Genetic control of chlamydospore formation in Candida albicans

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The chlamydospore is a distinctive morphological feature of the fungal pathogen Candida albicans that can be induced to form in oxygen-limited environments and has been reported in clinical specimens. Chlamydospores are not produced by the model yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, so there is limited understanding of the pathways that govern their development. Here, the results of a forward genetic approach that begins to define the genetic control of chlamydospore formation are described. Six genes – ISW2, MDS3, RIM13, RIM101, SCH9 and SUV3 – are required for efficient chlamydospore formation, based on the phenotypes of homozygous insertion mutants and reconstituted strains. Mutations in ISW2, SCH9 and SUV3 completely abolish chlamydospore formation. Mutations in RIM13, RIM101 and MDS3 delay normal chlamydospore formation. The involvement of alkaline pH-response regulators Rim13p and Mds3p in chlamydospore formation is unexpected in view of the fact that chlamydospores in the inducing conditions used here are repressed in alkaline media.

INTRODUCTION

Candida albicans is the most common fungal pathogen in humans. It exists as a commensal inhabitant of mucosal surfaces in most healthy individuals. However, alterations of host or environment can lead to overgrowth of the fungus and infection of the host (Odds, 1988).

One distinct property of C. albicans is its ability to produce chlamydospores, an ability shared only with the closely related species Candida dubliniensis. Chlamydospores are large, highly refractile cells with thick cell walls that form on the ends of elongated suspensor cells attached to hyphae and, occasionally, to pseudohyphae (Odds, 1988). They are rich in RNA (Vidotto et al., 1996) and can germinate in some cases (Jansons & Nickerson, 1970; McGinnis, 1980; Raudonis & Smith, 1982).

Chlamydospores can be induced to form under nutrient-poor oxygen-limited conditions at low temperatures (Calderone, 2002). In cornmeal agar, which is the typical inducing medium in vitro, both light and glucose inhibit chlamydospore formation while nitrogen has no effect (Dujardin et al., 1980a, b). Chlamydospores have been found in the lung of an AIDS patient (Chabasse et al., 1988), and thus may be relevant to infection. The ability to form chlamydospores is nearly universal among C. albicans clinical isolates (Al-Hedaithy & Fotedar, 2002), thus arguing that chlamydospores have a functional role in C. albicans biology.

The genetic requirements for chlamydospore formation are of interest for four reasons. First, chlamydospores are the output of a developmental process, so the system may be viewed as a model for development in other organisms. Second, it is unclear why the C. albicans genome has so many genes without close homologues in other organisms, and the possibility that they play a role in a C. albicans-specific process like chlamydospore formation may explain their presence. Third, some conserved regulatory pathways respond to well-defined signals, so identification of chlamydospore regulators may reveal the specific external signals that govern their formation. Finally, because chlamydospores form under growth conditions that are not routinely employed for C. albicans cultivation, their genetic requirements may reveal unique functional relationships, biological roles or regulatory signals that govern activity of known gene products.

The latter point has been most clearly reflected in the few known examples of chlamydospore regulators. Transcription
factor Efg1p was first characterized as a positive regulator of hyphae formation (Liu, 2001). Efg1p is required for chlamydospore formation but not for hyphal development in cornmeal agar upon oxygen limitation (Sonnenborn et al., 1999); in fact, the efg1Δ/efg1Δ mutant is hyperfilamentous. This observation was the first indication that Efg1p may function as a negative regulator of hyphae formation under some circumstances, a finding that was echoed in analysis of matrix-induced hyphae formation (Giusani et al., 2002). A second case is the MAP kinase Hog1p, which is activated by oxidative stress and promotes resistance to oxidants (Alonso-Monge et al., 1999). Like Efg1p, Hog1p is required for chlamydospore formation but not for hyphal development (Alonso-Monge et al., 2003). It is possible that chlamydospores are thus formed in response to oxidative stress. While the mechanistic roles in chlamydospore formation of Efg1p and Hog1p are not clearly understood, these findings have expanded our view of these key regulators and may ultimately prove relevant to understanding their roles as virulence factors.

*C. albicans* is a diploid organism that lacks a complete sexual cycle, and it has been difficult to apply genetic screens to the organism. Identification of *C. albicans* morphogenetic regulators has relied to a great extent on the use of *Saccharomyces cerevisiae* as both a biological model and surrogate host for identification of candidate genes (Liu, 2001; Berman & Sudbery, 2002). These benefits do not seem applicable to chlamydospore formation, which does not occur in *S. cerevisiae*. Recently, we developed a forward genetic strategy for gene discovery in *C. albicans* itself (Davis et al., 2002). We created a panel of 217 defined insertion mutants and screened them for phenotypic alterations. Our initial study reported identification of Mds3p as a new regulator of alkaline pH-induced hyphae formation. Here, we report an analysis of this mutant collection for defects in chlamydospore formation. Our results define a role for alkaline pH-response regulators in acidic conditions, suggest a functional connection between the Isw2p-dependent chromatin remodelling and Efg1p or Hog1p, and define a new phenotypic defect—the inability to form either hyphae or chlamydospores. Our results illustrate the utility of this mutant collection for phenotypic testing.

**METHODS**

**Yeast strains and media.** All *C. albicans* strains used in this study were derived from strain BWP17 (genotype ura3Δ::LMM434/ura3A::LMM434 arg4Δ::hisG/arg4::hisG his1::hisG/his1::hisG) (Wilson et al., 1999), and are listed in Table 1.

The His’ homozygous insertion mutant strains (GKO library) used for the *C. albicans* chlamydospore phenotypic screen were obtained from the library constructed in this laboratory by random transposon mutagenesis with the UAU1 cassette, as described previously (Davis et al., 2002).

*C. albicans* strains were grown in YPD plus uridine (2% dextrose, 2% Bacto Peptone, 1% yeast extract and 80 μg uridine ml⁻¹) at 30°C. Following transformations, selection was done on synthetic medium (2% dextrose, 6-7% yeast nitrogen base plus ammonium sulfate and the necessary auxotrophic requirements).

**Screening for chlamydospore formation.** The GKO library of 217 insertion mutants was screened as follows. Strains were grown overnight in YPD plus uridine at 30°C, diluted with YPD to an OD₆₀₀ value of 1-0, and consecutively serially diluted 1:10 three times. Then, 100 μl of the third serial dilution was plated onto cornmeal agar (17 g cornmeal agar l⁻¹ plus 80 μg histidine ml⁻¹ plus 0-33% Tween 80) under a glass coverslip to maintain a semi-anaerobic condition (Dalmau inoculation technique), and grown in the dark for 7 days at 25°C. Plates were examined over the following 21 days for chlamydospores. Four independent isolates from each disruption mutant were screened.

**Screening for filament formation in the embedded cell condition.** The screening of the chlamydospore defective mutants for filamentation ability was done using the embedded cells technique (Giusani et al., 2002) because this more closely resembles the burrowing of the filaments that occurs during chlamydospore formation. Strains were grown overnight in YPD plus uridine at 30°C, diluted with YPD plus uridine to 4×10⁶ c.f.u. ml⁻¹, cultured for 4 h at 30°C, and diluted to 400 c.f.u. ml⁻¹ in YPS plus uridine agar (1% sucrose, 2% Bacto Peptone, 1% yeast extract, 2% Bacto Agar and 80 μg uridine ml⁻¹); plates were poured with the cells mixed inside. Plates were incubated for 4 days at 37°C.

**Gene complementation.** The open reading frame (ORF) affected by each insertion was identified through the Stanford *Candida albicans* genome database (http://www-sequence.stanford.edu/group/candida). Complementing *SUV3* (orf19.4519), *SCH9* (orf19.829) and *ISW2* (orf19.7401) plasmids were made as follows. PCR was used to produce a fragment for *SUV3*, *SCH9* and *ISW2* from approximately 900 bps upstream of the ATG to approximately 400 bps downstream of the stop codon of the ORF for each gene template. (Primer sequences are given in Table 2.) These fragments were inserted into the pGEMT-Easy vector (Promega), which contains Nof/I sites flanking the insertion. The inserts were then released with Nof and ligated into Nof-digested dephosphorylated pDDB78, a *HIS1* vector (Sprecher et al., 2003), to generate plasmids pMLR101 (containing the *SUV3* insert), pCJN103 (containing the *SCH9* insert) and pCJN101 (containing the *ISW2* insert).

The complemented strains were constructed as follows. Strain MLR3 was constructed by transforming GKO443, the sew2Δ/suv3Δ homozygous insertion mutant, with the *Nrd1*-digested plasmid pMLR101 to histidine prototrophy. Strain CJN6 was constructed by transforming GKO781, the *sch9Δ/sch9* homozygous insertion mutant, with the *Nrd1*-digested plasmid pCJN103 to histidine prototrophy. Strain CJN1 was constructed by transforming GKO585, the *isw2Δ/isw2* homozygous insertion mutant, with the *Nrd1*-digested plasmid pCJN101 to histidine prototrophy. The unique *Nrd1* site in these plasmids lies in *HIS1* sequences; *Nrd1* digestion thus directs integration to the *HIS1* locus.

For comparison to the complemented strains, the mutant strains were made His’ by transforming each mutant with *Nrd1*-digested plasmid pDDB78 to histidine prototrophy. Strain CJN223 was derived from mutant GKO443; strain CJN19 was derived from mutant GKO781; strain CJN16 was derived from mutant GKO585.

**Suppression studies.** Plasmids pDDB61, containing the full-length *RIM101*, and pDDB71, containing an activated truncation of *RIM101* with a stop codon following amino acid N405 (*RIM101*Δ405), were used. The construction of these plasmids was described previously (Davis et al., 2000b). To target integration to the *RIM101* locus, pDDB61 and pDDB71 were digested with *Psp*MI. All strains constructed for the *RIM101* suppression study were generated by
Table 1. *C. albicans* strains used in this study

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<td>Wilson (1999)</td>
</tr>
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<td>Davis (2002)</td>
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</table>
RESULTS AND DISCUSSION

In order to identify regulators of chlamydospore formation, we screened a *C. albicans* homozygous insertion mutant strain library for strains defective in their ability to form chlamydospores. For most strains, chlamydospores typically formed at the end of suspensor cells coming off hyphal cells that burrowed into the agar. Chlamydospores consistently formed between 5 and 7 days in the dark after plating on cornmeal agar under a coverslip, which we refer to as chlamydospore-inducing (C-I) conditions below. Typical examples are seen for the reference strains DAY286 (His<sup>+</sup>) and DAY185 (His<sup>-</sup>) (Fig. 1A, B). Insertions in the genes *ISW2*, *MDS3*, *RIM101*, *RIM13*, *SCH9* and *SUV3* abolished or delayed chlamydospore formation. For each gene, we found that four independent insertion homozygotes had comparable phenotypic defects, and that introduction of a wild-type copy of the affected gene restored efficient chlamydospore formation. Therefore, the insertion mutation, not a secondary mutation elsewhere in the genome, is the cause of the phenotypic defect in each case.

### Chlamydospore formation defects in *suv3*, *sch9* and *isw2* mutants

The *C. albicans suv3/suv3* insertion mutant (CJN223) was unable to produce both chlamydospores and embedded hyphae under C-I conditions (Fig. 1C). In *S. cerevisiae*, Suv3p is a mitochondrial ATP-dependent RNA helicase that is required for mitochondrial biogenesis (Minczuk et al., 2002). We believe that *C. albicans suv3* mutants grow poorly on acetate, a non-fermentable carbon source (M. L. Richard, unpublished observation). Our observations thus suggest that mitochondrial function is required for both chlamydospore and hyphae formation under the oxygen-limited conditions used in the screen. Because mitochondria are a major source of oxygen radicals (Boveris & Cadenas, 1982), our findings are in keeping with the observation that the oxidative stress regulator Hog1p is required for chlamydospore formation.

The *C. albicans sch9/sch9* insertion mutant (CJN19) was also defective in producing both chlamydospores and embedded hyphae under C-I conditions (Fig. 1E). In *S. cerevisiae*, Sch9p is a protein kinase that has multiple roles in stress resistance, longevity and nutrient sensing (Fabrizio et al., 2001; Thevelein & de Winde, 1999). It is impossible to interpret the role that a global regulator like Sch9p may play in chlamydospore formation with confidence. However, considering that chlamydospores are thought to be a storage structure and that Sch9p is implicated in glycogen...
accumulation, it is possible that Sch9p is a component of a storage pathway required for chlamydospore formation.

The C. albicans isw2/isw2 insertion mutant (CJN16) was defective in chlamydospore formation but did produce embedded hyphae (Fig. 1G). In S. cerevisiae, Isw2p is part of a chromatin remodelling complex that is recruited to promoter regions by the repressor Ume6p (Goldmark et al., 2000; Fazzio et al., 2001). The similarity of the isw2/isw2, efg1/efg1 and hog1/hog1 mutant phenotypes is consistent with the simple hypothesis that C. albicans Isw2p may act in conjunction with either Efg1p or Hog1p. This hypothesis suggests that the isw2/isw2 mutant may have other phenotypes in common with efg1/efg1 or hog1/hog1 mutants. However, the isw2/isw2 mutant is not hyperfilamentous in a matrix-induced hyphae formation assay and is not hypersensitive to oxidative stresses (data not shown). Therefore, if Isw2p acts in conjunction with either Efg1p or Hog1p, its role may be limited to C-I conditions.

**Relationship of pH-response regulators and chlamydospore formation**

The rim13/rim13 and mds3/mds3 insertion mutant strains produced fewer chlamydospores and embedded hyphae than the reference strain (data not shown). In addition, chlamydospores were not produced by the mutants until 12 days, compared to 5–7 days for the reference strain (data not shown). We verified this defect with independently constructed deletion mutants (Fig. 2A, B, D, E) and corresponding strains with reconstituted wild-type alleles (Fig. 2C, F). Rim13p and Mds3p act in alkaline pH-response pathways in C. albicans and S. cerevisiae, yet C-I medium pH is 5-5. We determined that the medium remains approximately at pH 5-5 in the region of growth under the coverslip after chlamydospores are produced. Preparation of C-I medium at pH 8-0 abolished chlamydospore production by reference strains. These observations argue that Rim13p and Mds3p promote chlamydospore formation, not because of a metabolic alkalinization of C-I medium. Instead, Rim13p and Mds3p function in this context in acidic conditions.

The only known function of Rim13p in S. cerevisiae is to activate transcription factor Rim101p through proteolytic cleavage (Lamb et al., 2001; Lamb & Mitchell, 2003). Prior studies indicate that C. albicans uses a Rim101p processing pathway with similar gene products to promote alkaline pH-induced filamentation (Porta et al., 1999; Ramon et al., 1999; Davis et al., 2000b), and studies yet to be published indicate that C. albicans Rim13p acts in this pathway (D. Davis, unpublished data). If Rim13p promotes chlamydospore formation by stimulating Rim101p processing, then a rim101/rim101 mutant will be defective in chlamydospore formation. Indeed, we verified that a rim101/rim101 mutant has a delayed defect in chlamydospore and hyphae formation under C-I conditions (Fig. 2G, H) and that reconstitution of a Rim101 allele rescues the defect (Fig. 2I). A second prediction is that the rim13/rim13 mutant defect will be rescued by a RIM101-405 allele, which specifies a C-terminally truncated product that is active in the absence of proteolytic processing (Davis et al., 2000b). Indeed, we observed that introduction of the RIM101-405 allele into the rim13/rim13 mutant improved chlamydospore formation (Fig. 2K), whereas an extra copy of full-length RIM101 had no such effect (Fig. 2J). These results argue that Rim13p promotes chlamydospore formation by promoting Rim101p processing and activity.

**Utility of the C. albicans insertion mutant library**


Our results in this study illustrate the utility of the C. albicans insertion mutant library for analysis of C. albicans biological attributes. First, we have identified a role for alkaline pH-response regulators in an acidic growth condition, thus suggesting that a novel signal may activate these pathways. Second, phenotypic similarity suggested at first that Isw2p might have been a participant in either the Efg1p or the Hog1p pathways; this hypothesis was not supported by further phenotypic assays, but such observations may be useful with other genes in the future. Third, some of our negative results are informative; for example, the sla2/sla2 insertion mutant in the library is defective in hyphae formation under many growth conditions (Davis et al., 2002) as expected (Asleson et al., 2001), but not under C-I conditions. Fourth, we note that all of the chlamydospore-defective insertion mutants could be complemented, thus providing a further indication that secondary mutations do not pose an overwhelming problem for the UAU1 mutagenesis strategy (Enloe et al., 2000; Davis et al., 2002). Finally, extrapolation of our results to the entire C. albicans genome suggests that roughly 200 genes may be required in total for chlamydospore formation, an estimate that seems reasonable in comparison to S. cerevisiae sporulation (Veshorn & Pierce, 2000), for example.

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