Role of DNA Mismatch Repair and Double-Strand Break Repair in Genome Stability and Antifungal Drug Resistance in *Candida albicans*  

Melanie Legrand, Christine L. Chan, Peter A. Jauert, and David T. Kirkpatrick*  

Department of Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, Minnesota 55455  

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Drug resistance has become a major problem in the treatment of *Candida albicans* infections. Genome changes, such as aneuploidy, translocations, loss of heterozygosity, or point mutations, are often observed in clinical isolates that have become resistant to antifungal drugs. To determine whether these types of alterations result when DNA repair pathways are eliminated, we constructed yeast strains bearing deletions in six genes involved in mismatch repair (*MSH2* and *PMS1*) or double-strand break repair (*MRE11, RAD50, RAD52*, and *YKU80*). We show that the *mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ* mutants are slow growing and exhibit a wrinkly colony phenotype and that cultures of these mutants contain abundant elongated pseudohypha-like cells. These same mutants are susceptible to hydrogen peroxide, tetrabutyl hydrogen peroxide, UV radiation, camptothecin, ethylmethane sulfonate, and methylmethane sulfonate. The *msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yka80Δ/yka80Δ* mutants exhibit none of these phenotypes. We observed an increase in genome instability in *mre11Δ/mre11Δ* and *rad50Δ/rad50Δ* mutants by using a *GAL1/URA3* marker system to monitor the integrity of chromosome 1. We investigated the acquisition of drug resistance in the DNA repair mutants and found that deletion of *mre11Δ/mre11Δ, rad50Δ/rad50Δ*, or *rad52Δ/rad52Δ* leads to an increased susceptibility to fluconazole. Interestingly, we also observed an elevated frequency of appearance of drug-resistant colonies for both *msh2Δ/msh2Δ* and *pms1Δ/pms1Δ* (MMR mutants) and *rad50Δ/rad50Δ* (DSBR mutant). Our data demonstrate that defects in double-strand break repair lead to an increase in genome instability, while drug resistance arises more rapidly in *C. albicans* strains lacking mismatch repair proteins or proteins central to double-strand break repair.

*Candida albicans* is the single most important human fungal pathogen. Although *C. albicans* is normally a commensal, candidiasis may result when the host becomes debilitated or immunosuppressed. A 25 to 60% mortality rate associated with disseminated infection has been reported (11), due in part to the limited number of antifungal agents available and in part to increasing resistance to the most popularly used drug, fluconazole.

The three main classes of drugs used to treat *Candida* infections are polyenes (amphotericin B), azoles (fluconazole and related compounds), and echinocandins (caspofungin). Azoles, the most commonly used drugs, target the *ERG11* gene product, which encodes a lanosterol (C-14) demethylase required for ergosterol biosynthesis (30). Unfortunately, acquired resistance to azoles is becoming a serious clinical problem.

The development of azole resistance in *C. albicans* has been well characterized. Acquisition of resistance has been shown to be associated with point mutations in the *ERG11* gene or in the promoter region of drug transporters (17, 31). Several studies have shown that point mutations in the *ERG11* gene can result in amino acid alterations that change the protein’s affinity for fluconazole, while point mutations in the promoter of *CDR1* result in overexpression of the pump. Other studies demonstrated that gross chromosome rearrangements, such as chromosomal gain or loss or isochromosome formation (14, 25), occur in clinical isolates that have become drug resistant. However, the biological mechanisms that lead to these alterations have not been determined.

In many organisms, cells deficient in DNA mismatch repair (MMR) exhibit a mutator phenotype in which the rate of spontaneous mutation is greatly elevated (6, 10). The MMR pathway acts to remove bases that are mispaired as a result of a failure during replication, illustrated by the functional interaction of the MMR proteins with the DNA replication factor PCNA (5, 8). The MMR pathway also plays a role in maintaining the stability of certain types of repetitive DNA tracts (28). This pathway was first described in *Escherichia coli*, where MutS and MutL bind to the mismatch, activating the MutH endonuclease. Multiple MutS and MutL homologues have been characterized in *Saccharomyces cerevisiae* (13, 24). Null mutations in *MSH2* (MutS homolog) or *PMS1* (MutL homolog) cause an increase in base substitutions and insertion/deletion in simple sequence repeats in *S. cerevisiae* (23, 32).

Cells that are deficient in double-strand break (DSB) repair (DSBR) exhibit an elevated level of genome instability. DNA DSBs are the most dangerous form of DNA damage: failure to repair a DSB leads to loss of the fragment lacking a centromere, while improper repair can generate translocations, inversions, or deletions. Cells possess two major pathways for DSBR, homologous recombination (HR), which requires the presence of an intact second copy of the broken DNA, and nonhomologous end joining (NHEJ), in which the ends of the broken DNA molecules are religated (12, 15, 22). In *S. cerevisiae*, the RAD50 and MRE11 proteins are involved in the early steps of DSBR and are required for both HR and NHEJ (1).
In contrast, the RAD52 protein plays a central role in HR but does not act during NHEJ, while the YKU80 protein is required during NHEJ but not during HR (20).

MMR and DSBR play a major role in maintaining genome fidelity and stability. Because defects in the MMR pathway lead to point mutations and instability of repetitive DNA tracts, while defects in the DSBR pathway cause gross chromosome rearrangements, we investigated MMR and DSBR involvement in genome stability and antifungal drug resistance acquisition in C. albicans. We constructed strains bearing null mutations in both copies of six genes involved in MMR or DSBR and characterized their phenotypes by testing their sensitivity to several DNA-damaging agents, monitoring the integrity of chromosome 1 by using a GAL1/URA3 marker system, and determining their ability to become resistant to antifungal drugs.

In this study, we show that the mutants defective in HR (mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ) have a slow-growth phenotype and produce wrinkled colonies with pseudohypha-like cells whereas the colonies of the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants are smooth, appearing identical to wild-type colonies. We also observed that mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ strains are susceptible to hydrogen peroxide (H2O2), tetrabutyl hydrogen peroxide (TBHP), UV radiation, camptothecin, ethylmethane sulfonate (EMS), and methylmethane sulfonate (MMS), whereas the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants are not. There is an increase in the rate of appearance of 2-deoxyglactosone-resistant (2-DGγ) and 5-fluoroorotic acid-resistant (5-FOA+) colonies in the mre11Δ/mre11Δ and rad50Δ/rad50Δ mutants, reflecting an increase in genome instability in these mutants. Using E-test strips, we found that deletion of some of the DSBR genes leads to an increased susceptibility to some antifungal drugs. Interestingly, we saw an increase in the appearance of drug-resistant colonies inside the inhibition ellipse for both msh2Δ/msh2Δ and pms1Δ/pms1Δ (MMR mutants) and rad50Δ/rad50Δ (DSBR mutant).

### TABLE 1. Yeast strains used in this study

| Strain | Source | Phenotype | Relevant genotype |
|--------|--------|-----------|-------------------|
| C. albicans | SN76 | Ur4 Ura3 His1 | arg4::arg4 his1::his1 ura3::imm434 leu2-3,112 lys2::imm101 iro1::imm434 Arg4-1::Cd4 |
| DKCa39 | SN76 | Prototroph | rad52::Cd1 ura3::Cd4 |
| DKCa43 | DKCa39 | Prototroph | mre11::Cd1 ura3::Cd4 |
| DKCa61 | DKCa39 | Prototroph | msh2::Cd1 ura3::Cd4 |
| DKCa33 | DKCa39 | Prototroph | mre11::Cd1 ura3::Cd4 |
| DKCa98 | DKCa39 | Prototroph | msh2::Cd1 ura3::Cd4 |
| DKCa58 | DKCa39 | Prototroph | mre11::Cd1 ura3::Cd4 |
| DKCa95 | DKCa39 | Prototroph | msh2::Cd1 ura3::Cd4 |
| DKCa67 | DKCa39 | Prototroph | rad50::Cd1 ura3::Cd4 |
| DKCa127 | DKCa39 | Prototroph | rad50::Cd1 ura3::Cd4 |
| DKCa78 | DKCa39 | Prototroph | yku80::Cd1 ura3::Cd4 |
| DKCa112 | DKCa39 | Prototroph | rad52::Cd1 ura3::Cd4 |
| DKCa96 | DKCa39 | Prototroph | rad52::Cd1 ura3::Cd4 |
| DKCa97 | DKCa39 | Prototroph | rad52::Cd1 ura3::Cd4 |
| DKCa496 | DKCa43 | Arg+ | pms1::Cd1 ura3::Cd4 |
| DKCa511 | DKCa61 | Arg+ | msh2::Cd1 ura3::Cd4 |
| DKCa781 | DKCa98 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa225 | DKCa58 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa228 | DKCa95 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa456 | DKCa67 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa457 | DKCa127 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa461 | DKCa78 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa465 | DKCa112 | Arg+ | mre11::Cd1 ura3::Cd4 |
| DKCa2555 | DKCa96 | His+ | rad52::Cd1 ura3::Cd4 |

| S. cerevisiae | BY4743 | His+ Leu+ Ura- | MATα/a, his3Δ/ura3Δ, leu2Δ/leu2Δ, lys2Δ/lys2Δ, met5Δ/leu2Δ, ura3Δ/ura3Δ |
|--------------|--------|----------------|-------------------------------------------------|
| YMR224C | BY4743 | His+ Leu+ Ura- | mre11::Cd1 ura3Δ |
| YNL250W | BY4743 | His+ Leu+ Ura- | rad50Δ/rad50Δ |
| YMR106C | BY4743 | His+ Leu+ Ura- | yku80Δ/yku80Δ |
| YML032C | BY4743 | His+ Leu+ Ura- | rad52Δ/rad52Δ |

*S. cerevisiae* strains were maintained on YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 20 mg/liter uridine (YEPD+Ura) at 30°C.

**Materials and Methods**

**Strains and media.** The yeast strains used in this study are described in Table 1. *C. albicans* and *S. cerevisiae* strains were maintained on YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 20 mg/liter uridine (YEPD+Ura) at 30°C.

DKCa39 was constructed by replacing one of the GAL1 alleles on chromosome 1 in the SN76 strain (21) with the URA3 gene. The gait::URA3 cassette was amplified from AF14 (20) by PCR with oligonucleotides outside the GAL1 open reading frame (ORF) (CaGA11::1756-F and CaGA12::256-R). The PCR product was transformed into SN76, and transformants were selected on Ura+ plates.

**Gene disruption and reintegration of *C. albicans* wild-type genes.** To construct homozygous mutant strains, both alleles were deleted by HR by a PCR-based method.
TABLE 2. Oligonucleotides used in this work

| Primer name | Sequence 3' → 5' |
|-------------|-----------------|
| CaMSH2-KO-F | CTATAAAAGTGCAACATATTATAATTTTAAACCTGTTGACCTTGAATTCCAGG |
| CaMSH2-KO-R | AGTCGACATATTTGCTTCTGTTGACCTTGAATTCCAGG |
| CaPMS1-F | ATGTCCAAGGAATTTCAGTCTTTGTTGTTGATGCATCCAAATATATGGGA |
| CaMRE11-KO-F | ATGCCATTGGTAGAACGTATAGAACCAGGACCTGACACAATTAGGGTTCTAAC |
| CaMRE11-KO-R | TTATTTTCTTTTCCTGTTATTAAAGCTTCTGGAAAATATTCCTTTACTTTTACT |
| CaRAD52-KO-F | ATGAACTCTAGACCTGCACCTCCGCAACCACGACCACCACAACAACCTCTC |
| CaRAD52-KO-R | TGCATCTGTTGAATTTCAATACCCCGCCA |
| CaPMS1-F | TTGTATTATTAAACCAATATTTTCATTCGGCATTTCTACATGTCAATGCCCT |
| CaMSH2-KO-R | ATGTCTAGTACCAAAATAGATCTCAAATTCAGTGACTTGGCTGATGAAAGAC |

a Bold letters correspond to vector sequences from the plasmids containing the C. dublinensis HIS1 and ARG4 genes.
the dark. After 5 days, cells were examined under a Nikon E600 microscope to detect chlamydospore formation.

Colonies and colony morphology. To study the colony morphology of the mutants, frozen cells were streaked onto YEPD + Uri plates and incubated at 30°C for 48 h. Pictures of colonies on agar plates were taken with a Nikon CoolPIX900 camera attached to a Zeiss Stemi DRC microscope. To study the cell morphology of the mutants, cells from liquid cultures grown at 30°C overnight were examined under a Nikon E600 microscope.

Filamentation assay. To assay C. albicans hyphal growth, cells grown overnight were transferred to 5 ml of Spider medium (1% nutrient broth, 1% mannitol, 0.2% K$_2$HPO$_4$) or YEPD + Uri containing 10% adult bovine serum and grown in a 37°C shaker for 1 h. Cells were then examined under a Nikon E600 microscope.

DNA-damaging agent. (i) Oxidizing agent sensitivity. Two protocols were used to determine sensitivity to oxidizing agents. For the first, overnight cultures were diluted in water to an OD$_{560}$ of 2 and 10-fold dilutions were spotted onto YEPD + Uri plates containing 4 mM H$_2$O$_2$, 0.1 mM menadione, or 2 mM TBHP and incubated at 30°C for 24 h. For further H$_2$O$_2$ susceptibility characterization, we tested wild-type and mutant strains in liquid medium. Cells were inoculated into 2 ml of YEPD + Uri and grown overnight at 30°C. The next morning, 100 μl of the overnight cultures was inoculated into 5 ml of YEPD + Uri or 5 ml of YEPD + Uri plus 4 mM H$_2$O$_2$. Cultures were grown at 30°C for 2 h, and then 100 μl of a 1:10,000 or a 1:100,000 dilution was plated onto YEPD + Uri plates. After incubation at 30°C for 48 h, colonies were counted and the survival percentage was calculated for each strain. The assay was repeated four times.

(ii) UV sensitivity. Overnight cultures were diluted in water to an OD$_{560}$ of 2, and 10-fold dilutions were spotted onto YEPD + Uri plates. The plates were immediately irradiated with the indicated doses of UV light, wrapped in foil, and incubated at 30°C for 24 h.

(iii) Alkylating agents. Overnight cultures were diluted in water to an OD$_{560}$ of 2, and 10-fold dilutions were spotted onto YEPD + Uri plates containing 100 μM camptothecin, 0.03% EMS, or 0.01% MMC and incubated at 30°C for 24 h.

Chromosome 1 integrity. (i) Assay 1. Cells were grown overnight in YEPD + Uri broth at 30°C. Cells were then counted with a hemacytometer, and 100,000 cells were plated onto minimal 2-DG$^+$ and minimal 5-FOA$^+$ plates. Dilutions were also plated onto YEPD + Uri plates to confirm colony counting.

(ii) Assay II (fluctuation analysis). The appropriate strains were streaked onto YEPD + Uri for single colonies and incubated at 30°C for 3 days. For each strain, 20 overnight cultures were prepared by inoculating a unique single colony into 5 ml of YEPD + Uri. The next morning, the overnight cultures were diluted in water. Fifty or 100 μl of the 10$^{-6}$ dilution was plated onto YEPD + Uri plates, and 100 μl of the 10$^{-5}$ or 10$^{-6}$ dilution was plated onto 2-DG and 5-FOA with glass beads. Plates were incubated at 30°C. Colonies were counted on YEPD + Uri plates on day 2 and on 2-DG$^+$ and 5-FOA$^+$ plates on day 3. The rate of appearance of 2-DG$^+$ and 5-FOA$^+$ colonies was determined as described by Spell and Jinns-Robertson (29).

Characterization of 2-DG$^+$ and 5-FOA$^+$ colonies. For each strain, 20 2-DG$^+$ and 20 5-FOA$^+$ colonies were patched onto 2-DG$^+$ and 5-FOA$^+$ plates and incubated at 30°C for 2 days. Genomic DNA was extracted from these cells and screened by PCR to assess the presence or absence of the GAL1 and URA3 genes. The oligonucleotides CaGAL1-474-F, CaGAL1-256-R, and CaURA3-386-R were used in the same PCR mixture. If the GAL1 or URA3 gene was still present in 2-DG$^+$ cells or 5-FOA$^+$ cells, respectively, the PCR product was sequenced. If the GAL1 or URA3 gene was absent in 2-DG$^+$ cells or 5-FOA$^+$ cells, respectively, we used single-nucleotide polymorphism (SNP) analysis to determine the extent of the chromosomal alterations. The SNPs used (1322-2294 and F12n4) were located on chromosome 1 on both sides of the GAL1/URA3 locus, and both of the SNP sequences contained a restriction site (see Fig. 5). The regions containing the SNPs were amplified by PCR with the oligonucleotides AF-1322-2294-F/R and XU-F12n4-F/R. A 10-μl volume of the PCR product was digested with either the BclI or the HpaII enzyme, respectively, by adding 1 μl of the enzyme buffer and 0.5 μl of the enzyme. Digestion reaction mixtures were incubated at 37°C overnight and loaded onto a 2% agarose gel.

Drug resistance. Cells grown overnight were diluted to an OD$_{560}$ of 0.02 in 0.85% NaCl. To distribute the inoculum evenly, a sterile cotton-tipped applicator was soaked in the dilution and used to streak the entire agar surfaces of Casitone + Uri agar plates (0.5% yeast extract, 1% sodium citrate, 0.9% Bacto Casitone, 2% glucose, 2% agar, 20 mg/liter uridine). After the plates were allowed to dry, E-test strips were applied. Plates were incubated at 35°C for a week, and pictures were taken daily.

### RESULTS

**Sequence analysis.** To determine if the C. albicans genome contains orthologs of MSH2, PMS1, RAD50, MRE11, RAD52, or YKU80, BLAST searches (TBLASTN) of the C. albicans database (http://www.candidagenome.org/cgi-bin/nph-BLAST) were performed with the S. cerevisiae DNA repair protein sequence databases as queries. A single homologue for each of the genes was detected. In pairwise BLAST comparisons, the matches have e values that vary from 1.5e$^{-12}$ to 3.3e$^{-200}$ (Table 3). These results suggest that the C. albicans genes are likely to be orthologs of the S. cerevisiae DNA repair genes. The ORF number, chromosome location, gene size, and sequence identity are presented in Table 3. Each gene is named for its S. cerevisiae ortholog.

**Gene disruption.** To determine the role of DNA repair in the biology of C. albicans, we constructed disruptions in genes known to be involved in MMR and DSBR in S. cerevisiae. Because C. albicans is a diploid organism, both alleles of each gene must be deleted to generate null mutants. Gene disruptions in strain DKCa39 were carried out as described in Materials and Methods. When the gene of interest was closely flanked by an adjacent gene, the disruption included most of the ORF but left 79 bp at the 5$'$ end and the 3$'$ end in order to avoid changes in the expression of the flanking genes. When the gene of interest was not directly flanked by ORFs, the deletion included the entire ORF. The extent of each deletion is given in Table 3. A HIS1 disruption cassette was constructed and transformed into DKCa39 to generate heterozygotes. Two

### TABLE 3. Characteristics of C. albicans DNA repair genes

| Repair pathway | Gene | ORF | Chromosome | Size (nt) | Sequence deleted$^a$ | E value$^b$ | % Identity$^c$ |
|----------------|------|-----|------------|----------|---------------------|------------|--------------|
| MMR | MSH2 | orf19.3093 | 4 | 2,622 | 80–2543 | 3.9e$^{-183}$ | 41 |
| MMR | PMS1 | orf19.1605 | 3 | 2,331 | –94–2606 | 1.8e$^{-131}$ | 32 |
| DSBR | MRE11 | orf19.6915 | 7 | 2,049 | 80–1970 | 6.7e$^{-138}$ | 42 |
| DSBR | RAD50 | orf19.1648 | 3 | 3,999 | 80–3920 | 1.7e$^{-230}$ | 36 |
| NEHE | YKU80 | orf19.2383 | R | 1,830 | 80–1647 | 7.8e$^{-13}$ | 17 |
| HR | RAD52 | orf19.4208 | 6 | 1,695 | 80–1857 | 1.4e$^{-58}$ | 26 |

$^a$ Base 1 corresponds to the A of the ATG codon.

$^b$ With TBLASTN with S. cerevisiae homolog.

$^c$ Percent amino acid identity with S. cerevisiae homolog.

* C. albicans DNA repair mutants. 2007

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TABLE 4. Doubling times of C. albicans DNA repair mutants

| Strain | Repair pathway | Genotype | Mean doubling time (h) ± SD |
|--------|----------------|----------|----------------------------|
| DKCa39 | Wild type      | msh2Δ/msh2Δ | 1.09 ± 0.13                |
| DKCa33 | MMR            | msh2Δ/msh2Δ | 1.17 ± 0.13                |
| DKCa98 |                | msh2Δ/msh2Δ | 1.25 ± 0.11                |
| DKCa43 |                | pms1Δ/pms1Δ | 1.35 ± 0.08                |
| DKCa61 |                | pms1Δ/pms1Δ | 1.28 ± 0.23                |
| DKCa58 | DSBR (both HR and NHEJ) | mre11Δ/mre11Δ | 2.03 ± 0.77                |
| DKCa95 |                | mre11Δ/mre11Δ | 1.79 ± 0.44                |
| DKCa67 |                | rad50Δ/rad50Δ | 1.65 ± 0.2                 |
| DKCa127 |             | rad50Δ/rad50Δ | 1.46 ± 0.13                |
| DKCa96 | DSBR (HR only) | rad52Δ/rad52Δ | 1.78 ± 0.27                |
| DKCa97 |                | rad52Δ/rad52Δ | 1.94 ± 0.15                |
| DKCa78 | DSBR (NHEJ only) | yku80Δ/yku80Δ | 1.22 ± 0.08                |
| DKCa112 |              | yku80Δ/yku80Δ | 1.13 ± 0.13                |

We also observed that the colony morphologies of the HR null mutants differ significantly from that of the wild type. The mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ mutants exhibit a wrinkly phenotype on YEPD+Uri agar at 30°C, whereas colonies of the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants are smooth, identical to the wild-type colonies (Fig. 1 top). In YEPD+Uri broth, the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ cultures contain only yeast cells, similar to the parental strain, whereas the mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ cultures contain yeast cells but also abundant elongated pseudohypha-like cells (Fig. 1, bottom). The diameter-to-length ratio of the pseudohypha-like cells varied between the strains, with the mre11Δ/mre11Δ mutant exhibiting the largest ratio, followed by the rad50Δ/rad50Δ mutant and then the rad52Δ/rad52Δ strain.

On the basis of the colony morphologies of the HR mutants, we examined the degree of filamentation in the various strains in response to serum and in Spider medium at 37°C. True hyphae were observed in all of the mutants and the parental strain (data not shown).

We also investigated another morphological characteristic of C. albicans—its ability to produce large thick-walled spores called chlamydospores, whose function is unknown. We examined the degree of filamentation in the various strains in response to serum and in Spider medium at 37°C. True hyphae were observed in all of the mutants and the parental strain (data not shown).

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ined chlamydospore formation in the MMR and DSBR mutants; all are capable of producing chlamydospores. There was no delay in the appearance of the chlamydospores and no difference in their frequency or appearance (data not shown).

Susceptibility to DNA-damaging agents. In many organisms, different DNA repair mutants exhibit different susceptibilities to various types of DNA-damaging agents. We tested the responses of the MMR and DSBR mutants to numerous agents, including oxidizing agents, UV radiation, and alkylating agents, including compounds known to induce DSBs.

We tested responses to three oxidizing agents, TBHP, menadione, and H$_2$O$_2$ (Fig. 2). The mre11$Δ$/H9004/mre11$Δ$/H9004, rad50$Δ$/H9004/rad50$Δ$/H9004, and rad52$Δ$/rad52$Δ$ mutants are very susceptible to TBHP (at least 10-fold, 100-fold, and 100-fold growth inhibition, respectively). The rad52$Δ$/rad52$Δ$ mutants are slightly susceptible to menadione, while the mre11$Δ$/mre11$Δ$ and rad50$Δ$/rad50$Δ$ mutants are not. In our initial plate-based assay, none of the strains were affected by H$_2$O$_2$, but when we tested the strains with a more sensitive liquid assay, the pattern of susceptibility was very similar to the TBHP results, with the rad52$Δ$/rad52$Δ$ mutants being the most susceptible and the mre11$Δ$/mre11$Δ$ and rad50$Δ$/rad50$Δ$ mutants slightly less so (data not shown). Interestingly, we observed that C. albicans is more resistant to oxidizing agents than is S. cerevisiae (Fig. 2). This increased resistance may have been selected for during systemic infections, as macrophages kill invading organisms by challenge with reactive oxygen species. Finally, the msh2$Δ$/msh2$Δ$, pms1$Δ$/pms1$Δ$, and yku80$Δ$/yku80$Δ$ mutants are not affected by any of the oxidizing agents we tested (data not shown).

The mutants and control strains were also exposed to various intensities of UV radiation. The strains that are defective in genes involved in HR (mre11$Δ$/mre11$Δ$, rad50$Δ$/rad50$Δ$, and rad52$Δ$/rad52$Δ$) are affected by UV irradiation (Fig. 3), while the MMR msh2$Δ$/msh2$Δ$ and pms1$Δ$/pms1$Δ$ mutant strains and the NHEJ yku80$Δ$/yku80$Δ$ mutant strains are not (data not shown). The rad52$Δ$/rad52$Δ$ strain was most affected by UV exposure, while the rad50$Δ$/rad50$Δ$ strain was least affected.

In S. cerevisiae, DSBR mutants exhibit susceptibility to alkylating agents that introduce DSBs. In our study, the mre11$Δ$/mre11$Δ$, rad50$Δ$/rad50$Δ$, and rad52$Δ$/rad52$Δ$ mutants were very susceptible to camptothecin, EMS, and MMS. The msh2$Δ$/
msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants exhibited a wild-type level of sensitivity (Fig. 4 and data not shown).

For all of the phenotypes we tested, reintegration of a wild-type gene at the endogenous locus in the mutants restores the wild-type phenotype (data not shown).

**Chromosome instability assays.** To investigate the integrity of chromosome 1, we used a GAL1/URA3 marker system. The GAL1 locus is located on chromosome 1; we inserted a copy of URA3 into one allele of the GAL1 gene in order to allow us to readily distinguish between the two homologs. As this was done in the parental strain, the genotype of all of the resulting mutant derivatives is GAL1ΔGAL1Δ and rad50Δ/rad50Δ mutants by fluctuation analysis as described by Spell and Jinks-Robertson (29). The fluctuation assays showed that the rates of appearance of 2-DG' and 5-FOA' cells in the mre11Δ/mre11Δ and rad50Δ/rad50Δ mutants are significantly higher than in the parental strain (Table 6).

The mechanism of 2-DG or 5-FOA resistance acquisition in the parental strain and mutant strains was investigated by screening the genomic DNA of approximately 20 2-DG' and 20 5-FOA' colonies by PCR to test if the GAL1 or URA3 gene was still present (Table 7). When the gene was still present, the PCR product was sequenced to identify any point mutations.

**TABLE 5. Frequency of appearance of 2-DG' and 5-FOA' colonies in DNA repair mutant strains after 3 days**

| Strain | Mutation | 2-DG' colonies | 5-FOA' colonies |
|--------|----------|----------------|-----------------|
| DKCa39 | msh2Δ/msh2Δ | 2.4 ± 0.59 | 15 ± 0.88 |
| DKCa33 | msh2Δ/msh2Δ | 7.2 ± 2 (3) | 2.6 ± 0.75 (1.7) |
| DKCa43 | pms1Δ/pms1Δ | 1.8 ± 0.55 (0.7) | 1.3 ± 0.07 (0.9) |
| DKCa78 | yku80Δ/yku80Δ | 4.8 ± 2.6 (2) | 2.9 ± 1.5 (1.9) |
| DKCa96 | rad52Δ/rad52Δ | 0.74 ± 0.14 (0.4)b | 0.46 ± 0.43 (0.7)b |
| DKCa95 | mre11Δ/mre11Δ | 38 ± 13 (15.8) | 12 ± 1 (8) |
| DKCa127 | rad50Δ/rad50Δ | 26 ± 6 (10.8) | 13 ± 3.5 (8.7) |

*a Each frequency-of-appearance value (number of events per population) is the average of three independent experiments.

*b The data for DKCa96 were collected in a separate set of experiments in which the frequency of appearance of 2-DG' colonies in the wild type was 1.8 × 10⁻⁴ and the frequency of appearance of 5-FOA' colonies in the wild type was 6.1 × 10⁻⁴.
that inactivated the gene. When the PCR showed that the GAL1 or the URA3 gene was absent, SNP typing was used to determine the extent of the alterations. The 1322-2294 SNP is located near the telomere of chromosome 1, while the F12n4 SNP is located close to the putative centromere of chromosome 1 but on the opposite side of the GAL1 locus (Fig. 5).

Twenty 2-DG\(^r\) colonies and 20 5-FOA\(^r\) colonies were examined in the parental strain. All of the isolates had lost either the URA3 or the GAL1 gene, and SNP typing showed that the telomere-proximal 1322-2294 SNP had become homozygous for the same allele of the SNP, while the F12n4 SNP had remained heterozygous, indicating that a large portion of the left arm of chromosome 1 had undergone a loss-of-heterozygosity (LOH) event. The characterization of the 2-DG\(^r\) and 5-FOA\(^r\) colonies in the mutants showed a similar pattern—all of the resistant isolates had lost the gene and also become homozygous for the 1322-2294 SNP while retaining heterozygosity of F12n4. The only exceptions were found in the msh2\(^{Δ}\) msh2\(^{Δ}\), pms1\(^{Δ}\)/pms1\(^{Δ}\), and rad52\(^{Δ}\)/rad52\(^{Δ}\) mutants. In the msh2\(^{Δ}\)/msh2\(^{Δ}\) strain, 2 (12.5\%) of 16 5-FOA\(^r\) isolates retained the URA3 gene; both of these isolates also retained heterozygosity at both SNP locations. In the pms1\(^{Δ}\)/pms1\(^{Δ}\) strain, 2 of 20 2-DG\(^r\) and 3 of 20 5-FOA\(^r\) (12.5\% total) colonies retained the GAL1 or URA3 gene and remained heterozygous at both SNPs. Finally, 10\% (2 of 20) of the 2-DG\(^r\) isolates of the rad52\(^{Δ}\)/rad52\(^{Δ}\) strain exhibited the same pattern. For these isolates, the majority of the changes leading to 2-DG or 5-FOA resistance were alterations in repetitive tracts in the URA3 or GAL1 gene (as described in Table 7).

**Drug resistance.** To determine the roles that the DNA repair genes may play in acquisition of drug resistance, we tested the susceptibility of MMR and DSBR mutants to fluconazole with an E-test assay. The E-test assay consists of plastic strips containing a gradient of an antifungal agent, in this case, fluconazole. When the E-test strip is applied to an inoculated agar plate, the drug is immediately released from the plastic strip into the agar. After incubation, a symmetrical inhibition ellipse centered along the strip can be seen. The MIC is determined by the point where the ellipse intersects the strip; the drug concentration is given in intervals along the length of the strip.

The MICS for the MMR and DSBR mutants were determined after 48 h of exposure to fluconazole E-test strips. We observed that the MIC for the msh2\(^{Δ}\)/msh2\(^{Δ}\), pms1\(^{Δ}\)/pms1\(^{Δ}\), and yku80\(^{Δ}\)/yku80\(^{Δ}\) mutants was 0.5 \(µg/ml\), identical to that for the parental strain (data not shown). In contrast, the MICS for the mre11\(^{Δ}\)/mre11\(^{Δ}\), rad50\(^{Δ}\)/rad50\(^{Δ}\), and rad52\(^{Δ}\)/rad52\(^{Δ}\) mutants were 0.19, 0.19, and 0.094 \(µg/ml\), respectively (Fig. 6A), indicating that the total population of cells of these mutants was more susceptible to fluconazole than the parental cells were.

Interestingly, large bright colonies appeared within the inhibition ellipse of the msh2\(^{Δ}\)/msh2\(^{Δ}\), pms1\(^{Δ}\)/pms1\(^{Δ}\), and rad50\(^{Δ}\)/rad50\(^{Δ}\) mutants on days 2 through 4. As shown in Fig. 6B, we restested the drug resistance of msh2\(^{Δ}\)/msh2\(^{Δ}\) colonies that grew within the inhibition ellipse during testing with a fluconazole E-test strip. Two-thirds of the resistant isolates remained resistant to fluconazole, although the level of resistance varied between the isolates. In another test of fluconazole E-test strips, the MIC\(^s\) of A in the repetitive tract GTCAAAAAATTTG at nucleotides 823 to 828.

| Strain | Gene disruption | No. of 2-DG\(^r\) colonies | No. of 5-FOA\(^r\) colonies |
|--------|----------------|-----------------------------|-----------------------------|
| DKCa39 | None (wild type) | 20/20 | 0/20 |
| DKCa33 | msh2\(^{Δ}\)/msh2\(^{Δ}\) | 20/20 | 0/20 |
| DKCa43 | pms1\(^{Δ}\)/pms1\(^{Δ}\) | 18/20 | 2/20 |
| DKCa58 | mre11\(^{Δ}\)/mre11\(^{Δ}\) | 20/20 | 0/20 |
| DKCa67 | rad50\(^{Δ}\)/rad50\(^{Δ}\) | 20/20 | 0/20 |
| DKCa78 | yku80\(^{Δ}\)/yku80\(^{Δ}\) | 20/20 | 0/20 |
| DKCa96 | rad52\(^{Δ}\)/rad52\(^{Δ}\) | 18/20 | 2/20 |

\[a\] Both were insertions of A in the repetitive tract GTCAAAAAATTTG at nucleotides 823 to 828.

\[b\] Both were deletions of T in the repetitive tract CGTGGTTTATTG at nucleotides 188 to 191.

\[c\] Deletion of A in the repetitive tract TGGAAAAAAATCTG at nucleotides 793 to 799 and a change from A to C at nucleotide 94 (Thr32Pro).

\[d\] Insertion of A in the repetitive tract TGGAAAAAAATCTG at nucleotides 793 to 799, and a deletion of A in the repetitive tract TGGAAAAAAATCTG at nucleotides 793 to 799, and a change from T to G at nucleotide 548 (Val183Gly).

**DISCUSSION**

In the present work, we have investigated the function of DNA repair genes from *C. albicans* and the roles these genes play in genome stability and acquisition of drug resistance. We have shown that the HR mutants (mre11\(^{Δ}\)/mre11\(^{Δ}\), rad50\(^{Δ}\)/rad50\(^{Δ}\)/
rad50Δ, and rad52Δ/rad52Δ) are very susceptible to several DNA-damaging agents, whereas the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants are not. We also observed that the mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ mutants are slow growing and produce wrinkled colonies with pseudohypha-like cells in YEPD+Uri at 30°C. We observed increased genome instability in the mre11Δ/mre11Δ and rad50Δ/rad50Δ mutants with an assay for chromosome 1 integrity. Surprisingly, deletion of some of the DSBR genes leads to an increased susceptibility to some antifungal drugs. We also observed an elevated frequency of appearance of drug-resistant colonies of the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and rad50Δ/rad50Δ mutants inside the inhibition ellipse.

$MSH2$, $PMS1$, $MRE11$, $RAD50$, $YKU80$, and $RAD52$ are not essential genes in $C. albicans$, as we were able to construct null mutants for all of them. Nevertheless, $RAD50$ nulls were more difficult to obtain. This result is not likely to be due to the chromosomal location of $RAD50$; the ORF is not located near the centromere, telomeres, or one of the highly repetitive MRS elements, locations where gene disruptions can be more difficult to achieve. The difficulty in targeting the $RAD50$ sequence may be due to a local chromatin configu-

![FIG. 5. Chromosome 1 SNP locations. This diagram shows the position of SNPs (upward-pointing triangles) 1322-2294 and F12n4 relative to the $GAL1$ locus. SNP 1322-2294 is located in the middle of a Bcl restriction site. One of the alleles (cc atca [the bold letter represents the SNP]) contains the Bcl site, while the other (ccctca) does not. SNP F12n4 is located in the middle of an HpaII restriction site. One of the alleles (atcca) has the HpaII site, while the other (atctca) lacks it.](image)

![FIG. 6. Drug resistance in $C. albicans$ DNA repair mutants after 3 days. (A) Fluconazole (FL) E-test reading patterns for the parental strain and the HR mutants. The MICs shown are based on the scale of fluconazole concentrations imprinted on the E-test strip (in micrograms per milliliter). (B) Appearance of drug-resistant colonies inside the inhibition ellipse for the msh2Δ/msh2Δ DNA repair mutant strain. Arrows 1 to 6 point to large bright colonies within the inhibition ellipse of the msh2Δ/msh2Δ strain; these colonies are not present in the parental wild-type (WT) strain. Cells from these colonies were retested for sensitivity to fluconazole, as shown in parts 1 to 6. Four of the six initially fluconazole-resistant isolates were still resistant following retesting but exhibited various degrees of resistance.](image)
ration that makes the sequence less accessible, to a specific function of the RAD50 protein required for HR, or to the oligonucleotide sequences.

As shown in Fig. 4 and 5, mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ are affected by oxidizing agents, UV, camptothecin, EMS, and MMS, whereas the msh2Δ/msh2Δ, pms1Δ/pms1Δ, and yku80Δ/yku80Δ mutants are not. Previous studies have shown that C. albicans utilizes HR over NHEJ, while in mammals the reverse is found (16). We observed that mutations in genes involved in the HR pathway (MRE11, RAD50, and RAD52) affect the sensitivity of the mutants to DNA break-inducing compounds, while mutations in YKU80 (required for NHEJ) do not. From these data, we conclude that C. albicans preferentially uses HR to repair DNA breaks, in agreement with the work done by Larriba et al. (2, 4) on S. cerevisiae.

We find that the mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ mutants exhibit a slow-growth phenotype. In other organisms, various types of DNA damage activate specific cell cycle checkpoints that result in arrested cell cycle progression, providing more time for repair. Because the main DNA repair proteins are absent in these mutants, cells arrest for an even longer time to allow efficient DNA repair. This hypothesis has been confirmed in the rad52Δ/rad52Δ mutants by the Larriba group (3), who showed that deletion of RAD52 in C. albicans activates the DNA damage checkpoint and that cell cycle arrest generates a polarized-growth phenotype. As their rad52Δ/rad52Δ phenotype is comparable to the phenotypes we observed in our mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ mutants, it is likely that the cell cycle checkpoint arrest hypothesis is true for RAD50 and MRE11 as well.

Persistent DNA lesions in the absence of Mre11p, Rad50p, or Rad52p may trigger DNA checkpoints that result in changes in cell morphology. Work by other research groups has demonstrated that DNA checkpoint proteins are involved in morphological changes in response to a variety of DNA-damaging agents in C. albicans and other fungi (7, 18, 27).

A previous study (2) showed that mutations in LIG4, a gene shown to be involved in NHEJ in S. cerevisiae, impair myceliation in C. albicans. In our work, yku80Δ/yku80Δ NHEJ mutants did not show any defect in filament formation in response to and in Spider medium at 37°C. This observation suggests that the myceliation defect observed in lig4Δ/lig4Δ by Andaluz et al. is not the result of a defective NHEJ apparatus but rather may be due to a secondary function of the Lig4 protein in one of the signaling pathways controlling myceliation.

By using a GAL1/URA3 system on chromosome 1, we showed that deletion of MRE11 and RAD50 gives rise to an increased frequency of 2-DG' or 5-FOA' colonies compared to the parental strain when cells are grown on min-2-DG and min-5-FOA medium. This result indicates that the mre11Δ/mre11Δ and rad50Δ/rad50Δ mutants are more likely to lose GAL1 or URA3 function. The loss of GAL1 or URA3 function could be due to a point mutation in the ORF that would produce a nonfunctional protein or to the loss of the GAL1 or URA3 gene through gene conversion, BIR, reciprocal crossover, or segmental or total chromosome loss. To determine the relative frequencies of these events, we screened 20 2-DG' and 20 5-FOA' colonies of the parental, mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ strains by PCR to detect the presence of the GAL1 and URA3 sequences. We showed that the vast majority of the cells lost the GAL1 or URA3 gene. SNPs located on both sides of GAL1/URA3 were then used to distinguish among gene conversion, BIR, reciprocal crossovers, and segmental or total chromosome loss. If the flanking SNPs remained heterozygous in the 2-DG' or 5-FOA' strains, this would indicate that the cells lost the GAL1 or URA3 function by localized gene conversion. If the SNPs became homozygous, this would suggest that the cells underwent full-length chromosome loss. If one of the SNPs is still heterozygous while the other becomes homozygous, this would suggest that a segmental chromosome loss or BIR event took place. We observed that the majority of the 2-DG' and 5-FOA' colonies of the parental, mre11Δ/mre11Δ, rad50Δ/rad50Δ, and rad52Δ/rad52Δ strains lost GAL1 or URA3 function as a result of LOH events.

Because the GAL1/URA3 locus is located 450 kb away from the telomere, it is more likely that LOH results from BIR, in which one chromosomal arm is duplicated by using the homolog as a template, rather than segmental aneuploidy. The determination of the breakage point in all of these strains is ongoing work; the data will tell us whether there is a weak spot on chromosome 1 where chromosome breaks are favored in response to stress.

When we investigated the parental strain and the other mutants, we observed the same LOH mechanism for the appearance of 2-DG' and 5-FOA' colonies. On the basis of these results, we conclude that the spectrum of alterations on chromosome 1 is unchanged between the mutants and the parental strain, but the frequency of events is greatly increased in the strain, whereas the wild-type parental strain and strains bearing these strains appear within the inhibition ellipse of the E-test strip, whereas the wild-type parental strain and strains bearing homozygous deletions of other DNA repair genes do not exhibit this phenotype. Upon resteting, the resistant isolates exhibit various degrees of antifungal drug resistance. In other organisms, loss of MMR leads to a mutator phenotype; an increase in resistant isolates of the msh2Δ/msh2Δ and pms1Δ/pms1Δ mutants would be expected in these C. albicans mutants. The appearance of colonies within an E-test strip inhibition ellipse has been described in C. albicans previously; a heterogeneous population gave rise to resistant isolates, although the
isolates were only resistant transiently, possibly because of an epigenetic change in the expression of drug efflux pumps (19).

The increase in resistant isolates of the mre11Δ/mre11Δ and rad50Δ/rad50Δ strains, but not of the rad52Δ/rad52Δ or yku80Δ/yku80Δ disruptive, indicates that a complete loss of DSBR is required for an increase in the frequency of antifungal drug resistance—loss of either HR or NHEJ alone is not sufficient. With regard to RADS2, this result is surprising, given the colony morphology, DNA damage sensitivity, and antifungal drug sensitivity phenotypes associated with the loss of RADS2. Examination of antifungal drug resistance development in other HR-specific gene disruptions might address this issue. If this is the case, compounds that specifically inhibit RADS2, or possibly HR, may be effective as companion drugs during treatment for Candida infections, as they would increase susceptibility to the antifungal drug without increasing the frequency of appearance of antifungal drug-resistant cells.

Our drug studies link genome instability to acquisition of drug resistance, as we have shown that cells that are defective in DSBR are generally more sensitive to the antifungal agent fluconazole but are more likely to give rise to a subpopulation of cells that have acquired resistance to fluconazole. Another link between antifungal drug resistance and genome instability has been identified by the Berman group (25). They demonstrated that Candida cells that become resistant to antifungal drugs can harbor an isochromosomal derivative of chromosome 5. Isochromosomes are chromosomal variants in which both arms of the chromosome are identical. Such derivatives may arise by aberrant HR events between sister chromatids during DSBR. Further investigation of isochromosomal formation in DNA repair mutant strains might provide insight into the mechanisms of isochromosomal formation. Finally, a large proportion of patients infected with C. albicans are cancer treatment patients. Cancer patients are often treated with topo-isomerases inhibitors, which act against rapidly dividing cells. Fluconazole treatment of a C. albicans isolate from a cancer patient undergoing therapy with topoisomerase inhibitors, as inhibition of topoisomerases can lead to an increase in recombination.

Genome plasticity is a hallmark of C. albicans and is believed to generate diversity in an organism that propagates by clonal mitotic division, as Candida has not been demonstrated to undergo meiosis. Our data demonstrate that DNA repair pathways, the acquisition of drug resistance, and genome plasticity are linked. However, it is still unclear how C. albicans tolerates such drastic genome changes (isochromosome formation, aneuploidy, a high level of heterozygosity) and if these changes illustrate a global adaptive response of C. albicans to the various stresses the fungus encounters during the course of infection.

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