homozygous strains I had generated. As mentioned in the Introduction, very little is known about the frequency with which clinical isolates can mate and the effectiveness of the method used to recover recombinants is therefore difficult to establish. However, the results obtained indicate that it is sufficiently efficient to gauge the ability of clinical isolates to mate (see Results below and Discussion).

The methodology also proved superior to two other approaches I explored. Since opaque cells are the mating competent cells in *C. albicans* (Miller and Johnson,

2002), I conducted a white-opaque switching experiment using three strains (W43a-pNZ4, OD8916α-pNZ11 and W17α-pNZ11) and obtained opaque cells from all of them (Fig. 6.3). Several mating experiments were performed using these strains. I initially tried using these opaque cells to do the plate mating as described above but without success. The opaque cells were too sticky and could not be transferred efficiently by replica plating. Only a small percentage would be lifted off the source plate through binding to the velvet-covered disk used for transfer. Therefore insufficient cells could be replica plated from plate to plate (data not shown). I also tried liquid mating using opaque cells according to Lockhart's method (Lockhart et al., 2003), and plating the mating mixture on MA-NAT-MPA plates as described above. Unfortunately this was unsuccessful as well, probably because the mating efficiency between clinical isolates was too low and the selection system was not strong enough to obtain the recombinants among the limited number of cells that were plated on this medium (data not shown, refer to Fig. 6.2).

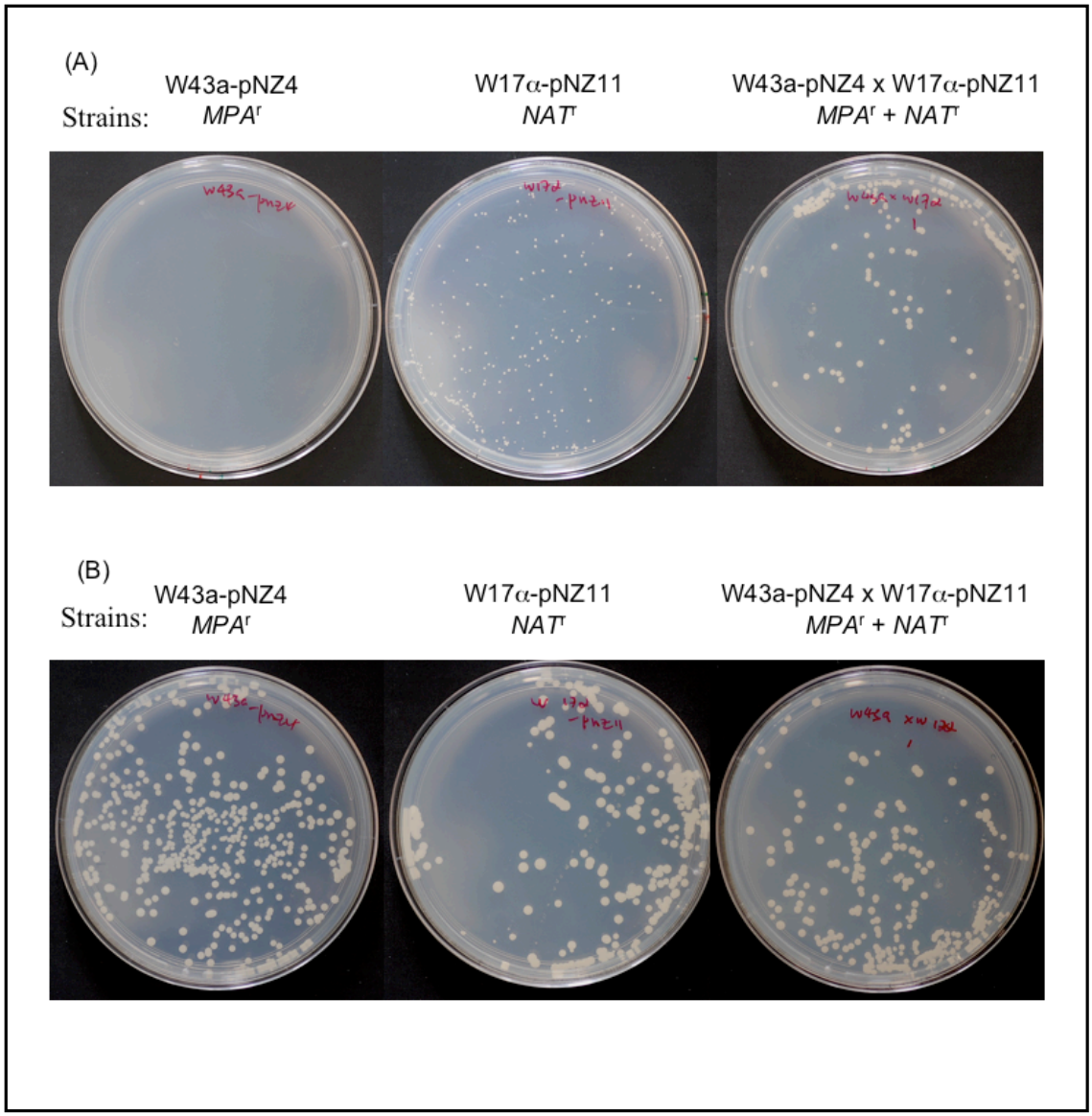


Figure 6.2. MPA/NAT dual selection on MA plates.

Equal volumes of cell suspensions of three strains, the MPA-resistant W43a-pNZ4 (*MPA*^{r}), the NAT-resistant W17α-pNZ11 (*NAT*^{r}) and the double resistant recombinant W43a-pNZ4xW17α-pNZ11 (*MPA*^{r}, *NAT*^{r}) were plated on MA+MPA+NAT plates containing 0.67% yeast nitrogen base with amino acid plus 2% glucose supplemented with MPA (5μg/ml) and NAT (200μg/ml) (A) and on MA plates containing no drugs (B) and incubated at 30^{o}C for 3 days. All strains can grow well on the MA plates. MA+MPA+NAT completely repress the growth of MPA-resistant strain W43a-pNZ4, and the NAT-resistant W17α-pNZ11 forms smaller colonies. The double marked strain W43a-pNZ4 X W17α-pNZ11 formed colonies on MA+MPA+NAT plates which were only slightly smaller than those on the drug-free plates.

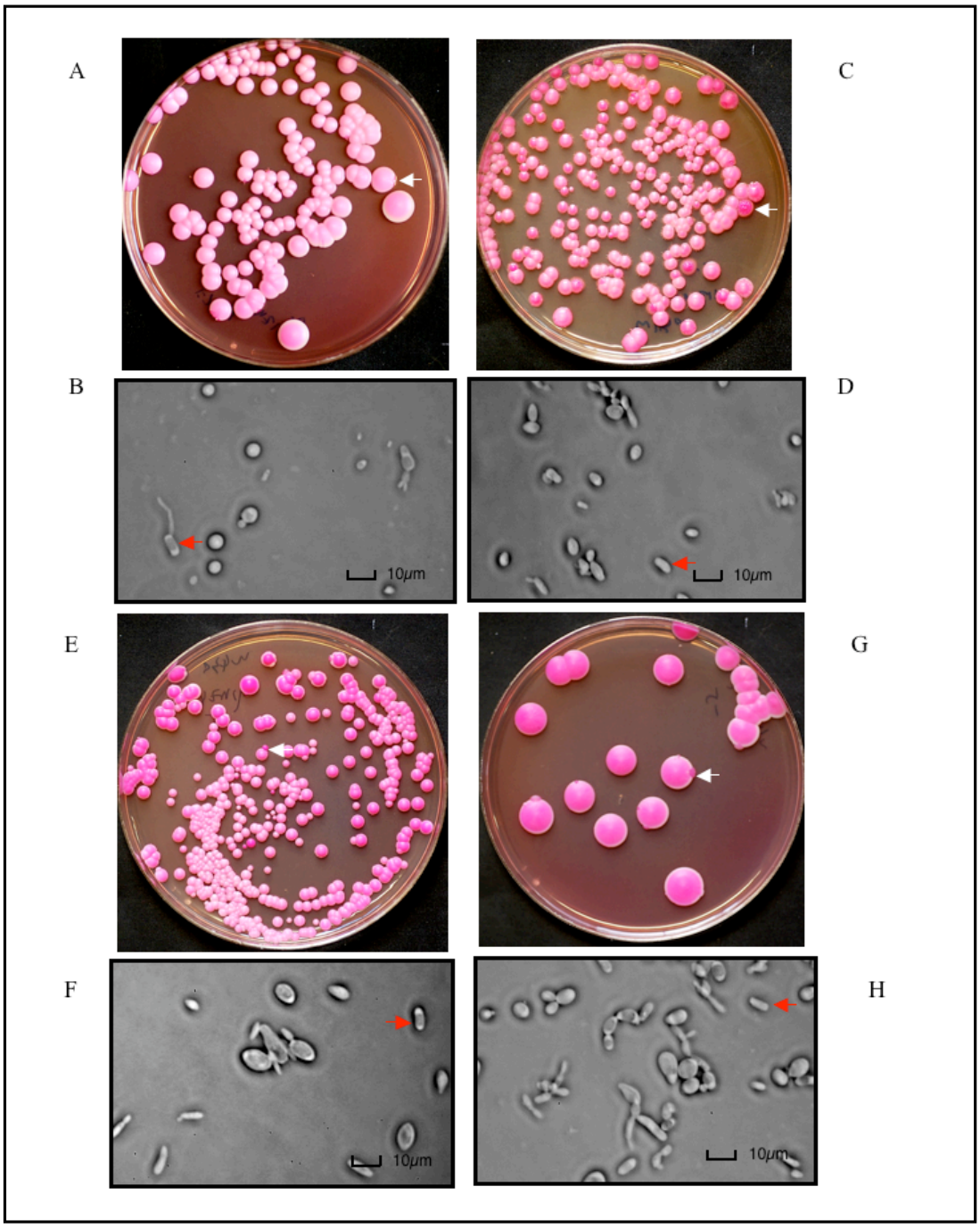


Figure 6.3. White / opaque cell transition detected on YPD-Phloxine B plates.

Four *MTL* homozygous strains OD8916α-pNZ11 (A, B); W43α-pNZ11 (C, D); W43a-pNZ4 (E, F) and FJ11α-pNZ11 (G, H) were plated on YPD-Phloxine B plates as described in section 2.7.1.4. and incubated at room temperature for 1 week. The putative opaque cells were picked from red sectors or colonies (arrows in A,C,E,G) and the presence of opaque cells was confirmed by microscopy (B, D, F, G; one example of a cell showing the characteristic elongated opaque cell morphology is marked by an arrow in each panel).

7.0. MATING BETWEEN CLINICAL ISOLATES

7.1 Introduction

This section describes how, by using the methodology I had developed, I assessed to what degree clinical *C. albicans* strains are still capable of generating viable recombinant lineages, by measuring the percentage of pairwise combinations of clinical isolates that is still capable of mating and of producing recombinant lineages.

7.2 Mating could occur between clinical isolates but not all combinations yielded recombinants

I tried to obtain recombinants (defined as cells containing both *MTL* alleles) from all 25 possible pairwise combinations of the five *MTL***a** and the five *MTL*α derivatives (Fig.3.4). For 11 combinations (boxes with red outline in Fig. 7.1) I obtained recombinants in a first mating attempt on YPD medium. Additional attempts on YPD did not yield recombinants from additional combinations.

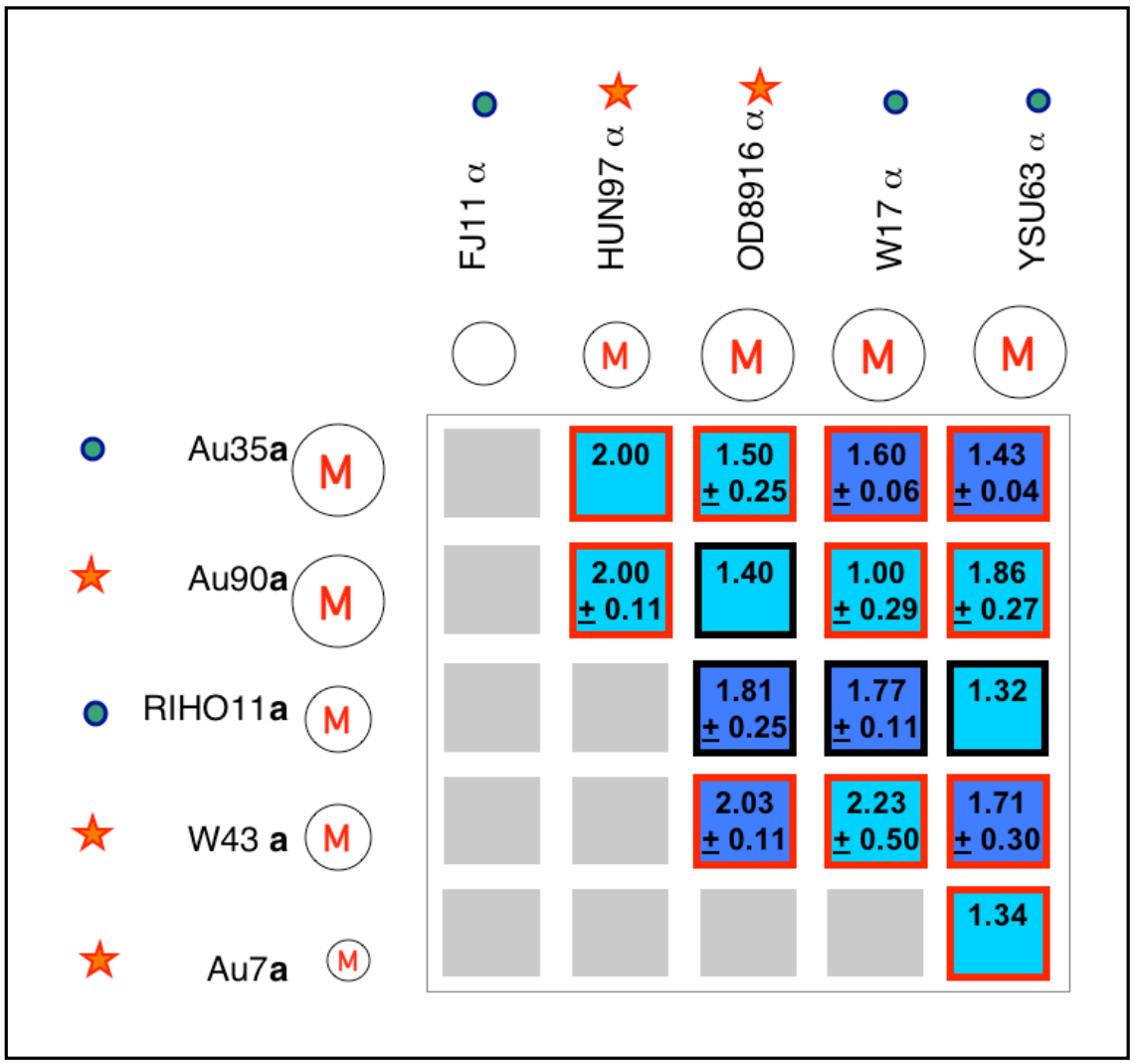


Figure 7.1. Outcome of matings between 25 combinations of *MTL* homozygous derivatives of 10 clinical isolates.

Names of the derivatives and their *MTL* alleles are listed on the left and the top of the figure. Dark blue squares indicate combinations yielding ≥7 recombinants per experiment and in which ≥10 % of all colonies tested were recombinants. Light blue boxes indicate combinations where fewer recombinants were obtained and grey boxes indicate combinations yielding no recombinants in ≥3 independent experiments (some crossings involving FJ11α were attempted only once after it was discovered that a genetic defect prevented it from mating). Numbers in boxes indicate the DNA content of recombinants (average ± SD where multiple recombinants was analyzed) relative to the average DNA content of the parents, determined by fluorescence-activated cell sorting (FACS). Boxes with a red outline indicate that recombinants were obtained from the first attempt of mating on YPD medium, while boxes with a black outline indicate that no recombinants were obtained from mating on YPD medium for at least two trials and the recombinants were obtained from mating on spider medium. Strains with big circles with M indicate this strain can mate with at least four other strains. Medium M: strains can mate with at least three other strains. Small M: strain can mate with only one other strain. Empty circle: strain can not mate with other strains. Strains labeled with stars are GPG strains and strains with circles are non-GPG strains.

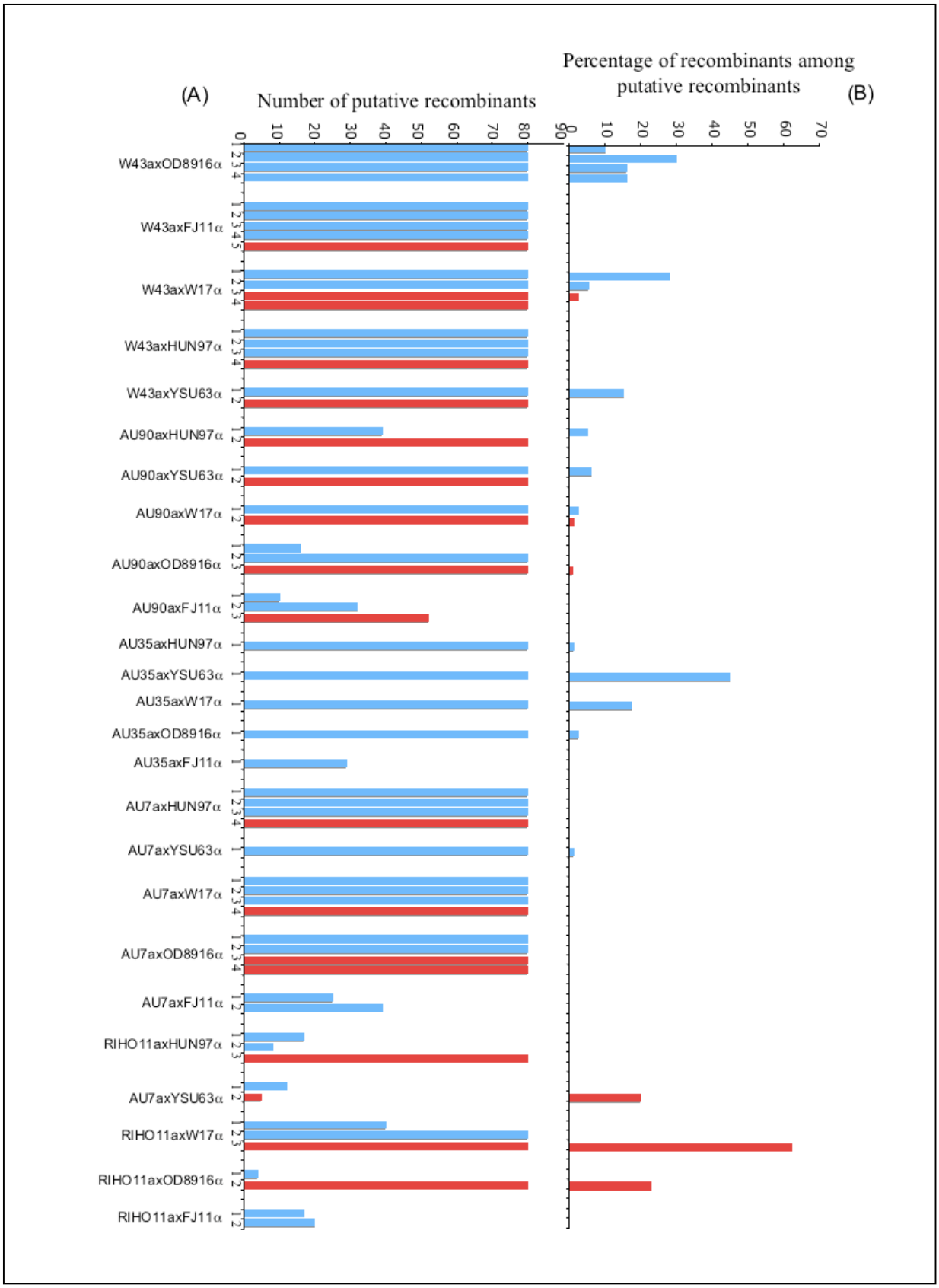

(A)
Number of putative recombinants
Percentage of recombinants among putative recombinants
(B)
W43axOD8916α
W43axFJ11α
W43axW17α
W43axHUN97α
W43axYSU63α
AU90axHUN97α
AU90axYSU63α
AU90axW17α
AU90axOD8916α
AU90axFJ11α
AU35axHUN97α
AU35axYSU63α
AU35axW17α
AU35axOD8916α
AU35axFJ11α
AU7axHUN97α
AU7axYSU63α
AU7axW17α
AU7axOD8916α
AU7axFJ11α
RIHO11axHUN97α
AU7axYSU63α
RIHO11axW17α
RIHO11axOD8916α
RIHO11axFJ11α

Figure 7.2. Efficiency of mating in the 25 mating experiments.

(A) Number of putative recombinants (above average size colonies on MM+MPA (I) (5μg/ml) plates) per experiment, for each mating experiment. When this number exceeded 80 the exact number was not determined. (B) Percentage of putative recombinants verified as true recombinants by PCR (i.e. containing both *MTL* alleles). True recombinants were obtained from 15 out of 25 combinations. Blue bars: mating on YPD plates. Red bars: mating on spider medium. Strains mated and numbers of experiments undertaken are listed on the left.

Repeat of the procedure on spider medium, which has been reported to increase nuclear fusion frequency between clinical isolates (Bennett et al., 2005), did not, in general, increase the number of recombinants, and reduced the ratio of true recombinants to putative recombinants (i.e. the number of PCR screens required to find true recombinants in five combinations (Fig. 7.2). Nevertheless, four combinations only mating on spider medium produced recombinants but three of them involved the same strain RIHO11, implying that spider medium is only more likely to yield recombinants for some specific combinations (boxes with black outlines in Fig. 7.1). Repeated mating attempts for the same strain combination yielded comparable results both in terms of the numbers of recombinants obtained (Fig 7.2a) and the frequency of recombinants relative to the number of large colonies on the initial MPA plates (putative recombinants; Fig 7.2b).

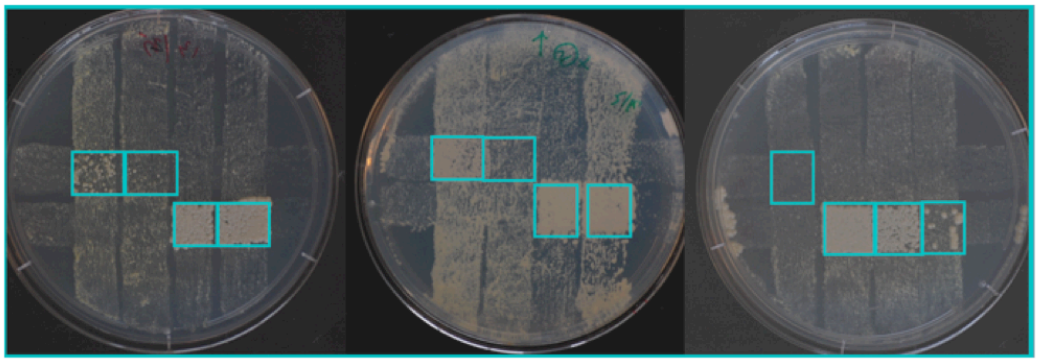
For 10 combinations no recombinants were obtained in at least three attempts (Fig.7.1). Five of these involved one strain, FJ11α. In mating involving this strain only very few large colonies appeared on the first minimal plus MPA medium (MM+MPA (I) in Fig. 6.1). Colonies from the final YPD plates did not contain both *MTL* alleles by PCR. FJ11α was also unable to mate with the universal tester strain 3710 (Magee et al., 2002) (section 2.12.3.). The tester strain is an MPA-resistant auxotrophic *MTL***a** derivative of the laboratory strain SC5314; when it mates with

an α prototrophic strain, the recombinants can be selected on minimal plates containing MPA. All other *MTL*α strains tested could mate with 3710 and all *MTL***a** strains tested could mate with universal tester strain 3685 (Magee et al., 2002), an MPA-resistant auxotrophic α derivative of the laboratory strain SC5314 (Fig. 7.3). To further investigate the reason for FJ11α's failure to mate I tried to mate the *MTL***a** derivative of FJ11 with universal tester strain 3685. This yielded recombinants, and thus it appeared likely that a defect in FJ11's *MTL*α locus was responsible for FJ11α's inability to mate. Indeed when FJ11's *MTL*α locus was sequenced (GenBank JN099704), I found that its *MTL* alpha 1 gene contained a stop-codon mutation (Fig. 7.4) that leads to truncation of the gene product which is essential for mating (Tsong et al., 2003).

For the remaining five unsuccessful matings each of the parents could mate with other strains and the universal tester strains (section 2.12.3.). Given the level of reproducibility of the method (Fig. 7.2) and the failure to recover recombinants in three attempts for each of these combinations (involving the PCR testing of several hundred colonies from patches on YPD+NAT plates except in some cases where only very few large colonies appeared on minimal plus MPA medium), the most likely explanation of these results is that the partners in these combinations are incompatible. Both *MTL* alleles were detected initially on patches from YPD+NAT plates, however, none of them was shown positive in the colonies on the final YPD plates (Fig. 6.1, also refer to section 2.12.2.).

As shown above different strain combinations gave different numbers of recombinants, reproducibly, which could be a quantitative indicator of mating

compatibility. I considered ways in which I could quantify the number of recombinants produced. However this does not seem feasible because it is very hard to calculate the total number of parental cells available for mating and there is no easy way to verify each putative recombinant on MM medium (large colonies, refer to Fig. 6.1).



	OD8916α	W43α	AU90a	FJ26a		YSU63α	FJ11α	W43a	AU35a		W17α	RIHO11a	FJ11a	KO2c-a
3710a	2	1				3	0				1			
3685α			3	3				3	3			3	2	2

Figure 7.3. Mating with universal tester strains to test the mating capability of clinical isolates.

MTL homozygous derivatives of clinical isolates (vertical stripes), were mated with tester strains 3710 and 3685 (horizontal stripes) and replica plated on MM+MPA (5 μg/ml) plates, allowing only growth of recombinants. The recombinants were further purified on MM+MPA (10 μg/ml) plates and verified by PCR for the presence of both *MTL* alleles (data not shown). The bottom panel is a graphic representation of the results, showing the names of the strains mated and the outcome. Scores were given to the clinical isolates according to their ability to mate with the universal tester strains. 0 indicates no colonies were obtained from the crossing square, 1 indicates less than 10 colonies were obtained from the crossing square, 2 indicates more than 10 but less than a full square of recombinants was obtained and 3 indicates that a full square of recombinants was obtained from the crossing.

Figure 7.4. Alignment of FJ11α *MTL*α1 and SC5314 *MTL*α1 (data from Stanford *C. albicans* database).

(A) DNA sequences: point mutation is marked red. (B) Amino acid sequences.

(A)

```
FJ11alphaMTL1     1 ATGGGAAATAAAAAAAAAACTAGAAAGACAGTACCTAAAGAATTCATTTC  50
MTLalpha1ORF      1 ATGGGAAATAAAAAAAAAACTAGAAAGACAGTACCTAAAGAATTCATTTC  50
                    **************************************************

FJ11alphaMTL1    51 TCTTTTTCGAGTACATTCTGGTCGCGATGCTCCAAGAAGAGACACAAGAG 100
MTLalpha1ORF     51 TCTTTTTCGAGTACATTCTGGTCGCGATGCTCCAAGAAGAGACACAAGAG 100
                    **************************************************

FJ11alphaMTL1   101 AAGTTCAAAAAAGTAAAAAGCATGGGTTTAGATTCACTTCGTTACCAGAT 150
MTLalpha1ORF    101 AAGTTCAAAAAAGTAAAAAGCATGGGTTTAGATTCACTTCGTTACCAGAT 150
                    **************************************************

FJ11alphaMTL1   151 CTTCCTGTTGCTAGTAATGCTTTACAAGAATTATTGCTTGAATATGGTTT 200
MTLalpha1ORF    151 CTTCCTGTTGCTAGTAATGCTTTACAAGAATTATTGCTTGAATATGGTTT 200
                    **************************************************

FJ11alphaMTL1   201 ACTAAATGACATAAAATGGGATTCAAAAGGTTTGAAGGCTAGTAAAAACA 250
MTLalpha1ORF    201 ACTAAATGACATAAAATGGGATTCAAAAGGTTTGAAGGCTAGTAAAAACA 250
                    **************************************************

FJ11alphaMTL1   251 AAAAGACAAAGCTAAAACCAATTAATTCTTTTATTGCATTTAGATCTTTT 300
MTLalpha1ORF    251 AAAAGACAAAGCTAAAACCAATTAATTCTTTTATTGCATTTAGATCTTTT 300
                    **************************************************

FJ11alphaMTL1   301 TACTCAAGAACTATTTCCAATCCGGAACACCAACGAGAACTATCATCTAA 350
MTLalpha1ORF    301 TACTCAAGAACTATTTCCAATCCGGAACACCAACGAGAACTATCATCTAA 350
                    **************************************************

FJ11alphaMTL1   351 ATTAGCGGATGTTTGAACTCAAGAATCAAATCAAGAAGTATGGAAACAAT 400
MTLalpha1ORF    351 ATTAGCGGATGTTTGGACTCAAGAATCAAATCAAGAAGTATGGAAACAAT 400
                    ********** ********************************

FJ11alphaMTL1   401 ACACACAATCATACAATAATTATTTACTTCTTCCTGATGCAAAATTGAAT 450
MTLalpha1ORF    401 ACACACAATCATACAATAATTATTTACTTCTTCCTGATGCAAAATTGAAT 450
                    **************************************************

FJ11alphaMTL1   451 TTTGTGGACTGGTTATGCGAGGCTTTGGATTACACGATTGATAATACAAC 500
MTLalpha1ORF    451 TTTGTGGACTGGTTATGCGAGGCTTTGGATTACACGATTGATAATACAAC 500
                    **************************************************

FJ11alphaMTL1   501 GCCCCAGATAGAAGATATTCTGTTAACTAGTTACAATCAGTTACTATCGG 550
MTLalpha1ORF    501 GCCCCAGATAGAAGATATTCTGTTAACTAGTTACAATCAGTTACTATCGG 550
                    **************************************************

FJ11alphaMTL1   551 GTACAATTGAGGATGTTTACATAATGAAGTAAAATATACAGCAAGTGATA 600
MTLalpha1ORF    551 GTACAATTGAGGATGTTTACATAATGAAGTAA 582
```

(B)

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FJ11alphaMTL1aa   1 MGNKKKTRKTVPKEFISLNRVHSGRDAPRRDTREVQKSKKHGNRFTSLPD  50
MTLalpha1aa       1 MGNKKKTRKTVPKEFISLNRVHSGRDAPRRDTREVQKSKKHGNRFTSLPD  50
                    **************************************************

FJ11alphaMTL1aa  51 LPVASNALQELLLEYGLLNDIKWDSKGLKASKNKKTKLKPINSNIANRSN 100
MTLalpha1aa      51 LPVASNALQELLLEYGLLNDIKWDSKGLKASKNKKTKLKPINSNIANRSN 100
                    **************************************************

FJ11alphaMTL1aa 101 YSRTISNPEHQRELSSKLADV---------------------------- 121
MTLalpha1aa     101 YSRTISNPEHQRELSSKLADVWTQESNQEVWKQYTQSYNNYLLLPDAKLN 150
                    ********************

FJ11alphaMTL1aa 122 ----------------------------------------- 121
MTLalpha1aa     151 NVDWLCEALDYTIDNTTPQIEDISLTSYNQLLSGTIEDVYIMK 19
```

Even though the method does not allow exact quantitation of the frequency with which matings produce recombinants, it was still possible to categorize compatibility levels on the basis that recombinants were easier to obtain from some strain combinations than others. I used three categories as shown in Figure 7.1. Dark blue squares indicate strain combinations yielding ≥7 recombinants per experiment and in which ≥10 % of all colonies tested were recombinants. Light blue boxes indicate combinations where fewer recombinants were obtained and grey boxes were combinations yielding no recombinants in ≥3 independent experiments (some crossings involving FJ11α were attempted only once after it was discovered that a genetic defect prevented it from mating). From Figure 7.1 it is evident that recombinants from seven matings were more easily obtained compared to the other eight matings. Mating of clinical isolates with universal mating tester strains was performed to validate the mating method (Fig. 7.3). It seems that if a strain could mate with the universal tester strain, this strain would be able to mate with at least one clinical isolate (Fig.7.5). Otherwise no strong correlation between the scores was detected. It seems that mating with universal test strains could not predict how well a strain would mate with other wild type isolates.

Using this compatibility categorization I could address the question of whether the GPG is protected from sex by barriers which prevent its members from mating. My results show that mating could happen both between GPG strains and between GPG and non-GPG strains (Fig 7.1). However it appears that incompatibility happens more often between GPG strains than between non-GPG strains (indeed all non-GPG strains capable of mating could mate with each other, while only half of GPG strains can mate with each other). In addition, 6 out of 7 more frequent matings (dark-blue combinations in Fig. 7.1) involve non-GPG strains. Thus, one reason why GPG strains are more clonal, could be that they are less likely to mate and produce recombinants. But the sample size was too small to show a statistically significant difference in mating ability between GPG and nonGPG groups.

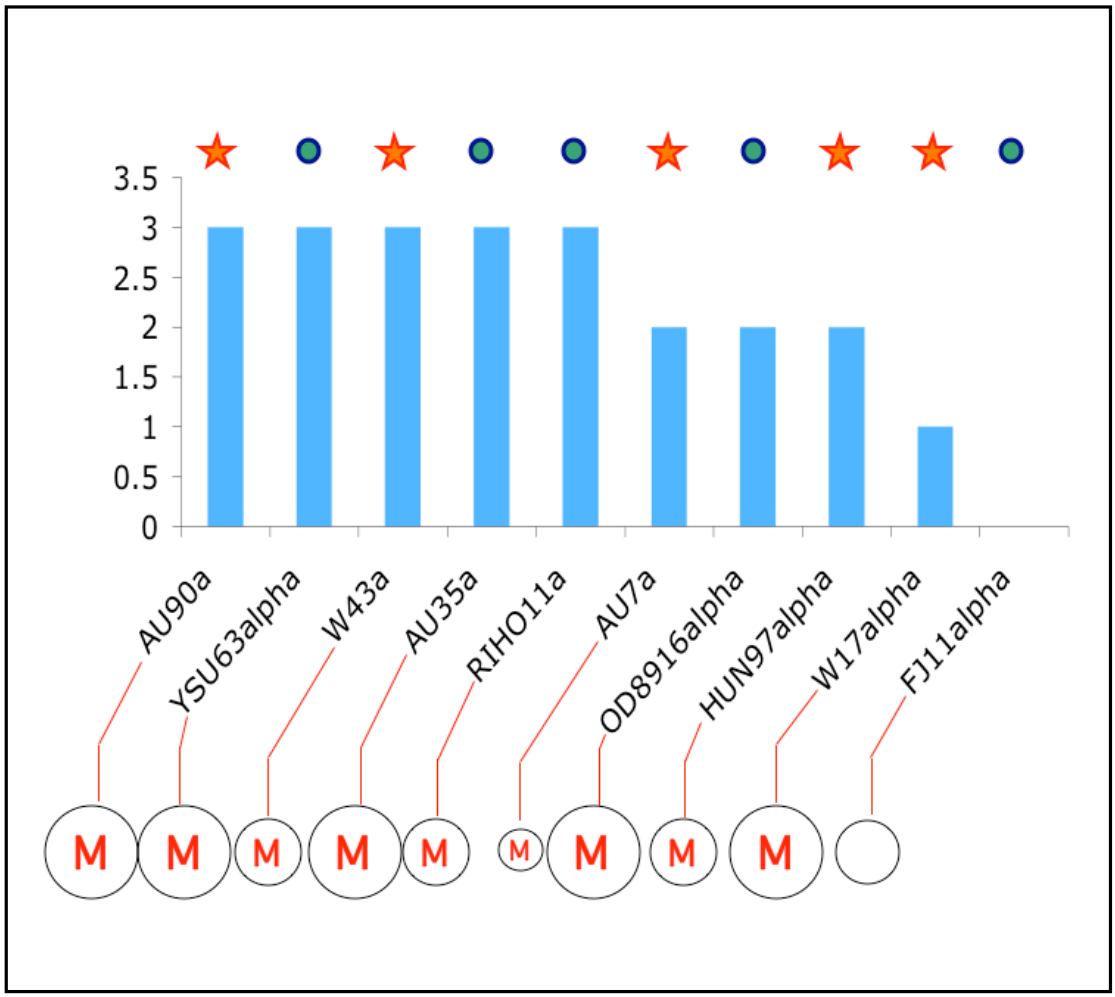


Figure 7.5. Bar graph showing semi-quantitative mating frequencies of clinical isolates with the universal tester strains.

Scores were given to the clinical isolates according to their ability to mate with the universal tester strains, as described in Fig. 7.3. (except for HUN97α and AU7a which were not shown in Fig. 7.3.) Different sizes M labels are used to denote ability of strains to mate with clinical isolates (as described in Fig. 7.2). GPG strains are labeled with stars non-GPG strains with circles.

7.3. Recombinants were usually mononucleate and often do not completely retain the parental genomes during the selection procedure

7.3.1. Recombinants were usually mononucleate with DNA content exceeding that of each parent

To eliminate the possibility that the recombinants are heterokaryons or products of transfer of a small number of chromosomes, two or three representative recombinants of each successful mating and their parental strains were examined for their DNA content by FACS analysis (section 2.14.1.) and for the number of nuclei by DAPI staining (section 2.13.1). Most parent strains had DNA contents similar to the lab strain SC5314, included as a control in some experiments. The exception was W17α. For this strain the FACS analysis indicated an increased DNA content. Furthermore fluorescence intensity was not as clearly distributed between two distinct peaks (G1 and G2 phases) as in other strains at the exponential phase (Fig. 7.6). Fluorescence microscopy revealed one factor that may contribute to this abnormality. Some W17α cells (<5%) were multinucleate (Fig. 7.7). For all other parental strains only mononucleate cells were observed.

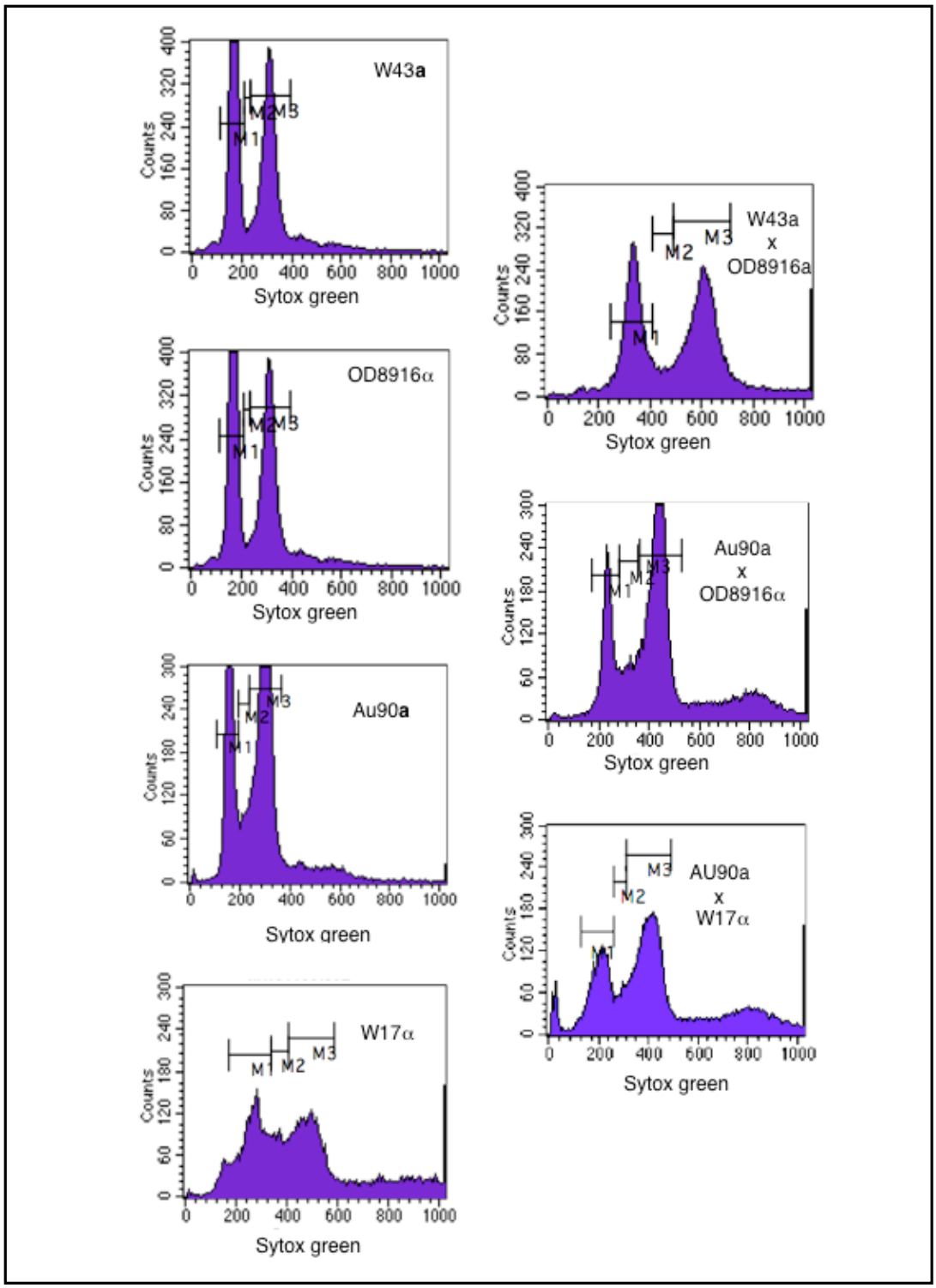


Figure 7.6. Examples of FACS analyses of parental strains and recombinants with SYTOX staining.

Four parents W43**a**, OD8916α, AU90**a**, W17α and their recombinants W43**a** x OD8916α, AU90**a** x OD8916α, AU90**a** x W17α are shown. M1 and M3 are peaks in the DNA content distribution corresponding to exponentially growing cells in their G1 and G2 phases, respectively. M2 corresponds to S phase cells. The peak regions were selected visually based on the shape of the histogram.

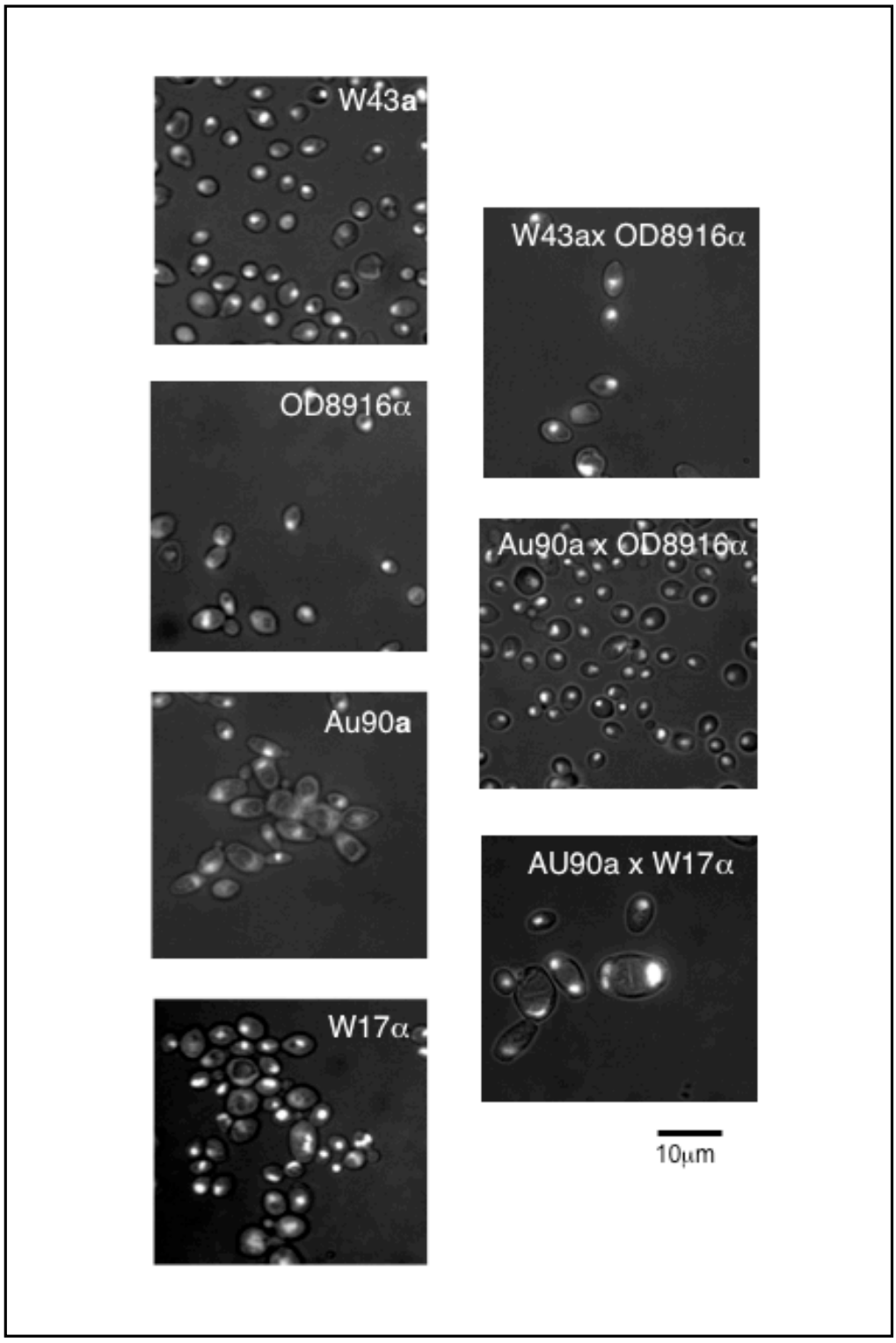


Figure 7.7. Examples of staining the nuclei of parental strains and recombinants with DAPI.

The strains are the same as in Fig. 7.6. The photographic images were generated by combining fluorescent images and bright field images.

Recombinants were, in general, mononucleate (Fig. 7.7 & Appendix 10.11), demonstrating that the two parental genomes had been united by karyogamy, i,e. verifying that they were true recombinants. However among some recombinants derived from W17α occasionally some multinucleate cells (<5% of the population) were detected (data not shown).

Most recombinant cells had DNA contents exceeding that of individual parents. Half of them had DNA contents close to the sum of that of both parents, but the other half contained considerably less than the sum of parental DNA contents (Fig. 7.1). I cannot exclude the possibility that differences in the ratio of mitochondrial to nuclear DNA between parents and recombinants contributed to these results, but the most likely explanation is that recombinants were originally tetraploid but then lost some parental chromosomes during the selection procedure.

7.3.2. Chromosome loss is unlikely to prevent recovery of recombinants

Dual drug resistance is crucial to the recovery of recombinants but selection is only against one drug at a time and thus during each round of selection, chromosome loss could lead to loss of the marker not under selection, in particular if that marker were to reduce fitness in the absence of the drug. Figure 7.8 shows the results of two three-primer multiplex PCRs with primer set TS2Fpr/pENOpr/TS1se and pACTFpf/CaACTpr/CaNATpr (refer to Table 2.7 for primer sequences), which can determine if chromosomes with or without drug resistance genes are present. If the marked chromosomes were still present, along with the unmarked chromosomes, two bands would be detected. The presence of resistance markers in parental strains and one or two representative recombinants from each crossing after the final step of the selection procedure (growth on YPD prior to PCR test for presence of both *MTL* alleles) was investigated. The vast majority of recombinants had retained the markers (Fig. 7.8). The PCR analysis showed that all parental strains contained the correct resistance cassettes and that *MPA*^{r}-cassette-marked and unmarked versions of chromosome 7 and *NAT*^{r}-marked and unmarked version of chromosome 1 were usually present in the recombinants. However, one of the recombinants that occasionally formed multinucleate cells (Au90**a** x W17α recombinants) had lost the markers, apparently a rearrangement had occurred in the region of chr7 into which the *MPA*^{r} cassette was integrated preventing any amplification of the region targeted by the PCR primers.

Not only the resistance markers were present in recombinants after drug resistance selection, I also assessed by PCR the stability of both resistance markers in the absence of selection in twenty-seven recombinants derived from all 15 successful matings after 100 generations of transfer in YPD medium without drugs (Fig. 7.8c). Only a small portion of marker loss was detectable from recombinants and the rate of marker loss was significantly lower than the rate of DNA content reduction of the recombinants (as will be described below in section 8.3). These results indicated that chromosome loss of the recombinants was nonrandom[1] but this would have little effect on the selection procedure, as copies of chromosomes with both drug resistance markers were retained after selection, most of them even retained after 100 generations of transfer on nonselective YPD medium.

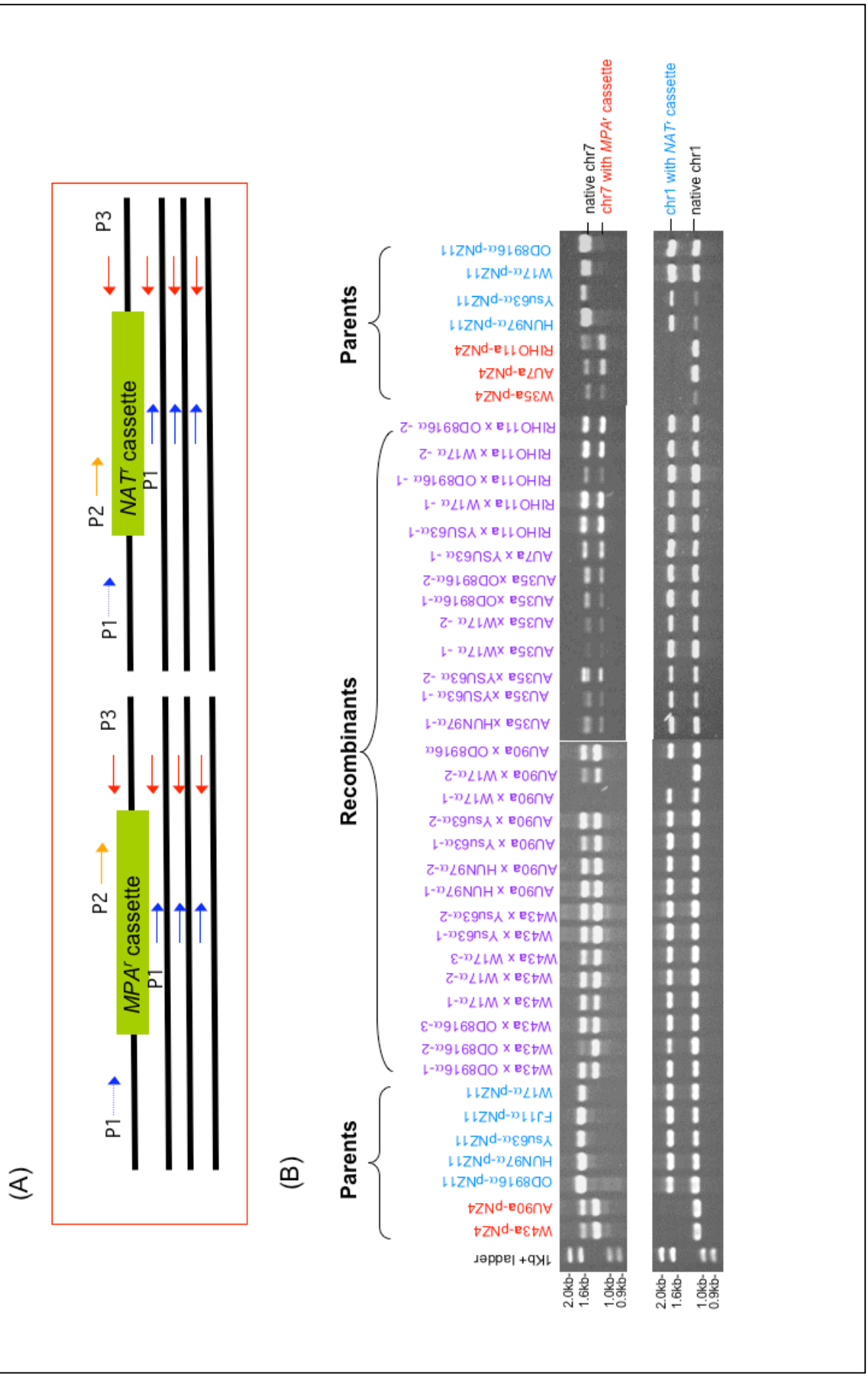
It is reported that *C. albicans* can undergo a parasexual cycle through random chromosome loss (Fig. 1.6) (Forche et al., 2008). Forche et al. reported that some of the progeny could be *MTL* homozygotes and were mating competent. However, this evidence was only discovered in recombinants arising from mating between the lab strain SC5314 derivatives, and in a stressful condition (Forche et al., 2008). To test if mating between clinical isolates resembles the same pattern, i.e. if some of the progeny would lost *MTL* heterozygousity, 27 single colonies were randomly picked on YPD plates, from six individual recombinants (of two recombinations), after these recombinants were serial transferred in YPD medium for 100 generations. Their *MTL* loci were checked by multiplex colony PCR (section 2.9.6). All of the 27 recombinants tested after serial propagation on YPD medium for 100 generations

[1] The actual process of chromosome loss was probably random. However, subsequent selection lead to predominance of clones with specific chromosome loss events is more frequent than others.

remained *MTL* heterozygous (Fig. 7.9). Therefore, *MTL* markers are also not lost because selection seems to favor *MTL* a/α in YPD medium.

Moreover, while chromosome loss occurred during the stepwise selection, there was no correlation between DNA content versus number of recombinants recovered (Fig. 7.11), as would be expected if rapid chromosome loss diminishes the chances of discovering recombinants with the selection procedure.

All of these evidences indicated that marker loss did not affect the selection procedure for recovering recombinants.


(A)
P1
P2
P3
MPAr cassette
NATr cassette
(B)
Parents
Recombinants
Parents
1Kb+ ladder
W43a-pNZ4
AU90a-pNZ4
OD8916α-pNZ11
HUN97α-pNZ11
Ysu63α-pNZ11
F111α-pNZ11
W17α-pNZ11
W43a x OD8916α-1
W43a x OD8916α-2
W43a x OD8916α-3
W43a x W17α-1
W43a x W17α-2
W43a x W17α-3
W43a x Ysu63α-1
W43a x Ysu63α-2
AU90a x HUN97α-1
AU90a x HUN97α-2
AU90a x Ysu63α-1
AU90a x Ysu63α-2
AU90a x W17α-1
AU90a x W17α-2
AU90a x OD8916α
AU35a xHUN97α-1
AU35a xYSU63α -1
AU35a xYSU63α -2
AU35a xW17α -1
AU35a xW17α -2
AU35a xOD8916α-1
AU35a xOD8916α-2
AU7a x YSU63α -1
RIHO11a x YSU63α-1
RIHO11a x W17α -1
RIHO11a x OD8916α -1
RIHO11a x W17α -2
RIHO11a x OD8916α -2
W35a-pNZ4
AU7a-pNZ4
RIHO11a-pNZ4
HUN97α-pNZ11
Ysu63α-pNZ11
W17α-pNZ11
OD8916α-pNZ11
native chr7
chr7 with MPAr cassette
chr1 with NATr cassette
native chr1
2.0kb-
1.6kb-
1.0kb-
0.9kb-

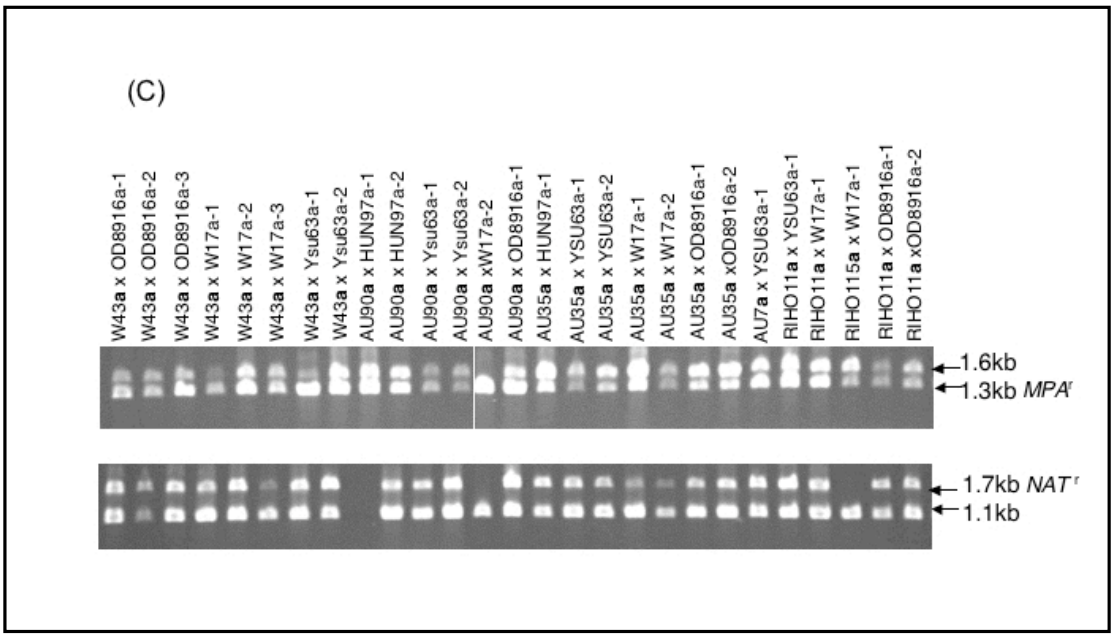


Figure 7.8. Presence of parental resistance markers in recombinants when first recovered after the mating procedures and after serial propagation for 100 generations.

(A) Principle of three-primer multiplex PCR used to determine if cells contain chromosomes with or without drug resistance genes. If the marker-bearing chromosome is retained, along with the unmarked second allele, two different-sized products are formed. Note: Both *MPA*^{r} and *NAT*^{r} cassettes were insertions. P1 also binds to the left of the resistance cassettes and should give a bigger PCR product in theory from the chromosomes with the resistance cassettes. However, this could not be detected using this PCR program. (B) PCR detection of resistance cassettes in single colonies of 28 representative recombinants and their parents from all successful matings (using primers TS2Fpr/pENOpr/TS1se for detection of the *MPA*^{r} cassette and primers pACTFpf/CaACTpr/CaNATpr for detection of the *NAT*^{r} cassette). Primer sequences are listed in Table 2.7. (C) PCR detection of resistance cassettes in 27 single recombinant colonies of 15 different recombinations after 100 generations transfer in YPD medium. The positions of the 1.3 kb and 1.7 kb products for the *MPA*^{r} and *NAT*^{r} cassettes are marked by arrows on the right sides of the gels, as are the sizes of the insertion site amplicons without inserted cassettes (1.6 kb and 1.1 kb, respectively).

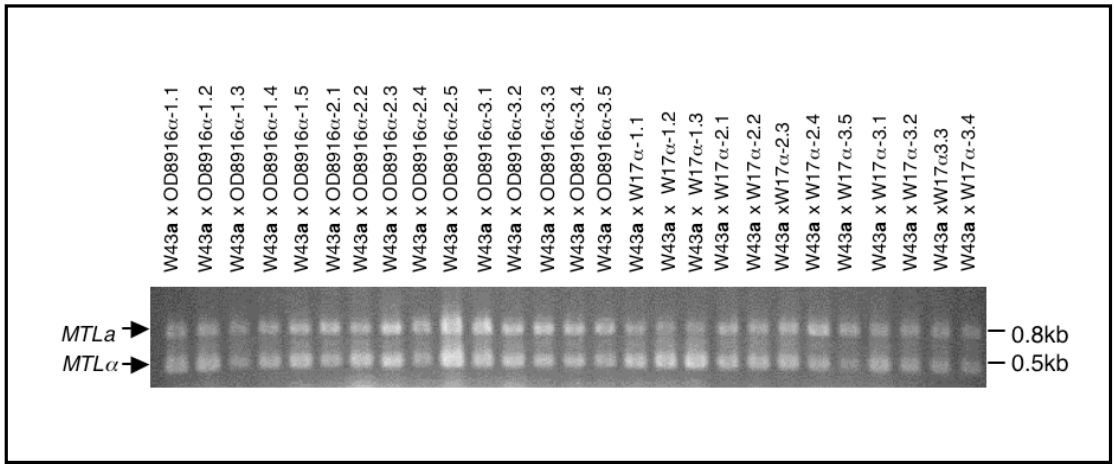


Figure 7.9. Both *MTL* alleles are retained in recombinants after 100 generations.

Both mating type loci (0.8 and 0.5 kb as indicated on the right and marked with arrows on the left) were detected in all 27 recombinants tested after serial propagation for 100 generations. The offspring from six recombinants arising from two matings were streaked and 3 to 5 individual colonies were tested from each recombinant. The names above the figure show the parents, followed by the numbers of the recombinants, followed by the number of the colony for the recombinant that was used.

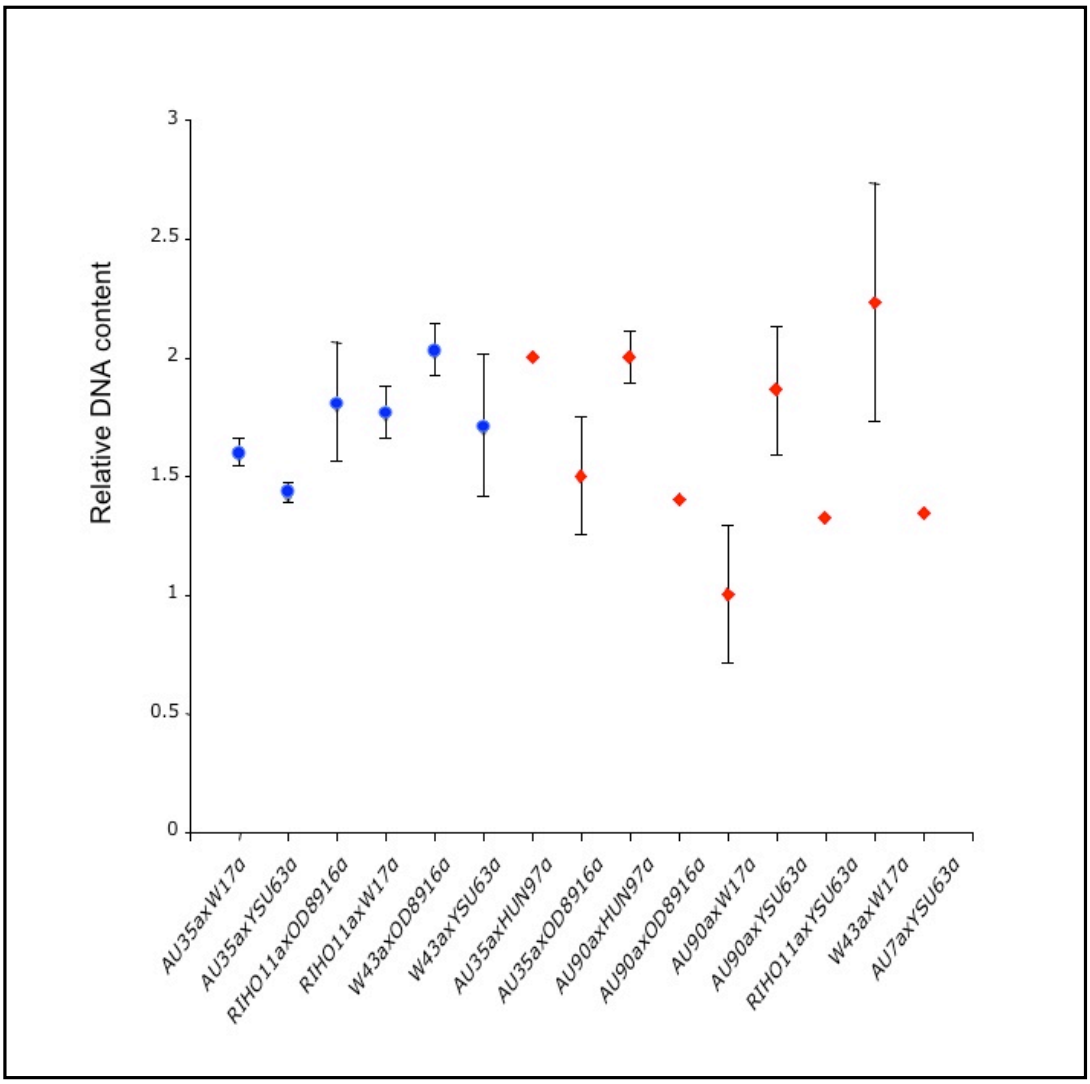


Figure 7.10. Relationship between the (average ± s.d, if more than 1 recombinants were obtained) DNA content of recombinants relative to that of the sum of their both parents and the mating efficiency of each successful mating.

Blue dots indicate combinations yielding ≥7 recombinants per experiment and in which ≥10 % of all colonies tested were recombinants. Red rhombus indicates combinations where fewer recombinants were obtained.

7.3.3. Chromosomes without drug resistance genes were lost frequently during selection

Figure 7.8 b shows that recombinants usually contained unmarked copies of chr1 and chr7. However, individual copies of chromosomes without drug resistance genes were lost frequently during the selection procedure. This was already suspected based on the DNA content of the recombinants. It was corroborated by experiments in which known heterozygosity in one parent's genome allowed the fate of one individual parental chromosome to be followed. Heterozygosity of other markers on the chromosomes used for the integration of resistance markers of one parent allowed identification of the retention of the non-resistance cassette-bearing copy of a chromosome without markers in recombinants as shown in Figure 7.11. Locus *SSR1* is located on chr 7, one copy of which also bears MPA^r; Locus MCEA1 is located on chromosome 1, one copy of which carries NAT^r. Figure 7.11 shows recombinants from two matings: W43*MTL*a x YSU63*MTL*α and AU90*MTL*a x YSU63*MTL*α. W43*MTL*a and AU90*MTL*a have two distinguishable alleles of MCEA1, while YSU63*MTL*α has two distinguishable alleles of *SSR1*. Amplification of the polymorphic MCEA1 region with primers MCEA1outpf2/MCEA1outpr2 on chr1 from parent strains W43*MTL***a** and AU90 *MTL***a** produces two different–sized PCR products, 2.8 kb and 2.6 kb; amplification from parent YSU63*MTL*α only produces the 2.6 kb product. Loss of the 2.8 kb product in W43*MTL***a** x YSU63*MTL*α and AU90*MTL***a** x YSU63*MTL*α recombinants indicates loss of the W43*MTL***a** and the AU90*MTL***a**–derived resistance cassette-free copy of chromosome 1. Likewise an YSU63*MTL*α-specific polymorphism detectable using primers SSR1-2/SSR1-3, in *SSR1* on chromosome1

generates a PCR product of 339 bp distinct from the one allele common to all three strains (267 bp) and another product (279 bp) common to the two *MTL***a** strains. Loss of the 339 bp product in W43*MTL***a** x YSU63*MTL*α and AU90*MTL***a** x YSU63*MTL*α recombinants indicates loss of the resistance cassette-free copy of the YSU63*MTL*α-derived chr7. It was found that approximately 50% of recombinants from the two combinations had lost one copy of chromosome 1 derived from parent W43*MTL***a** or Au90 *MTL***a** (Fig. 7.11a). Likewise, approximately 50 % of recombinants from the two crossings had lost one parental copy of chromosome 7 derived from YSU63*MTL*α (Fig. 7.11b)**.** Only three out of nine recombinants still retained both the unique copy of chromosome 1 from W43*MTL***a** or Au90 *MTL***a** and the unique copy of chr 7 present in YSU63*MTL*α, and two recombinants had simultaneously lost the unique copies of chr 1 and chr 7.

In one crossing, W43*MTL***a** x W17*MTL*α, polymorphisms at the *PNG2* locus (Zhang et al., 2010) allowed me to follow the fate of all four parental copies of chromosome 2 (Fig 7.12). *PNG2* is located on chromosome 2, bearing no selectable markers. Heterozygosity of two parents, W43*MTL***a** and W17*MTL*α, allows identification of individual parental chromosomes in recombinants. Figure 7.12 shows all four chromosomes were frequently lost, but loss was not random. Some recombinants retained all copies, some lost one and some two, with a significant bias (P=0.011; Freeman-Halton extension of Fisher test) towards loss of one particular W17 *MTL*α derived copy (394 bp on Fig. 7.12). Since there are no drug resistance markers on chromosome 2 this bias is most likely a result of selection as recombinants adapt to growth in YPD medium.

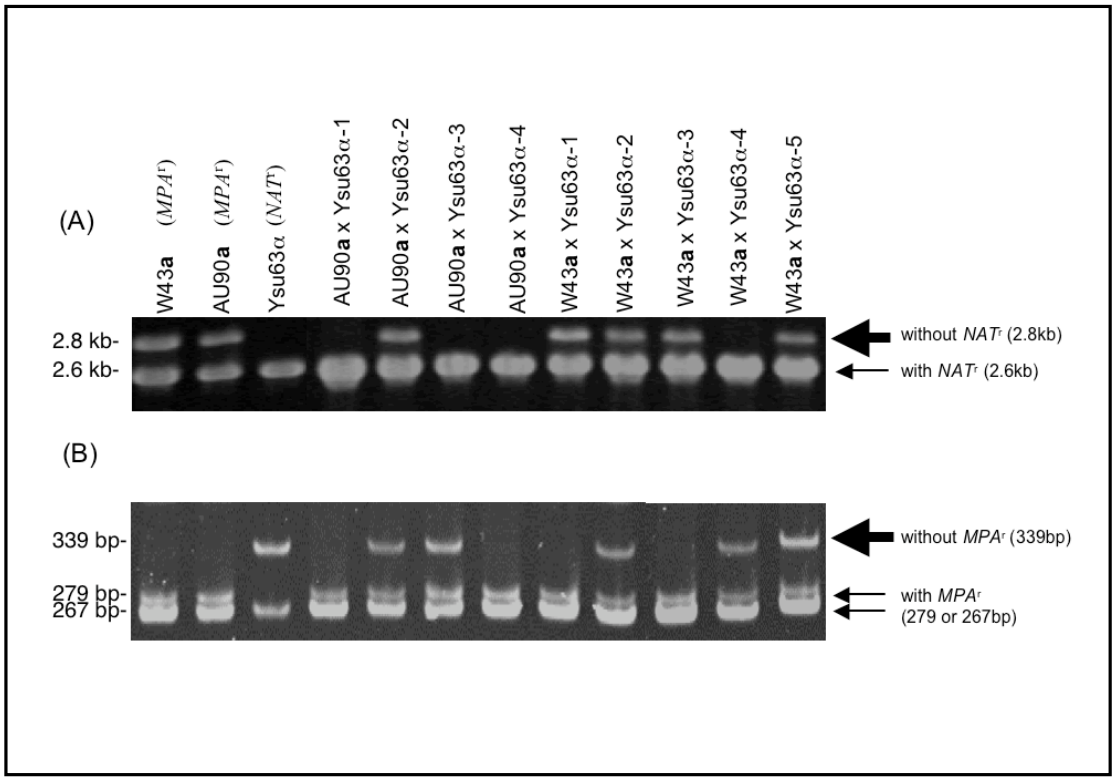


Figure 7.11. PCR analysis of retention of resistance cassette–free copies of chromosomes 1 and 7 in the offspring of two matings.

(A) Amplification of the polymorphic MCEA1 locus of chr 1 with primers MCEA1outpf2/MCEA1outpr2 (Zhang et al., 2009) from parent strains W43*MTL***a** and AU90 *MTL***a** produces two products of 2.8 kb and 2.6 kb. Amplification from parent YSU63*MTL*α (*NAT*^{r}) only produces the 2.6 kb product. Since the *NAT*^{r} cassette is integrated on chr 1 in YSU63*MTL*α (*NAT*^{r}), amplification of a 2.8 kb band in recombinants indicates retention of one, or several, copies of a non-resistance cassette-bearing copy of chr 1. (B) Likewise a YSU63*MTL*α-specific polymorphism detectable in *SSR1* on chr 7 (Zhou, 2011) using primers SSR1-2/SSR1-3, generates a PCR product of 339 bp distinct from the one allele common to all three strains (267 bp) and another product (279 bp) common to the two *MTL***a** strains. Since only W43*MTL***a** (*MPA*^{r}) and AU90*MTL***a** (*MPA*^{r}) have the *MPA*^{r} cassette integrated on chr 7, amplification of the YSU63*MTL*α (*NAT*^{r})–unique 339 bp PCR product in recombinants indicates presence of the non-resistance cassette-bearing copy of chr 7 derived from YSU63*MTL*α (*NAT*^{r}). Sizes of the products are marked on the right side of the gel. All primer sequences are listed in Table 2.7.

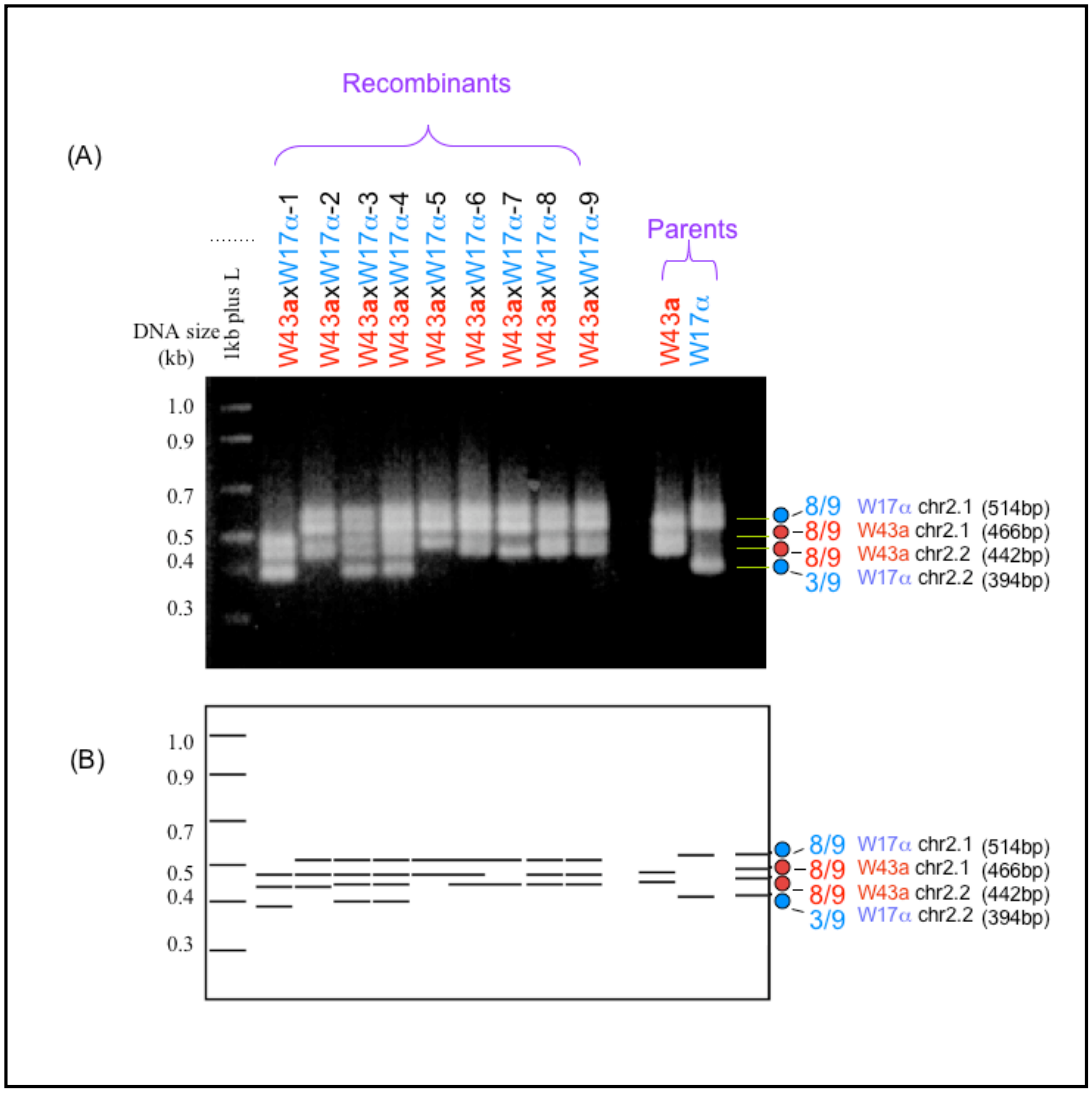


Figure 7.12. PCR analysis of retention of individual copies of chromosome 2 in offspring from the mating of W43*MTL*a and W17*MTL*α.

The strains W43*MTL*a and W17*MTL*α have four distinguishable alleles of *PNG2*, located on chromosome 2 (Zhang et al., 2010). (A) Image of a gel showing the four different *PNG2* alleles in 9 recombinants and the parents. On the right, markers indicate the position of the PCR products produced by the four different alleles, the fraction of recombinants retaining each allele, the origin of the allele and, in brackets), its size. (B) Since *PNG2* contains a repeat region generating additional artefactual bands (the top band from both of the parents), this panel shows a graphic representation showing only the four different allele-specific bands.

8.0. BENEFIT OF SEX IN *C. ALBICANS*

8.1. Introduction

As described in the previous section, most clinical isolates of *C. albicans* can mate in the laboratory. However, does sex still have any benefit or biological function for this yeast? There is a direct test to determine if sex still has a biological function, namely whether cells that choose sex over clonal reproduction improve, or at least do not diminish, the chance of passing on their genes to future generations. Therefore my objective in this section was to determine: if clinical isolates of *C. albicans* gain the ability to mate by losing one copy of the *MTL* locus, will this make it more or less likely that they will pass on their genes than continuing clonal propagation in the *MTL* heterozygous state. In other words, to identify if sex still had any benefit for *C. albicans*.

It is well known from the literature that sex is more likely to confer benefits, through the generation of new allele combinations, if adaptation to a novel environment is required (Otto and Lenormand, 2002). In the case of *C. albicans,* each tetraploid recombinant can draw on two parental genomes to increase fitness, through chromosome loss and duplication, mitotic recombination and differential expression of four parental alleles. Therefore, I tested if sex increases the chance of survival of parental genes in an environment novel to clinical isolates, YPD medium, using growth rate as a measure of fitness (fitness is commonly defined as the number of offspring a cell can produce. i.e. the ability of a cell to pass on its genes to the next generation (Orr, 2009), and by determining if the number of offspring produced by a recombinant is more than the parent can produce by continuing clonal reproduction.

To do this I used the recombinants described in the previous section (Table 2.1c), their *MTL* homozygous parents (Table 5.1) and the clinical isolates (Table 2.1a) from which they were derived.

8.2 It is difficult to demonstrate that in *C. albicans* sex increases the chance of homozygous parents' passing on their genes to future generations

In *C. albicans*, mating between two diploid cells generates tetraploid progeny, which over time becomes a diploid through chromosome loss (Bennett and Johnson, 2003). Therefore, the initial tetraploid cell only provides the starting material for creating the final diploid. Thus one would not necessarily expect the initial tetraploids to be better adapted to growth in laboratory media than their parents. Indeed they may initially be less fit because of negative epistasis between genes contributed from the parents (Khan et al., 2011). However, having four copies of each gene at their disposal, one would expect that by selectively discarding some of these copies, recombinants should eventually be able to generate a genotype with a fitness that exceeds that of the parents who do not have this additional genetic material as a resource for adaptation.

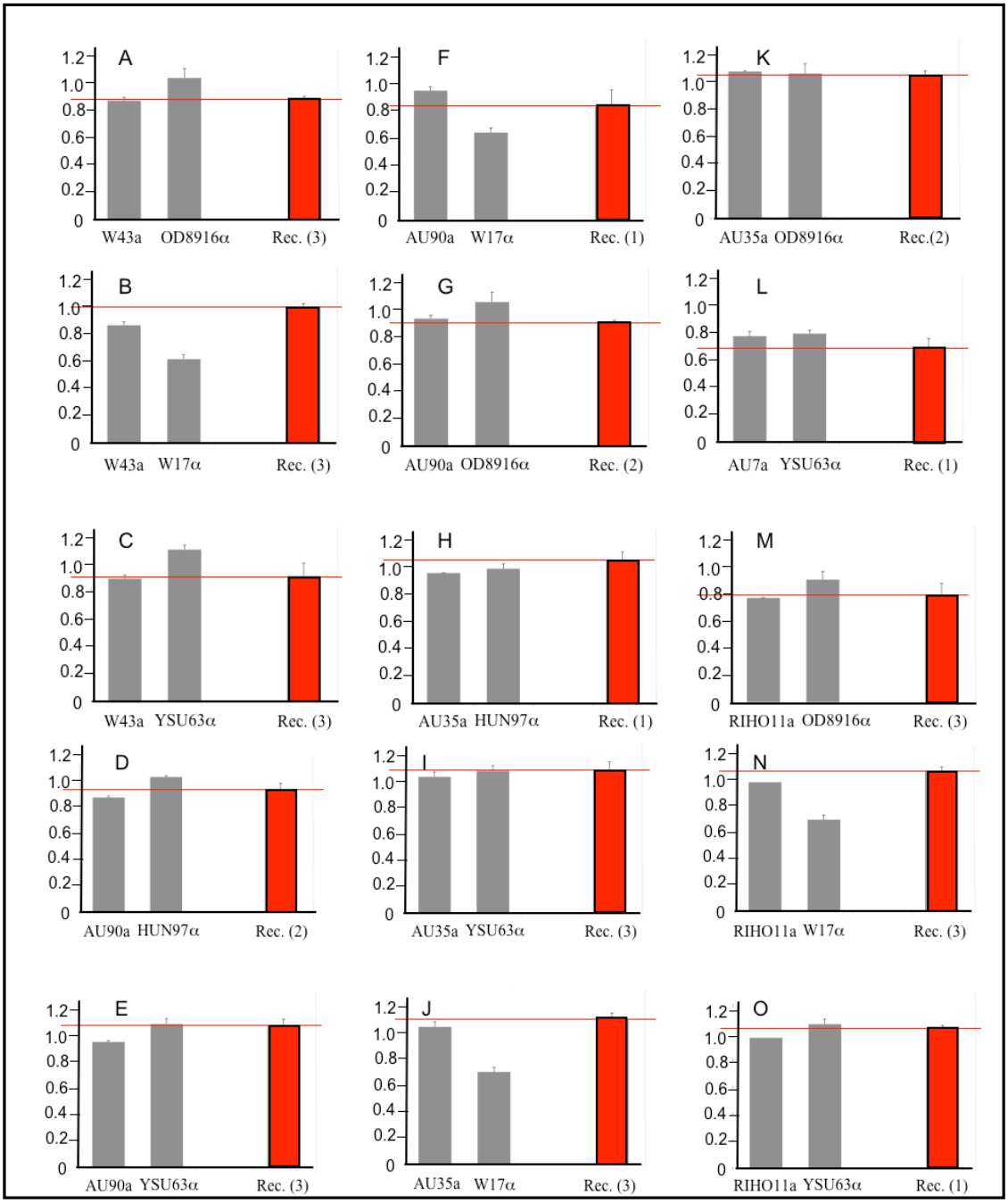


Figure 8.1. Comparison of the average growth rates (±std) of the recombinants and the pair of parents from which they were derived, for the 15 successful matings, when they just isolated.

(A) W43a x OD8916α, (B) W43a x 17α, (C) W43a x YSU63α, (D) AU90a x HUN97α, (E) AU90a x YSU63α, (F) AU90a x W17α, (G) AU90a x OD8916α, (H) AU35a x HUN97α, (I) AU35a x YSU63α, (J) AU35a x W17α, (K) AU35a x OD8916α, (L) AU7a x YSU63α, (M) RIHO11a x OD8916α, (N) RIHO11a x W17α, (O) RIHO11a x YSU63α. Each panel shows data for both parents (grey bars) and for recombinants (Rec.; red bars). In some cases the average growth rate of recombinants is based on several measurements for multiple recombinants, (the number of recombinants is shown in brackets). A red line indicates the average growth rate of the recombinants.

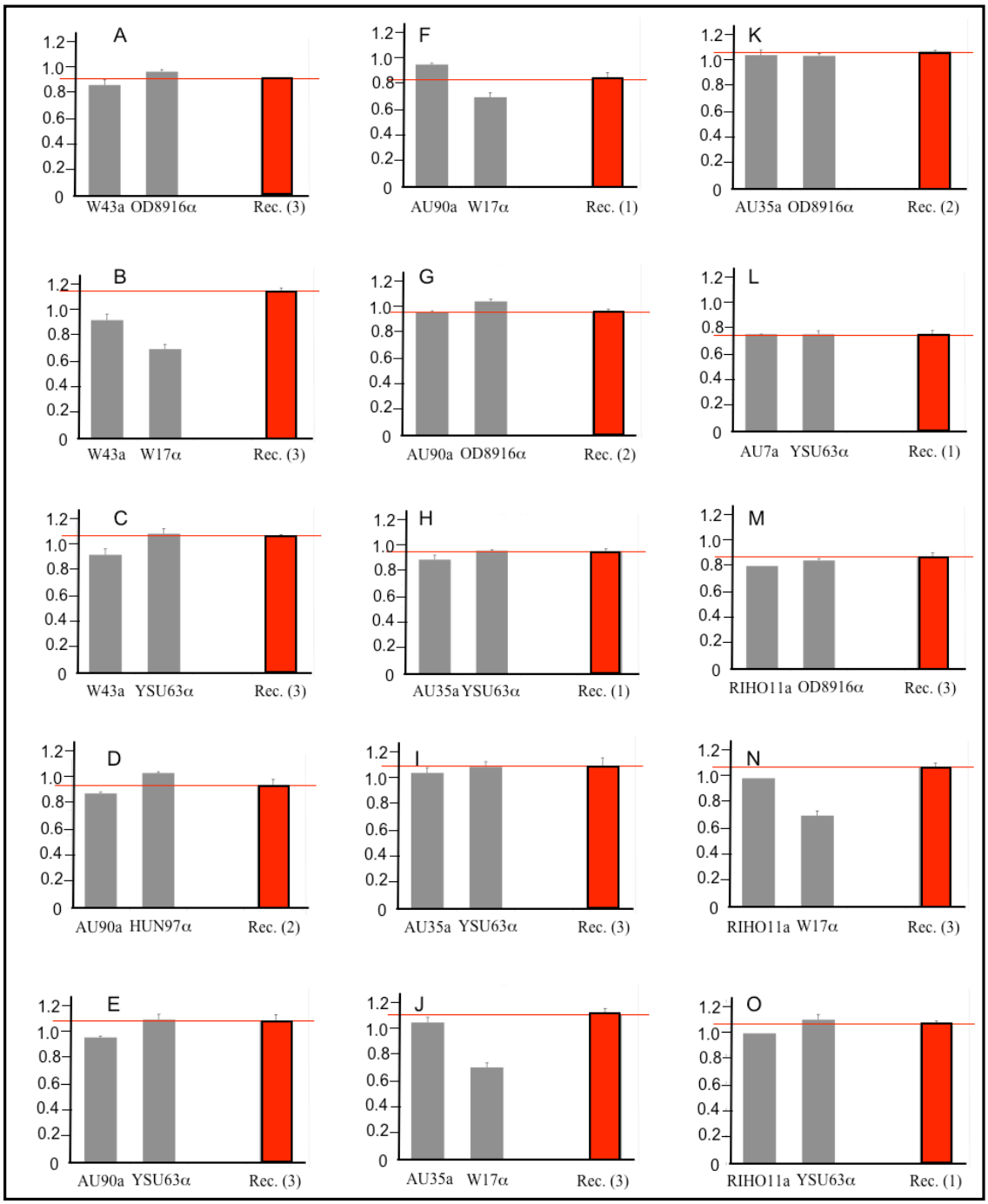


Figure 8.2. Comparison of the average growth rates (±std) of the recombinants and the pair of parents from which they were derived, for the 15 successful matings after propagation for 100 generations in YPD medium.

(A) W43a x OD8916α, (B) W43a x 17α, (C) W43a x YSU63α, (D) AU90a x HUN97α, (E) AU90a x YSU63α, (F) AU90a x W17α, (G) AU90a x OD8916α, (H) AU35a x HUN97α, (I) AU35a x YSU63α, (J) AU35a x W17α, (K) AU35a x OD8916α, (L) AU7a x YSU63α, (M) RIHO11a x OD8916α, (N) RIHO11a x W17α, (O) RIHO11a x YSU63α. Each panel shows data for both parents (grey bars) and for recombinants (Rec.; red bars). In some cases the average growth rate of recombinants is based on several measurements for multiple recombinants, (the number of recombinants is shown in brackets). A red line indicates the average growth rate of the recombinants.

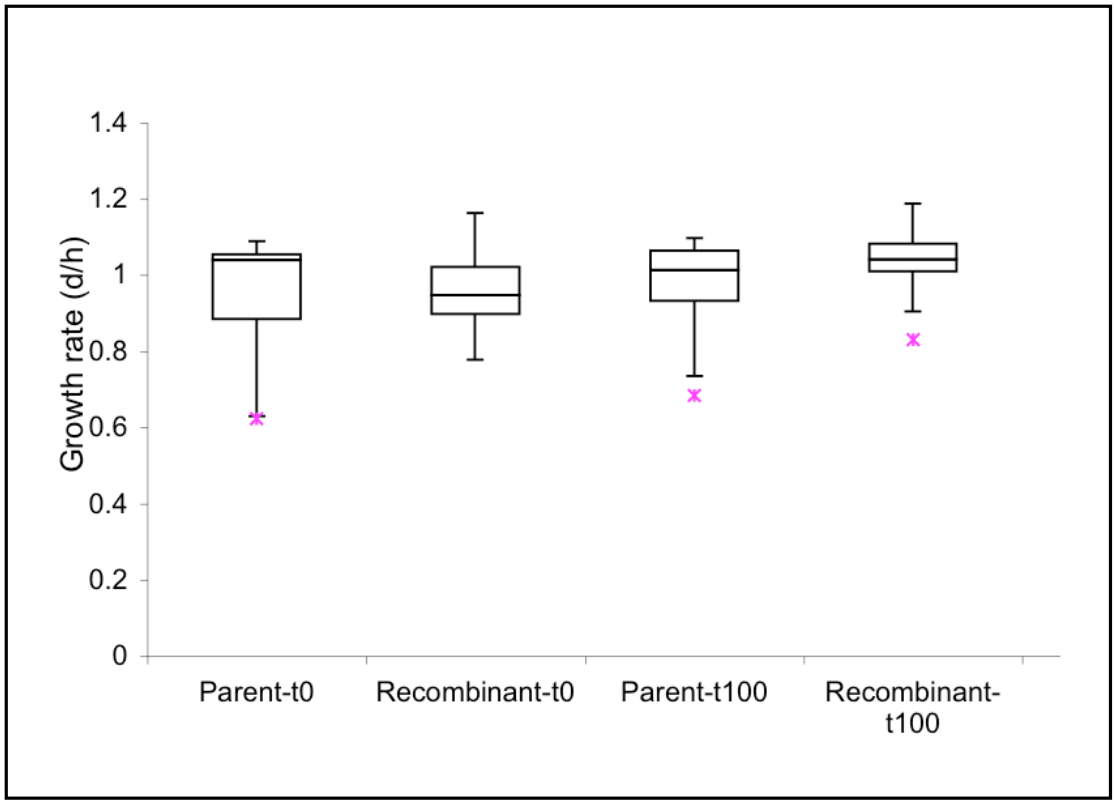


Figure 8.3. Median growth rates of parents and recombinants.

Box plot growth rate comparison between 9 drug resistance-marked homozygote parental strains and 34 recombinants derived from them before (t0) and after 100 generations growth in YPD (t100). Boxes indicate 75th and 25th percentile of the data with a line indicating the median. The "whiskers" indicate the minimum and maximum growth rates of each set, unless outliers (marked by asterisks) are present, in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. The asterisks outside the ends of the whiskers are the minimum outliers.

I compared the fitness (measured as growth rate) of 34 recombinants (Table 2.1c, up to 3 per mating, less if fewer recombinants had been recovered) recovered from the 15 successful matings (section 7.2) with that of their 9 parents (Table 2.1a, FJ11 was excluded since it produced no recombinants). When the growth rates of the recombinants were measured at the time of recovery (time point 0, termed t0; defined as when the recombinants were recovered from single colonies on YPD

medium, after two rounds of drug resistance selection (refer to Fig. 6.1. for details)). Twelve of the recombinants grew better than one of their parents, 11 grew better than both parents and 11 worse than both parents (Fig. 8.1). The median growth rate of the 34 recombinants was 9% lower than that of their 9 parents (Fig. 8.3, refer to section 2.7.1.5 for details of growth experiment). Only one parent, W17, with a very low growth rate compared to that of the other parents was consistently outperformed by its recombinant progeny.

In order to test if the difference in fitness between recombinants and parents was significant I used a randomization test described in section 2.15.2. The binominal test was not suitable because in most cases I used several recombinants from the same crossings (Table 2.1c) in the growth experiments and so these data were not independent. In the randomization test, we defined our test statistic D as the sum over all 34 recombinants of the fitness of the recombinant minus the average fitness of its two parents. Specifically, the null hypothesis was that there is no difference between the fitness of the recombinants and their parents ($D = 0$), and we wanted to test the two-sided alternative hypothesis ($D \neq 0$). At time 0, for the unshuffled data, D was 0.89. To find the distribution of D under the null hypothesis we randomized (shuffled) the 43 fitness values amongst both the parents and the recombinants. The randomization test showed that 272 out of 1000 randomizations gave a higher value of D than 0.89 (i.e. a p value of 0.272), i.e. the null hypothesis that the growth rate of recombinants is the same as that of parents was not rejected. Conducting the same analyses with the parent W17 and its progeny excluded gave $D = -1.28$ with a p-value of 0.886 (886 out of 1000 randomizations gave a higher value than the test statistic), i.e. the null hypothesis that the growth rates of recombinants is the same

as that of parents was again not rejected. Therefore, there was no significant difference in growth rate between the recombinants and their parents at this time point.

After propagation of 100 generations in YPD medium (Fig. 8.2) (time point 100, termed as t100) parental growth rates had changed little. Indeed the median parental growth rate had decreased by 2.5% (Fig. 8.3). For 6 out of 9 parents the growth rate measured was higher after 100 generations, but this is not a significant deviation from what is expected under the null hypothesis (4.5/9) that rates did not change. In contrast, after 100 generations the 34 recombinants seemed marginally fitter than their parents, as the recombinants' median growth rate had increased by 9.5%, and was 2.7% higher than that of the parents (Fig. 8.3). Of the recombinants 3 were less fit than both parents, 15 were fitter than 1 parent and 16 were fitter than both parents (Fig. 8.2). However, if W17 and its 10 recombinant descendants were excluded then 3 recombinants were less fit than both parents, 14 were fitter than 1 parent and 7 were fitter than both parents. Another randomization test was conducted. The test statistic for the unshuffled data was D = 2.98, and only 13 out of 1000 shuffles gave a higher value of *D* than 2.98 (i.e. a p value of 0.013). The null hypothesis was rejected in favour of the alternative hypothesis that recombinants have higher fitness. However conducting the same analyses with the parent W17 and its progeny excluded gave $D = 0.63$ with 146 out 1000 shuffles giving a higher value, so in this case the null hypothesis that $D = 0$ was not rejected.

To investigate if spontaneous chromosome loss happened during serial transfer of the recombinants in YPD medium, FACS analysis was performed to measure the

DNA content from the same set of representative recombinants of each successful mating before and after 100 generations of serial transfer in YPD medium (section 2.14.1). Comparison between the growth rate and the DNA content of each recombinant indicated that the increase of growth rate was associated with a reduction of DNA content for a given recombinant. Median DNA content of recombinant cells (and thus presumably their ploidy) had decreased by 21% (Fig. 8.4, Fig. 8.5, average DNA content decreased by 24%) after 100 generations, and several (11 out of 28) of the recombinants had reached DNA contents resembling that of diploid cells (the ratio, calculated by dividing the DNA content of one recombinant by the average DNA content of its two parents, was approximately 1; refer to section 2.14.2. for details). Fig.8.5 shows there was some correlation between reduction of ploidy and fitness gain of 28 recombinants before and after 100 generations of transfer on YPD medium (R=0.34). These results indicate that recombinants may benefit from selectively discarding some chromosomes.

In order to assess what additional fitness increases might be expected if recombinants were maintained in laboratory culture for even longer periods, I followed the kinetics of growth rate increase of 12 recombinants during 100 generations (Fig. 8.6). It appears that the fitness increased gradually over 100 generations and probably will continue to increase past t100 (Fig. 8.6). Nevertheless I stopped measuring the fitness after 100 generations because the benefit of recombination after 100 generations was small compared to the reduction of fitness caused by loss of *MTL* heterozygosity (see below in section 8.4.)

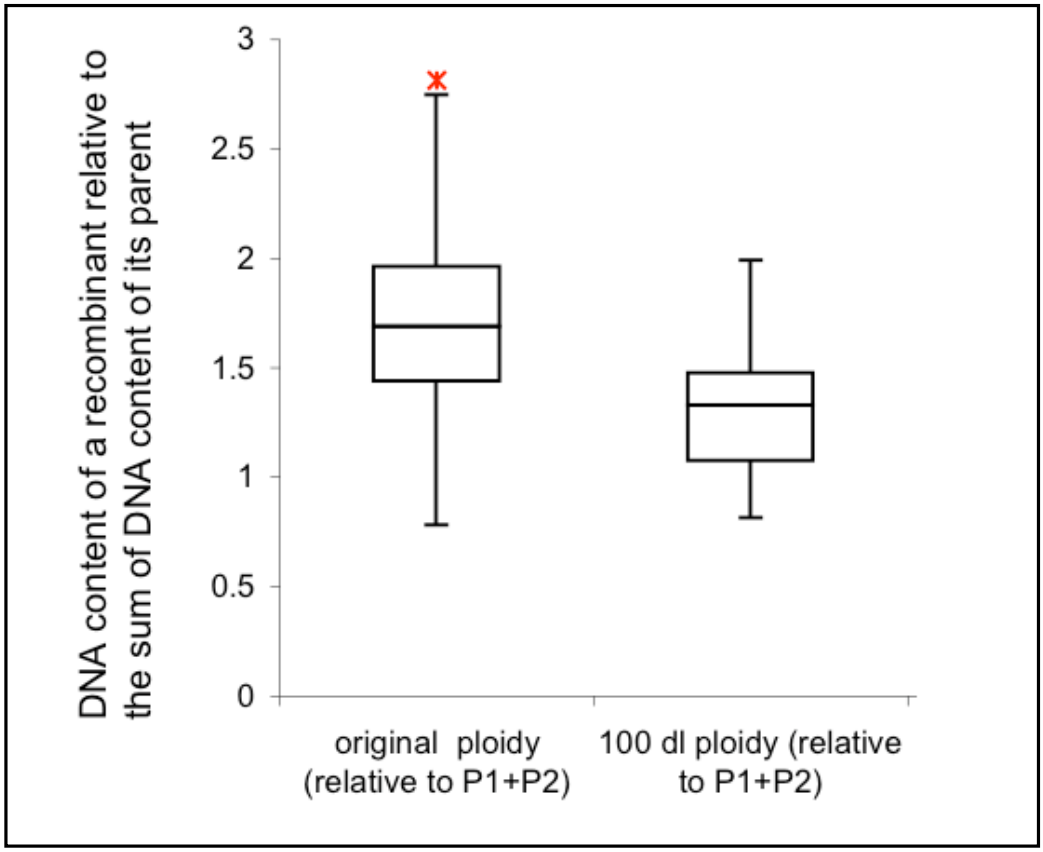


Figure 8.4. DNA content of 28 representative recombinants from the 15 successful matings relative to the average DNA content of their parents before and after 100 generations.

The recombinants are listed in Table 2.1c, except that W43a x YSU63α-3, AU90a x YSU63α-3, AU35a x YSU63α-3, AU35a x W17α-3, RIHO11a x OD8916α-3, RIHO11a x W17α-3 were not tested. The asterisk outside the ends of the whiskers is an outlier. DNA content was measured by FACS analysis (section 2.14.1) and calculated as described in section 2.14.2. Boxes indicate 75th and 25th percentile of the data with a line indicating the median. The "whiskers" indicate the minimum and maximum growth rates of each set, unless outliers (marked by asterisks) are present, in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. The asterisk outside the ends of the whiskers is the maximum outlier.

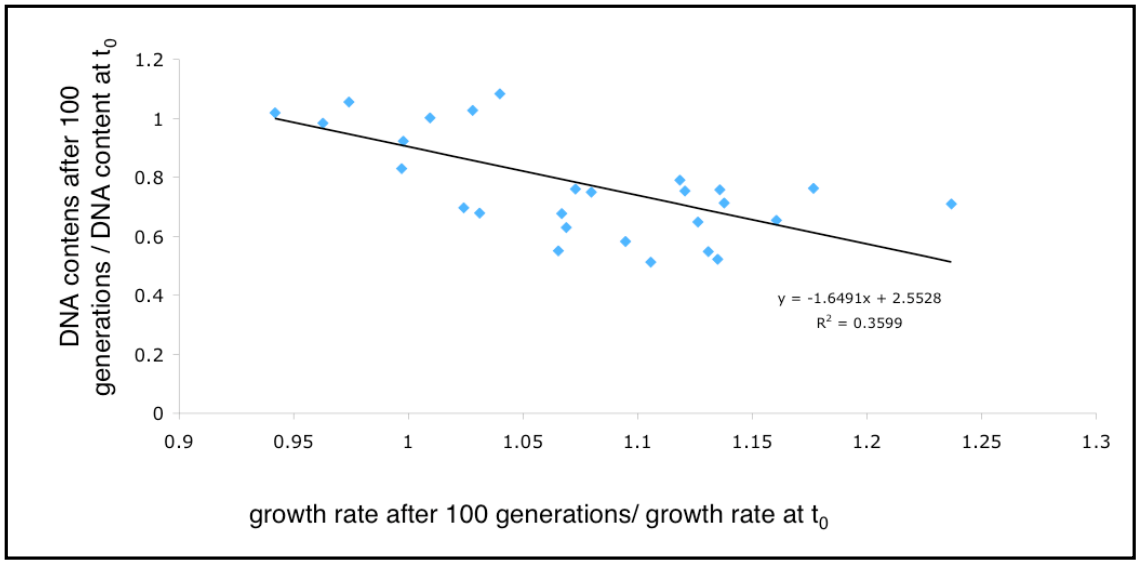


Figure 8.5. Relationship between the increase in the growth rate and decrease of the DNA content of recombinants after 100 generations serial transfer in YPD medium.

For each of 28 recombinants (the same strains as in Fig. 8.4), the ratio of DNA content at t_{100} (after serial propagation for 100 generations) and DNA content at t_0 (when first obtained) was plotted against the ratio of growth rate (doublings /h) at t_{100} to growth rate at t_0. A linear regression trend line (R^2=0.3599) is shown.

As the effect of sex could be different depending on each strain's genetic background and/or each combination of parental genomes, and because recombinants seemed initially less fit and were later possibly more fit, it is hard to measure the benefit of sex by just comparing the growth rates between the recombinants and their parents at two time points (t0 and t100). Therefore, I used simulation methods to estimate the benefit of sex for each strain, using the growth rates I had determined, to test if sex increased the chances of parents passing on their genes by recombination, by calculating the difference in the number of offspring between a cell that continues to propagate clonally and one that takes the path of sexual recombination for 100 generations, assuming the growth rates increased linearly during the time for the recombinants.

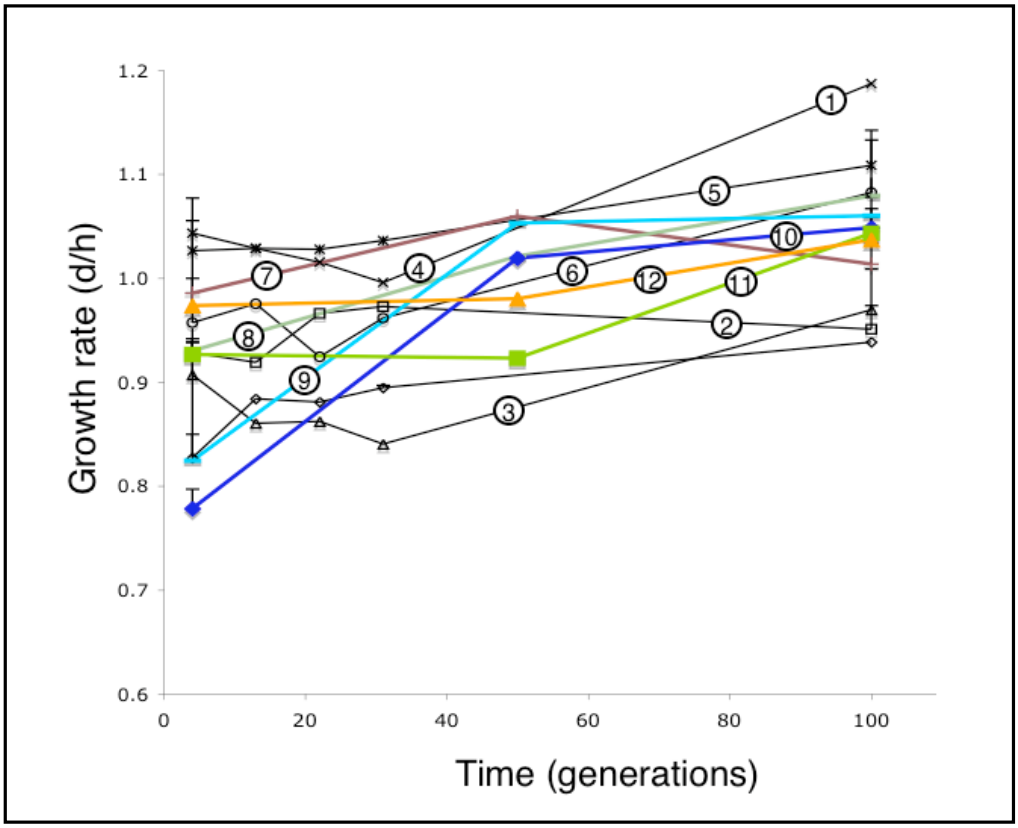


Figure 8.6. Changes in the growth rates of twelve recombinants during serial propagation over 100 generations.

Numbers indicate the different recombinants (1: W43**a** x OD8916α.1; 2: W43**a** x OD8916α.2; 3: W43**a** x OD8916α.3; 4: W43**a** x W17α.1; 5: W43**a** x W17α.2; 6: W43**a** x W17α.3; 7: RIHO11**a** x OD8916α.1; 8: RIHO11**a** x OD8916α.2; 9: RIHO11**a** x OD8916α.3; 10: W43**a** x YSU63α.1; 11: W43**a** x YSU63α.2; 12: W43**a** x YSU63α.3).

First I looked at the benefits of sex after 100 generations by testing if each clonally reproducing marked homozygote produced more progeny over 100 generations, than a recombinant (section 2.16.1). Then I calculated if sex conferred an advantage by comparing the sum of the progeny produced by both marked homozygous parental cells to the number of progeny produced by a recombinant (section 2.16.1).

When I compared the growth between each recombinant and one of its homozygous parents (i.e. 34 recombinants and each of the 9 parents), in 41 out of 68 cases the

recombinant produced more progeny over 100 generations (Fig. 8.7). Of these 41 cases, 32 involved three strains, W17*MTL*α, RIHO11*MTL*α or AU35*MTL***a**. One of these, AU35*MTL***a** always benefited from mating. All of the recombinants derived from it grew better than either of their parents. Strain W17*MTL*α developed a severe growth defect when the *NAT* resistance marker was introduced (which was not related to the marker itself, see details in section 8.4), which might explain why recombinants derived from this parent so often grew better than W17*MTL*α, but not necessarily better than the other parent (Fig. 8.7). Similarly, strain RIHO11*MTL*α changed its morphology when losing one copy of chromosome 5 to become a mating competent strain (it changed from a smooth colony phenotype to a "fuzzy" colony phenotype with a lower growth rate). When RIHO11*MTL*α mated with other strains, the recombinants formed smooth colonies and grew better than the "fuzzy" parent. These later two cases indicate that unfit parents can gain benefit from recombination. But this may also lead to an overestimation of the overall benefit of sex, since in our experiments both strains acquired the growth defect during laboratory manipulation before mating. Natural isolates with severe growth defects are likely to become extinct before having a chance to mate.

In summary, of all 34 recombinants only 13 produced more progeny than either parent after 100 generations. Nine of them involved the strain W17*MTL*α.

If two strains have sex instead of clonally reproducing, all of the progeny will be produced by the recombinant cell, while the number of progeny produced by the two strains continuing reproducing clonally will be the sum of the progeny produced by the two clonally reproducing cells. Therefore, to better assess whether

sex was superior to clonal reproduction, I did simulations to compare the sum of the progeny produced by both parental cells to the number of progeny produced by the recombinant over 100 generations. In 21/34 cases the recombinant progeny was smaller than the combined progeny of their clonally reproducing parents, in 13/34 cases it was larger (Fig. 8.8). But in one case the benefit was minimal (OD8916α x AU35a-1 - the number of recombinant progeny caught up after about 77 generations), and 9 of the combinations involved strain W17. Only three recombinants showed an obvious benefit of sex. In overall, I could not demonstrate an obvious benefit of sex for homozygous parents from these simulations.

From these data I found it difficult to demonstrate that sex increases the chance of parents' passing on their genes to future generations in *C. albicans*. Indeed, with most of the calculations, I can conclude that with some rare exceptions, sex does not increase the chance of homozygous parents' passing on their genes to future generations, unless the parent was a poorly growing strain. Only by one calculation (randomizing the growth rates between individual recombinants and the parents after 100 generations) can one demonstrate any benefit of sex.

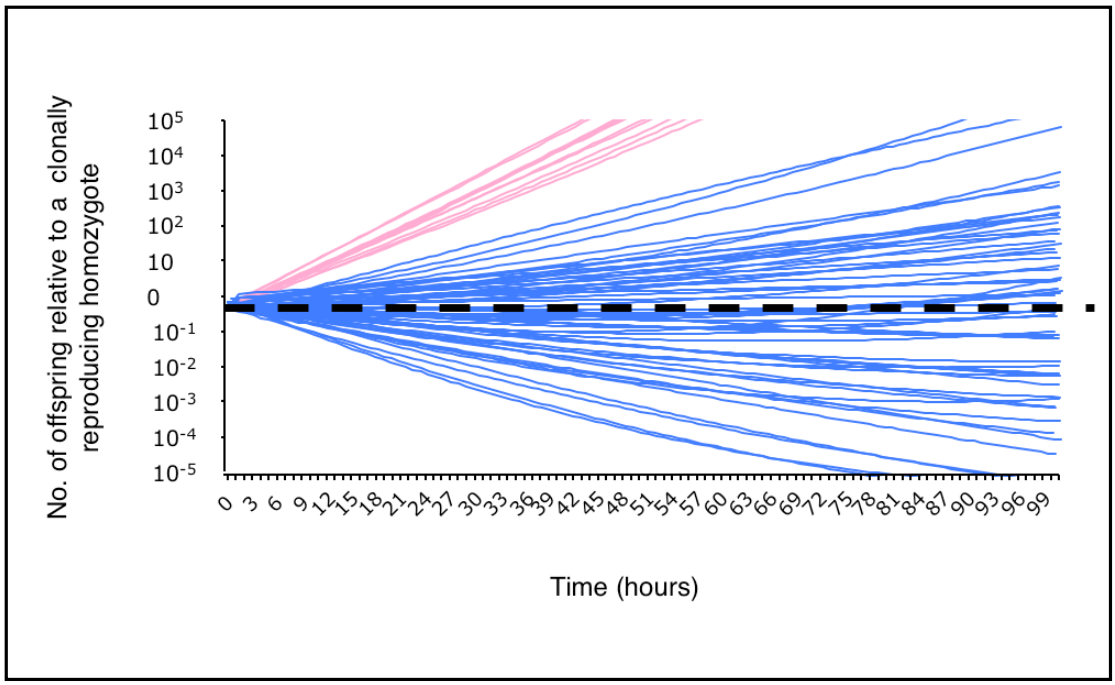


Figure 8.7. Simulation of the benefit of sex for individual strain (size of progeny from one cell engaging in sex relative to that of continued clonal reproduction).

Shown is a simulation of the number of offspring produced in YPD medium by one cell engaging in sex at t0 with a compatible partner. The number of offspring is expressed as a fraction of the offspring the homozygous cell could have produced by continued clonal reproduction. The simulation is based on growth rates experimentally determined in YPD medium (see section 2.16.1. for details). Pink lines: recombination involving strain W17. The number of offspring produced by 34 recombinants (names listed in Table 2.1c) was compared to that of each of their drug resistance-marked parent (Table 5.1).

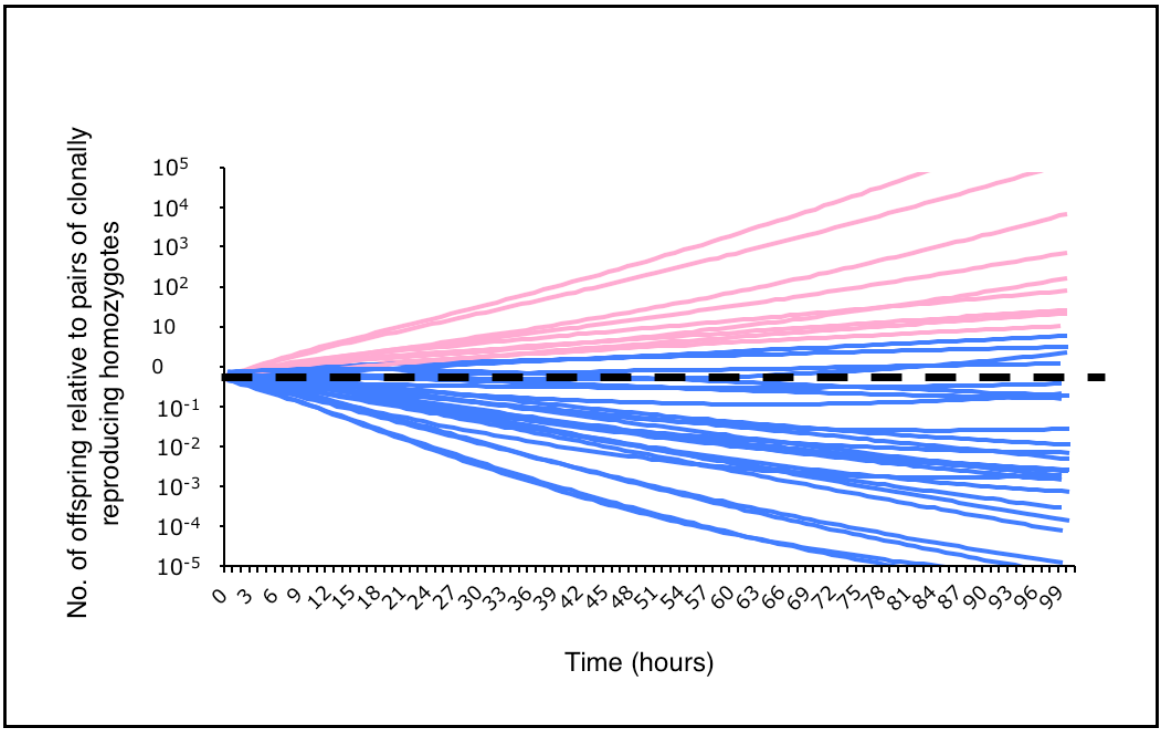


Figure 8.8. Simulation of the benefit of sex for pairs of strains (number of progeny of two cells engaging in sex relative to that of continued clonal reproduction).

Shown is a simulation of the number of offspring produced in YPD medium by two cells engaging in sex at t0. The number of offspring is expressed as a fraction of the offspring both the homozygous cells could have produced by continued clonal reproduction. The simulation is based on growth rates experimentally determined in YPD medium (see section 2.16.1. for details). Pink lines: recombination involving strain W17. The number of offspring produced by 34 recombinants (names listed in Table 2.1c) was compared to the sum of that of both of their drug resistance-marked parents (Table 5.1).

8.3 Possession of two resistance markers does not have an obvious negative impact on the fitness of recombinants

As described in section 5.0, to allow selection of recombinants after mating, each of the *MTL***a** parents were marked with an MPA resistances marker, while each of the *MTL*α parents were marked with a NAT resistance marker. Thus recombinants will have two drug resistance markers per cell but each parent only has one, and this could diminish recombinants' fitness compared to that of their parents and lead to an underestimate of the benefits of sex in the experiments in the previous section. I therefore tested in several ways if having both resistance markers in the recombinants impacted on their fitness.

First, three *MTL* homozygote strains (W43, W17 and OD8916) marked with both drug-resistance genes were generated as described in section 5.4, and their growth rates were each compared to the same three single-marked strains (W43 with the MPA resistance marker, W17 and OD8916 with the NAT resistance marker) (Table 8.1). The mean reduction in the growth rate of double-marked strains compared to that of single-marked strains was considerable (7.2 $\pm$ 12%), although this reduction was mainly caused by one strain (OD8916). This indicates that having two markers could have a negative fitness impact for some strains. However, after 100 generations serial transfer on YPD medium, the mean reduction of the growth rate of the three double-marked homozygous strains was 1.6 $\pm$ 2.6**%** compared to that of single marked homozygous strains. Furthermore, both of the markers were retained after 100 generations. This indicated that recovery of fitness was possible with both markers, and that the initial growth reduction for strain OD8916 with both markers

was possibly caused by other damage to the genome during second round of transformation (Bouchonville et al., 2009) and was not the direct result of possession of two markers.

Table 8.1. Average growth rates of single marked and double marked *MTL* homozygous strains when first isolated and after 100 generations of serial propagation

Strain name	Single Marked Doubling/h at t0 (average±std)*	Double Marked Doubling/h at t0 (average±std)*	Single Marked Doubling/h at t100 (average±std)*	Double Marked Doubling/h at t100 (average±std)*
W43a	0.87±0.03	0.86±0.02	0.90±0.04	0.91±0.03
OD8916α	1.04±0.07	0.82±0.06	1.01±0.06	0.97±0.07
W17α	0.62±0.03	0.63±0.01	0.68±0.04	0.67±0.03

* Based on experimental growth rates of 3 cultures for each strain.

Because two rounds of transformation (to generate the double-marked strains) could cause more damage than one round of transformation, and in the double-marked test strains there are two resistance markers per diploid cell while in a recombinant there would be two markers per tetraploid, or close to tetraploid cell, the above test may overestimate the impact of having two markers in the recombinants. A more accurate assessment of the fitness cost of two resistance markers was to test if one or both resistance markers in the recombinants would be lost during the recombinants' serial transfer in YPD medium for 100 generations. Like any other genes the two markers can be eliminated by recombination or chromosome loss if they have a negative impact on growth when recombinants are serially transferred in YPD medium. The reduction in the DNA content of the recombinants over 100

generations was already known (21%, refer to section 8.2), and one would expect that if markers had no greater fitness impact than that of other genes, the rate of marker loss would be roughly equal to DNA content reduction. If there was a negative impact for having one or two markers, or if there was a negative epistatic interaction between the markers, the rate of marker loss would be greater than DNA content reduction, as the chromosomes with the markers would be more likely to be lost during the serial transfer process.

The presence of both resistance markers was therefore checked by PCR in 5 single colonies from each of six recombinants (three W43a x OD8916α and three W43a x W17α) that had been transferred for 100 generations on YPD medium. All of the markers were still retained in the 30 colonies checked except for one colony of W43a x OD8916α, which lost a NAT resistance marker. The frequency of losing the MPA resistance marker was 0, and for *NAT* resistance marker it was 3%. Both frequencies were much lower than the rate of DNA content reduction over 100 generations (the median DNA content reduction of these 6 recombinants was 31% and the mean reduction was 36%). I expanded the experiment by checking if both resistance markers were retained after 100 generations by representative recombinants of all 15 successful matings. For each mating, one single colony was randomly picked from one or two representative recombinants and the presence of the resistance markers was checked by PCR. Again, MPA resistance markers were present in all 27 recombinants (0 lost) and the NAT marker was lost in only 2 out of 26 (8%) recombinants (Fig. 7.9c), while the median DNA content reduction of these recombinants was 21% (mean 24%). Both experiments imply that having either a

single or double resistance maker(s) in the recombinants had no significant negative impact on growth.

Therefore I could conclude that having two resistance markers in the recombinants did not interfere with measuring the benefit of sex, and that even individual markers apparently did not have a negative fitness impact.

8.4 Gaining mating competency reduces the chances of survival of *C. albicans'* genes

Previously (section 8.2) I have shown that it is difficult to demonstrate the benefit of sex for homozygous *C. albicans* parents. An additional factor that needs to be considered in evaluating the benefit of sex is the loss of *MTL* heterozygosity. As most *C. albicans* clinical isolates are *MTL* heterozygotes, to gain mating competency, a heterozygous strain has to lose its *MTL* heterozygosity and become an *MTL*-homozygous strain. It has been demonstrated that heterozygous clinical isolates are generally more virulent and have a competitive advantage over *MTL*-homozygous strains in colonizing hosts (Lockhart et al., 2005; Wu et al., 2007). The low prevalence of *MTL* homozygosity among clinical isolates and their virulence assessments suggest that loss of one *MTL* allele, necessary for mating competency, may also be associated with significant fitness loss. I therefore extended the analysis of the net benefits of sex to include the impact of gain of mating competency through *MTL* heterozygosity loss prior to mating on fitness.

8.4.1 Loss of *MTL* heterozygosity reduced the growth rate of parental strains

For all heterozygous clinical isolates tested in this study, the loss of one copy of chromosome 5 reduced growth rates, and usually substantially, after sorbose selection. I tested the growth rates, on YPD medium, of the ten wild type clinical isolates used in mating experiments and their ten homozygous derivatives immediately after their recovery from sorbose medium (section 2.7.1.3). The median growth rate of the homozygotes was 44% lower than that of the clinical isolates from which they were derived (Fig. 8.9). Pairwise growth rate comparison between the homozygotes and the heterozygotes also showed a median reduction of 44% and a mean reduction of 36 ± 16% by the homozygotes. A paired t-test showed that the *MTL*-homozygotes grew significantly slower than their heterozygous ancestors (paired t-test, two tailed $p = 5 \times 10^{-5}$).

The growth rate of the homozygotes recovered after culturing in YPD medium. After 30 generations of serial culture in YPD, the median growth rate of *MTL*-homozygous derivatives was only 3.7% lower than that of the heterozygous ancestors (Fig. 8.9), although the difference in the growth rate between the two groups was still statistically significant ($p = 0.03$ for two-tailed t-test paired). In pair-wise growth rate comparison of the two groups, the median and mean reduction of the *MTL*-homozygotes growth rates were 5% and 9 $\pm$ 9%, respectively. Serial propagation for a further 70 generations did not significantly increase growth rates. The median growth rate after 100 generations was 4% lower than that of the *MTL*-heterozygous ancestors and so it had reduced by 0.3% compared to the median

growth rate of the homozygotes after 30 generations (Fig. 8.9). For three strains I determined the time course of growth rate recovery more precisely (Fig. 8.10), and verified that, as a rule, most of the recovery occurred between 10 and 30 generations and that no significant increase in growth rate occurred after 30 generations in YPD.

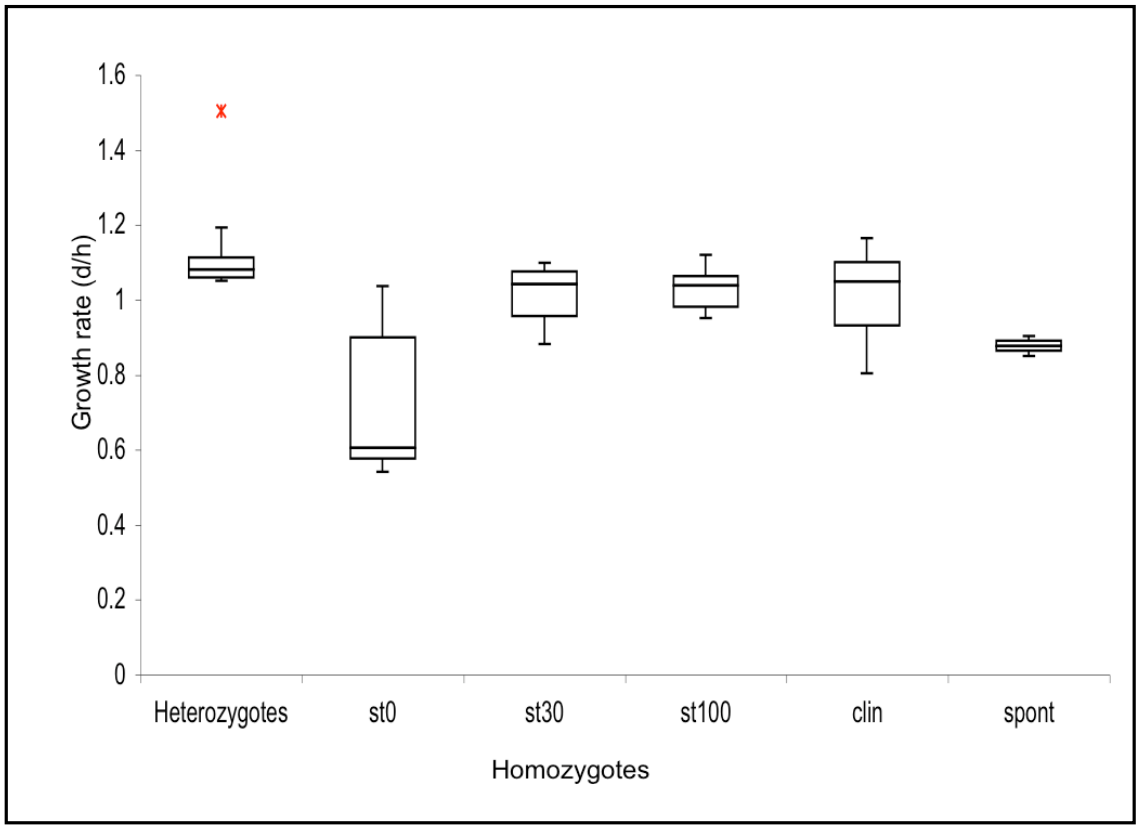


Figure 8.9. Growth rates of *MTL*-heterozygous and *MTL*-homozygous *C. albicans* strains.

Box and whisker plots of growth rates of: *MTL*-heterozygote clinical isolates; *MTL*-homozygotes (selected on sorbose (s)) after growth for 0, 30 or 100 generations (st0, st30, st100) in YPD; *MTL*-homozygous clinical isolates (clin, Table 2.2); and spontaneous *MTL*-homozygotes isolated on YPD medium (spont). Boxes indicate 75th and 25th percentile of the data with a line indicating the median. The "whiskers" indicate the minimum and maximum growth rates of each set, unless outliers (marked by asterisks) are present, in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. The asterisk outside the ends of the whiskers is an outlier.

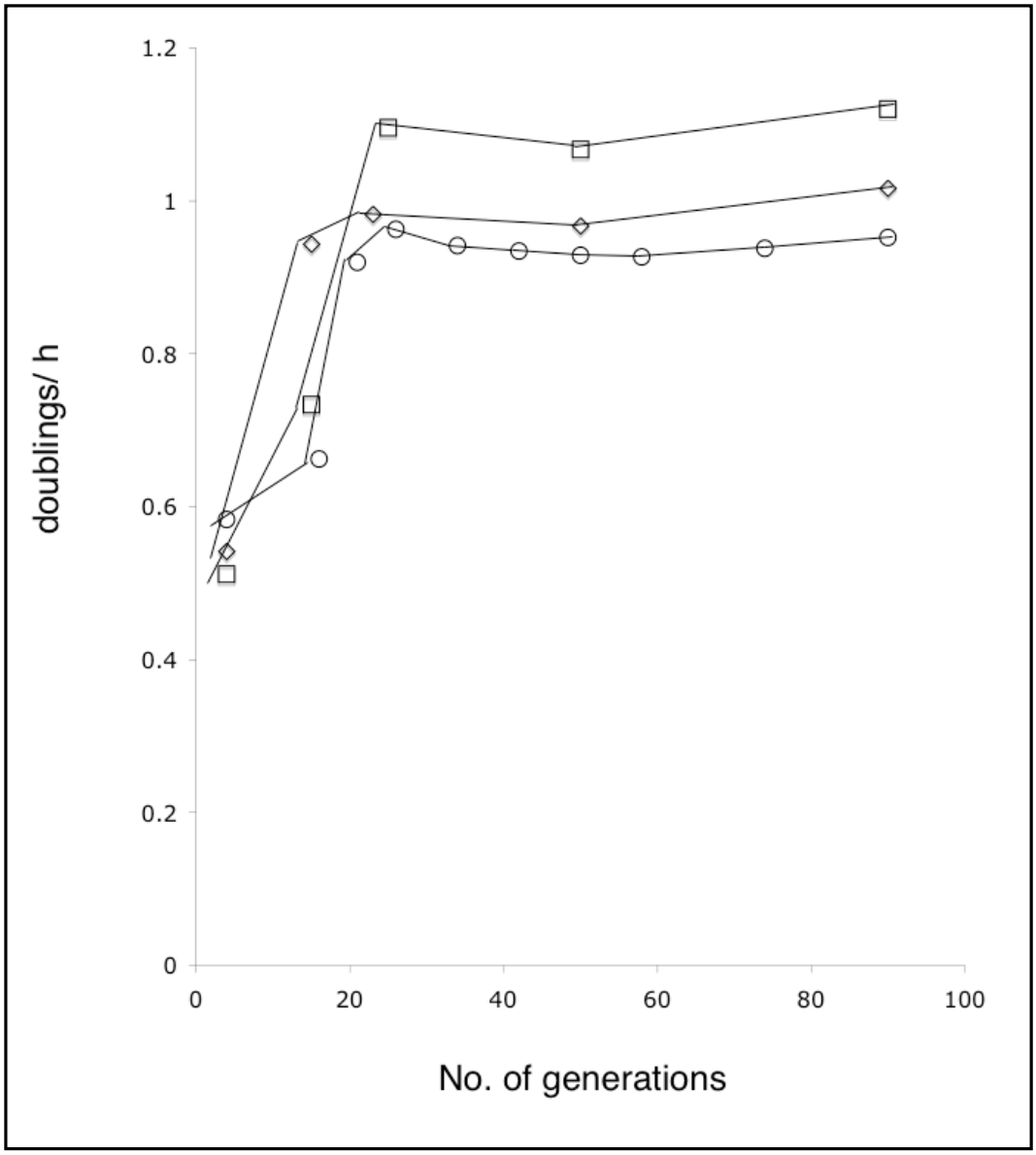


Figure 8.10. Increase in the growth rate of sorbose selection-derived *MTL*-homozygous derivatives of clinical isolates with culturing in YPD.

Growth rates of W43 (circles), OD8916 (diamonds) and W17 (squares) were measured during serial propagation in YPD for 100 generations.

In contrast to the growth recovery of *MTL*-homozygotes in YPD medium, the growth rates of the clinical *MTL* heterozygotes had changed little after propagation for 100 generations. The median growth rate of heterozygotes had increased by 1.7% after 100 generations (Fig. 8.11). For 7 out of 9 strains, the growth rate

measured was higher after 100 generations, but this is not a significant deviation from what is expected under the null hypothesis (5/10) that the rates did not change. This result implied that the growth recovery of sorbose-induced *MTL* homozygotes in YPD medium was not mainly caused by the adaptation to medium itself but related to the process of losing and duplicating one copy of chromosome 5, as aneuploidy is supposed to cause fitness reduction (Torres et al., 2008).

In nature, chromosome 5 loss is presumably not caused by exposure to sorbose, and exposure to sorbose might lead to other mutations that would reduce fitness in YPD and could contribute to, or explain, the fitness loss observed. Also, while the major natural mechanism of *MTL*-homozygosis in *C. albicans* is chromosome 5 loss followed by duplication of the homologous chromosome (Wu et al., 2005), *MTL*-homozygosis may not always be caused by chromosome 5 loss. Mitotic recombination or gene conversion also can play a role in this process (Wu et al., 2007; Wu et al., 2005). For these reasons the sorbose-selected *MTL*-homozygous derivatives could be less fit than naturally occurring homozygotes. To see if this was the case I carried out two experiments. In the first, I tried to obtain spontaneously arising *MTL*-homozygotes, by plating 4000-6000 colonies each of our 10 clinical isolates on YPD + phloxine B plates (see section2.5.1.7), and obtained 2 spontaneous homozygotes (W43α and OD8916α). The growth rates of the two *MTL* homozygotes were reduced by 20% (20 $\pm$ 0.5%) compared to the clinical isolates from which they were derived. This was less than the reduction typically seen after sorbose selection. However, unlike the sorbose-derived homozygotes, their growth rates did not increase over 30 generations (Fig. 8.12). It is unlikely that the phloxine B had altered the cells in some way, other than

chromosome 5 loss and duplication, to reduce growth rate, as no growth reduction was detected from YPD- phloxine B derived heterozygous cells (data not shown).

Since I only got two spontaneous *MTL*-homozygotes from our clinical isolates, in the second experiment I analyzed the growth rates of a set of 23 *MTL*-homozygous clinical isolates (Table 2.2) and compared these with the 10 *MTL*-heterozygous clinical isolates generated for mating experiments. The mean growth rate of these *MTL*-homozygous clinical isolates was 9.4 % lower than that of the wild type heterozygotes, a statistically significantly difference (t-test, equal var. $p < 0.05$), and the median growth rate was 3% lower than the median growth rate of our 10 clinical isolates (Fig. 8.9). Both mean and median assessments for the growth rate reduction of the wild type *MTL* homozygotes were comparable to the growth rate reduction of our sorbose-derived *MTL* homozygotes after 30 generations in YPD to the original *MTL* heterozygotes (Fig. 8. 9).

Comparing these data, it seems that, while the way in which I achieved *MTL* homozygosity may not be identical to what occurs naturally, if cells were analyzed >30 generations after the event, sorbose-induced loss of heterozygosity had a similar fitness impact to its long-term impact in naturally occurring *MTL* homozygotes. The quick recovery of *MTL*-homozygotes in YPD medium was probably related to duplication of chromosome 5. However, apparently even after a long period of recovery, loss of *MTL* heterozygosity still leads to a reduction in the growth rate compared to the heterozygous strains.

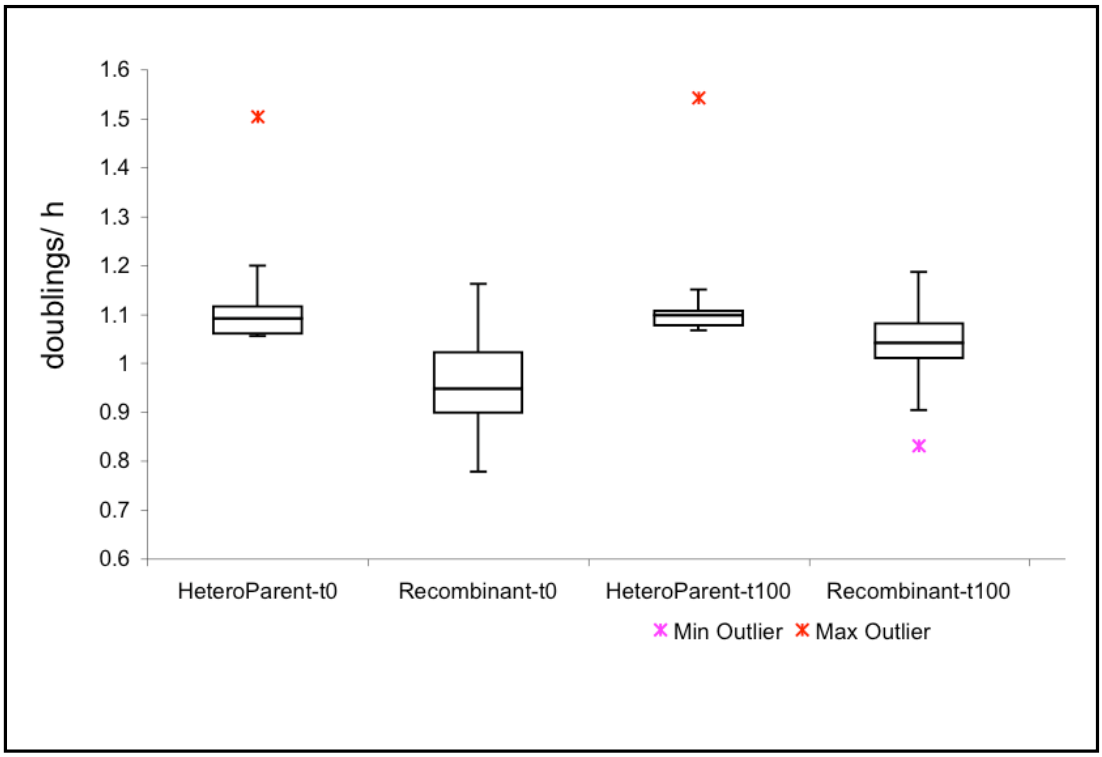


Figure 8.11. Growth rates of 9 clinical *MTL*-heterozygous parental strains and 34 recombinants' derivatives before (t0) and after 100 generations in YPD.

The strains used are listed in Table 2.1a&c. Box and whisker plots of growth rates of: *MTL*-heterozygote clinical isolates before and after serial transfer in YPD medium for 100 generations (t0 and t100), and recombinants before and after serial transfer in YPD medium for 100 generations (t0 and t100). Boxes indicate 75th and 25th percentile of the data with a line indicating the median. The "whiskers" indicate the minimum and maximum growth rates of each set, unless outliers (marked by asterisks) are present, in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. The asterisk outside the ends of the whiskers are outliers.

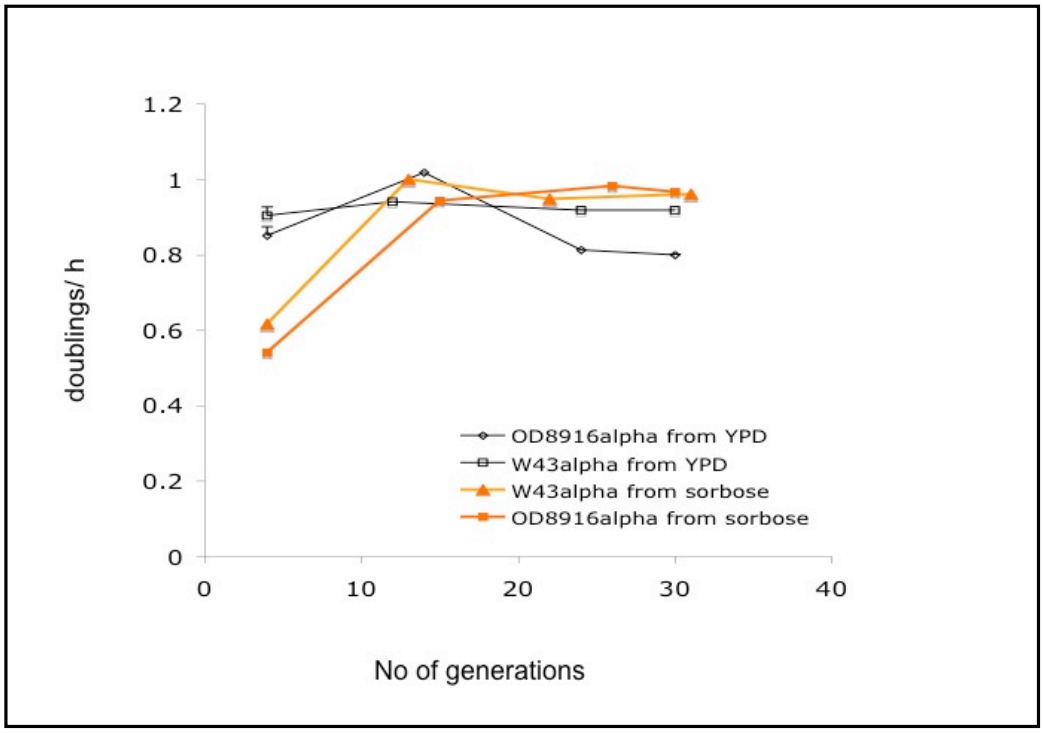


Figure 8.12. Comparison of recovery of growth rate in YPD of *MTL*-homozygous derivatives of W43 and OD8916 derived from YPD and sorbose, respectively, during serial propagation in YPD.

Growth rates of YPD derived W43*MTL*α and OD8916*MTL*α (black lines), and sorbose derived W43*MTL*α and OD8916*MTL*α (orange lines) were measured during serial propagation in YPD for 30 generations.

8.4.2 Most of the recombinants grow slower than their heterozygous clinical originals, even after 100 generations

Since loss of *MTL* heterozygosity leads to a reduction of fitness, but recombination may confer a small fitness benefit, it is necessary to verify the total cost-benefit for a heterozygous strain that takes the path of sexual reproduction, as most clinical isolates are heterozygotes. I therefore carried out an experiment to compare the fitness of the same 34 recombinants (refer to section 8.2) with that of their heterozygous ancestors, immediately after they were recovered and after 100 generations of growth in YPD (section 2.7.1.5). As predicted, in comparison to the original heterozygous clinical isolates the recombinants were of significantly reduced fitness. Upon initial recovery, only 1 of the recombinants grew better than one of their heterozygous ancestors, 4 of them grew better than both heterozygous ancestors and 29 of them grew worse than both heterozygous ancestors. The median growth rate of the 34 recombinants was 13% (14% if W17 and its recombinant descendants were omitted) lower than that of their 9 parents (the mean growth rate of recombinants was 11 $\pm$ 11% lower) (Fig. 8.11). With W17, randomization test showed at t0 recombinants versus original ancestors test statistic (D) = -4.35, p-value = 0.987 (i.e. 987 out of 1000 randomisations gave a higher value than the test statistic), i.e. recombinants have significantly lower growth rates than their parents. If any recombinants involving W17 versus original ancestors were excluded, the test statistic (D) = -3.21, p-value = 0.978 (978 out of 1000 randomisations gave a higher value than the test statistic), i.e. recombinants have significantly lower growth rates than their ancestors. Therefore, no matter with or without W17, the recombinants grew significantly worse than their heterozygous ancestors.

A comparison of growth rates between the recombinants and the heterozygous ancestors after 100 generations of serial transfer in YPD medium was also conducted. In this experiment, 3 of the recombinants grew better than one of their ancestors, 5 of them grew better than both ancestors and 26 of them grew worse than both ancestors. The median growth rate of the 34 recombinants was 5% (6% if W17 and its recombinant descendants were omitted) lower than that of their 9 ancestors (the mean growth rate of recombinants decreased by 7 $\pm$ 8% compared to that of its heterozygous ancestor - omitting W17 and its recombinant descendants made no difference to these figures) (Fig. 8.11). Using a randomization test for growth rates of recombinants versus original ancestors at t100, including W17 and its recombinant descendants, the test statistic (D) was -2.03, p-value = 0.09 (i.e. 90 out of 1000 randomisations gave a lower value than the test statistic), i.e. the null hypothesis that the growth rates of recombinants is the same as that of original ancestors can't be rejected (at the 5% level). If excluding W17 and any recombinants involving W17, randomization test showed that, test statistic (D) = -1.64, p-value = 0.096 (i.e. 96 out of 1000 randomisations gave a lower value than the test statistic), i.e. the null hypothesis that the growth rates of recombinants is the same as that of original parents also can't be rejected (at the 5% level). Therefore no matter with or without W17, results were not significant at the 5% level but were weakly significant at the 10% level. So it is fair to say that the recombinants grew almost significantly (close to, but not quite) worse than their original parents. This result is different from the fitness comparison between the recombinants and their homozygous parents (refer to section 8.2, Page 178), and is most possibly caused by the effect of losing *MTL* heterozygosity. Interestingly, 11 out of 28 recombinants

had reached to their diploid stage after 100 generations, but the mean and median growth rate of these diploid recombinants was 10% and 4% lower, respectively, compared to their heterozygous ancestors.

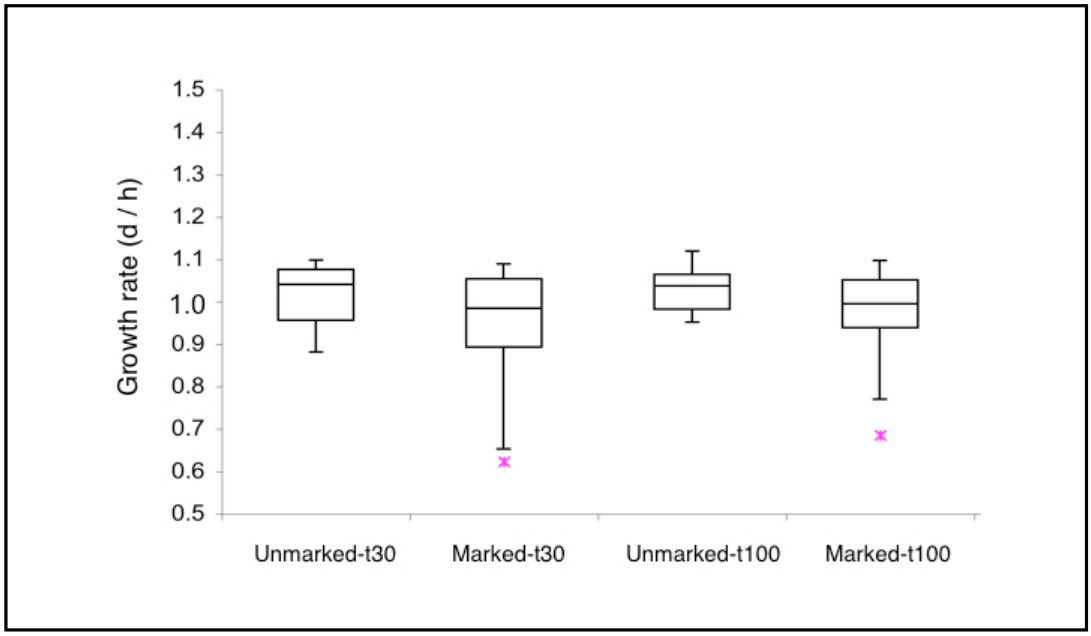


Figure 8.13. Effect of marker cassettes on growth rates of *MTL*-homozygotes.

Box plots showing growth rates of the 10 unmarked *MTL*-homozygotes and *MTL*-homozygotes marked with resistance cassettes 30 (t30) and 100 (t100) generations after recovery from sorbose plates. Boxes indicate 75th and 25th percentile of the data with a line indicating the median. The "whiskers" indicate the minimum and maximum growth rates of each set, unless outliers (marked by asterisks) are present, in which case the whiskers extend to a maximum of 1.5 times the inter-quartile range. The asterisks outside the ends of the whiskers are the minimum outliers of the growth rates of the *MPA^r* derivative of W17α (a second transformation of W17α with the *MPA^r* cassette was carried out and the resulting transformant had a similar growth rate (0.67 doublings per h after 30 generations; data not included in the figure). Omitting W17α, the median growth rates of the remaining marked and unmarked homozygotes were 1.046 and 1.041 doublings per h, respectively, at t30 and 1.016 and 1.038 doublings per h, respectively, at t100. The other strains used are listed in Table 5.1.

8.4.3 Introduction of the resistance markers had no significant impact on fitness in 9 of 10 tester strains:

As mentioned above, the growth rates of the recombinants were significantly lower than those of their heterozygous ancestors at time = 0, and possibly also after 100 generations. However, they were not significantly different to those of the homozygous parents at time = 0, and were even significantly higher than those of the homozygotes after 100 generations (see Fig. 8.3, section 8.2). One reason why the recombinants are less fit than the heterozygous clinical isolates from which they are derived, could be that the recombinants carry artificial drug resistance markers absent in the parents. To rule out the possibility that introduction of resistance markers in the homozygous parents would have a negative fitness impact on the parents and their progeny, I carried out an experiment to compare the fitness between the unmarked and marked parents.

Growth rates were compared between the 10 marked homozygotes (Table 5.1.) and the unmarked homozygotes grown for 30 generations in YPD medium. I conducted this comparison after 30 generations because the growth during manipulations associated with the introduction of markers was equivalent to approximately 30 generations. Thus both types of homozygotes had the same amount of time to adapt to YPD medium.

Across ten strains the median growth rate of marked homozygotes was 1.2% higher than that of the unmarked homozygotes. In pair wise comparisons between the two groups the mean growth rate of the marked strains was 1.84 $\pm$ 5.1% lower than the

unmarked homozygotes, but the median growth rate of the marked strains was higher than that of unmarked ones by 0.1% (Fig. 8.13). No significant difference between the growth rates of the two groups was detected (t-test, paired, two tailed). Growth rates measured for 4/10 marked homozygotes were lower than those of their unmarked parents, also indicating that there is no significant difference between the fitness of the two groups. Only in one strain, W17*MTL*α, the growth rate decreased significantly, by 43.1%, after transformation with the *NAT* resistance marker.

Since the growth rate of the *MTL*-homozygotes recovered during growth in YPD medium as described above (Fig.8.9), to make sure the comparison of the growth rates between the marked and unmarked strains were conducted at a stage where their growth rates had reached a plateau, I extended the experiment to compare their growth rates after a further 70 generations (t100). As shown in Figure 8.13, the median growth rate of unmarked homozygotes increased by 0.4% after a further 70 generations, while that of the marked ones decreased by 2%, after 100 generations. In both cases there were no significant differences in the growth rates between the two time points (paired t-test, two tailed). Moreover, the median growth rate of the marked homozygous strains (except for W17) was 1.7% lower than that of unmarked homozygous strains after a further 70 generations, and the mean and the median growth rates were reduced by 1.85 $\pm$ 2.7% and 0.02%, respectively, by pair-wise comparison of the two groups. No significant change in fitness between the marked and unmarked homozygotes (except for W17) was detected either for the measurement before and after the further 70 generations (t30 and t100). The growth rate of the marked W17*MTL*α was still 39% lower than that of its unmarked parent. Most of the resistance markers were retained after 100 generations of the marked

homozygotes in YPD medium (Fig. 7.9c; tested by PCR as described in section 7.3.2). All of these results suggest that introduction of the resistance markers had no significant impact on the fitness of the *MTL*-homozygous strains, except for W17.

The fitness reduction of strain W17*MTL*α caused by the introduction of the NAT resistance marker was probably not related to the actual cassette, because all of its mating progeny tested (22 individual colonies from 8 recombinants from 4 matings) retained the marker in the absence of selection after 100 generations transfer on YPD medium (section 8.3). Probably some other unknown damage to the genome was generated during transformation of strain W17*MTL*α with the marker. A second transformation of W17*MTL* α with the *NAT*^{r} cassette was carried out and the resulting transformant had a growth rate of 0.67 doublings per h after 30 generations, similar to that of the original one.

Because there was no significant difference between the fitness of unmarked and marked homozygous parents (except for W17), and because having one or two markers had no negative fitness impact on the recombinants, the difference between the fitness of marked homozygous parents and the heterozygous ancestors mentioned above (Fig. 8.3, Fig. 8.11) is probably almost exclusively caused by loss of *MTL* heterozygosity, with the exception of W17. If including W17, introduction of the resistance markers could have a small effect.

These results suggest that gaining mating competency reduces the fitness of *C. albicans* and thus reduces the chances of *C. albicans* cells passing on their genes to subsequent generations.

8.5. Overall, engaging in sex probably reduces the number of offspring produced by *C. albicans*

The above sections described how loss of *MTL* heterozygosity and mating affected fitness. Loss of one *MTL* locus reduced the growth rate of most of the clinical isolates. However in some cases recombination lead to a fitness gain after 100 generations, and additional gains may be possible. To better estimate the overall net benefit of taking the path of sexual recombination for clinical isolates, it was necessary to combine data from both analyses to estimate the total fitness effect of *MTL* heterozygosity loss plus mating in a final simulation. This would reveal for a cell that became *MTL* homozygous and mated, how many offspring it produced relative to the number of offspring that the same cell would have produced by clonal replication had it remained *MTL*-heterozygous. I calculated this for each of the clinical isolates whose *MTL*-homozygous derivatives had produced recombinants.

Because, as discovered in previous sections, any demonstrable benefit of sex would be small, I made assumptions that would maximize the benefit of sex in the simulations listed below, based on the growth data obtained from previous sections, namely: (i) that the growth rate of *MTL*-homozygotes corresponded to the rate measured 30 generations (in YPD) after recovery from sorbose selection, i.e. that there was no initial steeper decline of growth rate of *MTL*-homozygotes considered (section 8.4, Fig. 8.9, Fig. 8.10); (ii) that *MTL*-homozygotes found their mating-compatible partner 30 generations (i.e, approximately 30 hours) after loss of one *MTL* locus – given the low prevalence of *MTL* homozygous clinical isolates

(Lockhart et al., 2002) homozygotes will probably have to survive much longer before mating in their natural environment; and (iii) that the mating process only took the same amount of time that is required for one cell division (around 1 hour) – much shorter than the duration of the mating process in the laboratory which was more than 3 hours in liquid culture (Lockhart et al., 2003).

For the simulation, I wanted to link the data obtained with marked recombinants in section 8.2. with that obtained for their unmarked ancestors used in the earlier part of the simulation (section 8.4). Presence of the marker may have had an effect, albeit an extremely small effect, on fitness (see section 8.4). A marker factor was used to computationally adjust growth rates used in the simulation to compensate for this:

(Growth rate of marked recombinant at t_n)/(Growth rate of the marked parent) x (Growth rate of parental homozygote w/o resistance cassette)

(See details of simulation in section 2.16.3).

Thus, growth rates of recombinants were expressed as multiples of the growth rates of their unmarked parents in order to calculate relative growth rate differences and these were then used to estimate what the growth rates of recombinants resulting from the mating of unmarked homozygous cells would have been.

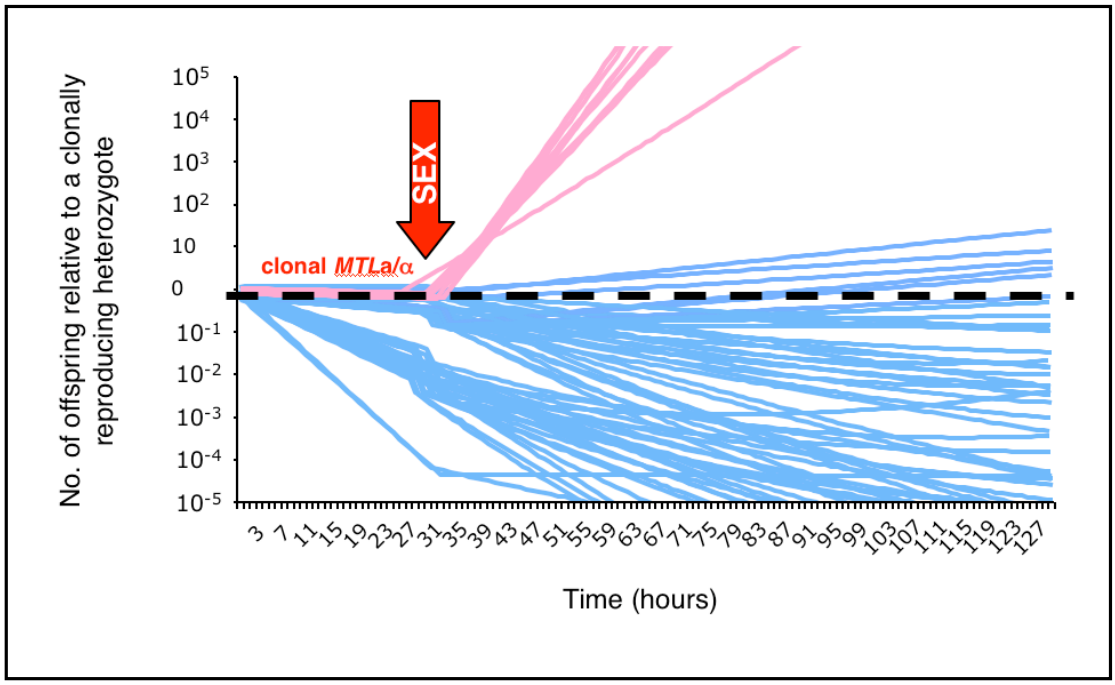


Figure 8.14. Simulation of the number of progeny produced by individual cells which became *MTL*-homozygous at t_0 and the progeny of which mated after 30 generations, (marked by arrow).

The number of progeny is relative to the progeny they could have produced by continued clonal propagation when remaining heterozygous (dashed line). The mating process is assumed to take the same time as one clonal cell division. The simulation is based on growth rates experimentally determined in YPD medium. The number of progeny from 34 recombinants (listed in Table 2.1c) from all 15 successful matings were each compared to the progeny from each of their parents. Calculations used growth rates of *MTL*-homozygotes without resistance cassettes. The effect of experimental manipulations used to achieve mating competency and recovery of recombinants was eliminated computationally (see section 2.16.3. for details). Pink lines: recombination involving strain W17.

Based on these assumptions, I calculated the net benefit of sex as the difference in the number of offspring between a cell that continues to propagate clonally and one that takes the path of sexual recombination for 130 generations. As with previous simulations, data from up to three recombinants per mating were used, leading to 68

comparisons (Fig. 8. 14; see section 2.16.3. for details). Figure 8.14 shows that after 130 generations (30 after *MTL* allele loss and 100 generations after mating), in 49 of the 68 comparisons the number of recombinant offspring was lower than that produced by the original *MTL* heterozygous cell that continued to reproduce clonally. Only in 19 of 68 comparisons was the number of offspring produced by the recombinant cell higher than that of its sister cell that continued to reproduce clonally 100 generations after mating.

Twelve of these 19 comparisons involved strain W17. As described previously (section 8.4), transformation of this strain with the NAT resistance marker had a big negative impact on W17*MTL*α's fitness, although this effect was not related to the resistance cassette itself, and mating greatly benefited W17*MTL*α, most likely because it allowed complementation of an unknown genetic defect that occurred directly before mating. Thus the calibration factor, designed to compensate only for the resistance cassette, probably vastly overcompensates any possible marker effect and as a result greatly inflates the estimate of offspring that an unmarked and genetically undamaged W17 would have produced. In other words, the simulation overestimates the benefit of mating for W17 (see below for a second type of simulation which avoids this problem). Omitting W17, in only 7 out of 48 comparisons did the number of offspring produced by a cell engaging in recombination exceed that produced by a cell that continued to reproduce clonally.

Comparing the fitness of a recombinant to only one of its parents may not be the most informative way to judge the benefits of sex. Parents differed in fitness and even if recombination would have no net benefit at all, one might expect the less-fit

parent of a mating pair to benefit, by giving it access to the 'better' genes of the fitter parent. In contrast recombination would be potentially a fitness cost to the fitter parent because it would mix its genes with 'inferior' genes of the less-fit parent. Nevertheless, in this scenario 50% of parents (the less fit members of each mating pair) would benefit from mating even if sex has no apparent benefit. However, as mentioned above, I found that much fewer than 50% of parents benefit from sex.

To obtain a more accurate picture of the overall cost-benefit ratio of taking the path of sexual recombination for clinical isolates, it is probably better to compare the progeny of pairs of cells that have lost an *MTL* allele and mated, with the progeny the same pair could have produced by remaining *MTL*-heterozygous and continuing clonal propagation.

For this simulation I made one additional assumption in favour of sex. Since the growth rates of two *MTL*-homozygotes that were to mate after 30 generations were different, there would, strictly speaking, be no mating partners for some of the mating competent cells of the fitter *MTL*-homozygote of the pair. However, I used the number of cells produced by the faster multiplying member of a pair at the time point of mating (30 generations after losing *MTL* heterozygosity) as the initial number of recombinants generated (see section 2.16.2).

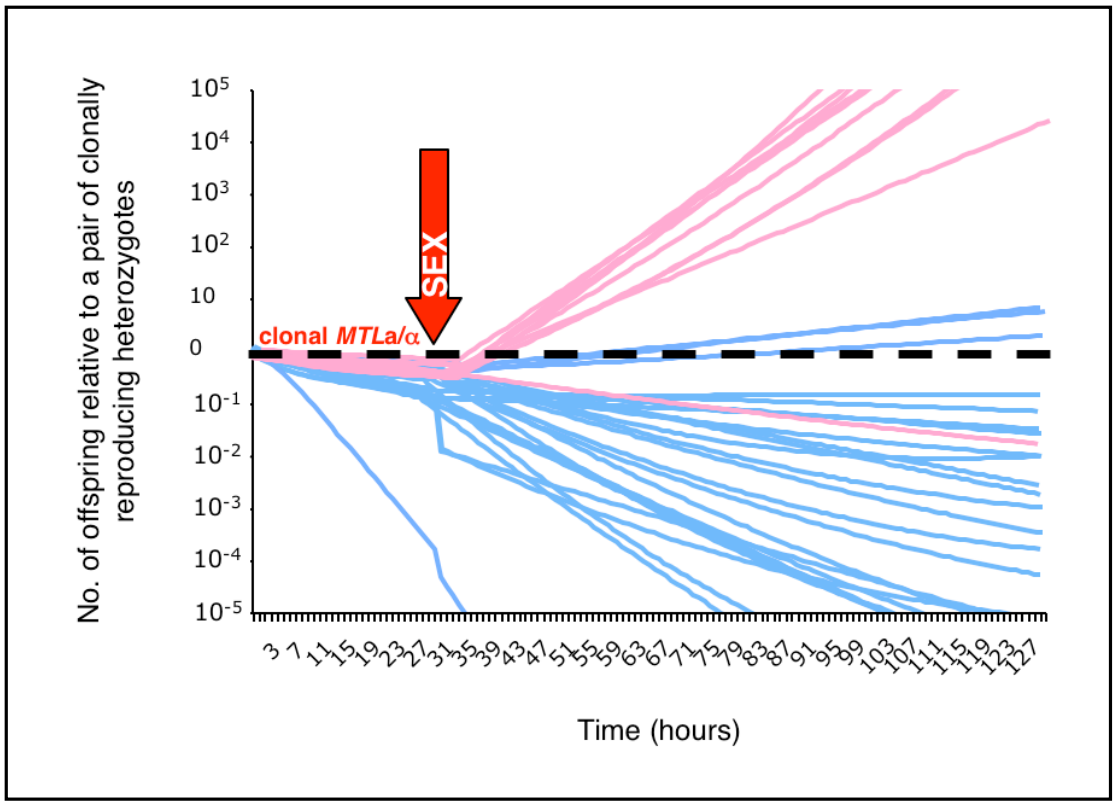


Figure 8.15. Simulation of the number of progeny produced by pairs of cells which became *MTL*-homozygous at t_0 and the progeny of which mated after 30 generations, (marked by arrow).

The number of progeny is relative to the progeny both cells could have produced by continued clonal propagation when remaining heterozygous (dashed line). The mating process is assumed to take the same time as one clonal cell division. The simulation is based on growth rates experimentally determined in YPD medium. The number of progeny from 34 recombinants (listed in Table 2.1c) from all 15 successful matings were each compared to the progeny from both of their parents. Calculations used growth rates of *MTL*-homozygotes without resistance cassettes before mating. The effect of experimental manipulations used to achieve mating competency and recovery of recombinants was eliminated computationally (see section 2.16.2. for details). Pink lines: recombination involving strain W17.

Figure 8.15 shows the overall impact of benefit of sex by comparing the number of progeny of two cells engaging in sex relative to that of continued clonal reproduction of both parents (section 2.16.2). In this simulation, for 22/34 combinations the cell number produced by recombinants did not exceed that of the

pair of clonally reproducing parents after 130 generations. For 12/34 combinations, there was an apparent benefit. But 9 cases involved strain W17 which could lead to an overestimation of the benefit of sex as described above. Excluding W17, and its recombinant descendents, only in 3/24 cases was there a benefit for sex. The median number of offspring (excluding W17) 130 generations after reaching mating competency and mating was only 0.04% that of a heterozygous clinical isolate which had continued to reproduce clonally. Even including W17, the median number of recombinant progeny after 130 generations was only 0.3% that of the clonal progeny arising from the same pair of isolates.

As mentioned earlier, the marker correction factor can be misleading if unknown genetic damage occurs during transformation. In addition, the above procedure requires the combination of growth data from two independently generated *MTL*-homozygotes[1] that may differ in fitness, a potential source of error. Since, as described in section 8.3 and 8.4, the presence of resistance markers actually had no readily demonstrable growth impact for recombinants and parents, it is possible to make an alternative simulation that omits the marker effect factor for the recombinants and uses the growth rates of marked homozygotes for 30 generations before mating (section 2.16.2. & 2.16.3). W17 would still benefit more from recombination than other strains in this simulation, but more realistically, would have to pay the price for its severe genetic defect by producing less progeny in the 30 generations of growth before finding a mating partner. Therefore, this alternative simulation seemed more appropriate.

[1] In some cases more than one unmarked homozygote was generated for a strain, only one of which was marked with resistance gene.

There were no obvious differences between these new simulations using the alternative calculations and the previous simulations with the expected exception of W17. (Fig. 8.16, Fig. 8.17). Excluding W17, in 6/48 cases, mating and recombination led to higher cell numbers than exclusively clonal reproduction (7/48 in previous simulations) for single strains and in 3/24 cases for pairs of strains (same as in previous simulation). When W17 was included, in only 6/68 matings did recombination lead to higher cell numbers for individual strains (19/68 in previous simulations) and in 3/34 cases for pairs of parents (12/34 in previous ones). The median number of offspring (excluding W17 and its descendents) 130 generations after reaching mating competency and mating was only 0.03% of that of a heterozygous clinical isolate which had continued to reproduce clonally, which was comparable to the original final simulation method described previously. And the median of the sizes of recombinant progenies after 130 generations was only 0.01 % of that of the clonal progeny of the same pair of isolates. Thus neither of these simulations could demonstrate an overall net benefit of sex over continued clonal propagation. On the contrary, achieving mating competency and mating appeared to significantly reduce clinical *C. albicans* isolates' chances to pass on their genes to future generations.

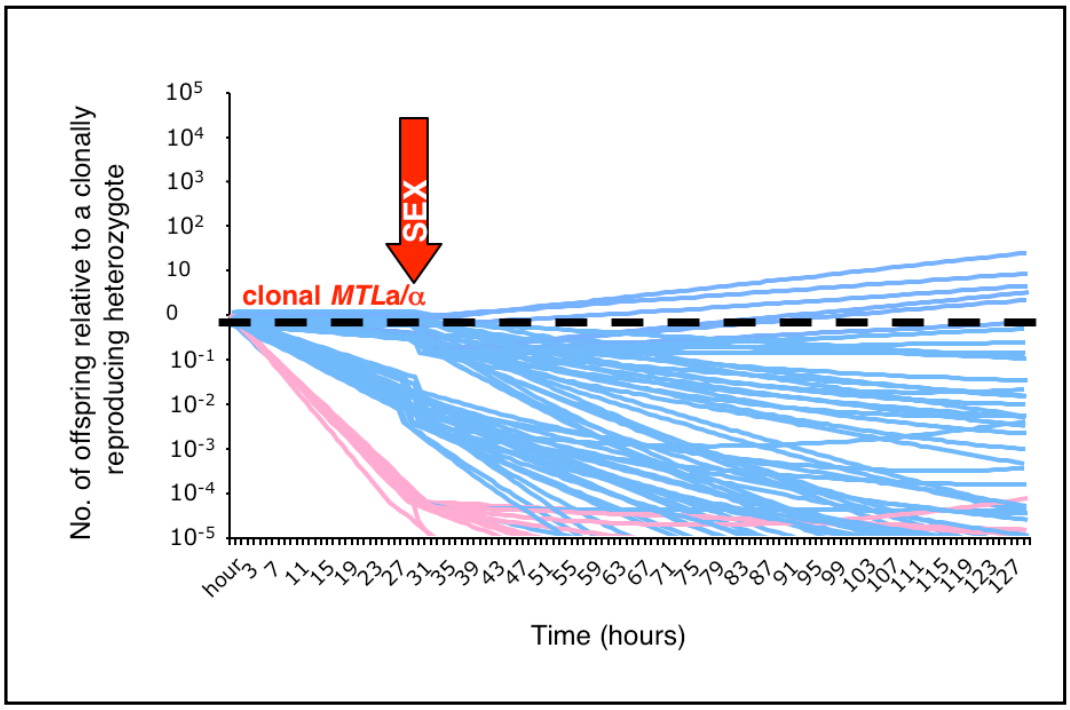


Figure 8.16. Alternative simulation of the number of progeny produced by individual cells which became *MTL*-homozygous at t_0 and the progeny of which mated after 30 generations, (marked by arrow).

The number of progeny is relative to the progeny each cell could have produced by continued clonal propagation when remaining heterozygous (dashed line). The mating process is assumed to take the same time as one clonal cell division. The simulation is based on growth rates experimentally determined in YPD medium. The number of progeny from 34 recombinants (listed in Table 2.1c) from all 15 successful matings were each compared to the progeny from each of their parents. Calculations used growth rates of *MTL*-homozygotes with resistance cassettes before mating. The effect of experimental manipulations used to achieve mating competency and recovery of recombinants was eliminated computationally (see section 2.16.3. for details). Pink lines: recombination involving strain W17.

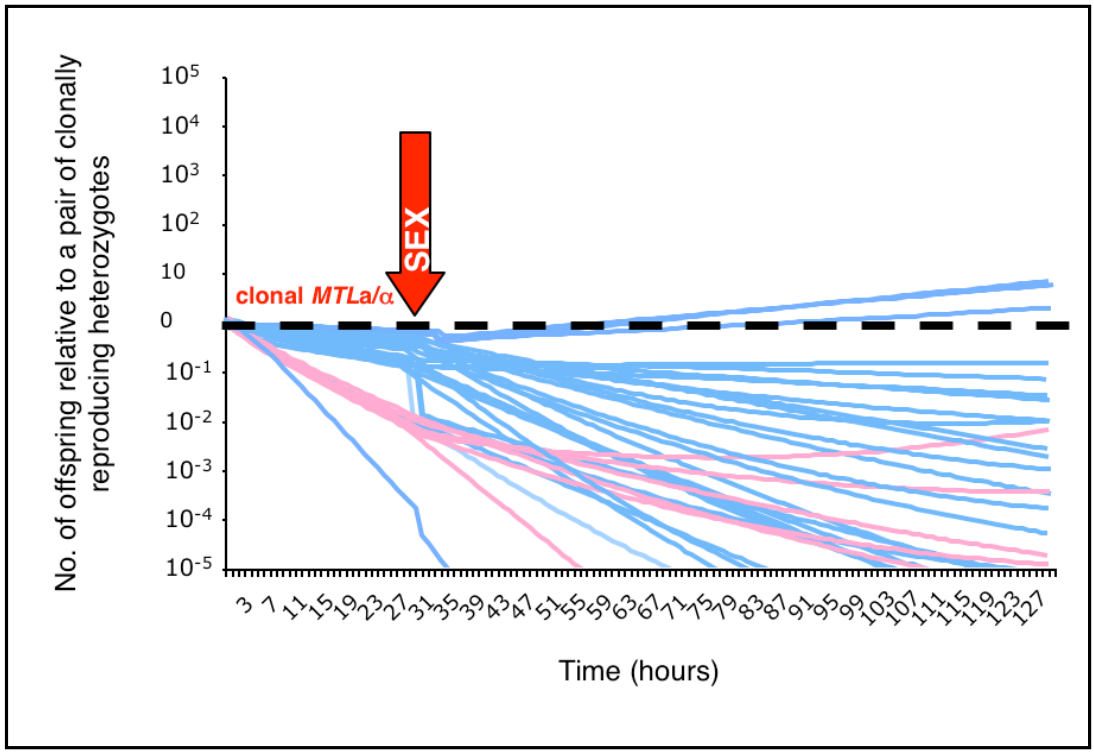


Figure 8.17. Alternative simulation of the number of progeny produced by pairs of cells which became *MTL*-homozygous at t_0 and the progeny of which mated after 30 generations, (marked by arrow).

The number of progeny is relative to the progeny both cells could have produced by continued clonal propagation when remaining heterozygous (dashed line). The mating process is assumed to take the same time as one clonal cell division. The simulation is based on growth rates experimentally determined in YPD medium. The number of progeny from 34 recombinants (listed in Table 2.1c) from all 15 successful matings were each compared to the progeny from both of their parents. Calculations used growth rates of *MTL*-homozygotes with resistance cassettes. The effect of experimental manipulations used to achieve mating competency and recovery of recombinants was eliminated computationally (see section 2.16.2. for details). Pink lines: recombination involving strain W17.

9.0 DISCUSSION

As described in the introduction, many researchers think that *C. albicans* uses covert and rare sex to generate new recombinant lineages in its natural environment, which help it to meet adaptive challenges (Alby and Bennett, 2010; Alby et al., 2009; Heitman, 2011). However, there is also conflicting evidence, suggesting that while *Candida* still possesses a marginally functional sexual machinery, the lineages created by sex may be inferior to clonal lineages and do not survive (section 1.3.6). The aim of my PhD project was to distinguish between these two possibilities, that is, to determine if *C. albicans* is likely to generate viable sexual recombinant lineages in its natural environment. Sex is expected to be more beneficial, by generation of new allele combinations from the parental genomes, if adaptation to a novel environment is required (de Visser and Elena, 2007). Therefore a comparison of the fitness of clinical isolates, their mating-competent *MTL*-homozygous derivatives and recombinants during adaptation to laboratory culture may help answer the question of whether there is a benefit for mating. If no net benefit of mating in laboratory culture is detectable, it is unlikely that mating would have any benefit in the human host.

A direct experimental test of the benefit of sex is measuring whether cells choosing sex over clonal reproduction improve their chances of passing on their genes to future generations. I therefore tested whether a benefit of sex was detectable in a set of ten clinical isolates (Table 2.1A), by mating the 10 strains in the laboratory in all possible combinations (Fig.3.4), and comparing the fitness of the resulting recombinants with that of their parents when they were adapting to grow in a new and unusual environment, laboratory culture (YPD medium).

I could not demonstrate a net benefit of sex under these conditions.

9.1 Not all clinical isolates can mate with each other

While previous studies have shown that *C. albicans* clinical isolates can mate in the laboratory, in most of these matings clinical isolates were mated with the auxotrophic derivatives from a laboratory strain SC5314 (Bennett et al., 2005; Legrand et al., 2004). The SC5314 derivatives, which are genetically almost identical, can mate easily with each other in the laboratory, and nuclear fusion occurs at a high efficiency during mating between strains derived from SC5314 (Bennett et al., 2005). However a small number of experiments with other strains suggests that differences in genetic background may often make clinical isolates incompatible or their mating machinery is not working that well with each other even if their mating type alleles are compatible (section 1.3.4). Such incompatibility may prevent mating or generate unfit recombinants. Clinical isolates may be much less capable of forming recombinants than the experiments with SC5314 suggest. This could be part of the explanation why there is little evidence of recombination in population analyses and why it is never seen in clinical samples.

As the frequency of completion of the mating process by nuclear fusion between clinical isolates is low (10%) (Bennett et al., 2005; Legrand et al., 2004), this makes it difficult to retrieve the small number of recombinants from a large number of parent cells without strong selective pressure. At the moment it is not clear if the few successes reported is because such matings were only rarely attempted or because recombinant retrieval is difficult and additional unsuccessful reports went

unreported. A better assessment of the frequency with which clinical isolates can mate requires a selection system for efficient recovery of rare recombinants arising from matings between *C. albicans* clinical strains. Auxotrophic markers are available for the lab strain SC5314, but are not the best choice for clinical isolates because achieving auxotrophy requires time-consuming disruption of both alleles of a particular biosynthesis gene. Two rounds of disruptions (transformations) also increase the chance of genetic damage to the strain (Bouchonville et al., 2009).

The introduction of two different drug resistance markers into parent strains should be a better way to allow selection of recombinants from mating between clinical isolates. Such markers are easier to introduce into strains than auxotrophic markers as they are dominant and, in addition, can be expected to interfere less with the fitness of recombinants in downstream experiments, as preliminary experiments showed auxotrophy reduced strains' growth rates even in rich medium (YPD) (data not shown). Only two drug resistance markers, *MPA*^{r} and N*AT*^{r,} were available in *C. albicans* at the start of this project (Shen et al., 2005). Two resistance vectors pNZ11 and pNZ4 were constructed as described in section 4.2 and 4.3. The *NAT*^{r} vector pNZ11 worked well and it was easy to get stable transformants with reasonable frequency. However, some problems with the transformation of the *MPA*^{r} vectors were encountered initially. I could not get stable integrated transformants using the plasmid 3408 constructed by Beckerman et al. (2001). This vector contains a native *IMH3* promoter, an *IMH3*^{r} gene from the strain 1161 that has three point mutations compared to the normal native *IMH3* gene, and confers MPA resistance, and a native *IMH3* terminator. Integration of this *IMH3* resistance cassette is directed to the native *IMH3* region by double or single cross over. This

was not easy to achieve as the *IMH3*^{r} is very similar to the native gene sequence and it was difficult to replace all of the three point mutations by double cross-over simultaneously. This vector also contains a putative ARS sequence in the *IMH3* promoter region which could promote multi-copy un-integrated high frequency transformation (Beckerman et al., 2001). This might be the reason why I could get transformants initially but after transfer of the resistant colonies on non-selective medium for several times, the resistance marker was lost. Also, the other *MPA*^{r} vector pNZ18 did not work. This vector contained a native *CaACT1* promoter plus the *IMH3*^{r} gene and the *IMH3* terminator from p3408 as described in section 4.3. Integration of the *IMH3* resistant cassette was supposed to be directed to the *ACT1* promoter region by single cross-over, or in the native *IMH3* region by double or single cross-over. I did not get any transformants with integration in the *ACT1* promoter region, probably because the *IMH3*^{r} gene plus the *IMH3* terminator (2kb) is much longer than the *ACT1* promoter (1kb) and most of the cross-over happened in the *IMH3* region. Transformation only worked when using a double cross-over strategy in which the plasmid contained two targeting sequences homologous to sequences in the *C. albicans* chromosome 7 (section 5.2). An additional advantage of this construction is that the tetracycline activator tetR-ScHAP4-WH11 in the cassette is under the control of the constitutive *CaENO1* promoter, and the MPA resistance-conferring *IMH3*^{r} ORF is under the control of a tetracycline-responsive promoter. This allows suppression of *IMH3*^{r} expression by tetracycline, in case the expression of an additional variant copy of the *IMH3* gene affected purine biosynthesis and thus fitness.

Transformation efficiencies of correct integration of pNZ11 and pNZ4 into the chosen target sites in *C. albicans* genome were reasonably high (section 5.2, 5.3),

making the two-dominant marker system easy to perform and efficient for marking different clinical isolates using only one round of transformation. The markers had usually no significant impact on fitness (section 8.4.3), except in one strain - W17*MTL*α. Transformation of strain W17*MTL*α with the *NAT*^{r} cassette resulted in a 43% reduction in growth rate. The reason for this remains unknown. A second W17*MTL*α clone was transformed with the *NAT*^{r} cassette but it acquired a similar growth defect (39% reduction compared to the unmarked W17*MTL*α). In both cases the cassette was inserted into the correct position, as with other strains, and did not disrupt any known native genes.

I developed a method to recover recombinants arising from the mating of derivatives of clinical isolates carrying the two drug resistance markers. I found no growth medium in which both MPA and NAT were effective for the recovery of rare recombinants, as described in section 6.2. The MA medium (0.67% Yeast Nitrogen Base with Amino Acids, 2% Glucose) supplemented with both MPA and NAT I developed allows dual selection. However, selection was only possible when the density of the cells was low (less than 100 colonies per 8 cm plate). Thus I used a stepwise selection procedure as described in section 6.2 to recover the recombinants.

I obtained recombinants from 15 out of 25 combinations of clinical isolates tested, providing an estimate of the frequency with which clinical isolate combinations are capable of mating of 60% (confidence interval 40%-80%). For a number of technical reasons this could be an over- or under-estimate of the true frequency with which clinical isolates can mate. One area of concern is that the drawn-out selection

procedure could lead to loss of some markers preventing recovery of recombinants in the final step. Indeed, the possibility that marker loss could be a problem was suggested by the observation that five combinations of parents did not yield recombinants even though each parent was capable of mating with other strains. In these mating attempts the markers from both parents could be detected frequently in the early stages of the second round selection on YPD+NAT (II) patches, but could not be detected from single colonies after the patches transferred to the final non-selective YPD (III) plate (Fig. 6.1). However in the five unsuccessful matings involving strain FJ11α, which is incapable of mating, parental markers were less frequently detected in the early stages of the selection on YPD+NAT (II) patches. Thus a more likely explanation than marker loss from recombinants is that in the five combinations that did not yield recombinants mating was initiated and cells fused, but nuclear fusion failed to occur and parental markers eventually segregated. This was also frequently observed in mating between clinical isolates reported by Lockhart et al. (2003). All of the 12 crossings conducted by these authors resulted in cell fusion, but nuclear fusion did not happen (Lockhart et al., 2003). I did not check the potential mating products by microscopy for zygote formation as I used white cells for mating and the zygote formation frequency would be too low to be observed.

Also, I found no evidence for rapid drug resistance markers or *MTL* heterozygosity loss and both drug resistance markers and *MTL* heterozygosity were retained more frequently than expected by chance, and DNA content of recombinant was not positively correlated with recombinant recovered (section 7.3.2). All of these

observations indicate that the selection procedure effectively recovers recombinants and is not the cause of failure for recovering recombinants from some matings.

In summary, it seems most likely that failure to obtain recombinants with the procedure developed reflects the inability of strain combinations to initiate or complete the entire mating process. In one strain (FJ11α) this was due to a general inability to mate. The mating failures of combinations of the remaining strains seem to be caused by combinations of specific incompatibilities, since all of these strains could mate with other clinical parents (Fig. 7.2.) or the universal tester strains.

Such incompatibilities may be medium-specific or merely reduce recombination frequency beyond the threshold of detection in my method. Several trials were undertaken to increase the mating frequency by using Spider medium as an alternative medium. In experiments mainly conducted with SC5314, Spider medium could increase nuclear fusion frequency (Bennett et al., 2005). Spider medium contains mannitol and YPD contains glucose as the carbon source. Bennett et al. (2005) claimed that media containing mannitol generate more mating products than media containing glucose, probably because Gpa1/Gpa2 signaling pathway as sensing glucose and inhibting mating. Although in my mating experiments using Spider medium the number of putative recombinants on the first MM+MPA plates increased about ten-fold compared to mating on YPD medium, stable recombinants were rare at the final stage after two rounds of drug resistance selection. For 13 pairs of isolates mating was attempted both on YPD and Spider medium, for three of them there were no recombinants from mating on Spider medium while there

were recombinants from mating on YPD medium. For two of them the frequency of recombinants from mating on YPD medium was higher (percentage of recombinants obtained from the final YPD plates tested by PCR relative to the putative recombinants picked from MM+MPA (I)) than that on Spider medium (28% compared to 2.5%, and 2.5% compared to 0.9%). For four of them recombinants were obtained from mating on Spider medium but not on YPD medium. However three of these involved one strain RIHO11 (which will be discussed below), and one had a mating frequency less than 1%; (1 recombinant in 110 putative recombinants) on Spider but not on YPD medium (< 1 %; or < 1/ 86). In the remaining three experiments no recombinants were obtained on either YPD or spider medium (Fig. 7.2 & Appendix 10.9). In conclusion, Spider medium is not better than YPD.

The low frequency of recombinants obtained from mating on Spider medium is probably due to the fact that the recombinants can more easily lose their chromosomes on Spider medium than on YPD medium. Other researchers have also reported that tetraploids lose chromosomes more slowly on YPD than on Spider medium (Forche et al., 2008). Possibly *MTL* heterozygosity is also not strongly selected for on Spider medium. These markers used for selection may often be lost when using Spider medium. While mating on YPD usually gives better recombinant yields, some strains may mate better on Spider medium. SC5314 may be such a strain (Bennett et al., 2005). In my experiments only one strain RIHO11 mated on Spider medium consistently better than on YPD medium, and recombinants involving RIHO11 (3 out of 4 combinations) were only obtained from mating on Spider medium (section 7.2). This strain underwent a morphological alteration after

loss of *MTL* heterozygosity following sorbose selection and grew only in the hyphal form. Mating between this strain and other strains was hard to be detected on YPD medium because RIHO11**a** was very sticky and hard to be transfered from plate to plate using my method. There were very few resistant colonies on MM+MPA (I) plates (Fig. 6.1) and none of them were verifiable as recombinants. However, it was easier to get recombinants from mating between RIHO11*MTL***a** and other strains on Spider medium (Fig. 7.2) except one combination (RIHO11*MTL***a** x HUN97*MTL*α). RIHO11*MTL***a** still grew in hyphal form on Spider medium and also could not be replica plated efficiently, but I could get more resistant colonies on MM+MPA (I) plates (putative recombinants) if mating happened on Spider medium, and the frequencies of confirmed recombinants relative to the putative recombinants were reasonably high (Fig. 7.2b). Another possible reason for the low frequency of recombinants recovered from Spider medium using my method is that the frequency of white-opaque switching is low compared to that of on YPD medium, as my method using opaque cells spontaneously arising from white cells for mating, and it was reported that mating was better on Spider medium when using opaque cells (Bennett et al., 2005). But this could not explain why I could get much more MPA-resistant colonies from the first round MPA selection on Spider compared to on YPD medium. In any event, to get a more accurate estimation of mating ability for clinical isolates, Spider medium should be used as an additional mating medium to recover recombinants if no recombinants were obtained using the standard YPD medium.

In *C. albicans*, opaque cells are the mating competent cells. Cells have to undergo a phenotypic switch to the opaque phase to become mating competent. Miller and

Johnson discovered in 2002 that the mating frequency of opaque cells is 10^6 times higher than that of white cells, by mixing cells of each auxotroph parent strain in a liquid culture and then deposited the mating mixture onto nitrocellulose filters, followed by selecting the recombinants on minimal medium (Miller and Johnson, 2002). I tested the plate mating method using opaque cells. However the opaque cells were too sticky and could not be replica plated on to other plates for mating and selection. My method presumably uses the rare opaque cells which spontaneously arise from white cells in YPD medium during the 7 days of incubation at room temperature for mating. While using opaque cells could increase mating frequency in theory, it was not practicable with the plate mating system. Instead, using white cells I could get a satisfactory mating frequency (section 7.2), and probably this system is more similar to the situation that might happen in nature.

I also tried mating in liquid culture by using either white cells or opaque cells and selecting the mating products using dual drug selection on MA medium. This method failed (data not shown), probably because the mating frequency between clinical isolates is very low and it is hard to isolate recombinants from liquid culture mating, as a biofilm cannot be formed in liquid culture, and contact between cells is important for mating. Other researchers (Lockhart et al., 2003) used liquid culture to mate *C. albicans* opaque cells from clinical isolates and checked the zygote formation between these cells with a microscope. However, as mentioned above, while they found that cell fusion (efficiency not reported) occurred between clinical isolates, nuclear fusion was not detected. This was most likely because the nuclear fusion efficiency between clinical isolates was too low and it was hard to isolate a

true recombinant with both parental markers without an efficient method. Indeed Bennett et al (2005) observed nuclear fusion in two recombinants from 20 zygotes from the same pair of clinical isolates with which Lockhart et al (2002) failed to obtain recombinants, by mixing opaque **a** and α cells and depositing the mixtures on nitrocellulose filters on solid medium (Bennett et al., 2005). Therefore mating happened on solid medium. They picked individual zygotes with a microscope from 24 h mating mixes using a glass needle attached to a micromanipulator, incubated them on YPD, and then re-streaked for single colonies. PCR was used to analyze the *MTL* configuration of these colony-purified progeny (Bennett et al., 2005). I did not try this method. As described in the introduction, only one case of successful mating between unmarked clinical isolates has been reported so far, probably because mating frequency between clinical isolates is quite low and an efficient selection system is required.

A quantitative mating method developed by Miller and Johnson (Miller and Johnson, 2002) has been modified and used by other researchers (Alby et al., 2009; Bennett et al., 2005; Huang et al., 2009; Schaefer et al., 2007). This method uses auxotrophic markers for mating cells that are SC5314 derivatives. Strains with different auxotrophic markers are grown in the opaque phase at 25°C overnight in liquid medium and approximately 3×10^7 cells of each strain mixed and deposited onto 0.8 μm nitrocellulose filters. The filters are then grown on the surface of YPD plates for 1–4 days at 23°C. Cells are collected from the filters and plated onto minimal medium to select for recombinants. I did not try this method either. As explained earlier, I decided against use of auxotrophic markers. Also, I did not find a suitable medium to do the dual drug resistant selection in one round, which would

have allowed me to modify the auxotrophic marker selection method for use with my drug resistance markers. In summary, it seems that the plate mating using white cells works for the two - marker system better than the other alternative methods I tried.

My method, using repetition on Spider medium if necessary, may give a more realistic assessment of mating frequency than previous methods. The mating frequencies determined by my method are comparable to what others have observed, or higher. This is supported by a comparison between the frequency of recombinant-generating combinations observed in this study (60%; confidence interval 40%-80%) and data reported by others. Reported frequencies range from 73% to 100%, when clinical isolates were tested for their ability to mate with SC5314 (Bennett et al., 2005; Legrand et al., 2004) and 50% (one pair out of two pairs tested) for mating with other clinical isolates (Bennett et al., 2005).

My experiments suggest that the ability to mate with SC5314 is not a true reflection of their ability to mate with other clinical isolates. A strain's mating capacity can be detected by mating it with the universal mating tester strains (which are *MTL* homozygous *MPA*^{r} auxotrophic derivatives of SC5314, refer to section 7.2), but even if a strain can mate with SC5314, it may not be able to mate with other strains, and there is no good correlation between the ability of a strain to mate with SC5314 and its ability to mate with other clinical isolates (Fig. 7.5). SC5314 is apparently a super-mater; it can mate with most strains tested by different research groups with high nuclear fusion frequencies. My mating method is more informative because it measures the mating frequency between different combinations of clinical isolates,

which has not been reported, except by Bennett et al. (2005) who reported mating between only two pairs of clinical isolates.

9.2 Mating barriers between clinical isolates

It appears as though some mating barriers exist between clinical isolates. First of all, one out of ten (FJ11*MTL*α) of strains tested was completely incapable of mating with *MTL***a** isolates because of a mutation in the mating type locus. Similarly, other researchers found that 1 out of 7 naturally occurring *MTL* homozygous strains could no longer mate due to a mutation, different from the one in FJ11, in its *MTLα1* gene (Legrand et al., 2004).

Secondly, barriers could be caused by incompatibilities of genetic background between strains, as I found mating incompatibility between specific strain combinations. Previous studies have suggested that *C. albicans* isolates can be assigned to at least five main clades (clade I, II, III, SA, E), based on genetic similarities (Odds et al., 2007; Schmid et al., 1999; Soll and Pujol, 2003). Mating may happen less between general-purpose genotype (GPG) strains (clade I). This group of strains belongs to a ubiquitous genotype that exceeds other genotypes in prevalence in a wide variety of hosts and circumstances (Schmid et al., 1999; Zhang et al., 2009). It is believed that general-purpose genotypes cannot survive if the allele combinations responsible for their competitive advantage under a wide variety of circumstances are broken up by sexual recombination (Forbes et al., 1997; Lynch, 1984), and indeed the population structure of GPG strains appears more clonal than that of the remainder of the species (Holland et al., 2002). My results

show that the GPG strains tested could mate both with other GPG strains and with non-GPG strains. However incompatibility (lack of detectable recombinants) occurred slightly more often in matings between GPG strains (50%, 3 out of 6 combinations) than in matings between non-GPG strains (33%, 2 out of 6 combinations). Also 6 out of 7 higher frequency matings (percentage of confirmed recombinants compared to number of potential recombinants tested > 10%) involved non-GPG strains. Thus one reason why GPG strains are more clonal is that they may be less likely to mate. However, the sample size was too small to reach any firm conclusions. Other researchers demonstrated that the ability to mate did not appear to correlate with the relatedness of the strains in a given cross (Bennett et al., 2005). They demonstrated that the nuclear fusion efficiencies from 6 strains mating within clade I varied from 32% to 93%, respectively, while mating between an isolate from clade I (GPG) and an isolate from clade II or clade SA showed rates of nuclear fusion of 58% and 32%, respectively (*C. albicans* clade designations are taken from Lockhart et al. (2003)). However, this was only judged by mating clinical isolates with SC5314 derivatives. As described earlier, mating with SC5314 may not necessarily give a true reflection of the mating ability of a strain. For the two pairs of matings between clinical isolates, one pair was a mating between clade I (GPG) and the other pair was between clade I (GPG) and clade II. These authors obtained no recombinants from the first pair (nuclear fusion efficiency was less than 6%) but got two recombinants from the second pair (nuclear fusion efficiency was 10%) (Bennett et al., 2005). Mating within GPG strains did thus have a lower frequency of nuclear fusion than mating between GPG strains and strains from other groups. But again the sample size was too small to reject chance as an explanation for the observed differences. In any event, factors other than the inability to mate

are probably more important in protecting the GPG-specific allele combinations, such as low fitness of *MTL* homozygotes and recombinants (see below).

I found another possible mating barrier in *C. albicans*, namely that each strain preferentially lost one allele of chromosome 5 (chr 5) – at least when sorbose selection was used. Since chr 5 loss is apparently the predominant mechanism by which homozygosity at the *MTL* locus arises, as judged from an analysis of spontaneously occurring *MTL* homozygous isolates (Wu et al., 2005), preferential loss of one copy of chr 5 could be caused by recessive lethal mutations on chr 5. Chr 5 has a large heterozygous region and several reports from other researchers have shown that recessive lethal mutations occur on other chromosomes (Sarachek and Weber, 1986; Whelan and Soll, 1982). Presumably a lethal mutation on one chromosome allele that is masked by the other allele and would be uncovered if the masking allele is lost. Consequently only the allele with the lethal mutation can be lost by sorbose selection. In this case one would only ever find one mating type. But this evidence is different from what happened in strain SC5314 and some other strains (W43 and FJ11 in our study), as both *MTL* locus could be lost during sorbose selection in these strains, indicating there is no presence of recessive lethal alleles on chr 5 (Forche et al., 2008). Probably only some strains carry recessive lethal alleles on chr5. However if strains preferentially lost one allele of chr 5 in nature this preference would only diminish mating frequency to the same degree as differences in mating type loci in fungi with a fully functional sexual cycle.

Also it is uncertain how strong a barrier against mating preferential chr 5 loss could be, as it was reported recently that both *MTL*a or *MTL*α strains of *C. albicans* are

capable of same sex mating (refer to 1.2.2.1) with *MTL*a or *MTL*α strains in the laboratory, implicating the potential for genetic exchange even within unisexual populations of the organism (Alby et al., 2009).

Lastly, in contrast to lab strain SC5314, in which chr 5 (containing *MTL* locus) loss was random in recombinants and could generate *MTL***a**/**a**, *MTL*α/α and *MTL***a**/α progenies (Forche et al., 2008), chromosome loss in the recombinants derived from mating between clinical isolates was not random[1], and *MTL* heterozygosity was strongly selected on YPD medium (see below), as it seems to be in nature (Lockhart et al., 2002). Thus mating seems to predominantly generate *MTL* a/α progeny, incapable of mating.

9.3 The fate of parental genes in recombinants

The tetraploid cells generated during mating of *C. albicans* have not as yet been observed to undergo meiosis. However, it has been shown that they can return to the diploid state by random chromosome loss via a parasexual process (Forche et al., 2008). The recombinants obtained by using my mating method were usually mononucleate and with a DNA content exceeding that of their parents, as verified by DAPI staining and FACS analysis, indicating that they were generated by the fusion of parental nuclei. *C. albicans* is considered a diploid yeast (Odds, 1988). However, not all of the clinical isolates are strictly diploid (Selmecki et al., 2010). Nine out of ten of my parental strains appeared to be diploid, by FACS analysis.

[1] The actual process of chromosome loss is probably random. However, subsequent selection which leads to predominance of clones with certain chromosome loss events is non-random.

They had similar DNA contents to the typical diploid strain SC5314. One strain, W17α, appeared to have a greater DNA content than other strains as determined by FACS analysis and some of the cells contain two nuclei (Fig. 7.7). In this case the recombinants generated by this parent were sometimes abnormal as well, some of them were multinucleate and the ploidy of these recombinants was hard to define by FACS analysis.

In more than half of the recombinants, the DNA content was close to that expected for tetraploids, i.e. comparable to the sum of the parents' DNA contents (Fig. 7.1). However, the DNA content of several recombinants was considerably less than the sum of the DNA contents of both parents (Fig. 7.1), indicating that recombinants were often no longer tetraploid at the time of isolation.

Another interesting finding is that the recombinants seem to always retained their *MTL* heterozygosity when they returned to their diploid stage, implying that chromosome loss was not completely random and *MTL* heterozygosity could be important to fitness for *C. albicans* clinical isolates. All the recombinants tested after serial propagation on YPD medium for 100 generations remained *MTL* heterozygous (Fig. 7.10). Also, even though recombinants were propagated in medium without MPA or NAT, loss of resistance cassettes occurred less often rather than more often than expected by chance (section 8.3). The reason for the selection of the retention of resistance markers on YPD medium is unknown. Non-random loss of chromosomes was further confirmed in some recombinants by the absence of parental gene markers. For one of these, *PNG2,* a specific allele loss was nonrandom even though it is not on the same chromosomes as the drug resistance

cassettes: an indication of non drug-related selection in laboratory culture. This result is apparently in conflict with a previous study (Forche et al., 2008), in which the tetraploid recombinants derived from the lab strain SC53145 efficiently returned to the diploid state by random chromosome loss and generated both *MTL*a and *MTL*α strains thus completing a parasexual cycle. This result is however only reported for mating between SC5314 derivatives and was quite different to the results with my clinical strains, suggesting once again that mating involving SC5314 is not a true reflection of mating between clinical isolates.

In conclusion, nuclear fusion occurs between clinical isolates during mating and spontaneous chromosome loss happens in recombinants derived from clinical isolates. However, this loss is not completely random[2], and fitness gain (not usually involving discarding drug resistance markers) is a driver of chromosome loss. As shown in the section 8.2, I observed a fitness increase of the recombinants during adapting to YPD medium over 100 generations which was correlated with the reduction of their DNA content (Fig. 8.5) that was expected to be the result of ploidy reduction of the tetraploid recombinants. It seems that the growth rates of the recombinants increase gradually, probably associated with the rate of chromosome loss for each strain.

It would be interesting to know how many chromosomes have been lost and whether heterozygosity is preferentially retained for most chromosomes. In general, it has been suggested that heterozygosity is favored (and analysis of sequenced

[2] The actual process of chromosome loss is probably random. However, subsequent selection which leads to predominance of clones with specific chromosome loss events is non-random.

SC5314 and WO1 supports this hypothesis, as most chromosomes are heterozygous).

9.4. Sex diminished parents' chances to pass on their genes to future generations.

In biology, fitness describes how successful an organism is at passing on its genes to the next generation. The more likely that an individual is able to survive and live longer to reproduce more, the higher is the fitness of that individual (Orr, 2009). The reproductive strategy of an organism is aimed at producing as many offspring as possible in the long term (Orr, 2009). A direct experimental test of the biological function of sex is whether cells benefit from choosing sex over clonal reproduction by improving their chances of passing on their genes to future generations.

In this study, a comparison of the fitness of clinical isolates, their mating-competent derivatives and recombinants, over 100 generations in laboratory culture showed that there is no net benefit of sex in *C. albicans*, but rather that it severely reduces the chances of the survival of parental genes. I used growth rates in liquid YPD medium as a fitness indicator. In such an artificial environment sex should be more advantageous than in the host as accelerated adaptation to altered environments, by generation of new allele combinations from the parental genomes, is held to be one of the main benefits of sex (de Visser and Elena, 2007), as described in the introduction.

I could not carry out a direct competition experiment to compare the success of clonal versus sexual recombination in laboratory culture, relying upon parental strains to mate and compete with recombinants in the same experiment. Because the mating frequency between clinical isolates was very low (approximately 1 recombinant was obtained from 10^6 to 10^7 cells when I combined mating-compatible combinations of white cells of clinical isolates' *MTL* homozygous derivatives (data not shown)), mating would be extremely rare if I just grew up sets of clinical isolates and expected their spontaneous occurring *MTL* homozygotes to mate. Analyzing how these could compete with their heterozygous ancestors in the same serial culture would be infeasible because of their small numbers. The same argument applies to animal model experiments as well. Instead, to be able to demonstrate the overall net benefit of sex, I compared the growth rates of each of the recombinants and their parents separately, in YPD medium, as described in result section 8.2, and then used several simulation methods to estimate the total fitness effect of the loss of *MTL* heterozygosity plus mating (section 8.5).

The growth rates of the recombinants and their parents had to be adjusted for these simulations. Several factors that may affect the fitness of the recombinants and their parents were considered. As described in the introduction, the first step towards mating for a heterozygous clinical isolate is loss of *MTL* heterozygosity. Loss of one mating type locus via sorbose selection was associated with a severe decrease in fitness (section 8.4). After 30 generations the growth rate was usually close to that of the original parent. I did not analyze the karyotypes of these derivatives, but sorbose selection typically brings about the loss of one copy of chr 5, often followed

by the duplication of the remaining copy (Kabir et al., 2005). The initial loss of one copy of chr 5 is the most probable cause of the initial fitness loss. The subsequent chr 5 duplication, plus possibly additional genome rearrangements, as occur both in association with sorbose selection (Kabir et al., 2005) and in naturally occurring *MTL* homozygous isolates (Wu et al., 2005), may be instrumental in restoring fitness.

Previous studies revealed that loss of one copy of chr 5 followed by duplication is the predominant mechanism by which spontaneous homozygosis at the *MTL* locus arises in the laboratory (Wu et al., 2005). However, Wu and his colleagues suggested that in nature, such *MTL* homozygous are rapidly removed due to the loss of competitiveness with their *MTL* heterozygous parent strains (Wu et al., 2007), and that therefore most natural *MTL*-homozygous strains which have been isolated have arisen by an alternative mechanism, namely through multiple mitotic cross-overs outside the *MTL* locus (Wu et al., 2007). They reached this conclusion by testing the heterozygosity for non-*MTL* genes along chr 5 in 11 natural *MTL* homozygous strains and some degree of heterozygosity were detected, although natural a/a or α/α strains are less polymorphic along chr 5 than natural a/α strains. They also found that the great majority of polymorphisms were shared between natural *MTL*-heterozygous and natural *MTL*-homozygous strains, indicating the markers employed were good indicators of ancestral heterozygosities, not recent mutations, suggesting the heterozygosity of non-*MTL* sites in natural *MTL*-homozygous strains evolved before *MTL*-homozygosis.

While I did not directly establish this, my sorbose-derived *MTL* homozygotes are likely to have arisen from losing of one copy of chr5 followed by duplication (Kabir et al., 2005), and may thus not be representative of the most commonly isolated *MTL*-homozygotes. Also, sorbose may select for other mutations that may affect fitness. However their fitness, initially reduced by about 44%, recovered to a level of 8.6% (median reduction around 3%) lower than that of their *MTL*-heterozygous ancestors after serial propagation, whereas that of my two spontaneous *MTL*-homozygous derivatives remained reduced by 20% compared to the original heterozygotes. Also all naturally occurring homozygote isolates I analyzed (including all of the 11 *MTL* homozygous strains described in Wu et al, 2007 paper[3]) had a growth reduction of 9.3% (mean) or 3% (median) compared to the heterozygotes used in this project.

So, it is fair to say that the sorbose-induced loss of chr 5 has, if anything, a lower impact after 30 generations than spontaneous loss seen in clinical *MTL*-homozygous isolates. And that probably spontaneous chr 5 loss has an initial impact that is beyond what we see in the natural homozygotes. Therefore, if I assume in my simulations that the reduction of growth rate of each of my isolates upon chr 5 loss was equivalent to the growth rate measured 30 generations later, that was a very generous assumption of what is probably happening in the natural environment.

In addition, assuming that the natural isolates obtained by Wu et al. (2007) were indeed generated by mitotic recombination, my fitness data cast doubt on their

[3] The 11 *MTL* homozygous strains described in Wu et al, 2007 paper which I analysed actually had a growth reduction of 10.6% (mean) or 8% (median) compared to the heterozygotes used in this project.

assumption that such *MTL* homozygotes are fitter and compare better with their heterozygous ancestors than those derived by chr 5 loss.

Another factor that makes it difficult to compare the fitness of clonally reproducing clinical isolates with those of *MTL* homozygotes and recombinants is the need to genetically modify the *MTL* homozygotes prior to mating in order to be able to select recombinants. Both the resistance cassettes themselves and genetic damage incurred during transformation could reduce fitness.

My results show that the presence of resistance markers had no readily demonstrable growth impact for the recombinants and the parents. Most of the recombinants tested retained both of the resistance markers after 100 generations, and the rates of marker loss (0 for the MPA resistance marker, 8% for the NAT resistance marker) were lower than the rate of median DNA content reduction (21%). This lower than expected loss of markers indicates the absence of a negative effect of individual markers on fitness or negative epistasis between the resistance markers (section 8.3). In the parental strains, transformation of the *MTL*-homozygous derivatives with the NAT^r or the MPA^r marker had no significant impact on growth rate for 9 of the 10 parental strains, except for one strain W17, in which a severe growth defect occurred during transformation (section 8.4). Nevertheless in the growth simulation, two calculations were carried out in case there was a small marker effect on the growth rates of the recombinants and their parents. In the initial calculation, I combined the data from resistance cassette-less *MTL* homozygotes with those from recombinants obtained by mating of parents containing resistance cassettes. To link the two analyses, growth rates of

recombinants were expressed as multiples of the growth rates of their resistance cassette-marked parents. These factors were then multiplied by the growth rate of unmarked *MTL* homozygous derivatives of the same strain to estimate what the growth rate of the recombinants without resistance cassettes would have been (section 8.5). However, since there was no significant marker effect on growth rates for homozygous parents, other than W17, and since the marker correction factor can be misleading if genetic damage occurs during transformation (in W17*MTL*α), this simulation method overestimates the benefit of sex in W17. The alternative simulation omits the marker effect factor for the recombinants and uses the growth rates of marked homozygotes for 30 generations before mating. There were no obvious differences between the two calculations except for W17, for which using the alternative simulation method is more realistic, as mentioned in section 8.5. In conclusion, by using both calculation methods the marker effect can be corrected for allowing me to make a fair comparison of growth simulations in culture.

Mating, followed by subsequent chromosome loss may help cells to adapt in laboratory culture. It is believed that the sexual cycle of *C. albicans* is completed by returning initially tetraploid recombinants to diploidy, through spontaneous chromosome loss (Forche et al., 2008). Unlike their parents, tetraploid recombinants can draw on two parental genomes to generate, by selective chromosome loss and retention, allele combinations that increase their fitness in a novel environment. Thus the recombinants should have a long-term advantage over parents in adapting to YPD medium by selectively discarding parental chromosomes. As described in section 9.3, the fitness increase in the recombinants did correlate with the reduction in their DNA contents. Interestingly, it was evident that if there was no growth

increase for a given recombinant, the DNA content of this recombinant did not reduce (for example, the recombinants involving strain AU35), even if the recombinant was a tetraploid. In contrast, DNA content reduction did not necessarily lead to an increase of growth rate. This is expected as some chromosome combinations may have no negative epistasis between genes contributed from their parents. Indeed, 11 out of the 28 recombinants tested had reached their diploid stage after 100 generations of transfer in YPD medium, determined by FACS analysis. The mean and median growth rate reduction of these diploid recombinants was 10% and 4%, respectively, compared to their heterozygous ancestors (section 8.4.2). Thus even if the recombinants have an advantage over parents in adapting to YPD medium, not only would the fitness reduction at the early tetraploid stage lead them to be out-competed by their diploid parents, but also even when returned to diploidy can they not compete with the *MTL*-heterozygous ancestors of their parents.

After serial propagation for 100 generations, the recombinants were equally likely to grow marginally faster or marginally slower than their homozygous parents. But with only very rare exceptions did they grow faster than the original heterozygous clinical isolates. As mentioned in section 8.5, it seems that in the big picture, we could not see a net benefit for sex, even at best, and under the most favorable circumstances and assumptions. My results suggested that overall, the fitness increase of the recombinants with time did not eliminate the fitness differential between the original clinical isolates and their recombinants. With rare exceptions, sexual recombinants' fitness was not sufficiently high to compensate for the cost. The median number of offspring (excluding W17 and its recombinant descendents)

130 generations after reaching mating competency and mating was less than 0.05% (0.03% to 0.04%) of that of a heterozygous clinical isolate which had continued to reproduce clonally, measured by using either of the simulation methods.

There may however be a slight long-term advantage to recombination for some strains, at least in an 'YPD universe'. Three strains, AU35, HUN97 and YSU63 did not lose fitness when they became *MTL* homozygous, and when AU35*MTL*a mated with either HUN97*MTL*α or YSU63*MTL*α, the recombinants produced more progeny (over 130 generations) than either of their parents or the sum of both parents (Fig. 8.14 and 8.15). However, even after 100 generations of transfer on YPD medium, most of the recombinants derived from strain AU35 (6/7) did not reduce their DNA content like other recombinants. They retained the ploidy they had on isolation: they were tetraploid or triploid. If this happened in nature, one would expect that tetraploid, or close to tetraploid, clinical isolates would be discovered in nature, which has not yet been reported for a long time until recently, two natural tetraploids of *C. albicans* has been discovered (unpublished report). It is not known if these tetraploids are the result of mating or some other chromosomal event. The tetraploids grow poorly on laboratory medium, probably indicating that selective pressures in nature may be very different from those in the lab, and that 'sick' strains may still be able to propagate for some time in nature. However, the extremely low frequencies of tetraploids obtained in nature suggest these strains can not compete with the diploids and probably will be quickly eliminated by genetic drift. And also if one mating generates highly successful tetraploids, these cells are probably not able to mate ever again. Thus these advantages may be unique to laboratory culture in the short term and do not support a continuing role for sex in

nature, or even in an 'YPD universe', if these tetraploid strains were the only positive outcome of sex.

For strain RIHO11, sorbose selection changed its morphology and very much reduced its growth rate. The morphology of the recombinants derived from this strain recovered the normal morphology and growth rate. Therefore this strain would sometimes get benefit from mating with certain other strains. The strain W17*MTL*α (which got a severe growth defect after being marked with a *NAT*^{r}) got the most benefit from sex, as shown in Fig. 8.14 and 8.15 (pink lines). However, as shown in Fig. 8.16 and 8.17, this strain could be eliminated quickly before it had a chance to mate, as it would produce much fewer progeny before it found a mating partner.

Therefore, even if there was a long-term net benefit for some strains, the price of mating competency was usually a severe reduction in fitness that would tend to eliminate the mating competent strain before this benefit could be realized. Control experiments established that this was not (usually) the result of the introduction of drug resistance cassettes and other manipulations of the strains, necessary for obtaining recombinants.

It may be argued that recombinants' growth rates may continue to increase further after 130 generations. Indeed it is possible that all clinical isolates that are capable of mating could generate recombinant progeny, which could eventually be better adapted than, and outnumber, their clonal progeny. However the benefits of such hypothetical long-term gain are insubstantial compared to a number of

considerations listed below, which indicate that sex confers even fewer benefits in its natural environment to *C. albicans* than the data calculated in result section 8.5 suggest.

One consideration is that in my calculations I used growth rates for homozygotes measured after 30 generations of serial propagation (result section 8.5). I did this because these rates were comparable to those of naturally occurring *MTL* homozygotes. However I found that loss of *MTL* heterozygosity initially leads to a more severe decline of growth rates (median reduction was 44% compared to their heterozygous ancestors); therefore my calculation probably inflates the number of mating cells at 30 generations and thus of the recombinant progeny.

Also, it is apparent from Fig. 8.9 that due to the slow growth of homozygotes, any extension of the time between loss of *MTL* heterozygosity and mating will further reduce the eventual number of the recombinant progeny and thus the benefit of sex. In my simulations *MTL* heterozygotes mated after 30 generations. In the host it will probably take much longer before they can find a suitable partner and can mate. *MTL* homozygotes are rare among clinical isolates (prevalence of 3-10% (Legrand et al., 2004; Lockhart et al., 2002), only ~ 60% of pairwise combinations of *MTL* homozygotes of opposite mating type may be capable of producing recombinants (this study,(Bennett et al., 2005; Lockhart et al., 2003), and only if both have also undergone an epigenetic switch to the mating-competent opaque phenotype (Heitman, 2010). Even when mating is initiated, only a small fraction of encounters

between cells that are capable of forming recombinants actually yield recombinants (this study, Bennett et al., 2005; Lockhart et al., 2003).

When mating occurs, one new cell is formed while clonally propagating cells continue to multiply exponentially, a well-known intrinsic cost of sex (de Visser and Elena, 2007). My results (Fig. 8.14 to 8.17) may not show the full severity of this effect because my calculations assume that mating only takes as long as one clonal cell division, an underestimate, at least under laboratory conditions (Lockhart et al., 2003).

It would be informative to test if recombinants can compete with their parents in an animal model of colonization by *C. albicans*. In the laboratory culture, some recombinants eventually grow faster than their homozygous parents – albeit by 130 generations usually not (yet?) faster than their *MTL* heterozygous ancestors (section 8.4). It is uncertain whether such relative fitness gains will occur in the natural environment to which the parents are already adapted; if so, they are expected to be less pronounced than the gains I observed (de Visser and Elena, 2007). Indeed our collaborators at the University of Otago co-inoculated the oral cavities of rats with pairs of *MTL***a** and *MTL*α strains. Recombinants were sometimes observable in samples taken 7-days post-inoculation, but their numbers decreased over time, while overall *C. albicans* colonization levels increased (E. Rodrigues and R. D. Cannon, personal communication). This indicates that in a more natural environment, recombinants could not compete with their parents.

Lastly, in my simulations, cells that chose sexual reproduction cannot become extinct. In the host however, a given host niche can only support a limited number of cells which compete with each other for resources. Fitness loss will reduce the percentage of cells of the less fit genotype in the population, especially if their frequency in the population is small, as is likely to be the case for *MTL* homozygotes and recombinants. This will increase the risk of its extinction, which is in direct inverse proportion to its frequency (Kimura and Ohta, 1969). Thus in an environment containing both clonally and sexually reproducing cells, gaining mating competency and mating would further reduce the benefit of sex beyond that indicated by my calculations.

As described above, in my experiment, I tested how much net benefit mating may be, by looking at growth rates of strains in YPD medium. Since sex should be most beneficial when a species encounters a novel environment, i.e. sex would be more likely to be beneficial in helping *C. albicans* adapting to the strange world of YPD-filled flasks than it would be in their usual environment-human host, by generating novel allele combinations from the parental genomes. The sexual recombinants should have the ability to grow as fast as possible in a very rich medium. However, my results suggested that with rare exceptions, sexual recombinants' fitness was insufficient to compensate for the cost during adaptation to YPD medium. In general, a net benefit of sex was not observed under this condition. As argued above, the benefits of sex are likely to be even smaller in the human host. However, it has been proposed that organisms that can produce both sexual and asexual

progenies are expected to mate more frequently when stressed, and such stress-induced mating is predicted to facilitate adaptation. A study carried out by Goddard et al. (2005) provides experimental support for this model in *S. cerevisiae*. Asexual cells grew as well as sexual cells in rich medium (a low glucose YPD medium) but were not as fit as populations allowed to undergo sexual reproduction in a harsher condition (elevated temperature combined with osmotic stress). This result suggests that there is a connection between stress and selection of recombinant strains (Goddard et al., 2005). Also, Berman and Hadany (2012) proposed that parasex could occur much more frequently under stressful conditions in *C.albicans* to generate high levels of diversity, helping this yeast to adapt to different host niches (Berman and Hadany, 2012), based on assuming that stress could induce *MTL* homozygosis, white-opaque switching, initiating mating and promoting chromosome loss. It can be argued that under more stressful conditions the benefit of sex would be detectable in *C. albicans* and ideally, I should have examined growth under a variety of more selective conditions to determine if mating products could provide an advantage under harsher environmental conditions. However, as will argue below, I think even under stressful conditions, sex may still have little benefit in *C.albicans*.

Firstly, the benefit of sex would be most apparent during cells adapting to a novel environment. However, it is claimed by Goddard et al. (2005) that no adaptation was observed either for sexual cells and asexual cells in the benign medium, but markedly adaptation was observed in the harsh medium, indicating both type of cells were well adapted to the benign environment tested. Obviously the benign

medium (a low glucose YPD medium) is very close to the environment of the lab strains (and *S. cerevisiae*'s natural environment) that was used so that there was no possible benefit. Only in a harsher condition (elevated temperature combined with osmotic stress), adaptation and the benefit of sex was detected (Goddard et al., 2005). In my case, adaptation and fitness gain was observed in YPD medium for sexual recombinants. This evidence indicates that YPD medium can be used to detect the benefit of sex, as it did provide some selection pressure. Sex should help the cells to make the adaptation (fitness gain) faster than clonal reproduction, if it is still functional. What I am investigating is if sex helps in adaptation to a novel environment. The direction of this adaptation is irrelevant. So YPD is an appropriate system for me as is the harsh medium for Goddard. Maybe a harsh medium may allow even more fitness gains in *Candida* than YPD. Not because it is harsher, but because it is more different to the host than YPD. But if so that would mean that such experiments might lead to wrong conclusions because the overestimation –even more than YPD already does, the benefits of sex.

Secondly, the experiments carried out by Goddard et al. (2005) used the model strain *S. cerevisiae*. This strain has a functional sexual machinery including meiotic recombination. This is the most important aspect of sex. The mating products of S. cerevisiae do not have to become tetraploids first. However, *C.albicans* apparently lost the ability to undergo meiosis and the mating of two diploid cells generates tetraploids. As described previously, the tetraploids normally have a growth reduction compared to their diploid ancestors. Probably this is why in the benign medium, sexual lineages can grow as good as asexual lineages in *S. cerevisiae* but not in *C.albicans*. This evidence at least implied that sex has no disadvantage in *S.*

cerevisiae under normal conditions, but this is not the case for *C. albicans* in which engaging in sex severely diminished the chance of cells to pass on genes to future generations, as described in this study. Also the disadvantages to sex are largely caused by events prior to mating in *C. albicans*. Mating did improve adaptation in YPD medium, however, this benefit appear not sufficient to compensate its cost, especially the cost of loss of *MTL* heterozygosity. There is no reason to believe that *MTL* homozygosity loss has less consequence in a harsh medium.

Thirdly, probably it will provide more solid evidence if I have tested fitness of my strains in a more harsh environment, but previous studies carried out by other researchers revealed that it is probably not a necessity. This is because: (1) viability loss associated with returning to diploidy was detected in *C. albicans* in a harsh medium. Even stress conditions lead to more frequent parasex in *C.albicans* as hypothesized by Berman and Hadany (2012), this does not necessary mean the sexual lineages would compete their parents, as previous studies carried out by Bennett et al. (2003) revealed that in a stress medium (pro-spo medium), stress led to cell death for tetraploids while they were losing their chromosomes to reach the diploid stage (Bennett and Johnson, 2003). The number of viable cells obtained from pre-spo plates was <1% after 2 days for tetraplod cells while no chromosome lost were observed from diploids. This is a indirect evidence that in a more stressful condition, even mating could occur more frequently (which has no experimental support so far), the sexual recombinant lineages may still not be able to compete their parents in the laboratory. (2) Stress conditions also increase rates of mitotic recombination in *C. albicans*, which is measured as loss of heterozygosity (LOH) at

specific loci. Forche et al. (2011) proposed that “this "stress-induced LOH" facilitates the rapid adaptation of *C. albicans*, which does not undergo meiosis, to changing environments within the host” (Forche et al., 2011). Therefore, through LOH, adaptation is also faster for asexual cells. So that might reduce the benefit of sex in a harsh medium.

Is it possible that same-sex mating confers more benefit than *MTL*a/*MTL*α mating? It has been reported that *C. albicans* can undergo same sex mating in the laboratory (Alby et al., 2009; Heitman, 2011). Probably *C. albicans* uses this mechanism to promote homothallic mating instead of heterothallic mating in nature to reduce the level of recombination. Another important human fugal pathogen *C. neoformans* has a similar same-sex mating cycle (Lin et al., 2007). This specialized sexual cycle could play a role in survival of these strains as opportunistic human fungal pathogens (Alby et al., 2009; Heitman, 2011 ; O'Gorman et al., 2009). However, my results showed that loss of *MTL* heterozygosity significantly reduced the fitness of a given strain and that this seems by far the largest cost of sex. It thus seems unlikely that same sex mating has a net benefit for *C. albicans*.

9.5. Conclusions

The results presented in this study show that not all clinical isolates can mate with each other. Mating barriers exist between clinical isolates and presumably would restrict mating for *C. albicans* in the human host. For the pairs that can mate, sex

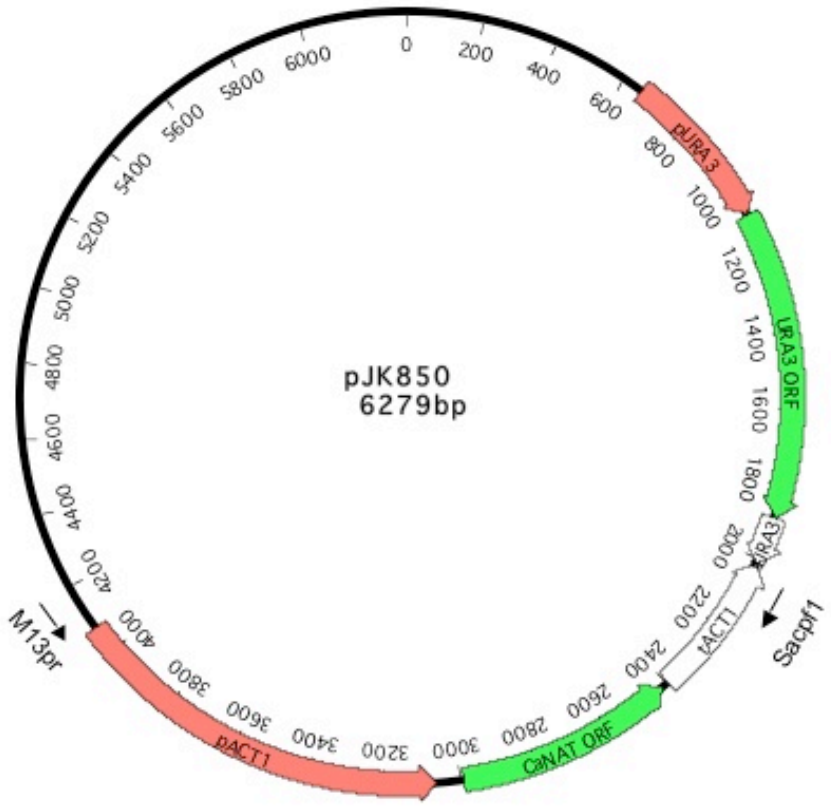
assists mating-competent *C. albicans* strains to adapt to a novel environment and can compensate for genetic defects. Nevertheless, in most cases I was unable to demonstrate that sex increases the chances of parents passing on their genes to future generations, in spite of experimental conditions favoring sex over clonality and making assumptions that would favor recombinants. With rare exceptions, sexual recombinants' fitness was not sufficiently high to compensate for this cost, even in an artificial environment, in which the benefit of sex should be larger than in environments to which the parents are already adapted. There may be a slight advantage to some recombination. However even if this were the case, genetic drift coupled with the initial fitness loss would make it unlikely that rare spontaneous *MTL*-homozygous variants in a population would survive long enough to mate and any recombinant offspring remain long enough to realize this benefit.

My results do not support the hypothesis that sex is still a viable reproductive strategy for *C. albicans*. A more likely explanation for its residual ability to mate, and slight signatures of recombination in its population structure, is that sex has lost its function so recently that neither the mutational decay of the sex machinery nor the decay of the signal of recombination in genetic marker distributions used in population studies are yet complete. A similar explanation might apply to other fungal pathogens for which a limited ability to mate has recently been demonstrated, such as *C. neoformans* (Kwon-Chung, 1975; Kwon-Chung, 1976; Lin et al., 2007) and *A. fumigatus* (O'Gorman et al., 2009).

10.0. APPENDICES

10.1. Appendix 10.1. Plasmid pJK850

(Shen et al., 2005)



10.2. Appendix 10.2. Plasmid pBlueScriptII KS(+)

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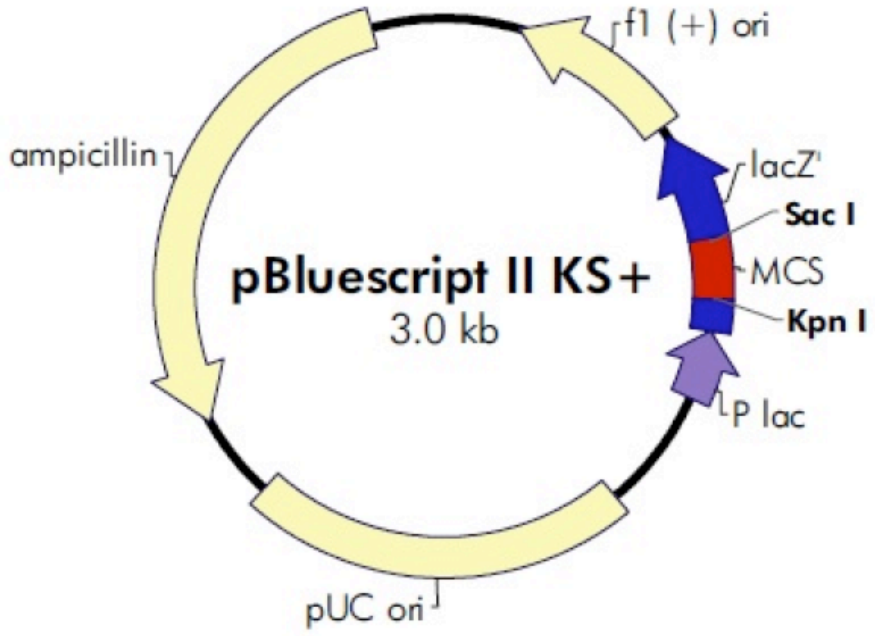

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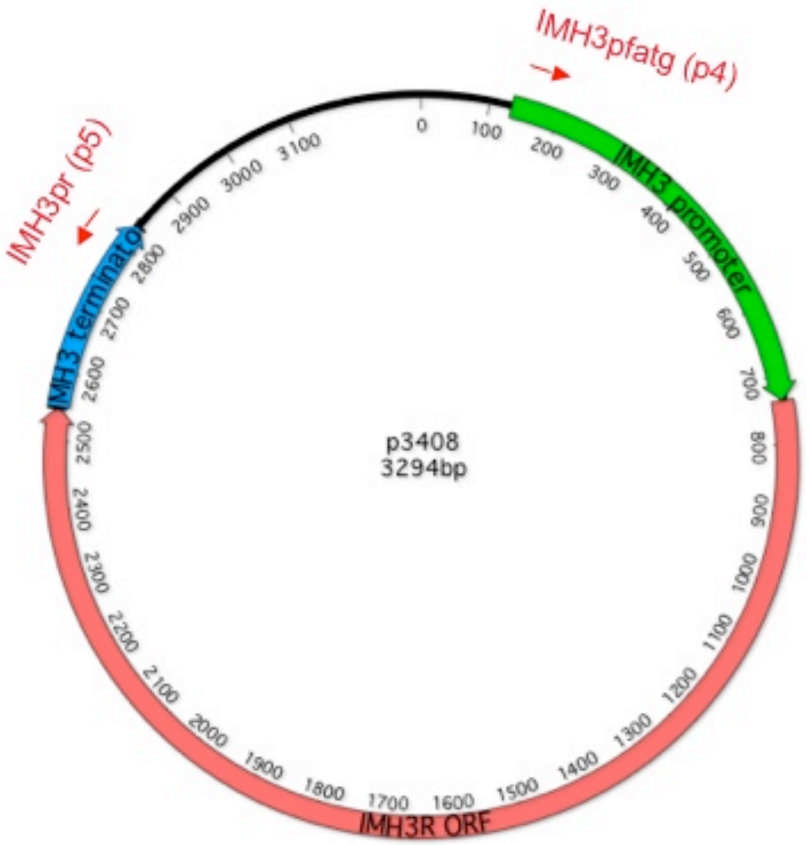
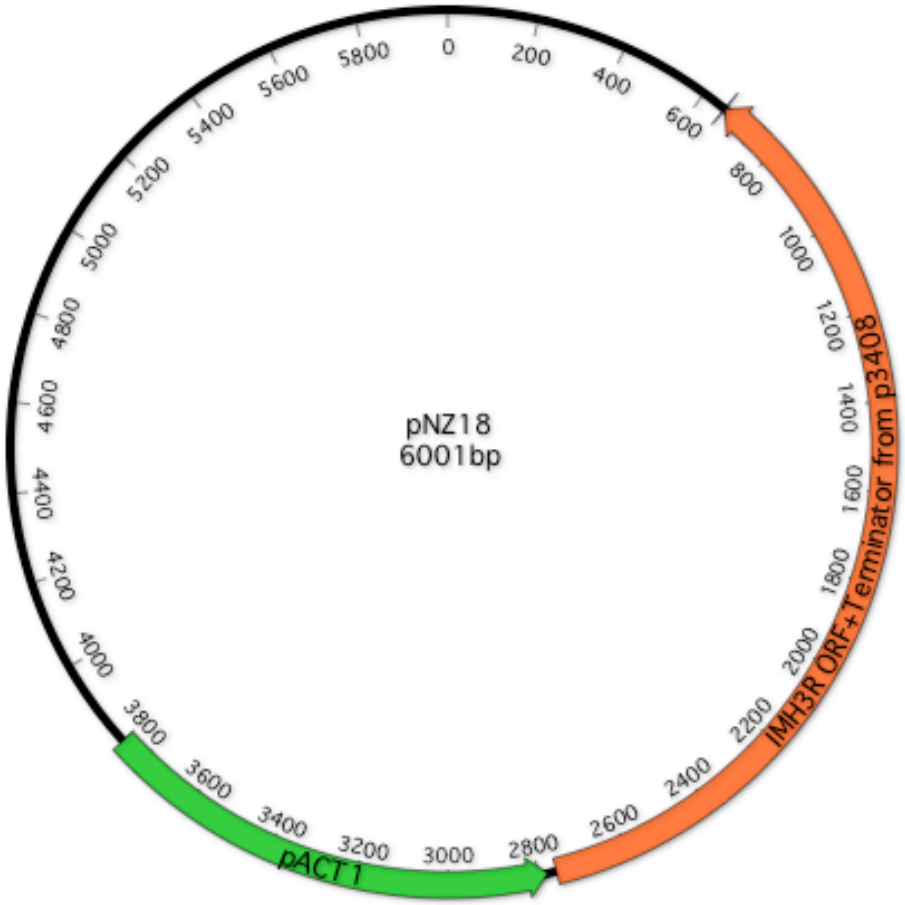
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
lac promoter 817–938
pUC origin 1158–1825
ampicillin resistance (bla) ORF 1976–2833

10.3. Appendix 10.3. Plasmid plasmid p3408

(Beckerman et al., 2001)

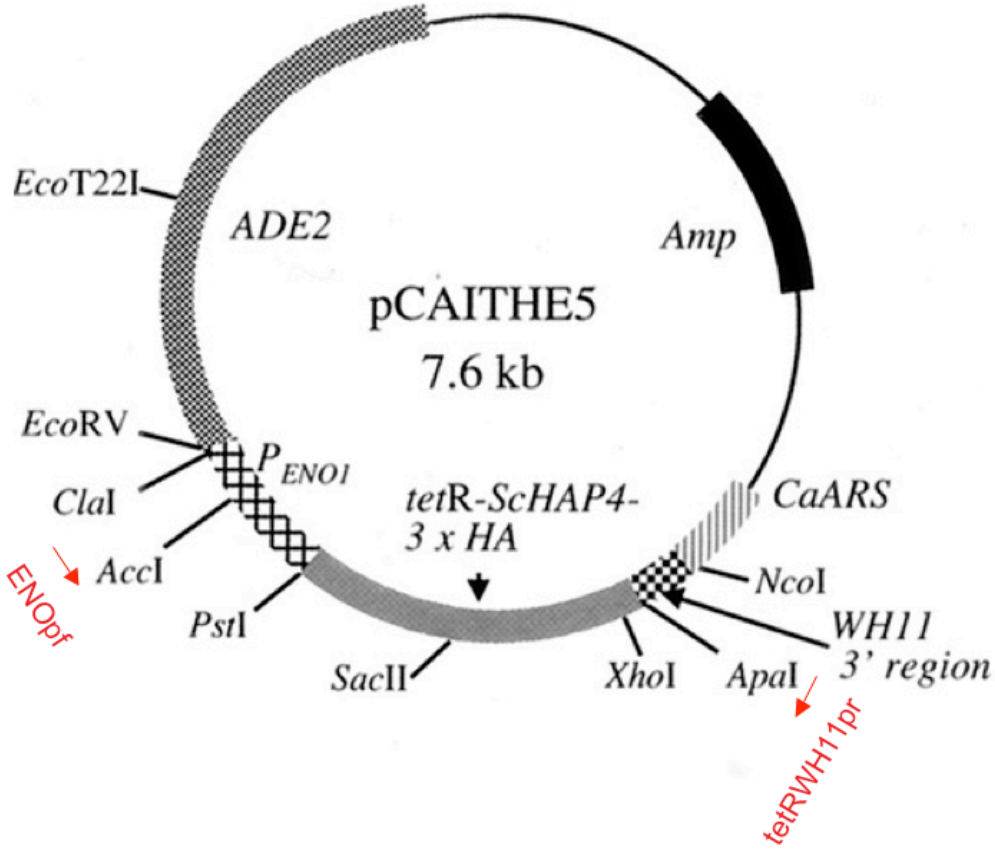


10.4. Appendix 10.4. Plasmid pNZ18



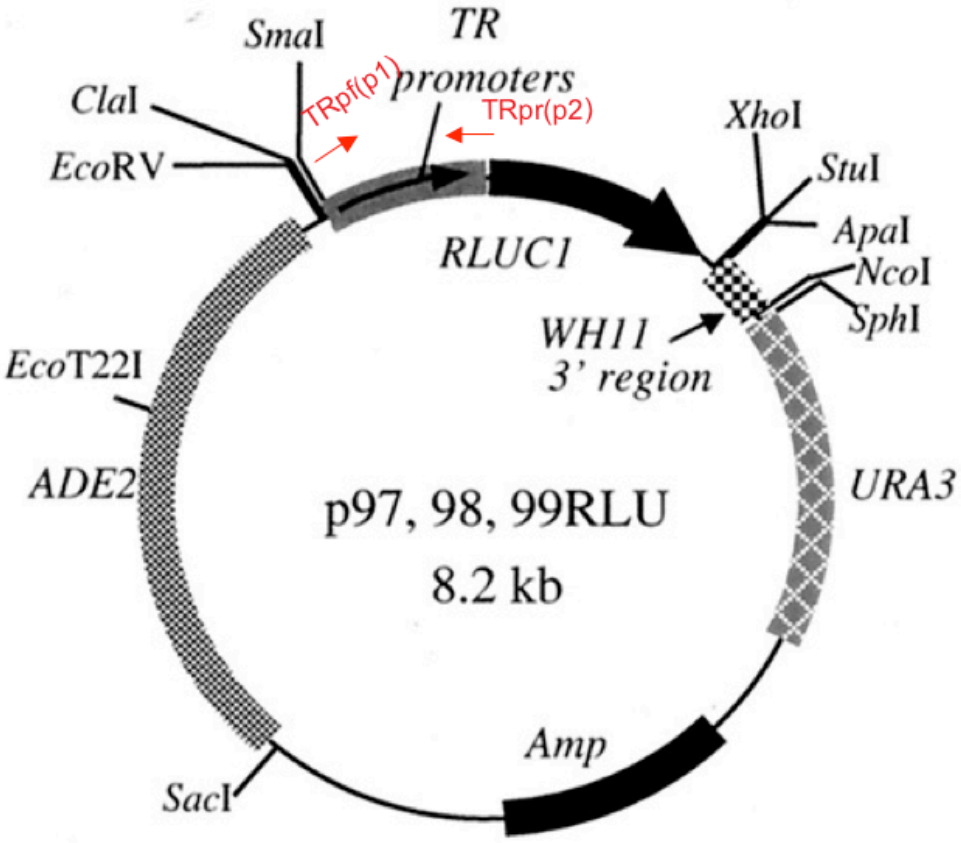
10.5. Appendix 10.5. Plasmid pCAITHE5

(Nakayama et al., 2000)



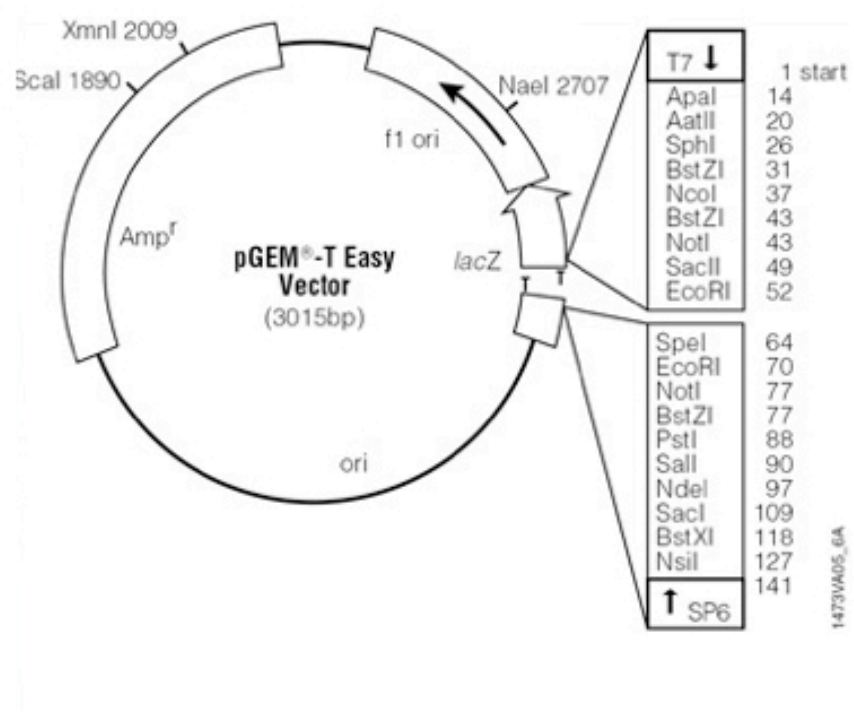
10.6. Appendix 10.6. Plasmid p99RLU

(Nakayama et al., 2000)



10.7. Appendix 10.7. Plasmid pGemT-Easy

Source: http://www.promega.com/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol/



10.8. Appendix 10.8. Sequence of plasmid pNZ11 (GenBank: FJ804173.1)

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            other sequences; artificial sequences; vectors.
REFERENCE   1  (bases 1 to 4925)
  AUTHORS   Zhang,N., Magee,B.B., Magee,P.T., Cannon,R. and Schmid,J.
  TITLE     A method for mating clinical Candida albicans isolates
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 4925)
  AUTHORS   Zhang,N., Magee,B.B., Magee,P.T., Cannon,R. and Schmid,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-MAR-2009) IMBS, Massey University, Riddet Road,
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                     /note="Candida albicans tACT1"
     CDS             complement(1063..1635)
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10.9. Appendix 10.9. Sequence of plasmid pNZ4 (GenBank: FJ804172.1)

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            other sequences; artificial sequences; vectors.
REFERENCE   1  (bases 1 to 9809)
  AUTHORS   Zhang,N., Magee,B.B., Magee,P.T., Cannon,R. and Schmid,J.
  TITLE     A method for mating clinical Candida albicans isolates
  JOURNAL   Unpublished
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  AUTHORS   Zhang,N., Magee,B.B., Magee,P.T., Cannon,R. and Schmid,J.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-MAR-2009) IMBS, Massey University, Riddet Road,
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10.10. Appendix 10.10. Table of mating experiments

Table 10.1. Results of mating experiments

Crosses[1]	Experiment	Number of recombinants obtained (No. of colonies tested[2])
W43**a** x OD8916α	1	5 (50)
	2	45 (150)
	3	8 (50)
	4	4 (25)
W43**a** x FJ11α	1	0 (150)
	2	0 (50)
	3	0 (50)
	4	0 (90)
	5	0 (78)[3]
W43**a** x W17α	1	16 (57)
	2	4 (74)
	3	2 (80)[3]
	4	0 (26)[3]
W43**a** x HUN97α	1	0 (98)
	2	0 (100)
	3	0 (80)
	4	0 (152)[3]
W43**a** x YSU63α	1	15 (98)
	2	0 (52)[3]

AU90**a** x HUN97α	1	2 (39)
	2	0 (111)[3]
AU90**a** x YSU63α	1	6 (98)
	2	0 (52)[3]
AU90**a** x W17α	1	1 (40)
	2	1 (84)[3]
AU90**a** x OD8916α	1	0 (16)
	2	0 (86)
	3	1 (110)[3]
AU90**a** x FJ11α	1	0 (10)
	2	0 (32)
	3	0 (52)[3]
AU35**a** x HUN97α	1	1 (80)
AU35**a** x YSU63α	1	36 (80)
AU35**a** x W17α	1	14 (80)
AU35**a** x OD8916α	1	2 (80)
AU35**a** x FJ11α	1	0 (29)
AU7**a** x HUN97α	1	0 (80)
	2	0 (140)
	3	0 (81)
	4	0 (117)[3]
AU7**a** x YSU63α	1	1(80)
AU7**a** x W17α	1	0 (120)
	2	0 (110)
	3	0 (80)

	4	0 (120)[3]
AU7**a** x OD8916α	1	0 (24)
	2	0 (140)
	3	0 (136)[3]
	4	0 (80)[3]
AU7**a** x FJ11α	1	0 (25)
	2	0 (39)
RIHO11**a** x HUN97α	1	0 (17)
	2	0 (8)
	3	0 (24)[3]
RIHO11**a** x YSU63α	1	0 (12)
	2	1 (5)[3]
RIHO11**a** x W17α	1	0 (40)
	2	0 (80)
	3	25 (40)[3]
RIHO11**a** x OD8916α	1	0 (4)
	2	6 (26)[3]
RIHO11**a** x FJ11α	1	0 (17)
	2	0 (20)

[1] Isolates were induced to lose one copy of chromosome 5 by plating on sorbose, eliminating either the *MTL***a** or the *MTL*α allele; the remaining allele is shown.
[2] Recombinants verified by PCR to contain both *MTL* alleles and both parental resistance cassettes. Numbers in brackets are the numbers of above average size colonies on MPA plates tested.
[3] Mating on the alternative, spider, medium.

10.11. Appendix 10.11. Table of FACS analysis

Table 10.2. Comparison of DNA content and number of nuclei in parents and recombinants

Strain or recombinant	FACS G1 peak[1] (Sum of parental DNA contents)	FACS G2 peak[1] (Sum of parental DNA contents)	Recombinant DNA content relative to parental[2]	nuclei/cell[3]
OD8916α	168	312	na	1
AU90**a**	158	299	na	1
W43**a**	169	309	na	1
AU35a	135	246	na	1
AU7a	139	248	na	1
RIHO11a*	--	--	na	1
HUN97α	158	314	na	1
YSU63α	146	278	na	1
FJ11α	143	249	na	1
W17α	188-270[4]	365-482[4]	na	≥1
HUN97α	121	223	na	1
YSU63α	128	231	na	1
W17α	139	270	na	≥1
OD8916 α	140	254	na	1
W43**a** x OD8916α	331 (337)	600 (621)	1.9	1
	336 (337)	616 (621)	2.0	nd
	362 (337)	674 (621)	2.2	nd
W43**a** x W17α	341 (357-439)	634 (674-	1.6-1.9	1

		791)		
		658 (674-		
	362 (357-439)	791)	1.7-2.0	nd
		952 (674-		
	499 (357-439)	791)	2.3-2.8	1
W43**a** x YSU63α	235 (315)	442 (587)	1.5	1
	303 (315)	567 (587)	1.9	nd
AU90**a** x HUN97α	334 (316)	634 (613)	2.1	1
	314 (316)	578 (613)	1.9	nd
AU90**a** x YSU63α	321 (304)	571 (577)	2.0	1
	256 (304)	474 (577)	1.7	nd
	207 (346-	399 (664-		
AU90**a** x W17α	428)[4]	781)[4]	0.8-1.2[4]	≥1
	141 (346-	249 (664-		
	428)[4]	781)[4]	0.6-0.8[4]	nd
AU90**a** x OD8916α	235 (326)	432 (611)	1.4	1
AU35**a** x HUN97α	250 (256)	458 (469)	2.0	1
AU35**a** x YSU63α	189 (263)	345 (477)	1.45	1
	183 (263)	331 (477)	1.4	1
AU35**a** x W17α	211 (274)	419 (516)	1.5	≥1
	231 (274)	422 (516)	1.5	1
AU35**a** x OD8916α	183 (275)	327 (500)	1.3	1
	230 (275)	419 (500)	1.7	1
AU7**a** x YSU63α	176 (267)	325 (479)	1.4	1
RIHO11**a** x	157 (256)	280 (462)	1.2	1

YSU63α				
RIHO11**a** x W17α	255 (278)	505 (540)	1.8	1
	243 (278)	445 (540)	1.8	1
RIHO11**a** x				
OD8916α	158 (280)	282 (508)	1.1	1
	168 (280)	310 (508)	1.2	1

[1]Median channel fluorescence of M1 and M3 peaks in FACS analyses (see Fig. 7.6 for examples) representing DNA content of cells in G1 and G2 phases in exponentially growing cultures. Results shown were obtained in two separate experiments. Recombinants and their parents were assessed in the same experiment and one strain measured in both experiments had identical DNA content on both occasions. Numbers in brackets indicate the sum of parental G1 and G2 DNA contents

[2] DNA content of recombinants divided by average DNA content of parents; numbers shown are the average of G1 and G2 peak ratios. A value of 2.0 indicates that the recombinant's DNA content per cell is equivalent to the sum of DNA contents of two parental cells.

[3] Determined by nuclear staining and fluorescence microscopy of cell suspensions from patch cultures on YPD plates.

[4] Cultures from one of three patches of W17α tested had an elevated cellular DNA content (G1: 270 units and G2: 482 units)

na: not applicable; nd: not determined

* RIHO11**a** forms hyphae in YPD medium and the DNA content can not be measured using FACS.

red: second FACS

10.12. Appendix 10.12. Raw data of FACS experiments of recombinants and their parents.

A Table of the raw data of FACS experiments of recombinants and their parents is included in the CD.

10.13. Appendix 10.13. Raw data of all growth experiments

A Table of the raw data of all growth experiments is included in the CD.

11.0. REFERENCES

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