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Genetic interactions among homologous recombination mutants in *Candida albicans*

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ABSTRACT

rad52-ΔΔ and, to a lesser extent, *rad51*-ΔΔ deletants of *Candida albicans* displayed slow growth and aberrant filamentous morphology whereas *rad59*-ΔΔ mutants, both by growth rate and morphology resembled wild type. In this study, we have constructed pair-wise double deletants to analyze genetic interactions among these homologous recombination (HR) proteins that affect growth and morphology traits. When grown in liquid YPD medium, double mutant *rad51*-ΔΔ *rad59*-ΔΔ exhibited growth rates, cell and colony morphologies, and plating efficiencies that were not significantly different from those observed for *rad51*-ΔΔ. The same was true for *rad52*-ΔΔ *rad59*-ΔΔ compared to *rad52*-ΔΔ. Slow growth and decreased plating efficiency were caused, at least in part, by a decreased viability, as deduced from FUN1 staining. Flow cytometry and microscopic studies of filamentous mutant populations revealed major changes in cell ploidy, size and morphology, whereas DAPI staining identified complex nuclear rearrangements in yeast and filamentous cells. These phenotypes were not observed in the *rad59*-ΔΔ mutant populations. Our results show that abolishing Rad51 functions induces the appearance of a subpopulation of aberrant yeast and filamentous forms with increased cell size and ploidy. The size of this complex subpopulation was exacerbated in *rad52*-ΔΔ mutants. The combination of filamentous cell morphology and viability phenotypes was reflected on the colony morphology of the respective mutants. We conclude that the *rad52* mutation is epistatic to *rad51* for all the morphological traits analyzed. We discuss these results in the light of the several functions of these recombination genes.

1. Introduction

The opportunistic fungal pathogen *Candida albicans* is a diploid organism that can grow as unicellular yeast and in two filamentous multicellular forms-pseudohyphae and hyphae. Several lines of evidence suggest that environmental cues determine transition between these morphological (Berman, 2006; Shapiro et al., 2011; Sudbery et al., 2004; Sudbery, 2011). In addition, *C. albicans* can polarize growth and generate a filamentous phenotype in response to several genotoxins that activate the DNA-damage checkpoint (MMS, UV light), cause replication stress (hydroxyurea) (Loll-Krippleber et al., 2014; Shi et al., 2007), or interfere with the spindle body function (nocodazole) (Bai et al., 2002). Growth polarization has also been reported in strains deficient in DNA repair. These include deletion mutants lacking homologous recombination (HR) genes (*RAD52*, *RAD51*, and *RAD54*), exonucleases genes (*RAD50* and *MRE11*) involved in resection of double strand breaks to generate ssDNA, the substrate for Rad51 binding (Andaluz et al., 2006; Hoot et al., 2011; Legrand et al., 2007), or chromatin-modifying genes, such as *RTT109* or *HAT1* (Lopes da Rosa et al., 2010; Tscherner et al., 2012). Growth polarization in response to genotoxins appears to be mediated by both the DNA-replication and DNA-damage checkpoint pathways, since deletion of effector kinase *RAD53* abolishes filamentation (Loll-Krippleber et al., 2014; Shi et al., 2007). Very little is known about the events that trigger filamentation in HR, exonucleases, or chromatin-modifying mutants. Although there are indications that *rad52* polarization is preceded by activation of the DNA-damage checkpoint (Andaluz et al., 2006), identification of the triggering lesions or the signaling pathway involved is missing.

In *Saccharomyces cerevisiae*, HR uses the *RAD52* epistasis group of genes (*RAD51, RAD52, RAD54, RAD55, RAD57,* RAD59, and the MRX complex formed by *RAD50*, *MRE11*, and *XRS2*). Rad51, assisted by its paralogs Rad55 and Rad57 and stimulated by Rad54, is required for all recombination mechanisms involving strand invasion such

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as gene conversion (GC) crossovers (CO), and most break-induced replication (BIR) events (Davis and Symington, 2004; Kang and Symington, 2000; Krogh and Symington, 2004; Malkova et al., 1996). Rad52 plays a central role in HR as the *rad52* mutation is epistatic to mutation in any other gene of the epistasis group. First, Rad52 has a mediator activity in loading Rad51 onto ssDNA to form the invading nucleofilament; second, it has an annealing activity between complementary ssDNA (Ivanov et al., 1996; Nimonkar et al., 2009; Sugiyama et al., 1998), which is essential for the second end capture in the double-strand break-repair (DSBR) model as well as for other non-conservative recombination events (Krogh and Symington, 2004; Paques and Haber, 1999; Prado et al., 2003; Symington, 2002). Rad51- and Rad52-independent pathways of recombination have been reported in *S. cerevisiae*. Whereas the latter remains poorly characterized (Coic et al., 2008; Pohl and Nickoloff, 2008), Rad51-independent mechanisms, such as single strand annealing (SSA) and a minor BIR pathway, have been investigated in some detail (Ivanov et al., 1996; Mortensen et al., 1996). Both SSA and Rad51-independent BIR require Rad52, Rad59, and the MRX complex. Rad59 is a Rad52 paralog, first identified in *S. cerevisiae*, which displays high similarity to the N-terminal half of Rad52. Rad59 is thought to function as an accessory factor in SSA by potentiating the single-strand annealing activity of Rad52. *RAD52*, when present in more than one copy, suppresses the radiation sensitivity and recombination defects of rad59 mutants, but not vice versa (Bai and Symington, 1996).

Epistatic and synergistic interactions between mutations in HR genes have been extensively studied in *S. cerevisiae* by analyzing phenotypic traits such as -ray sensitivity, recombination rates, sporulation efficiency, and mating type switching (Paques and Haber, 1999; Krogh and Symington, 2004). Since HR mutants of *S. cerevisiae* do not display any aberrant filamentous phenotype, genetic interactions among HR genes affecting morphology have not been analyzed yet. However, morphological control mechanisms are important for adaptation of fungal cell pathogens to stress and antifungal drugs as well as for their virulence and survival in the host (Harrison et al., 2014; Kadosh, 2013). We previously reported that *rad52*-ΔΔ and, to a lesser extent, *rad51*-ΔΔ cells from *C. albicans* exhibit severe phenotypes including a decreased growth rate and a filamentous morphology, whereas *rad59*-ΔΔ cells showed wild type phenotypes (Andaluz et al., 2006; Ciudad et al., 2004; Garcia-Prieto et al., 2010). In the present study, we have extended the phenotypic analysis of single mutants. In addition, because the genetic interactions between *RAD51*, *RAD52* and *RAD59* in several HR-dependent processes in *S. cerevisiae*, we have constructed pairwise-double deletants to establish synergistic as well as epistatic relationships between those mutations that could affect growth rate, and cell and colony morphologies. Based on colony morphology in several solid media, as well as loss of cell viability and changes in ploidy, and filamentation ability we conclude that *rad52* mutation was epistatic to *rad51*. Besides, our phenotypic analysis strongly suggests that *rad52* and *rad51* mutations were epistatic to *rad59*. We discuss these results on the light of the several functions of these recombination genes.

2. Materials and methods

2.1. Strains and culture media

The strains used in this study are shown in Table 1. They were routinely grown in YPD medium or synthetic complete (SC) medium (Andaluz et al., 2006). Uri^+ prototrophs were selected on SC plates lacking uridine. Uri^- segregants were selected on YPD plates supplemented with 5-fluoroorotic acid (5-FOA). The haploid strains W303 and its *rad52* derivative (LSY1029-13B), both *ADE2 RAD5*, were kindly provided by Lorraine Symington, from Columbia University. To inspect for hyphal growth stationary phase cultures were inoculated on agar plates with SPIDER medium, M199 medium, pH 7,9 (Gifco BRL, Life technologies), or Leés medium (Lee et al., 1975; Liu et al., 1994), prepared as described before (Andaluz et al., 2006). Colonies were visualized with the naked eye and then microscopically inspected. To induce germinative tubes, exponentially growing cells in YPD were supplemented with calf serum (10% final concentration) and incubated a 37 °C for 1–2 h.

2.2. DNA extraction and analysis and cell transformation

Extraction of genomic DNA, PFGE, and Southern blot hybridization have been described before (Andaluz et al., 2007; Gomez-Raja et al., 2008). *C. albicans* cells were transformed by electroporation in a BTX electroporation system (Andaluz et al., 2006) or using the lithium acetate method (Walther and Wendland, 2003). Plugs for PFGE were prepared and subsequently treated with proteinase K (1 mg/ml) at 55 °C for 24 h.

2.3. Construction of double mutants rad51-ΔΔ rad59-ΔΔ, rad52-ΔΔ rad59-ΔΔ and rad51-ΔΔ rad52-ΔΔ

To construct the double mutant *rad59*-ΔΔ *rad52*-ΔΔ, both alleles of *RAD52* were sequentially disrupted in the *rad59*-ΔΔ background, as previously described (Ciudad et al., 2004; Garcia-Prieto et al., 2010). The analysis of the resulting strains by Southern blot using the Rad52 disruption cassette as a probe is shown in Supplemental Fig. S1. To construct the double mutant *rad59*-ΔΔ *rad51*-ΔΔ, both alleles of *RAD51* were disrupted sequentially in the *RAD59/rad59::hisG* background. The resulting strains were verified by Southern blot using a 891 bp *Eco*RI/*Spe*I probe from the *RAD51* clone (Garcia-Prieto et al., 2010). Then the second allele of *RAD59* was disrupted as described above (Supplemental Fig. S2).

To construct the *rad51*-ΔΔ *rad52*-ΔΔ double mutant, both *RAD52* alleles were sequentially disrupted in a *rad51::hisG/rad51::hisG* background, as previously described (Ciudad et al., 2004). Constructs were verified by Southern blot using the *RAD52* disrupting cassette as a probe (Supplemental Fig. S3A).

Single and double mutant strains conserved essentially the PFGE karyotype of the parental CAF2-1 strains (Supplemental Fig. S3B), apart from changes in the sizes of both ChrR homologs, suggesting that gene disruptions did not generate gross chromosomal rearrangements detected by this technique.

2.4. Determination of growth rate and cell morphology

Liquid cultures grown until OD_{600} of 6 were used to inoculate 250 ml flasks containing 50 ml of the indicated liquid medium at an OD_{600} of 0.05. After 2 h at 30 °C with shaking samples were taken every 30 min and OD_{600} calculated from dilutions with $DO_{600} < 0.4$. Because some strains had a filamentous phenotype, the ratio between DO_{600} and CFUs was calculated for each strain within the OD_{600} range 0–0.4. The cell morphology of fresh cells was inspected microscopically using a Nikon Eclipse 600 fluorescence microscope with a 60 × DIC objective. A CC-12 digital camera interfaced with Soft Imaging System software was used for imaging.

2.5. Cell staining procedures

Aliquots (100 μl) from overnight cultures of the indicated strains ($DO_{600} \approx 5$) were pelleted, resuspended in Phloxine B (5 mg/ml) and observed under the microscope. DAPI and Calcofluor