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Parasexuality and Ploidy Change in *Candida tropicalis*

Riyad N. H. Seervai,* Stephen K. Jones, Jr., Matthew P. Hirakawa, Allison M. Porman, Richard J. Bennett

Department of Microbiology and Immunology, Brown University, Providence, Rhode Island, USA

***Candida* species exhibit a variety of ploidy states and modes of sexual reproduction. Most species possess the requisite genes for sexual reproduction, recombination, and meiosis, yet only a few have been reported to undergo a complete sexual cycle including mating and sporulation. *Candida albicans*, the most studied *Candida* species and a prevalent human fungal pathogen, completes its sexual cycle via a parasexual process of concerted chromosome loss rather than a conventional meiosis. In this study, we examine ploidy changes in *Candida tropicalis*, a closely related species to *C. albicans* that was recently revealed to undergo sexual mating. *C. tropicalis* diploid cells mate to form tetraploid cells, and we show that these can be induced to undergo chromosome loss to regenerate diploid forms by growth on sorbose medium. The diploid products are themselves mating competent, thereby establishing a parasexual cycle in this species for the first time. Extended incubation (>120 generations) of *C. tropicalis* tetraploid cells under rich culture conditions also resulted in instability of the tetraploid form and a gradual reduction in ploidy back to the diploid state. The fitness levels of *C. tropicalis* diploid and tetraploid cells were compared, and diploid cells exhibited increased fitness relative to tetraploid cells *in vitro*, despite diploid and tetraploid cells having similar doubling times. Collectively, these experiments demonstrate distinct pathways by which a parasexual cycle can occur in *C. tropicalis* and indicate that nonmeiotic mechanisms drive ploidy changes in this prevalent human pathogen.**

Many yeast species can reproduce by both sexual and asexual mechanisms. Asexual reproduction is generally utilized when conditions are favorable for growth, while sexual reproduction is favored when nutrients are limiting (1). Sexual reproduction can generate increased levels of genomic diversity that promote adaptation to a changing or hostile environment (2). However, sex is also associated with an increased cost relative to asexual reproduction (3, 4). The ability to switch between sexual and asexual modes of reproduction (facultative sex) allows for reallocation of resources to sexual reproduction during periods of low fitness and also provides a mechanism for coping with deleterious mutations (5, 6).

Saccharomyces cerevisiae, the most widely studied of the ascomycetes, has served as a model organism for the study of mating and meiosis in fungi. It exhibits a mating cycle in which yeast cells alternate between haploid and diploid phases. Mating between haploid *MAT***a** and *MAT*α cells generates diploid **a**/α cells that can subsequently undergo meiosis to form haploid ascospores (7, 8). Similar to *S. cerevisiae*, several human fungal pathogens have been shown to exhibit extant sexual programs (4, 8, 9). *Cryptococcus neoformans*, a basidiomycete that is a causative agent of fungal meningoencephalitis, exhibits both homothallic (same-sex) and heterothallic (intersex) mating (10, 11). This has direct implications for pathogenesis as sexual spores of *C. neoformans* are infectious propagules in animal models of infection (12). Furthermore, phylogenetic analysis of the sibling species *Cryptococcus gattii* has implicated α-α same-sex mating in the generation of a highly virulent isolate responsible for an ongoing outbreak that initiated on Vancouver Island (13–15). *Aspergillus fumigatus*, a prevalent airborne, ascomycetous pathogen, was also recently shown to undergo a complete sexual program complete with the formation of recombinant ascospores (16).

Candida species represent an important genus of human pathogens and these ascomycetes vary in their potential to undergo sexual reproduction. Several clinically relevant species reside within the so-called *Candida* clade, a group of species that translate the CUG codon as serine instead of leucine, as in the universal genetic code (17, 18). Within this clade, *Candida lusitaniae*, *Candida guilliermondii*, *Debaryomyces hansenii*, and *Lodderomyces elongisporus* have been reported to undergo full sexual cycles that include sporulation (19–23). In the case of *C. lusitaniae*, studies reveal that although this species lacks several conserved meiosis genes, it completes its sexual cycle through Spo11-dependent recombination and sporulation (17, 20). The meiotic process leads to high levels of aneuploidy, and resulting asci contain a dyad of ascospores rather than the conventional tetrads formed by *S. cerevisiae* (20).

The most prevalent human fungal pathogen is *Candida albicans*, a *Candida* clade member that undergoes both homothallic and heterothallic mating (24–26). Mating in this species is regulated by a phenotypic switch in which cells must transition from the conventional white state to the alternative opaque state to become mating competent (27). *C. albicans* possesses a number of genes implicated in meiosis (17, 28) but has not been observed to undergo a conventional meiotic program. In its place, ploidy reduction occurs through a process of concerted chromosome loss that results in high levels of chromosomal aneuploidy (29, 30). This parasexual chromosome loss has been proposed to promote adaptation through the generation of a large pool of genetically

Received 25 May 2013 **Accepted** 3 October 2013

Published ahead of print 11 October 2013

Address correspondence to Richard J. Bennett, Richard_Bennett@brown.edu.

* Present address: Riyad N. H. Seervai, Laboratory of Genome Maintenance, The Rockefeller University, New York, New York, USA.

R.N.H.S. and S.K.J. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00128-13.

doi:10.1128/EC.00128-13

diverse progeny (31). Recombination during the parasexual process is dependent on the conserved meiosis-specific protein Spo11, indicating parallels with a conventional meiosis (30). Furthermore, *C. albicans* has recently been shown to form viable haploid cells that can undergo either auto-diploidization or mating to return to the diploid state (32).

Candida tropicalis is also a clinically relevant *Candida* clade species and is rapidly emerging as a major cause of candidiasis on a global scale (33). Until recently, *C. tropicalis* strains were believed to be asexual, but it is now apparent that **a** and α diploid cells can undergo efficient mating to generate tetraploid **a**/α cells (34, 35). *C. tropicalis* mating is controlled by a white-opaque transition related to that in *C. albicans*. However, it is not known if *C. tropicalis* cells can complete a sexual or parasexual program to return to the diploid state.

In this study, we tested the ability of genetically marked tetraploid strains of *C. tropicalis* to undergo a conventional meiosis or a parasexual (nonmeiotic) reduction in ploidy. We show that tetraploid cells can be induced to undergo a parasexual process when grown on sorbose medium. The resulting progeny are diploid, and **a** or α cells are capable of remating to form tetraploid cells, thus establishing a complete parasexual cycle for *C. tropicalis*. We also observed that *C. tropicalis* tetraploid cells are unstable during prolonged propagation in rich medium, eventually returning to the diploid state after ~240 generations. Furthermore, we show that *C. tropicalis* diploid cells outcompete tetraploid cells during prolonged coculture *in vitro*. Taken together, our work compares the fitness of diploid and tetraploid states in *C. tropicalis* and demonstrates that this species can complete a parasexual cycle via distinct ploidy reduction pathways.

TABLE 1 Strains used in this study

Strain	Genotype	*MTL* type	Ploidy
CAY2059	*arg4/arg4*	**a/a**	Diploid
CAY2060	*arg4/arg4*	**a/a**	Diploid
CAY2881	*his1/his1 GAL1/gal1::SAT1*	α/α	Diploid
CAY3031	*his1/his1 GAL1/gal1*	α/α	Diploid
CAY3371	*his1/his1 gal1/gal1::SAT1*	α/α	Diploid
CAY3372	*his1/his1 gal1/gal1::SAT1*	α/α	Diploid
CAY3373	*his1/his1/HIS1/HIS1 arg4/arg4/ARG4/ ARG4 gal1/gal1::SAT1/GAL1/GAL1*	**a/a**/α/α	Tetraploid
CAY3374	*his1/his1/HIS1/HIS1 arg4/arg4/ARG4/ ARG4 gal1/gal1::SAT1/GAL1/GAL1*	**a/a**/α/α	Tetraploid
CAY3375	*his1/his1/HIS1/HIS1 arg4/arg4/ARG4/ ARG4 gal1/gal1::SAT1/GAL1/GAL1*	**a/a**/α/α	Tetraploid
CAY4090	*his1/HIS1 arg4/ARG4 gal1::SAT1/GAL1*	**a**/α	Diploid
CAY4276	*his1/HIS1 arg4/ARG4 gal1/GAL1*	**a**/α	Diploid
CAY4277	*his1/HIS1 arg4/ARG4 gal1/GAL1*	**a**/α	Diploid
CAY4281	*his1/HIS1 arg4/ARG4 gal1::SAT1/GAL1*	**a**/α	Diploid
CAY4283	*his1/HIS1 arg4/ARG4 gal1::SAT1/GAL1*	**a**/α	Diploid
CAY4311	*his1/HIS1 arg4/ARG4 gal1/GAL1*	**a**/α	Diploid
CAY4539	*his1/HIS1 arg4/ARG4 gal1/GAL1 ura3::SAT1/URA3*	**a**/α	Diploid
CAY4888	*his1/his1/HIS1/HIS1 arg4/arg4/ARG4/ ARG4 gal1/gal1/GAL1/GAL1*	**a/a**/α/α	Tetraploid
CAY4890	*his1/his1/HIS1/HIS1 arg4/arg4/ARG4/ ARG4 gal1/gal1/GAL1/GAL1*	**a/a**/α/α	Tetraploid

MATERIALS AND METHODS

Media. Standard media were prepared as previously described (36, 37). Yeast extract peptone dextrose (YPD) contained 1% yeast extract, 2% peptone, and 2% glucose. YPD medium was supplemented with 200 μg/ml nourseothricin (YPD-NAT) to select for nourseothricin-resistant (NAT^R) strains (38). Sporulation medium #1 consisted of 1% potassium acetate, 0.05% dextrose, 0.1% yeast extract, and 0.01% complete amino acid mix. Sporulation medium #2 was Gorodkowa medium and consisted of 1% peptone, 0.5% NaCl, and 0.1% glucose. Potato dextrose agar (PDA) medium was 3.9% Difco potato dextrose agar and 0.07% tartaric acid. Minimal medium was 0.7% yeast nitrogen base (YNB) and 2% glucose. Sorbitol medium contained 18.2% sorbitol, 0.7% YNB, 2% glucose, complete amino acids, and 25 μg/ml uridine. Malt extract medium was 3% malt extract and 0.5% peptone. Sheep's blood agar was Columbia agar with 5% sheep's blood (Becton, Dickinson, and Co). V8 medium contained 5% V8 juice and 0.05% KH_2PO_4 (pH 7.0). Serum medium was YPD medium supplemented with 10% fetal bovine serum. Low-phosphate medium contained YNB without phosphate (CYN6701; Formedium, Ltd., Hunstanton, England) and KH_2PO_4 (10 μM) and was adjusted to pH 4.7 with 3 M HCl. Synthetic low-ammonium dextrose (SLAD) medium was YNB (without amino acids or ammonium sulfate) supplemented with 50 μM ammonium sulfate and 2% glucose. Sorbose medium contained 0.7% yeast nitrogen base, 2% sorbose, and 25 μg/ml uridine (with or without amino acids). 2-Deoxygalactose (2-DOG) and presporulation media were prepared as described previously (29). Solid media contained 2% agar.

Strain and plasmid construction. All strains used in this study are listed in Table 1. Gene deletions in *C. tropicalis* strains were performed using the *SAT1* flipper strategy (38). Strains were transformed with ~1 μg of DNA using a modified electroporation protocol, as described previously (20, 34). In brief, cells were grown overnight at 30°C in 3 ml of YPD medium and diluted into 50-ml YPD cultures, which were grown overnight at 30°C. Samples were centrifuged and resuspended in 0.1 M lithium acetate, 7.5 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 10 mM dithiothreitol for 1 h at room temperature. After cells were washed twice with water and once with 1 M sorbitol, they were resuspended in the liquid that remained after decantation. For each transformation, ~50 μl of cells was mixed with 10 to 15 μl of DNA, and the mixture was electroporated at 1.8 kV using a MicroPulsor Electroporator (Bio-Rad). The cells were immediately resuspended in 1 ml of cold 1 M sorbitol, followed by a recovery period of 4 h at 30°C in 1 ml of YPD medium. Finally, cells were plated onto YPD medium containing 200 μg/ml nourseothricin (YPD-NAT) to select for cells that had incorporated the construct into the genome through homologous recombination. PCR was used to confirm proper integration into the correct locus. In order to flip out the *SAT1* marker from the genome, cells were grown in liquid yeast extract-peptone (YP) medium supplemented with 2% maltose for 5 days at 30°C and plated on YPD medium containing 20 μg/ml nourseothricin. Small colonies were replica patched onto YPD and YPD-NAT plates to confirm loss of nourseothricin resistance. For double mutants, the transformation process was repeated, and PCR was used to confirm loss of the open reading frame (ORF).

In order to delete genes using the *SAT1* flipper, the 5′ and 3′ regions of the gene of interest were PCR amplified and cloned into the plasmid pSFS2a (38). The 5′ flank of the target gene was digested with ApaI/XhoI, and the 3′ flank was digested with SacI/SacII and cloned into pSFS2a. The resulting plasmid was digested with ApaI/SacI in order to liberate the deletion cassette. Correct integration of the cassette into the genome was confirmed by PCR. All oligonucleotides used in this study are listed in Table 2.

***C. tropicalis* mating assay.** Mating in *C. tropicalis* was carried out using two methods. In the first, strains with deletions at the *HIS1* or *ARG4* locus were streaked onto YPD medium, and cells were resuspended in liquid Spider medium and grown for 3 to 5 days at 30°C. Following incubation, equal amounts (optical density at 600 nm [OD_{600}] of 0.5 or ~10^7 cells) of each strain were mixed together in 100 μl of YPD medium and pipetted onto 0.8-μm-pore-size nitrocellulose filters on the surface of

TABLE 2 Oligonucleotides used in this study

Oligonucleotide no.	Oligonucleotide name	Sequence
562	CtMTLα2 Check 1	TAA AAC ATT AAG CAT AGA GGA CAA AGA A
563	CtMTLα2 Check 2	AAC TTC AAA TGC AAA ATG TAA AAC ATA C
564	CtMTL**a**1 Check 1	GGA TAA AGA GAG CTT AAG TTC AGA AGA G
565	CtMTL**a**1 Check 2	AAG TAT CAT CTG TCT TAT CTG ATT CCT TC
641	CtHIS1 (ApaI) −900	GGA GCG GGG CCC ACC TCA AGA GAT GAA TGA CA
642	CtHIS1 (XhoI) 0	GCC GGC TCG AGA ACT CTA AAC TAT GAT TGT ATT C
643	CtHIS1 (SacII) +900	GGA GCG CCG CGG AGT TCA TCT GTA TAA TAG TAC A
644	CtHIS1 (SacI) +1,800	GGC CGC GAG CTC TCC ACA CAT TCA TAA ATG TTC A
645	CtHIS1 5′ Check −950	CCA AGG ACA ACA CTC GTG CAG T
646	CtHIS1 3′ Check + 1,900	CAA ATT GAT CAC TTC TCT AGT CGA
647	CtHIS1 5′ ORF +200	AGG GTA ATT GTG ATT TGG GTA
648	CtHIS1 3′ ORF +600	CTT GGA AGA AAC CAA GTG AG
657	CtARG4 (ApaI) −900	GGA GCG GGG CCC TGA ATG TGT TGG TGG TGA TG
658	CtARG4 (XhoI) 0	GCC GGC CTC GAG GAC ATT GTT GTT AGA ATA AGT T
659	CtARG4 (NotI) +1,400	GGA GCG GCG GCC GCA CAT TCA TTC TCC TTC CCT CTC
660	CtARG4 (SacII) +2,300	GGC CGC CGC GGG AAT ATT GAT TGT TGA TTG TTG
661	CtARG4 5′ Check −950	GAC GTT GTT TAC CCT TGG TAT C
662	CtARG4 3′ Check + 2400	TTG TGG AAT GTA ATT TGC ACA C
663	CtARG4 5′ ORF +200	TCA GGA TTA GAA GAA ATT CGT G
664	CtARG4 3′ ORF +600	TGG ACT TTG ATT AAC TCT GGT T
1046	CtGAL1 (ApaI) −900	GGC GCC GGG CCC GAA ACG AAG ACC TAA TTC ACC C
1047	CtGAL1 (XhoI) +50	GCC GGC CTC GAG GGT TCA TTA GAA TGA GGA TCA G
1048	CtGAL1 (SacII) +1,200	GGA GCG CCG CGG GAA TCA CAG GGC TGA TTT AG
1049	CtGAL1 (SacI) +2,150	GGC CGC GAG CTC CGT TAA TTG TCA CCT ATC TAG AC
1050	CtGAL1 5′ Check −950	GGG ATC TAG TCA GGG AAG TTC
1051	CtGAL1 3′ Check + 2200	CTT CTA ATA CCC CAT AGT AC
1052	CtGAL1 5′ ORF +250	CAT CAT AAC CAA CAC AGA TAG C
1053	CtGAL1 3′ ORF +650	CGT AAA CTG CTT GTA CCA ATG
1054	CtADE2 (ApaI) −950	GGC GCC GGG CCC CCT GCG TAT TCT TAC GTA CA
1055	CtADE2 (XhoI) 0	GCC GGC CTC GAG TGA CCA CCT CCT AAA ATA CC
1056	CtADE2 (SacII) +1,500	GGA GCG CCG CGG GAG GTA TTC CAG TAG CTA CTG
1057	CtADE2 (SacI) +2,500	GGC CGC GAG CTC TCG CAC CAT GCG ATT CCT TG
1058	CtADE2 5′ check	CGA CCT GCG TAT TCT TAC GT
1059	CtADE2 3′ check	GAG ACA ATT GCT ACG CTG CG
2295	CtURA3 (ApaI) −450	GGC GCC GGG CCC GAA ATC TAT CAA GCT TCA TGT GAC
2296	CtURA3 (XhoI) 0	GCC GGC CTC GAG GGA TGT TTA GAT GCT CTT TC
2297	CtURA3 (SacII) +750	GGC GCC CCG CGG GAT ATA GAG AAG CCG GTT GG
2298	CtURA3 (SacI) +1300	CCG GCG AGC TCG GGA TGA TGA TCA AGT TGA TG
2299	CtURA3 5′ ORF −800	GGA GAC TAC TCC ATC ATC GT
2300	CtURA3 3′ ORF +1,500	GTG TAT TTG CAG GTT ATG GG

Spider medium plates. Plates were incubated at 30°C for 5 days, after which cells were collected from the filters and streaked onto His^-/Arg^- plates in order to select for successful mating products. PCR using primers directed at *MTL***a**1 and *MTL*α2 (Table 2) were used to confirm the presence of both mating types in the mating products. Ploidy was checked using flow cytometry as described below. The second method used the same steps, except that cells from YPD medium were streaked directly onto plates containing Spider medium and grown at room temperature for 5 days.

Flow cytometric analysis. Cytometric analysis of cells was performed essentially as described previously (25). Cells were grown in 500 μl of YPD medium to an OD_{600} of ~0.5 and fixed in 70% ethanol at 4°C overnight. Cells were then washed twice in water and resuspended in 0.5 mM Tris-HCl, pH 8.0, followed by sonication at low power. Following this, cells were resuspended in freshly prepared 2 mg/ml RNase A solution in 0.5 mM Tris-HCl, pH 8.0 (heated to 98°C for 10 min and allowed to slowly cool to room temperature). Samples were incubated at 37°C for 2 h, centrifuged, and resuspended in freshly prepared 5 mg/ml pepsin in 55 mM HCl at 37°C for 45 min. They were then washed with 0.5 mM Tris-HCl, pH 7.5, sonicated at low power, and resuspended in 200 μl of 0.5 mM Tris-HCl, pH 7.5. Fifty microliters of each sample was stained with 500 μl of Sytox Green dye (1 μM) at 4°C for 1 to 48 h. A total of 10,000 cells from each sample were run on a MACSQuant Analyzer 10 (Miltenyi Biotech), and data were analyzed using FlowJo (Tree Star Inc.).

Chromosome loss assay. Chromosome loss in *C. tropicalis* was measured using a method similar to that described for *C. albicans* (29). Tetraploid strains CAY3373, CAY3374, and CAY3375 were grown on YPD medium, streaked onto test medium, and incubated at 30°C or 37°C for 8 to 10 days. Cells were then removed from the plates and resuspended in water. The OD_{600} was measured and used to plate ~10^3 cells on YPD medium and ~10^5 cells on 2-DOG plates. The percentage of chromosome loss was calculated as described previously (29).

Batch culture evolution assay. *C. tropicalis* diploid and tetraploid strains were maintained in a manner similar to that used for *S. cerevisiae* (39). Frozen stocks were streaked onto YPD medium and subsequently grown overnight in liquid YPD medium. The overnight culture (100 μl) was diluted into 10 ml of YPD medium and grown at 30°C. Three replicates of each cell line were maintained. Cells were diluted 1:100 (100 μl of culture into a fresh tube of 10 ml of YPD medium) daily (24 h ± 1 h) for 42 days. Such dilution allows for approximately 6.64 ($\log_2$ 100) mitotic

divisions before the population returns to stationary phase (39). A 500-μl sample was taken from each line every 3 days (72 h ± 1 h) and frozen in 500 μl of 1:1 YPD medium–50% glycerol at −80°C for subsequent cytometric analysis. For analysis of ploidy change, control diploid and tetraploid cells were used to define gates for regions where diploid G_1 and tetraploid G_2 peaks were present. The percentage of total cells present in the peaks was calculated by dividing the number of cells in the gated region by the total cell count.

***In vitro* competition assay.** Diploid and tetraploid strains were compared for fitness in coculture experiments. The frozen stock of each strain was grown on YPD plates overnight at 30°C and subsequently grown in 3 to 5 ml of YPD cultures overnight at 30°C. A hemocytometer was used to determine the concentration of cells present. Competing strains were then mixed together in equal amounts of ~10^7 CFU in 10 ml of YPD medium. Two independent cultures were used for each assay in each independent experiment. Cells were diluted 1:100 (100 μl of culture into a fresh tube of 10 ml of YPD medium) daily (24 h ± 1 h) for 7 days. This allowed for a maximum of ~6.64 divisions each day. After 1 week, an equal number of cells were plated onto YPD and YPD-NAT plates, and the percentage of NAT^R cells was calculated.

***In vivo* competition assay.** Female BALB/c mice (18 to 20g; Charles River Laboratories) were made neutropenic by intraperitoneal injection of 200 μg of anti-Gr1 monoclonal antibody (clone: RB6-8C5; BioXCell) 24 h prior to infection. *C. tropicalis* strains were grown overnight in liquid YPD medium at 30°C. On the day of infection, *C. tropicalis* cells were diluted to an OD_{600} of 0.2 into 10 ml of fresh YPD medium and grown at 30°C for 5 h. Log phase cells were collected and washed three times in sterile phosphate-buffered saline (PBS). Cell concentrations were measured with a hemocytometer. Mice were infected with a mixture of two *C. tropicalis* strains at a ~50:50 ratio, with a total inoculum of ~1.0×10^6 CFU by injection into the lateral tail vein. Each *C. tropicalis* competition mixture included one isolate resistant to nourseothricin (NAT^R) and one isolate sensitive to nourseothricin (NAT^S). Dilutions of the inoculum were plated onto YPD agar and YPD-NAT agar to confirm initial CFU counts. Mice were euthanized at 48 h postinfection, and the liver, spleen, brain, and kidneys were isolated from each mouse. The organs were homogenized using a gentleMACS Dissociator. Dilutions of each tissue homogenate were plated onto YPD medium and incubated at 30°C for 24 h to calculate the total fungal burden from each organ. Colonies were then replica plated from YPD medium onto YPD-NAT medium to calculate the percentage of the recovered *C. tropicalis* population that included NAT resistance. Four to eight mice were used for each combination of *C. tropicalis* strains. Neutropenia was monitored by collecting blood daily through retro-orbital bleeding of mice. Blood samples were stained with fluorescein isothiocyanate (FITC)-labeled anti-Gr1-antibody, and neutrophil populations were identified by FITC staining, cell size, and cell complexity using flow cytometry. Neutropenia in mice treated with the anti-Gr1 antibody was confirmed 4 days following injection with the antibody (data not shown). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University.

Measurement of doubling times. Strains used in the competition assay were streaked onto YPD plates and grown overnight at 30°C and subsequently resuspended in liquid YPD medium. Cells were diluted to an OD_{600} of 0.2 in 1 ml of YPD medium, and four 150-μl samples of each strain were transferred into a 96-well plate. The plate was incubated at 30°C in a Synergy HT multimode plate reader (BioTek), and the OD_{600} was measured every 30 min. To calculate the doubling time of each strain, readings were averaged and plotted against time as a semilogarithmic graph.

Statistical analysis. Statistical analysis was completed using the PAST (Paleontological Statistics Software for Education and Data Analysis) software package. Overall, parametric tests were used whenever data sets were found to be normally distributed via the Anderson-Darling method; otherwise, nonparametric tests were used. A *P* value of 0.05 was used throughout as a measure of significant difference. For the chromosome loss assay, a Bonferroni-corrected Mann-Whitney U test was used. For the batch culture evolution experiments, Tukey's test was used for comparisons between diploid and tetraploid samples on the same given day. The *in vitro* competition data were compared using an independent-samples *t* test. The *in vivo* competition data were compared using one-way analysis of variance (ANOVA).

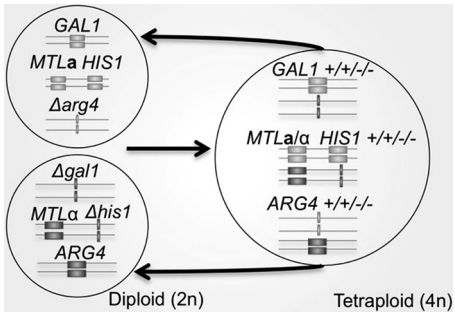

FIG 1 A genetic selection to monitor ploidy reduction in *C. tropicalis*. Diploid **a/a** (CAY2059 and CAY2060) and α/α (CAY3371 and CAY3372) *C. tropicalis* cells were mated to generate tetraploid strains (CAY3373, CAY3374, and CAY3375) that were heterozygous at the *GAL1*, *MTL*, *HIS1*, and *ARG4* loci. Loss of *GAL1* function was screened for by selection on 2-deoxygalactose (2-DOG) medium, which selects for Δ*gal1* cells. Chromosomal positions of genes are implicated based on chromosome positioning in the related species *C. albicans*. Linkage of *MTL* and *HIS1* loci in *C. tropicalis* was also demonstrated in the present studies.

RESULTS

A screen for ploidy reduction in *C. tropicalis* tetraploids. Previous studies showed that *C. tropicalis* **a** and α diploid cells are able to mate to form stable **a**/α tetraploid cells (34, 35). In order to test for conditions that result in a reduction of ploidy in *C. tropicalis* tetraploid cells, we constructed a genetically marked tetraploid strain as outlined in Fig. 1. Diploid α/α strains were constructed that were homozygous mutants for *HIS1* and *GAL1* genes (*MTL*α/α Δ*gal1*/Δ*gal1* Δ*his1*/Δ*his1*) and mated to diploid **a/a** cells in which both copies of the *ARG4* gene had been deleted (*MTL***a**/**a** Δ*arg4*/Δ*arg4*). The resulting tetraploids were therefore heterozygous at the *MTL*, *GAL1*, *HIS1*, and *ARG4* loci (Fig. 1). We note that the chromosomal locations of these loci have not been determined for *C. tropicalis* although each locus resides on a different supercontig (17). Three tetraploid strains (CAY3373, CAY3374, and CAY3375) resulting from independent mating experiments (see Fig. S1 in the supplemental material) were used to screen for chromosome loss. *S. cerevisiae* and *C. albicans* strains expressing *GAL1* are unable to grow on medium containing 2-deoxygalactose (2-DOG) as the carbon source (40, 41), and we similarly used 2-DOG in positive selection for *C. tropicalis* cells that had lost both copies of the wild-type *GAL1* allele.

The genetically marked tetraploid strains were grown under a series of environmental conditions to identify those that could induce either parasexual chromosome loss or meiosis in *C. tropicalis*. As a proxy for ploidy reduction, we initially monitored loss of *GAL1* alleles via selection on 2-DOG medium. A total of 14 different laboratory media were tested, including rich (YPD), *S. cerevisiae* presporulation, sorbose, potato dextrose agar (PDA), malt extract (malt), sorbitol, YPD-serum, low-phosphate, low-nitrogen (SLAD), and blood agar media. Cultures were grown at 30°C and 37°C for 8 to 10 days and subsequently plated onto YPD medium to determine the total number of viable cells and onto

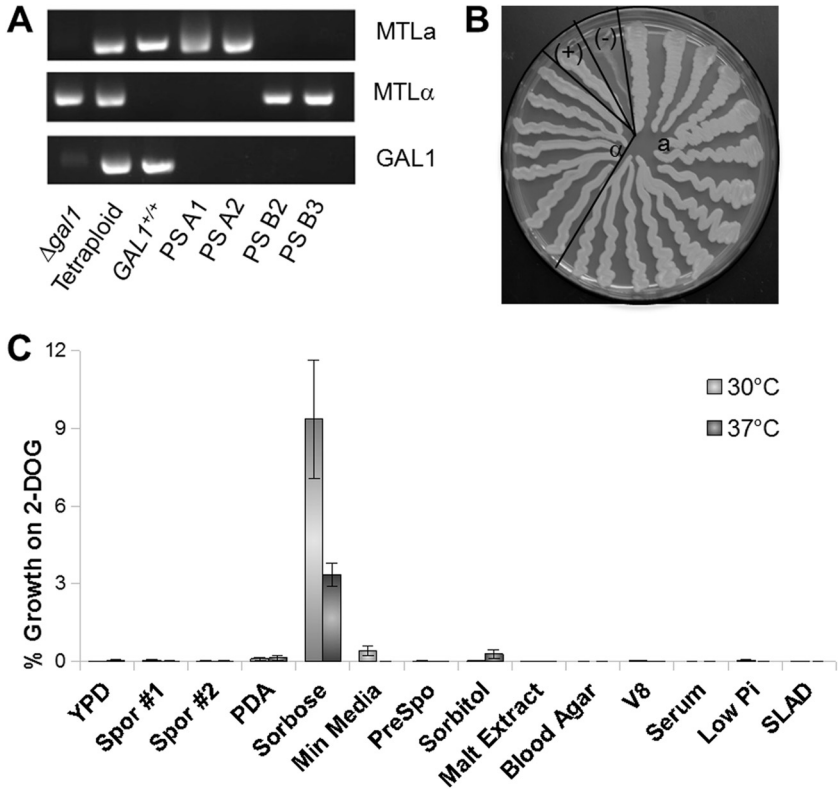

FIG 2 Chromosome loss in *C. tropicalis* tetraploid strains. (A) A Δ*gal1 MTL*α strain (CAY3371) was mated with a *GAL1*$^{+/+}$ *MTL***a** strain (CAY2059) to generate the starting tetraploid strain (CAY3373) that was heterozygous at the *GAL1* and *MTL* loci. Cells grown on sorbose medium for 8 to 10 days and selected on 2-DOG medium are shown as parasexual (PS) products. Parasexual colonies were screened for cells containing only *MTL***a** (PS A1 and PS A2) or *MTL*α (PS B2 and PS B3) loci. (B) A 2-DOG plate showing selection of Δ*gal1* cells homozygous at the *MTL* locus after growth on sorbose for 8 to 10 days. CAY3371 (Δ*gal1 MTL*α) and CAY3373 (the parental tetraploid) are shown as positive and negative controls, respectively. (C) Graph showing tetraploid stability under various medium conditions at 30°C and 37°C. The *y* axis represents a linear scale of percentage growth on 2-DOG medium. Bars represent means ± standard errors ($n = 2$ for minimal medium, sorbitol, malt extract, blood agar, V8, serum, low P_i, and SLAD; $n = 3$ for sporulation medium 1; $n = 4$ for sporulation medium 2, PDA, and presporulation medium; $n = 5$ for YPD medium; $n = 7$ for sorbose).

2-DOG medium to determine the number of cells that had lost *GAL1*.

Of all the media tested, sorbose medium induced the greatest formation of Δ*gal1*/Δ*gal1* cells from *C. tropicalis* tetraploid cells (Fig. 2C). For cells grown at 30°C on sorbose medium, 9.4% of viable cells were resistant to 2-DOG (2-DOG^R) compared to 0.0028% of those from YPD medium ($P = 2.3E{-}6$). Similarly, at 37°C, 3.3% of viable cells taken from sorbose medium were found to be 2-DOG^R, compared to 0.039% of cells from YPD medium ($P = 1.9E{-}4$). Analysis of cells grown on other types of laboratory media generally revealed low rates of formation of 2-DOG^R colonies, similar to those of cells grown on YPD medium. For example, testing on *S. cerevisiae* sporulation medium at 30°C and 37°C resulted in 0.058% and 0.019% of cells becoming 2-DOG^R, respectively. These data indicate that growth on sorbose medium leads to increased rates of *gal1*$^-$ colony formation in *C. tropicalis* tetraploid strains relative to other culture conditions. Interestingly, growth on presporulation medium led to negligible loss of *GAL1*, which was not significantly different from growth on YPD medium at 30°C ($P = 1.0$) and 37°C ($P = 1.0$). This finding contrasts with results in *C. albicans*, where incubation on presporulation medium at 37°C led to high rates of *GAL1* loss from tetraploid cells (29, 30).

Tetraploid cells recovered from sorbose medium have reduced ploidy. Growth on 2-DOG medium is indicative of loss of the wild-type *GAL1* alleles but is not necessarily demonstrative of chromosome loss. In order to characterize the chromosome content in progeny cells selected on 2-DOG medium, we first analyzed the genotype of 2-DOG^R cells at the *MTL* locus. PCR was used to identify cells that had lost both copies of *MTL*α (two such progeny are shown in Fig. 2A). Since we observed that *MTL* and *HIS1* loci are genetically linked (these loci are also linked in *C. albicans*, where they both reside on chromosome 5), α progeny were identified by an inability to grow on medium lacking histidine. In total, we observed that 13 out of 198 2-DOG^R colonies (6.6%) were *MTL***a**, with the rest of the colonies being heterozygous at the *MTL* locus, while 7 out of 460 colonies (1.5%) were *MTL*α. Cells were also directly picked from colonies on sorbose plates and restreaked on YPD medium, and 8 colonies out of 84 (9.52%) were *MTL***a** by PCR screening. We identified a single *MTL*α isolate directly from sorbose plates out of 376 tested (0.27%). These experiments establish that *MTL* alleles were concomitantly lost during growth on sorbose medium and that chromosome loss was not completely random as *MTL***a** cells were generated at a higher frequency than *MTL*α cells. The reason for the bias in *MTL* loss is not known; however, it is possible that His$^-$ cells are selected against during growth on sorbose medium (even though this medium was supplemented with amino acids), thereby promoting the recovery of *MTL***a** cells over *MTL*α cells.

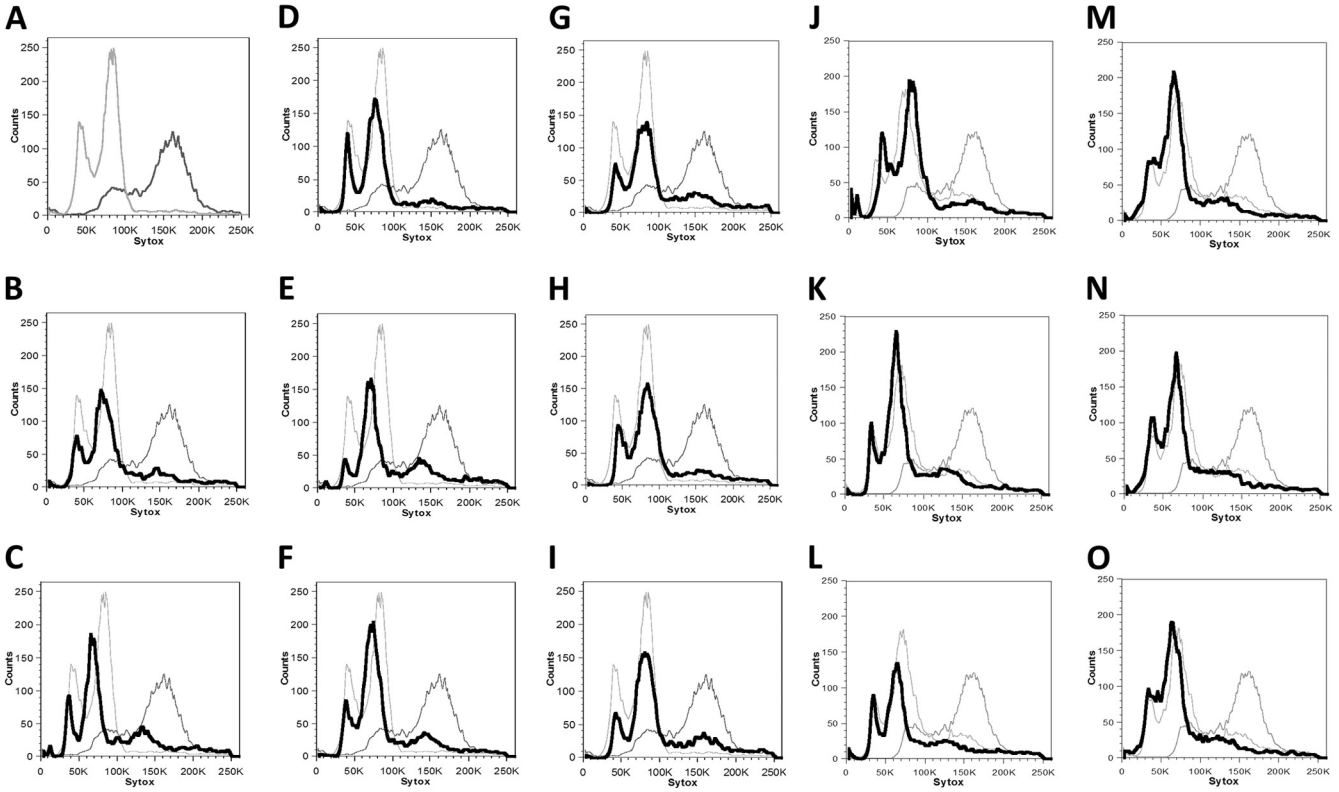

FIG 3 Cell cytometric analysis of parasexual cells. The *x* axis (Sytox) represents a linear scale of fluorescence, and the *y* axis represents a linear scale of cell number. (A) Comparison of the initial Δ*gal1* diploid strain (CAY3371; light gray) with the starting tetraploid (CAY3373; dark gray). These strains are also included for reference in panels B to O. Eight independent colonies were isolated from 2-DOG selection after growth on sorbose for 8 to 10 days and identified as *MTL***a** (B to E) or *MTL*α (F to I) isolates and catalogued as parasexual strains A1 to A4 and B2 to B5, respectively (Table 3). (J to O) Cell cytometric analysis of tetraploid cells exposed to sorbose and selected for loss of *gal1* only, taken from plates grown at 30°C (parasexual strains F1, F2, and F7 in panels J to L, respectively) and 37°C (parasexual strains F9, F11, and G1 in panels M to O, respectively). Samples in panels A to I and J to O were analyzed on separate days, and thus, control diploid and tetraploid plots show minor differences.

We next used flow cytometry to determine the overall ploidy of selected parasexual progeny. Eight 2-DOG^R progeny colonies that were homozygous at the *MTL* locus, consisting of four that were *MTL***a** (Fig. 3B to E) and four that were *MTL*α (Fig. 3F to I), were chosen for cytometric analysis. While small variations in ploidy states were observed between the parasexual progeny, all of the progeny were similar in DNA content to control diploid strains. We also examined the ploidy of colonies selected directly from 2-DOG plates with unknown *MTL* status. Again, all samples analyzed showed peaks that were similar to those in the control diploid strain (Fig. 3J to O), which has a ploidy profile similar to that of other parental diploid strains (see Fig. S1 in the supplemental material). Overall, this analysis establishes that growth on sorbose medium leads to increased rates of ploidy reduction in *C. tropicalis*, resulting in progeny cells that are diploid, or close to diploid, in DNA content.

Parasexual progeny cells are mating competent. Given that the progeny cells arising from sorbose medium include **a** and α diploid cell types, we tested whether these cells could undergo additional rounds of mating to reform tetraploid cells. *MTL*α progeny that were Δ*his1*/Δ*his1* were mated with a control *MTL***a** strain that is auxotrophic for arginine (CAY2060). Within 3 to 5 days, cocultured cells began to exhibit a mating response (Fig. 4A) and formed zygotes (Fig. 4B), indicative of productive mating. Mating cells were also plated to His$^-$/Arg$^-$ medium, and the resulting colonies were identified as *MTL***a**/α cells (Fig. 4C). The ploidy states of these mating products were examined using flow cytometry and showed peaks close to those of a tetraploid control (Fig. 4D to G). Remated tetraploids could also be induced to undergo chromosome loss when grown on sorbose medium at 30°C or 37°C, again as indicated by the generation of 2-DOG-resistant colonies (data not shown). Together, these experiments demonstrate that a parasexual cycle can be established for *C. tropicalis*, in which cells transition between diploid and tetraploid states.

C. tropicalis* tetraploid cells become diploid during prolonged culture in rich medium.** As shown above, chromosome loss in *C. tropicalis* tetraploid cells is facilitated by growth on sorbose medium over a period of 8 to 10 days. Studies in *S. cerevisiae* have shown that tetraploid, triploid, and haploid strains trend toward the diploid state when they are cultured over multiple generations, and that the process of chromosome loss from tetraploid to diploid is concerted across the genome (39, 42). We therefore addressed whether ploidy reduction in *C. tropicalis* tetraploid cells might also occur during extended culture in YPD medium. A genetically marked tetraploid strain (CAY3373) and a control *MTLa**/α diploid strain (CAY4311) were grown separately in YPD medium in three replicate lines for 42 days at 30°C. Cells were diluted back 1:100 (100 μl into 10 ml of YPD medium) every 24 h allowing for ~6.6 generations each day, and samples were frozen down every 72 h for subsequent analysis. Flow cytometry showed

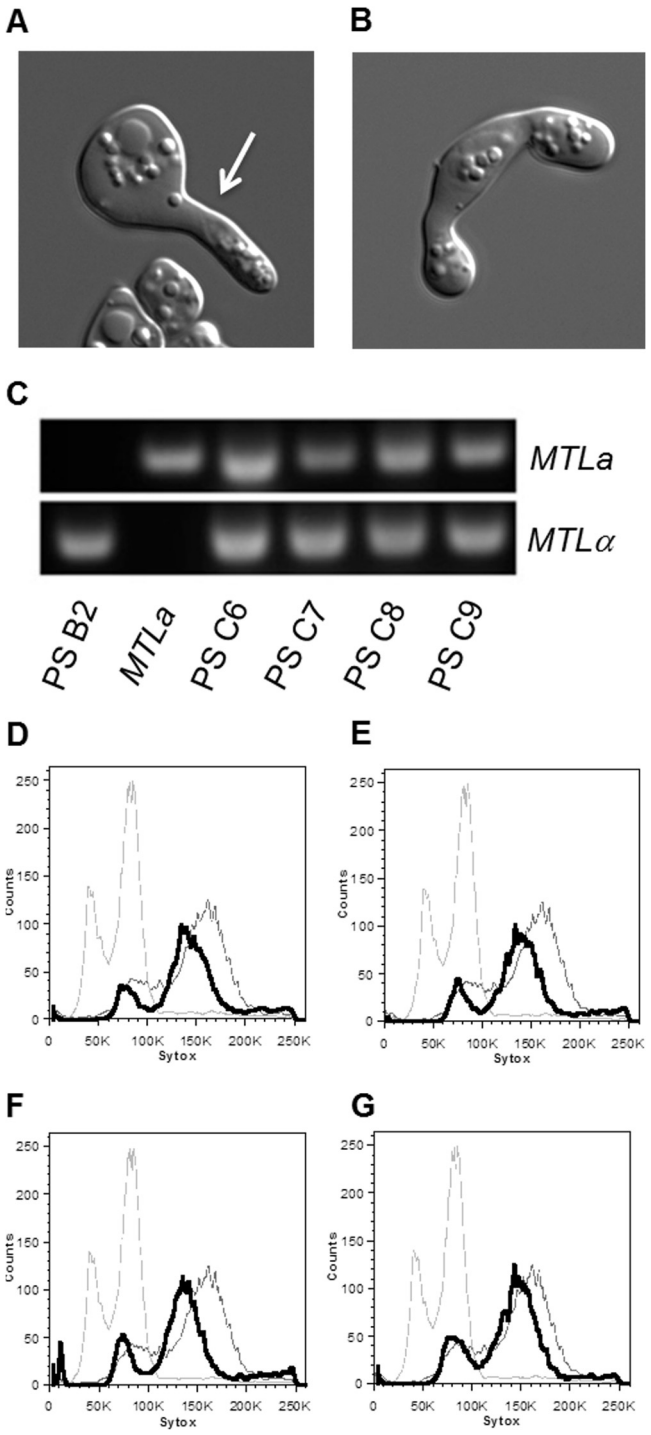

FIG 4 *C. tropicalis* parasexual progeny are mating competent. (A and B) Representative images of mating projections (A) and zygotes (B) seen during mating of parasexual progeny with wild-type strains. (C) Characterization of *MTL* loci from four parasexual remating products (parasexual strains [PS] C6 to C9), compared with the parasexual parent (B2) and *MTL***a** parental strain (CAY2060). (D to G) Cell cytometric data showing ploidy of parasexual remating products PS C6 (D), PS C7 (E), PS C8 (F), and PS C9 (G). The *x* axis (Sytox) represents a linear scale of fluorescence, and the *y* axis (counts) represents a linear scale of cell number. Diploid (light gray) and tetraploid (dark gray) controls are shown in the background as described in the legend of Fig. 3.

that all three *C. tropicalis* tetraploid cell cultures lost ploidy over the course of the experiment and returned to the diploid state (Fig. 5A to C). Interestingly, the temporal profiles of the independent tetraploid lineages showed very similar patterns of ploidy reduction. G_1 and G_2 peaks were distinct up to day 18 (120 generations), and then multiple peaks were observed between generations 120 and 140. Distinct peaks started to reappear around day 24 (160 generations) and eventually reached a defined diploid state by 36 days (240 generations). Three independent diploid cultures were also propagated in YPD medium for multiple generations, and two of the three showed a steady diploid state across the period of the experiment (Fig. 5D). One diploid lineage unexpectedly showed a trend toward increased ploidy (data not shown).

To quantify changes in ploidy, we measured the percentage of cells within the diploid G_1 peak (G_1^{Dip}) and tetraploid G_2 peak (G_2^{Tet}) relative to control strains. As predicted for tetraploid strains, G_1^{Dip} showed an increasing trend over time, while G_2^{Tet} decreased to a basal level by 36 days/240 generations (Fig. 5E). Diploid strains showed static trends for both of these parameters, as expected for a stable diploid population, while tetraploid strains converged toward the diploid state by the 42-day time point, or 279 generations (Fig. 5E). The difference in G_1^{Dip} between the diploid and tetraploid lines was significant until approximately day 30/200 generations (average *P* of 0.012), and this significance was lost over the final 12-day period. The G_2^{Tet} difference remained statistically significant until day 33/220 generations (average *P* of 0.0046). Our analysis shows that *C. tropicalis* tetraploid cells, while generally stable, begin to lose chromosomes during prolonged culture in rich medium, becoming aneuploid and eventually returning to the diploid state in a manner similar to that described in *S. cerevisiae*.

***C. tropicalis* diploid cells outcompete tetraploid cells during coculture *in vitro*.** To investigate the conversion of tetraploid populations to diploid populations, we determined if there was a fitness advantage to propagating in the diploid state. To compare the fitness of tetraploid and diploid cells, we maintained cultures containing cells of both ploidy states in YPD medium for 7 days at 30°C, diluting them 1:100 every 24 h. A nourseothricin-resistant (NAT^R) diploid strain was grown in competition with a nourseothricin-sensitive (NAT^S) tetraploid strain, or, conversely, a NAT^R tetraploid strain was grown in competition with a NAT^S diploid strain. Nourseothricin resistance served as a selectable marker for determining the fraction of the population that derived from the diploid or tetraploid strain (Fig. 6A). In both cases, the diploid strain used was a prototrophic precursor to the *MTL*α strain used to construct the tetraploid strain.

Following culture for 7 days in YPD medium, cells were plated onto YPD plates with or without nourseothricin to distinguish NAT^R and NAT^S colonies. The proportion of tetraploid cells showed a statistically significant decrease from day 0 to day 7 ($P = 0.005$), comprising only 20% of the population recovered on day 7 despite representing 50% of the initial inoculum (Fig. 6A). Furthermore, occasional NAT^R diploid cells were recovered despite entering the experiment as tetraploid, while the opposite was not true (data not shown). We conclude that *C. tropicalis* diploid cells hold a significant fitness advantage over tetraploid cells *in vitro* and that this is independent of the NAT marker used in these studies, given that tetraploid populations decreased in both NAT^R and NAT^S configurations.

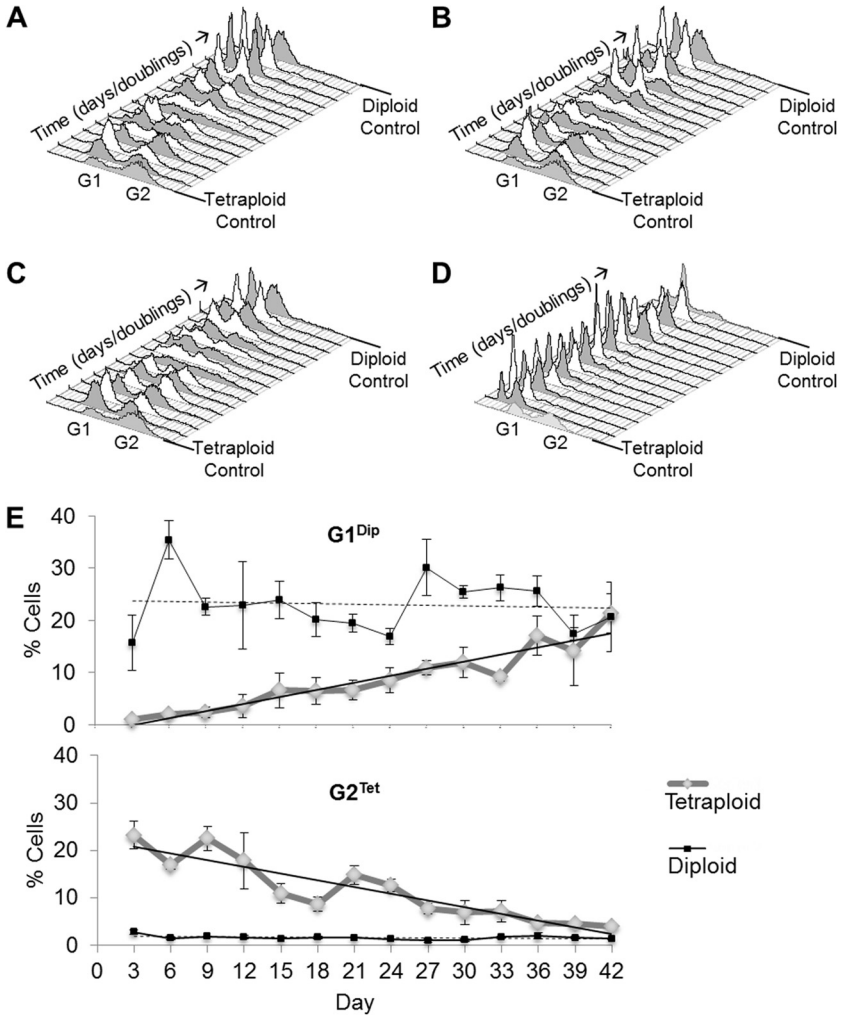

FIG 5 Ploidy reduction in *C. tropicalis* tetraploid strains. (A to C) Cytometric data showing gradual ploidy change from the tetraploid (4n) state to the diploid (2n) state, as evidenced by the leftward shift of G_1 and G_2 peaks in three independent lines. (D) Cytometric data showing stability of a diploid strain under the same conditions. The *x* axes represent a linear scale of fluorescence, the *y* axes represent a linear scale of cell number, and the *z* axes represent a linear scale of time in days. Control tetraploid (front) and diploid (back) strains are shown on either end for reference. (E) Changes in the G_1^{Dip} peak and G_2^{Tet} peak during passage of tetraploid strains. Values for control diploid strains are shown in black. The *y* axis represents the proportion of total cells contained within the given peak with reference to a control strain. The solid and dashed black lines represent the best-fit lines for tetraploids and diploids, respectively. Values represent means ± standard deviations ($n = 3$ for tetraploids).

To determine if these results were due to differential growth rates between diploid and tetraploid strains, we analyzed the doubling times for each of the strains used in the competition assay. No significant differences in growth rates were observed between diploid and tetraploid strains, as both doubled every 60 min during exponential growth. We also determined that diploid and tetraploid strains were able to grow to the same maximum optical densities in saturated cultures (Fig. 6B and data not shown). These results indicate that the increased fitness of diploid strains is not simply the consequence of faster growth under these culture conditions.

To determine if a fitness advantage of diploid cells was also observed *in vivo*, we performed a competition experiment using an established murine model for systemic infection (43). Mice were infected via the tail vein with mixed populations of diploid and tetraploid cells, and cells were subsequently recovered from the brain, kidney, liver, and spleen. After 2 days of infection, we saw no change in the relative proportions of diploid and tetraploid strains between the inoculum and the recovered cells (Fig. 6C and D). Thus, the initial NAT^R diploid inoculum (44.4%) showed similar frequencies in the brain (37.0%), kidney (25.9%), liver (58.5%), and spleen (45.5%) following infection (Fig. 6D). Similarly, the initial frequency of NAT^R tetraploid strains (26.2%) was not significantly different when they were recovered from any of the infected organs (brain, 10.8%; kidney, 12.5%; liver, 12.7%; spleen, 14.5%) (Fig. 6C). Longer time points during *in vivo* infection were not possible as *C. tropicalis* strains led to murine morbidity, with the result that mice had to be euthanized.

DISCUSSION

C. tropicalis was recently discovered to have an extant mating program in which diploid cells fuse to form tetraploid products (34, 35). In this study, we used a genetic selection to identify conditions that induce *C. tropicalis* tetraploid cells to return to the diploid

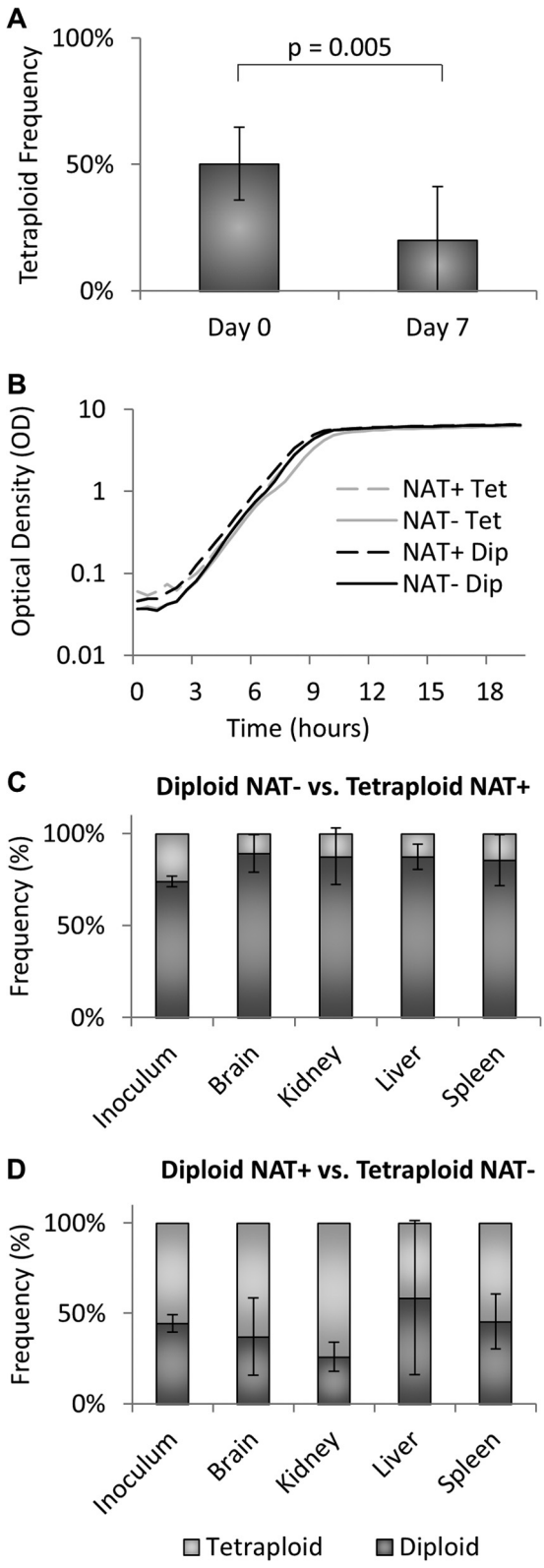

FIG 6 *C. tropicalis* ploidy competition assays. (A) Coculture of NAT^R diploid with NAT^S tetraploid strains and NAT^R tetraploid with NAT^S diploid strains. The *y* axis represents the percentage of tetraploid cells present at the beginning (day 0) and end (day 7) of the experiment. Bars represent means ± standard deviations. NAT^R diploid strains (CAY4090, CAY4281, and CAY4282) were competed with NAT^S tetraploid strains (CAY4888 and CAY4890), while NAT^R tetraploid strains (CAY3373 and CAY3374) were competed with NAT^S diploid strains (CAY3031, CAY4276, and CAY4277) ($P = 0.005$; $n = 8$). (B) Growth curve for strains used in the assay. The *x* axis represents a linear scale of time in hours, and the *y* axis represents a logarithmic scale of optical density (OD) as a measure of cell concentration. The experiment was carried out twice, with one representative figure shown here. (C and D) *In vivo* competition assay showing population frequencies of NAT^R diploids versus NAT^S tetraploids (C) and NAT^R tetraploids versus NAT^S diploids (D) in murine brain, kidney, liver, and spleen tissue after 48 h, compared to the initial inoculum. Bars represent means ± standard deviations for 4 to 8 mice in independent experiments.

state. Populations of *C. tropicalis* tetraploid cells were stable for multiple generations when grown on diverse laboratory media but exhibited chromosome instability when propagated for extended periods in rich (YPD) medium. In addition, tetraploid cells could also be induced to return to the diploid state by selective growth on sorbose medium. The diploid products of chromosome reduction were mating competent, thereby establishing that *C. tropicalis* can undergo a parasexual program.

The efficiency of chromosome loss in *C. tropicalis* tetraploid cells was highest when cells were grown on sorbose medium; 3 to 9% of *C. tropicalis* tetraploid cells became 2-DOG^R when incubated on this medium. Assuming a random segregation of chromosomes (and no loss of *GAL1* due to recombination), this indicates that 18 to 54% of sorbose-selected colonies became disomic for this chromosome. In contrast, only 0.003 to 0.04% of *C. tropicalis* cells grown on YPD medium lost *GAL1*, a difference of 2 to 3 orders of magnitude. Flow cytometric analysis of Δ*gal1* progeny confirmed that most chromosomes had become disomic during growth on sorbose medium, and thus parasexual progeny exhibited a ploidy similar to that of control diploid cells.

Sorbose medium has also been shown to induce concerted chromosome loss in tetraploid cells of the related species *C. albicans* (29, 30). In *C. albicans*, growth on sorbose medium is stressful as most cells in the population are unable to survive on this medium (29, 44). We saw a similar stress response in *C. tropicalis*, with reduced cell viability on sorbose medium. The survival rate was even lower when cells were grown on sorbose without amino acids (1 to 6%) although there was no difference in loss of the *GAL1* marker between the two conditions. In both *Candida* species, colonies that survive stressful growth on sorbose medium have frequently undergone parasexual chromosome loss. *C. albicans* tetraploid cells were also previously shown to exhibit chromosome instability when incubated on *S. cerevisiae* presporulation medium at 37°C (29). In contrast, growth on presporulation medium did not result in high rates of chromosome loss in *C. tropicalis*. Furthermore, whereas presporulation medium induced high rates of cell death in both *C. albicans* and *C. tropicalis* tetraploid cells at 2 days, only *C. tropicalis* cells recovered viability after 4 days of growth on this medium (data not shown). We surmise that chromosome instability is associated with cell stress, and induction of the parasexual program may be used to survive hostile environmental conditions, as further discussed below.

Notably, none of the conditions tested in this study were found to induce sporulation in *C. tropicalis* tetraploid cells, and we thus found no evidence for a meiotic program in this species. Identification of a conventional meiosis in *C. albicans* has been a long-standing goal as it would allow the use of classical genetics in this important human pathogen (45, 46). Similarly, we now report

TABLE 3 Parasexual strains in this study

PS group and strain no.[a]	Cell type or genotype	Isolate no.	Original strain(s) (growth condition)
Selected progeny tested for *MTL* type			
*MTL***a/a**			
A1	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	1	CA3373 (sorbose at 30°C)
A2	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	2	CA3373 (sorbose at 30°C)
A3	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	3	CA3373 (sorbose at 30°C)
A4	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	4	CA3373 (sorbose at 30°C)
A5	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	5	CA3373 (sorbose at 30°C)
A6	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	6	CA3373 (sorbose at 30°C)
A7	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	7	CA3373 (sorbose at 37°C)
A8	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	8	CA3373 (sorbose at 37°C)
A9	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	9	CA3373 (sorbose at 37°C)
A10	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	10	CA3373 (sorbose at 37°C)
A11	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	11	CA3374 (sorbose at 30°C)
A12	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	12	CA3374 (sorbose at 37°C)
B1	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	13	CA3375 (sorbose at 30°C)
*MTL*α/α			
B2	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	23	CA3373 (sorbose at 30°C)
B3	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	25	CA3373 (sorbose at 30°C)
B4	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	242	CA3373 (sorbose at 30°C)
B5	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	283	CA3373 (sorbose at 30°C)
B6	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	302	CA3374 (sorbose at 30°C)
B7	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	320	CA3374 (sorbose at 30°C)
B8	*MTL*α/α Δ*gal1*/Δ*gal1*::*SAT1* Δ*his1*/Δ*his1*	336	CA3374 (sorbose at 30°C)
Progeny from sorbose tested for *MTL* type			
B9	*MTL***a/a** Δ*gal1*/Δ*gal1*::*SAT1*	1	
B10	*MTL***a/a**	2	CA3373 (sorbose at 37°C)
B11	*MTL***a/a**	3	CA3373 (sorbose at 37°C)
B12	*MTL***a/a**	4	CA3373 (sorbose at 37°C)
C1	*MTL***a/a**	7	CA3373 (sorbose at 37°C)
C2	*MTL***a/a**	9	CA3374 (sorbose at 37°C)
C3	*MTL***a/a**	10	CA3375 (sorbose at 37°C)
C4	*MTL***a/a**	11	CA3375 (sorbose at 37°C)
Randomly selected progeny from 2-DOG			
F1	Δ*gal1*/Δ*gal1*::*SAT1*	1	CA3373 (sorbose at 30°C)
F2	Δ*gal1*/Δ*gal1*::*SAT1*	2	CA3373 (sorbose at 30°C)
F3	Δ*gal1*/Δ*gal1*::*SAT1*	3	CA3373 (sorbose at 30°C)
F4	Δ*gal1*/Δ*gal1*::*SAT1*	4	CA3373 (sorbose at 30°C)
F5	Δ*gal1*/Δ*gal1*::*SAT1*	5	CA3373 (sorbose at 30°C)
F6	Δ*gal1*/Δ*gal1*::*SAT1*	6	CA3373 (sorbose at 30°C)
F7	Δ*gal1*/Δ*gal1*::*SAT1*	7	CA3373 (sorbose at 30°C)
F8	Δ*gal1*/Δ*gal1*::*SAT1*	8	CA3373 (sorbose at 30°C)
F9	Δ*gal1*/Δ*gal1*::*SAT1*	1	CA3373 (sorbose at 37°C)
F10	Δ*gal1*/Δ*gal1*::*SAT1*	2	CA3373 (sorbose at 37°C)
F11	Δ*gal1*/Δ*gal1*::*SAT1*	3	CA3373 (sorbose at 37°C)
F12	Δ*gal1*/Δ*gal1*::*SAT1*	4	CA3373 (sorbose at 37°C)
G1	Δ*gal1*/Δ*gal1*::*SAT1*	5	CA3373 (sorbose at 37°C)
G2	Δ*gal1*/Δ*gal1*::*SAT1*	7	CA3373 (sorbose at 37°C)
G3	Δ*gal1*/Δ*gal1*::*SAT1*	8	CA3373 (sorbose at 37°C)
G4	Δ*gal1*/Δ*gal1*::*SAT1*	9	CA3373 (sorbose at 37°C)
Mating products			
C5	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	5	B2 × CAY2060; mating product G
C6	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	6	B2 × CAY2060; mating product G
C7	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	7	B2 × CAY2060; mating product G
C8	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	19	B3 × CAY2060; mating product H
C9	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	20	B3 × CAY2060; mating product H
C10	*MTL***a/a**/α/α *his1*/*his1*/*HIS1*/*HIS1 arg4*/*arg4*/*ARG4*/*ARG4 gal1*/*gal1*::*SAT1*/*GAL1*/*GAL1*	21	B3 × CAY2060; mating product H
Haploid screen			
D1	*Gal1*$^-$	1	CAY4539 (2μM H_2O_2)
D2	*Gal1*$^-$ *Ura3*$^-$	2	CAY4539 (H_2O_2)
D3	*Gal1*$^-$	3	CAY4539 (H_2O_2)
D4	*Gal1*$^-$	4	CAY4539 (H_2O_2)
D5	*Gal1*$^-$ *Arg4*$^-$	5	CAY4539 (H_2O_2)
D6	*Gal1*$^-$ *Ura3*$^-$	6	CAY4539 (H_2O_2)
D7	*Gal1*$^-$	7	CAY4539 (H_2O_2)
D8	*Gal1*$^-$	8	CAY4539 (H_2O_2)
D9	*Gal1*$^-$	9	CAY4539 (H_2O_2)
D10	*Gal1*$^-$	11	CAY4539 (H_2O_2)
D11	*Gal1*$^-$ *Ura3*$^-$ *Arg4*$^-$	12	CAY4539 (H_2O_2)
D12	*Gal1*$^-$ *Arg4*$^-$	13	CAY4539 (H_2O_2)
E1	*Gal1*$^-$ *Ura3*$^-$	15	CAY4539 (42°C)
E2	*Gal1*$^-$	16	CAY4539 (42°C)
E3	*Gal1*$^-$ *Arg4*$^-$	17	CAY4539 (42°C)
E4	*Gal1*$^-$	19	CAY4539 (42°C)
E5	*Gal1*$^-$ *Ura3*$^-$	20	CAY4539 (42°C)
E6	*Gal1*$^-$	21	CAY4539 (42°C)
E7	*Gal1*$^-$	22	CAY4539 (42°C)

[a] PS, parasexual.

that *C. tropicalis* appears to undergo a parasexual cycle rather than a true sexual cycle culminating in meiosis. Growth of *C. tropicalis* tetraploid cells on sorbose medium induced an efficient reduction in ploidy, but the surviving colonies on this medium did not produce any spore-like cells. It therefore remains to be seen if a cryptic meiotic program exists for either *C. albicans* or *C. tropicalis*.

C. tropicalis tetraploid cells were also found to undergo a reduction in ploidy to the diploid state during prolonged culture in YPD medium. Strikingly, each of three independent tetraploid lines showed a similar trend toward diploidy via intermediate aneuploid states. This ploidy change began around day 18 in YPD culture following approximately 120 generations of propagation. Since cells were cultured under rich growth conditions, it therefore appears that the parasexual process in *Candida* species does not take place exclusively under conditions of stress (30, 31), an important factor in considering where chromosome loss may occur in host environments.

Studies of genomic states in *S. cerevisiae* have established that this organism is also capable of undergoing asexual changes in ploidy. *S. cerevisiae* cells preferentially propagate as diploid cells, and prolonged culture of haploid, triploid, or tetraploid cells results in their convergence toward the diploid state (39, 42, 47). This genomic convergence is independent of mating or meiosis and may occur by chromosome nondisjunction during mitosis (e.g., to drive a ploidy reduction) or by endoreduplication (e.g., to drive a ploidy increase). *Aspergillus nidulans*, a primarily haploid ascomycete, also undergoes a reduction in ploidy (from diploid to haploid) via a parasexual mechanism when grown continuously in standard medium (48). It is therefore apparent that extended culture can induce ploidy changes in diverse fungal species although the precise molecular mechanisms driving parasexual ploidy changes remain to be elucidated.

The fitness of *C. tropicalis* diploid and tetraploid cells was also directly compared in this study. When cocultured in YPD medium for 7 days, diploid cells showed enhanced fitness over tetraploid cells. This is in spite of the fact that diploid and tetraploid cells showed indistinguishable growth rates *in vitro*. However, coculture in a murine model of infection failed to demonstrate an enhanced fitness of diploids, which may be due to the limited time period (2 days) used for *in vivo* experiments. It therefore appears that diploid cells exhibit increased fitness over tetraploid cells but that tetraploid cells can stably survive and propagate in *in vivo* niches, at least for a defined period of time. In *S. cerevisiae*, haploid cell populations were also shown to be overtaken by diploid cells even though the investigators failed to pinpoint any overt fitness advantage of diploids over haploids (47). Other studies have also failed to identify specific fitness advantages of diploid strains over haploid strains of *S. cerevisiae* (49, 50). We conclude that *C. tropicalis* diploid cells hold a cryptic fitness advantage over tetraploid cells but that the nature of the fitness advantage is currently unknown.

Recent studies have revealed that *C. albicans* diploid cells can also undergo a ploidy reduction to the haploid state *in vitro* and *in vivo* (32). This discovery was surprising as *C. albicans* was thought to be an obligate diploid due to the presence of multiple recessive lethal alleles on different chromosome homologs (51, 52). The formation of *C. albicans* haploids presumably occurs via a nonmeiotic mechanism as very few chromosomal crossovers were detected in haploid cells (32). To test if *C. tropicalis* can also exist in the haploid state, we screened for *C. tropicalis* haploid cells using a diploid strain heterozygous for *GAL1*, *HIS1*, *ARG4*, and *URA3*. We observed loss of several genetic markers in a subset of the population (Table 3) but have so far been unable to obtain *C. tropicalis* haploid cells under a variety of *in vitro* culture conditions (data not shown).

What are the consequences of parasexuality for the lifestyles of *Candida* species? Berman and Hadany (31) have proposed that diverse progeny resulting from parasexuality would have the potential to better survive and adapt to stressful environments. In support of this model, the investigators note that many of the steps in the *C. albicans* parasexual cycle are induced by stress, and thus parasexuality can generate recombinant forms precisely when needed for adaptation to a hostile environment. Preliminary experiments in *C. albicans* support this hypothesis as they show that parasexual progeny can better resist stressful conditions *in vitro* than parental diploid cells (M. P. Hirakawa and R. J. Bennett, unpublished observations). We note that both stressful culture conditions (growth on sorbose) and nonstressful conditions (prolonged culture in YPD medium) induce parasexual chromosome loss in *C. tropicalis* tetraploid cells. It therefore remains an intriguing question as to the precise conditions that induce the parasexual cycle in nature and the role of this cycle in generating recombinant forms that can promote adaptation to environmental niches.

ACKNOWLEDGMENTS

S.K.J. and A.M.P. were supported by predoctoral fellowships F31DE022701 and F31DE022703, respectively. M.P.H. was supported by a Graduate Assistance in Areas of National Need Training Grant P200A100100. This work was supported by National Institutes of Health grant AI081704 (to R.J.B.) and by National Science Foundation Grant MCB1021120 (to R.J.B). R.J.B. also holds an Investigator in the Pathogenesis of Infectious Disease Award from the Burroughs Wellcome Fund.

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