Candida albicans Mds3p, a Conserved Regulator of pH Responses and Virulence Identified Through Insertional Mutagenesis

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Manuscript received July 26, 2002
Accepted for publication September 13, 2002

ABSTRACT

Candida albicans is a commensal fungus that causes diverse infections after antibiotic use or immune debilitation. Gene discovery has been limited because the organism is an asexual diploid. We have developed a strategy that yields random homozygous insertion mutants. The strategy has permitted identification of several prospective essential genes. Many of these genes are homologous to nonessential *Saccharomyces cerevisiae* genes, and some have no *S. cerevisiae* homolog. These findings may expand the range of antifungal drug targets. We have also identified new genes required for pH-dependent filamentation, a trait previously associated with virulence. One newly identified gene, *MDS3*, is required for expression in alkaline media of two filamentation-associated genes, *HWP1* and *ECE1*, but is not required for expression of other pH-response genes. In *S. cerevisiae*, the two *MDS3* homologs are required for growth in alkaline media, thus arguing that Mds3p function in adaptation to external pH changes is conserved. Epistasis tests show that Mds3p contributes to virulence and alkaline pH responses independently of the well-characterized Rim101p pH-response pathway.

CANDIDA albicans is the most frequently encountered fungal pathogen of humans (Fidel and Sobel 1996; Patterson 1999; Farah *et al.* 2000; Ellepola and Samaranayake 2001). Although it is a benign inhabitant of mucosal surfaces in most individuals, it is a significant cause of infection when host or environmental factors are permissive. *C. albicans* can infect any tissue and can cause morbidity and mortality despite treatment with existing therapies. The diverse infection capability of this organism distinguishes it from many well-understood pathogens. These features have focused interest on the definition of genes that govern *C. albicans* survival in the host and pathogenicity (Calderone and Fonzi 2001; Navarro-Garcia *et al.* 2001).

Most known *C. albicans* survival and pathogenicity genes have been identified through their expression patterns or properties of their deduced gene products (Navarro-Garcia *et al.* 2001). Functional screens for *C. albicans* genes have been based on heterologous expression in the budding yeast *Saccharomyces cerevisiae* or overexpression in *C. albicans* (Whiteway *et al.* 1992; Fu *et al.* 1998; Brown *et al.* 1999; Lane *et al.* 2001). Ultimately, gene function is deduced from disruption mutations that cause loss-of-function defects. However, it has been largely impossible to identify genes through an initial screen of gene disruption mutants. The main impediments to gene disruption screens—that *C. albicans* is diploid and lacks a sexual cycle—require mutations to be homozygous, an objective that demands involved genetic manipulations (Pla *et al.* 1996).

We describe here a gene disruption screen in *C. albicans*. We have developed an insertional mutagenesis strategy that is based on the *UAU1* cassette, a gene disruption marker that permits selection of homozygous mutants from heterozygotes (Enloe *et al.* 2000). The screen incorporates *C. albicans* genomic sequence information (Tzung *et al.* 2001) to streamline analytical methods and to eliminate noncoding region insertions, whose properties are less predictable than those of coding region insertions. We have used this strategy to identify new genes that govern pH-dependent filamentation, a trait previously tied to virulence (Davis *et al.* 2000b). Properties of the newly identified *MDS3* gene indicate that it acts through a pathway distinct from the previously described *RIM101* pathway (Porta *et al.* 1999; Ramon *et al.* 1999; Davis *et al.* 2000b; El Barkani *et al.* 2000) and that both pathways govern pH-dependent expression of filamentation genes.

MATERIALS AND METHODS

Library construction: *C. albicans* genomic DNA from strain CAI4 (Fonzi and Irwin 1993) was digested to completion with *Spe*I, and gel-purified 4- to 12-kbp DNA fragments were ligated into *Spe*I-digested, phosphatased vector pDDB124 [a ligation product of Promega (Madison, WI) vector pGEM-t

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easy]. The library is composed of 16,000 clones with an average insert of 6 kbp.

The *Tn7-UAU1* transposon donor plasmid pAED98 was constructed as follows. The *Not*I site of plasmid pGPS3 (New England Biolabs, Beverly, MA) was destroyed by digestion and filling in to create plasmid pGPS3Δ*Not*I. The *UAU1* cassette (Enloe *et al.* 2000) was amplified in a PCR to introduce flanking *Spe*I sites (primers 5′*Spe*IpBME101clone and 3′*Stu*IpBME101clone) and cloned into pGEM-t easy. The *UAU1* insert was released with *Spe*I and inserted into the *Spe*I site of pGPS3Δ*Not*I to generate plasmid pAED98.

Mutagenesis was performed using the GPS-M mutagenesis system (New England Biolabs) according to the manufacturer's instructions, using 20 ng of pAED98 and 80 ng of the genomic library. pAED98 was inactivated after transposition through PI-*Sce*I digestion. Transposon-mutagenized library plasmids were transformed into DH10B Max Efficiency cells (GIBCO, Grand Island, NY) and recovered by selection on LB + AMP + KAN plates. Plasmids were recovered from individual colonies using the Millipore (Bedford, MA) Montage miniprep system. For each plasmid, one transposon junction was sequenced using Primer S followed by BLASTN analysis against the Candida genomic database (http://www.stanford.edu/group/candida/index.html).

Identification of homozygous mutants: Each *Tn7-UAU1*-mutagenized plasmid insert was released through *Not*I digestion and transformed into *C. albicans* strain BWP17 (Wilson *et al.* 1999), selecting Arg+ transformants. Integration was thus directed by the genomic sequence flanking the insertion. For each plasmid, up to 12 transformants were patched onto YPD plates, grown at 30° for 2 days, and then replica plated to SC-Arg-Ura plates. One Arg+ Ura+ isolate from each patch was screened by whole-cell PCR for the presence of a transposon insertion allele and the absence of a wild-type allele. When no segregant lacking a wild-type allele was identified, the selection was repeated with additional independent transformants. For each gene in this study, between 10 and 35 transformants were subjected to Arg+ Ura+ selection.

***MDS3* sequence:** Two neighboring open reading frames (ORFs; 6.8871 and 6.8872) in assembly 6 of the *C. albicans* genomic sequence have homology to neighboring portions of *S. cerevisiae MDS3*. Our sequencing of both strands of the intergenic region from strain BWP17 revealed a C residue in place of the T in the predicted stop codon of ORF 6.8871, which yields a single contiguous ORF composed of 6.8871 and 6.8872. This 557-base sequence, which includes two other single-base-pair changes as well, has been submitted to GenBank (accession number AY150166). The entire *MDS3* open reading frame is 4152 bp long and contains 1384 codons.

Plasmids: The *MDS3* reconstruction plasmid (pVIN103) was generated as follows. Plasmid pGEMT-HIS1 (Wilson *et al.* 1999) was digested with *Sal*I and *Nsi*I and religated with an *Srf*I linker to generate plasmid pVIN101. PCR was used to generate a 2640-bp fragment (from −1148 to +1484 relative to ATG of *MDS3*) with a unique artificial *Not*I site at +882 bp. This fragment was blunt-end ligated into *Srf*I-digested pVIN101 to generate plasmid pVIN103.

***C. albicans* strains:** All *C. albicans* strains were derived from strain BWP17 (genotype *ura3*Δ*::*λ*imm434/ura3*Δ*::*λ*imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*; Wilson *et al.* 1999). Strains DAY25 (*rim101/rim101*) and DAY185 (*RIM101/RIM101*) have been described previously (Davis *et al.* 2000b).

Strain DAY286, a Ura+ Arg+ His− reference strain, was created by transformation of strain BWP17 with *Not*I-digested plasmid pRS-Arg-Ura-BN, as described previously (Davis *et al.* 2000b).

Strain VIC21 was generated by transforming GKO9, the original *mds3/mds3* homozygous insertion mutant, to histidine prototrophy with *Not*I-digested plasmid pVIN103 to direct integration to the *MDS3* locus. GKO9 was also transformed with *Nru*I-digested pVIN101 to generate strain VIC25.

Strain VIC16, the *rim101/rim101 mds3/mds3* double mutant, was constructed by transforming strain VIC18 (*rim101::dpl200/rim101::dpl200*), a derivative of strain BWP45 (Wilson *et al.* 2000), with *Not*I-digested pVIN104, the *mds3::Tn7-UAU1* insertion plasmid. Arg+ transformants were subjected to Arg+ Ura+ selection and PCR screening for loss of the *MDS3* allele, as described above.

Strains VIC28, VIC30, and VIC31 were generated by transforming strain VIC16 to histidine prototrophy with *Nru*I-digested pVIN101 (*HIS1* vector), *Not*I-digested pVIN103 (*HIS1-MDS3*), and *Ppu*MI-digested pDDB61 (*HIS1-RIM101*; Davis *et al.* 2000b), respectively.

Strain VIC3, the *mds3*Δ*/mds3*Δ mutant, was generated as follows. Strain BWP17 was subjected to consecutive rounds of transformation with PCR products *mds3::ARG4* and *mds3::URA3* using primers MDS3-5DR and MDS3-3DR. Each deletion-insertion removes *MDS3* codons 2–1375.

Media: Media for *C. albicans*, *S. cerevisiae*, and *Escherichia coli* followed standard recipes (Lee *et al.* 1975; Natarajan and Datta 1993; Liu *et al.* 1994; Davis *et al.* 2000b; Lamb *et al.* 2001; Sambrook and Russell 2001).

Virulence testing in the murine model of hematogenously disseminated candidiasis: Male Balb/c mice weighing 23–28 g were inoculated via the tail vein with 10^6 vegetative cells (blastospores) of each strain of *C. albicans* and monitored for survival twice daily. The survival of mice infected with the various strains was compared, using the log-rank test with the Bonferroni adjustment for multiple comparisons (Curran-Everett 2000).

RESULTS

Creation of homozygous insertion mutants: The *UAU1* marker cassette permits selection for homozygous insertion mutations (Enloe *et al.* 2000). It includes a functional *ARG4* gene and can recombine to excise *ARG4* and yield a functional *URA3* gene (Figure 1A). Transformants heterozygous for a *UAU1* insertion at a *C. albicans* genomic locus (*orf::UAU1/ORF*) may be selected as Arg+ transformants of marked *C. albicans* strain BWP17 (Wilson *et al.* 1999). These transformants yield Arg+ Ura+ segregants in two genotypic classes: homozygotes (*orf::URA3/orf::UAU1*) and triplication derivatives (*orf::URA3/orf::UAU1/ORF*). We found previously that *UAU1* insertions in nonessential genes yielded 7–30% homozygotes among Arg+ Ura+ segregants; insertions in essential genes yielded only viable triplication derivatives (Enloe *et al.* 2000).

To create a set of homozygous insertion mutants, the *UAU1* cassette was incorporated into a *Tn7* transposon, and *Tn7-UAU1* was inserted into a *C. albicans* genomic library through transposition *in vitro* (Figure 1A). We sequenced 756 insertion sites and compared them to the *C. albicans* genomic sequence database. Among these insertions, 425 lay in annotated *C. albicans* ORFs, representing 353 different ORFs. A group of 253 insertions were transformed into *C. albicans* strain BWP17 after excision from vector sequences. For each insertion, several Arg+ transformants were isolated and subjected to

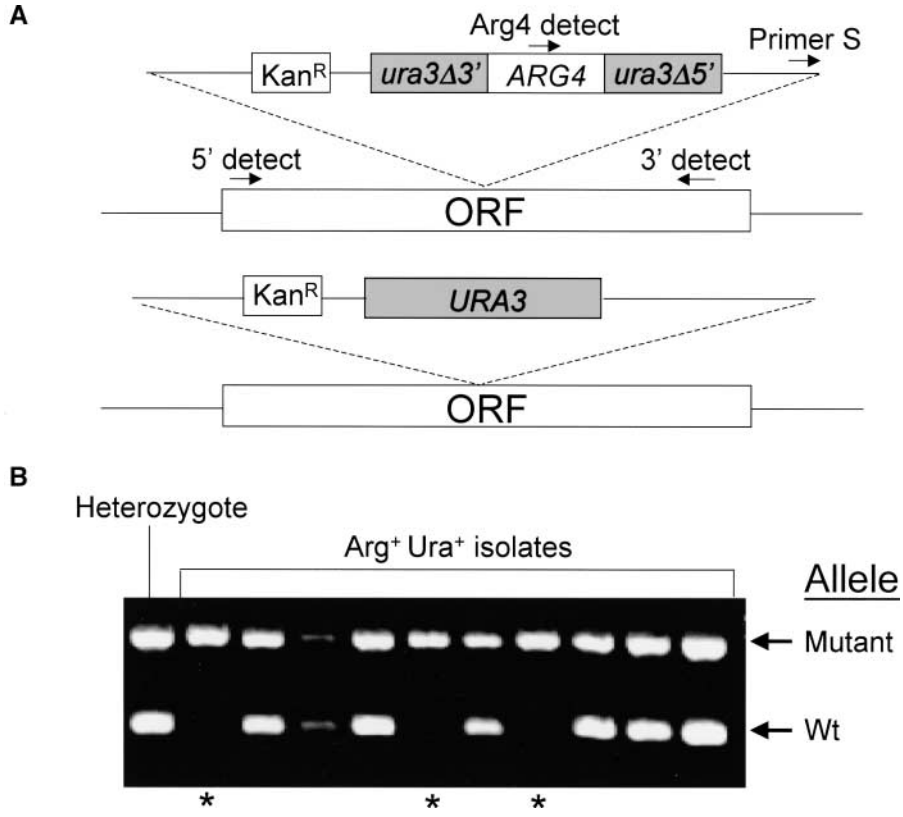

FIGURE 1.—Structure and characterization of *Tn7-UAU1* insertions. (A) Diagram of *orf::Tn7-UAU1* (top) and *orf::Tn7-URA3* (bottom) insertions. Insertions of *Tn7-UAU1* were created through *in vitro* transposition into a *C. albicans* genomic library, and insertion sites were determined by DNA sequencing with Primer S. Insertions into annotated ORFs were transformed into *C. albicans*, and Arg+ transformants were subjected to Arg+ Ura+ selection to generate *orf::Tn7-UAU1/orf::Tn7-URA3* recombinants. PCR with insertion primer Arg4 detect and ORF-specific primers 5′ detect and 3′ detect were then used to verify presence of the *orf::Tn7-UAU1* allele and absence of the wild-type *ORF* allele. (B) PCR analysis of recombinants from *mds3::Tn7-UAU1/MDS3* transformants. A total of 10 independent transformants with the *mds3::Tn7-UAU1* insertion were subjected to Arg+ Ura+ selection, and one Arg+ Ura+ isolate from each transformant was analyzed by PCR. The leftmost lane shows results with an *mds3::Tn7-UAU1/MDS3* heterozygote, included as a control. Lanes marked with an asterisk represent homozygotes that have lost the wild-type *MDS3* allele. Mutant, *mds3::Tn7-UAU1* allele; Wt, *MDS3* allele. Note that the *mds3::Tn7-URA3* insertion is too large to amplify efficiently with this set of primers.

Arg+ Ura+ selection. Homozygous insertion mutants were then distinguished from triplication derivatives by a PCR test with flanking primers (Figure 1B). The results of these manipulations are presented in Figure 2. For 217 of the insertions, at least one homozygote was isolated. In this group, the median frequency of homozygotes was ~40% of Arg+ Ura+ segregants. For 36 of the insertions, only triplication derivatives were found among Arg+ Ura+ segregants from 10 to 35 transformants of each.

Insertions in genes that are essential for growth under our plating conditions should yield only Arg+ Ura+ triplication derivatives. This explanation probably accounts for most of the 36 homozygote-negative insertions (see Figure 2 legend). One argument is that 32 of these *C. albicans* genes have homologs in *S. cerevisiae*, of which 20 are essential, so it seems reasonable that many of the *C. albicans* genes are essential. A second argument is that failure to yield homozygotes is not restricted to a particular insertion in an ORF, as illustrated by two genes in which we recovered two independent ORF insertions, *CDC60* and *SNU114*. In these cases, neither of the insertions yielded homozygous segregants. Finally, we argue that failure to yield homozygotes is not simply a consequence of the general location in the genome, as illustrated by our observations with *BMH1*. Here we recovered one insertion within the ORF and a second insertion within 3′ noncoding sequences, 67 bp beyond the ORF stop codon. No homozygotes were recovered from the ORF insertion (0/20), but several (5/11) were recovered from the 3′ sequence insertion, which is unlikely to abolish gene function. In fact, a recent study shows that *C. albicans BMH1* is essential for viability (COGNETTI *et al.* 2002). These observations argue that the failure to yield homozygotes is a consequence of the insertion's effect on gene function, not its general chromosomal location.

We found that insertions in four *C. albicans* genes that lack clear *S. cerevisiae* homologs (ORFs 6.3847, 6.1131, 6.7513, and 6.534) failed to yield homozygous segregants. Absence of these genes from *S. cerevisiae* may indicate that they function in *C. albicans*-specific growth control pathways or that their functions are carried out by structurally divergent genes in *S. cerevisiae*. More detailed analysis of these gene products may reveal unique aspects of *C. albicans* cell physiology that might be exploited for therapeutic and diagnostic purposes.

Identification of pH-response-defective mutants: To identify possible pH-response regulators, we screened for insertion homozygotes that were defective in hyphae formation at pH 8 in liquid TC199 medium. Insertions associated with this phenotype lay in three genes: *SLA2*,

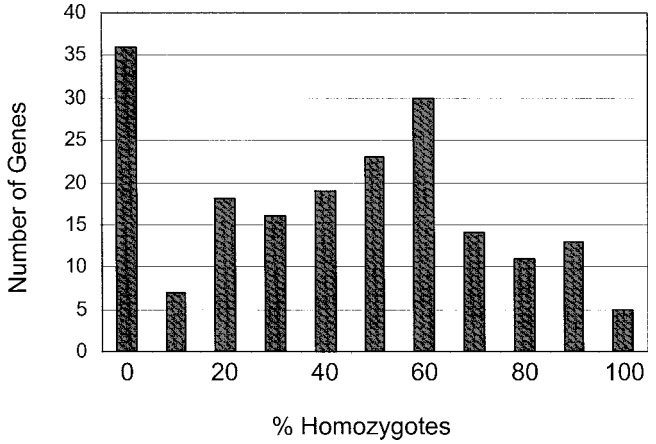

FIGURE 2.—Frequency of homozygote isolation from *UAU1* insertion heterozygotes. *Tn7-UAU1* insertion site sequences were compared to the *C. albicans* genomic sequence to identify insertions within or near ORFs. Segregants carrying two copies of the insertion were selected and homozygotes were identified by PCR tests for the wild-type copy of the insertion site. Insertions in these genes and ORFs did not yield homozygotes: *CDC60*, *YHR186*, *YDR196*, *ERG9*, *DIM1*, *ARP9*, *SNU114*, *CDC37*, *GCD11*, *ERG20*, *SMC3*, *DBP8*, *YJR072*, *TAF145*, *GPI8*, *RFC1*, *PSA1*, *ALG1*, *POP4*, *YBL004*, *MSW1*, *CLN3*, *VPS16*, *AFG3*, *ZUO1*, *ASF1*, *BMH1*, *YJL207*, *POS5*, *HGT1/RGT2*, *BCY1*, and *CEM1*; and ORFs 6.3847, 6.1131, 6.7513, and 6.534.

RIM13, and *MDS3*. *SLA2* is known to be required for filamentation (ASLESON *et al.* 2001). *RIM13* specifies a homolog of *Aspergillus nidulans* PalB and *S. cerevisiae* Rim13p, which are proteases required for PacC and Rim101p processing, respectively. Mutations in *C. albicans RIM13* are expected to cause defects in alkaline pH-induced hyphal formation, as do mutations in other Rim101p-processing pathway genes (PORTA *et al.* 1999; DAVIS *et al.* 2000b). *MDS3* specifies a 1383-residue protein with a kelch-like protein interaction domain (residues 112–167). The *Tn7-UAU1* insertion mutation lies at *MDS3* codon 82. *C. albicans* Mds3p has two *S. cerevisiae* homologs, Mds3p and Pmd1p (27 and 31% identity, respectively), both of which also have N-terminal kelch-like domains. The *S. cerevisiae* homologs function as inhibitors of meiosis (BENNI and NEIGEBORN 1997) and have no known role in pH-response or filamentation regulatory pathways.

C. albicans is a diploid, and strains are heterozygous for uncharacterized mutations that can become homozygous during strain manipulation, particularly during the selection for mitotic recombination that we employ with *UAU1* insertions. We used three genetic tests—mitotic cosegregation, reconstitution, and *de novo* mutant construction—to establish that the *mds3* insertion mutation rather than an uncharacterized mutation is the cause of the defect in filamentation in TC199 pH 8 medium. First, we isolated 23 *mds3::UAU1/mds3::URA3* homozygous mitotic segregants, each from an independent heterozygous transformant, and found that all homozygotes were defective in filamentation in TC199 pH 8 medium. Thus the filamentation defect and homozygous mutant genotype cosegregate through mitotic recombination. Second, we transformed a *HIS1-MDS3* plasmid carrying much of the *MDS3* gene into an *mds3::UAU1/mds3::URA3* strain. Integration of *HIS1-MDS3* at a disrupted *mds3* allele reconstituted an intact *MDS3* allele and restored filamentation in TC199 pH 8 medium; integration of the *HIS1* vector alone had no effect (Figure 3, A–C). Thus a wild-type phenotype is restored by reconstitution of a wild-type *MDS3* allele. Third, we used conventional methods (WILSON *et al.* 1999) to create two *mds3*Δ*::ARG4/mds3*Δ*::URA3* strains. The *mds3*Δ deletion removes codons 2–1375. These deletion mutants were defective in filamentation in TC199 pH 8 medium (Figure 3, compare columns D and E; and data not shown). These findings verify that the *mds3* insertion mutation is the cause of the mutant phenotype. In addition, we infer that the *mds3* insertion causes a loss of gene function because it is recessive and causes the same phenotype as an extensive *mds3* deletion. Therefore, Mds3p has a positive role in filamentation in TC199 pH 8 medium.

Role of Mds3p in alkaline pH responses: Mds3p may have a general role in filamentation or may be required more specifically for filamentation under certain conditions. We observed that *mds3/mds3* mutants are defective in filamentation in three alkaline media: TC199 pH 8 liquid medium (see above; Figure 3), Spider plates (pH 7.5; data not shown), and Lee's liquid medium (pH 7.5; data not shown). Titration of TC199, Spider, or Lee's media to pH 4 prevents filamentation of wild-type *C. albicans* (data not shown), thus indicating that alkaline pH is required to promote filamentation in these media. However, the mutants were induced to undergo filamentation in liquid serum (Figure 3, B and E) or in synthetic GlcNAc liquid medium at pH 4 (data not shown). Although serum is not acidic, it contains inducers of filamentation that are active at pH 6 (FENG *et al.* 1999). These observations indicate that Mds3p is not required for filamentation under all conditions, but is required in several media in which alkaline pH induces filamentation.

To determine whether Mds3p is required for transcription of alkaline pH-induced genes, we carried out Northern analysis with cells grown at pH 8 (Figure 4). Both *mds3/mds3* insertion and deletion homozygotes were defective in expression of two filamentation genes, *HWP1* and *ECE1* (Figure 4, lanes 4 and 6), compared to wild-type strains (Figure 4, lanes 2 and 5). Reconstitution of *MDS3* in the insertion mutant restored *HWP1* and *ECE1* expression (Figure 4, lane 3). In contrast, the *mds3/mds3* mutant strains expressed the alkaline pH-induced genes *PHR1* (Figure 4) and *PRA1* (data not shown) at levels comparable to those of wild-type strains. A control *rim101/rim101* mutant was defective in expression of *HWP1*, *ECE1*, and *PHR1* (Figure 4, lane 1). The

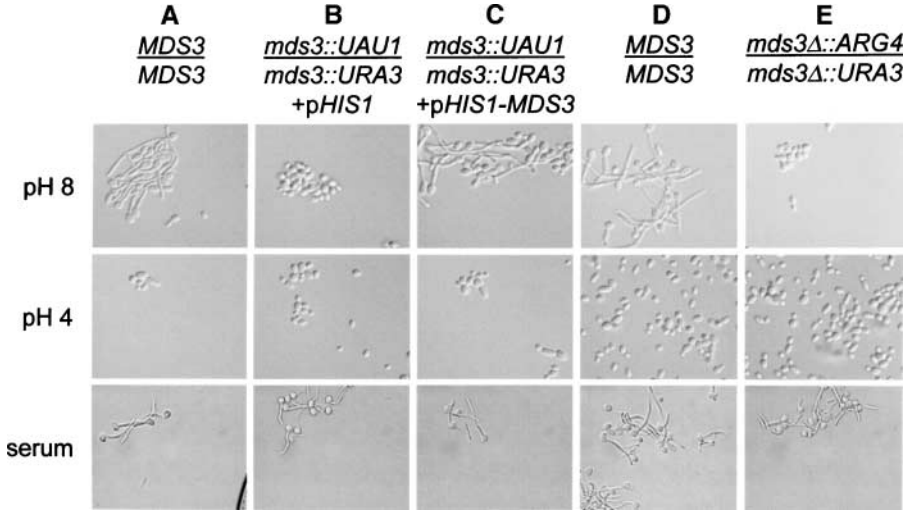

FIGURE 3.—Filamentation response of wild-type and mutant strains. Cells were incubated for 6 hr at 37° in TC199 pH 8 (top row), TC199 pH 4 (middle row), and serum (bottom row) and photographed. Strains were DAY185 (A), VIC25 (B), VIC21 (C), DAY286 (D), and VIC3 (E).

rim101/rim101 mutant overexpressed the alkaline pH-repressed gene *PHR2*, as reported previously (DAVIS *et al.* 2000b); the *mds3/mds3* mutant strains did not (Figure 4). These findings indicate that Mds3p is required for expression of two filamentation genes at alkaline pH, but that it is not required for all alkaline pH-induced transcription.

Several pH-response regulators are required for proteolytic removal of the Rim101p C-terminal region, which activates Rim101p. Mutations in these genes are suppressed by the *RIM101-405* allele, which specifies a truncated Rim101p derivative (DAVIS *et al.* 2000a,b). Introduction of the *RIM101-405* allele into *mds3/mds3* strains did not restore alkaline pH-induced filamentation (data not shown), thus arguing that Mds3p does not act in the Rim101p proteolysis pathway. Therefore, Mds3p may act downstream of Rim101p to govern a subset of Rim101p-dependent processes, or Mds3p may act in a parallel, Rim101p-independent alkaline pH-response pathway.

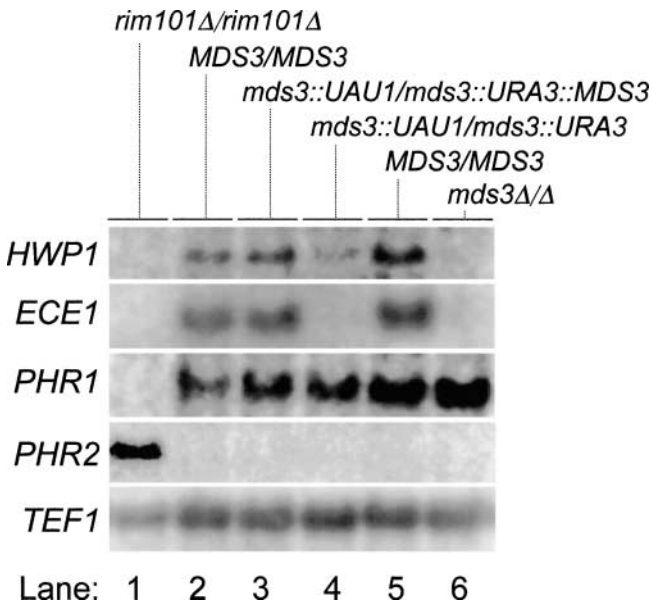

FIGURE 4.—Transcript accumulation at alkaline pH. RNA was prepared from cells grown at 37° in TC199 pH 8 medium and used for Northern analysis. The strains were DAY25 (lane 1), DAY185 (lane 2), VIC21 (lane 3), VIC25 (lane 4), DAY286 (lane 5), and VIC3 (lane 6), with relevant genotypes indicated. Strains were prototrophic (lanes 1–4) or His− (lanes 5 and 6).

The model in which Mds3p acts in parallel with Rim101p predicts that *rim101/rim101 mds3/mds3* double mutants may have a phenotype more severe than that of either single mutant. We observed that *mds3/mds3* mutants and *rim101/rim101* mutants had a mild growth defect at pH 9 (doubling times of 3 and 4 hr, respectively, compared to 2 hr for the wild type); a double mutant had a severe growth defect (7-hr doubling time; Figure 5A). Also, each single mutant had a mild growth defect in the presence of LiCl; the double mutant had a severe growth defect (Figure 5A). Introduction of a wild-type *RIM101* or *MDS3* allele improved growth of the double mutant on these media (data not shown), thus confirming that each mutation contributes to the severity of the defect. These findings argue that Rim101p and Mds3p act in parallel pathways to govern ion and alkaline pH tolerance.

Mds3p requirement for host-pathogen interaction: To determine whether Mds3p has a role in infection, we compared the abilities of wild-type and prototrophic mutant strains to cause a lethal disseminated infection in a mouse-tail-vein injection model. The median survival of mice infected with the wild-type strain was 3 days, the median survival of mice infected with the *mds3/mds3* strains was 5 days ($P < 0.0001$ *vs.* the wild-type strain), and the median survival of mice infected with the *mds3/mds3* + p*MDS3* reconstituted strains was 3 days (Figure 6A). Therefore, Mds3p is required for full virulence in this assay. A *rim101/rim101* strain was also

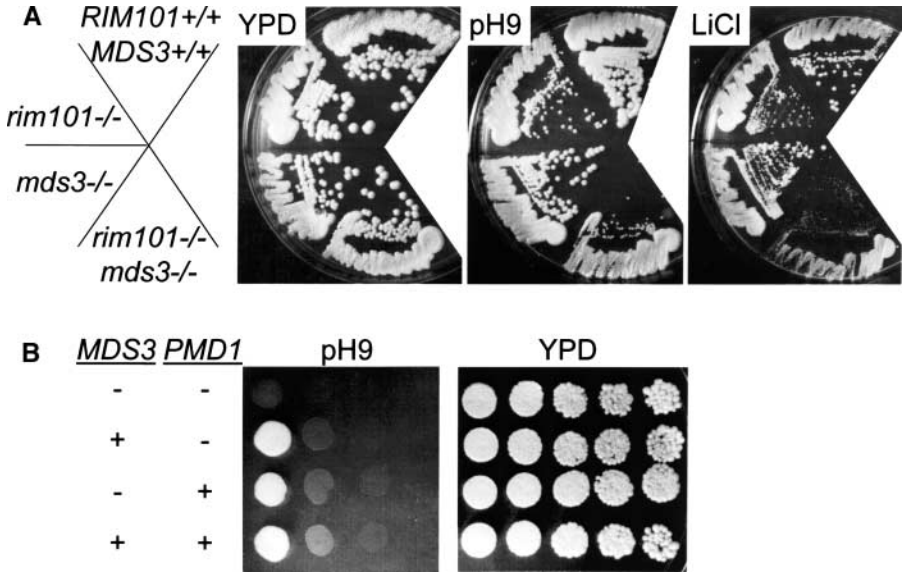

FIGURE 5.—Growth properties of *C. albicans* and *S. cerevisiae* mutant strains. (A) *C. albicans* strains (from top to bottom: DAY185, DAY25, VIC25, and VIC28) were streaked on the surface of control YPD medium, YPD pH 9 medium, and YPD LiCl medium. Plates were photographed after 3 days at 37°. (B) *S. cerevisiae* strains homozygous for *mds3* and *pmd1* mutations as indicated (left) were spotted in a fivefold dilution series on the surfaces of YPD pH 9 and control YPD plates, which were photographed after 2–3 days at 30°. Strains (from top to bottom) are LNY890, LNY899, LNY748, and LNY746 (BENNI and NEIGEBORN 1997).

attenuated in virulence. Mice infected with this strain had a median survival of 12 days ($P < 0.0001$ *vs.* the wild-type strain; Figure 6A), in keeping with prior studies (DAVIS *et al.* 2000a). The *rim101/rim101 mds3/mds3* double mutants were more severely attenuated than either single mutant as the median survival of mice infected with the double mutant was 24 days ($P < 0.0001$ *vs.* the *mds3/mds3* mutants; $P = 0.014$ *vs.* the *rim101/rim101* mutant; Figure 6B). We confirmed that each mutation contributed to attenuation of the double mutant, because reconstitution with p*MDS3* and p*RIM101* plasmids improved virulence, yielding a median survival of 9 and 6 days, respectively ($P < 0.001$ and $P < 0.0001$, respectively, compared to the *rim101/rim101 mds3/mds3* strains; Figure 6B). These findings indicate that Mds3p and Rim101p make independent contributions to *C. albicans* virulence.

Conservation of Mds3p function in *S. cerevisiae*: The *S. cerevisiae* Mds3p homologs, Mds3p and Pmd1p, function as negative regulators of meiosis (BENNI and NEIGEBORN 1997). To determine whether they also govern alkaline pH responses, we compared growth of wild-type, single-, and double-mutant strains on pH 9 medium (Figure 5B). The wild-type and each single mutant grew equally well, but the *mds3 pmd1* double mutant grew poorly at pH 9. All strains grew comparably on control YPD medium (Figure 5B). Our results indicate that *S. cerevisiae* Mds3p and Pmd1p promote a response to alkaline conditions.

DISCUSSION

We have described here a function-based gene discovery strategy for *C. albicans*. The strategy involves selection of homozygous insertion mutants from heterozygotes and thus streamlines large-scale isolation of defined homozygous mutants in this asexual diploid organism. The selection properties of the *UAU1* cassette derive from its genetic structure and not from specific features of the markers themselves (ENLOE *et al.* 2000), so this strategy may be adapted for mutagenesis of other diploid organisms and cell lines.

Our analysis indicates that the *C. albicans* essential gene set is more distinct from that of *S. cerevisiae* than previously thought. Among the insertions that failed to yield homozygotes, one-third were in genes whose *S. cerevisiae* homolog is nonessential. Only a few genes, such as *SNF1* and *FKS1*, were known previously as essential in *C. albicans* yet nonessential in *S. cerevisiae* (DOUGLAS *et al.* 1997; MIO *et al.* 1997; PETTER *et al.* 1997). Such genes are of interest for two reasons. First, their properties suggest that *S. cerevisiae* may have a redundant or compensatory pathway that permits survival of the corresponding mutant. Thus this gene class may motivate identification of new biological mechanisms in *S. cerevisiae*. Second, because antifungal drug targets have been chosen from among essential *S. cerevisiae* gene products, this gene class may expand the spectrum of gene products and pathways that serve as exploitable drug targets.

The main limitation in our mutagenesis strategy stems from the requirement for mitotic recombination or gene conversion to generate a homozygous mutant. Thus we may be unable to recover homozygous insertions in a nonessential gene if there is a nearby heterozygous lethal mutation. We have tried to minimize this problem by using independent heterozygous transformants for selection: We expect that we will recover insertions in both alleles of a gene. So, while some transformants may have the *UAU1* insertion and a lethal mutation on the same copy of the chromosome (and thus the homozygote may be nonrecoverable), other transformants will

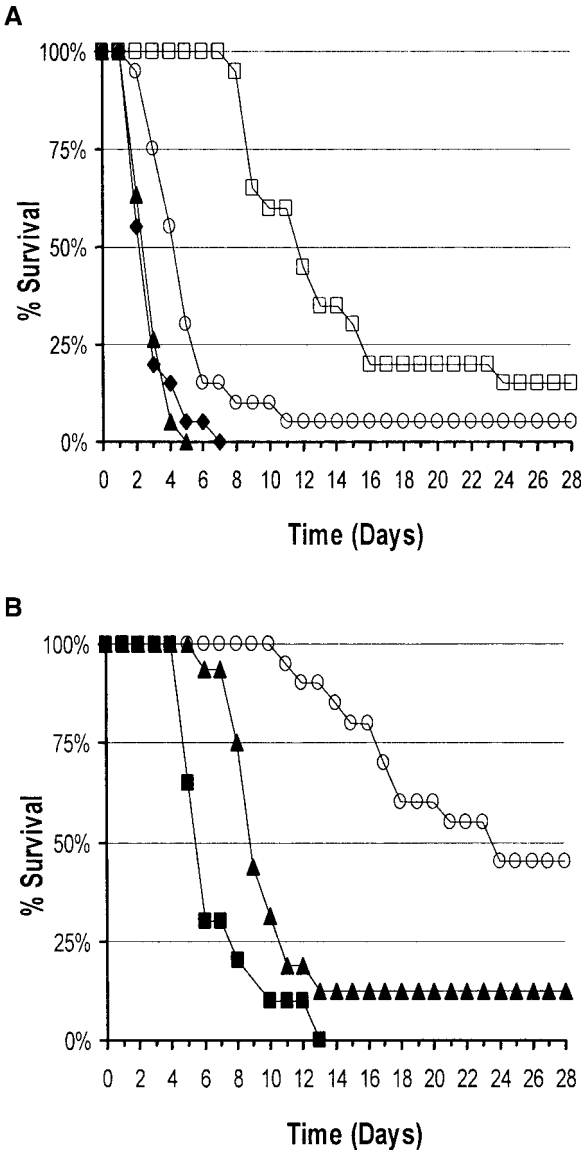

FIGURE 6.—*C. albicans* virulence assays. Balb/c mice were inoculated with 10^6 fungal cells, and survival was monitored over time. All *C. albicans* strains used in these experiments were prototrophic. (A) Survival of mice infected with (◆) wild-type (n = 20), (○) *mds3/mds3* (n = 20), (▲) *mds3/mds3::MDS3* (n = 19), and (□) *rim101/rim101* (n = 20) strains of *C. albicans*. (B) Survival of mice infected with (○) *rim101/rim101 mds3/mds3* (n = 20), (▲) *rim101/rim101 mds3/mds3::MDS3* (n = 16), and (■) *rim101/rim101::RIM101 mds3/mds3* (n = 20) strains. The combined data from two independent experiments are presented. Mice were infected with all strains used in each experiment on the same day whereas the data in A and B are plotted separately to aid visualization. Each experiment used independently constructed *mds3/mds3*, *mds3/mds3* p*MDS3*, *rim101/rim101 mds3/mds3*, *rim101/rim101 mds3/mds3::MDS3*, and *rim101/rim101::RIM101 mds3/mds3* strains.

have the insertion and lethal mutation on the opposite allelic copies of the chromosome. Also, the genes in which we could not recover insertions are plausible essential genes. For example, most are homologs of genes that are essential in *S. cerevisiae*. Among the others, many act in well-understood pathways that are plausibly essential. For example, Vps16p is required for vacuolar biogenesis; while it is not essential in *S. cerevisiae* (BRYANT and STEVENS 1998), it is essential in Arabidopsis (ROJO *et al.* 2001) and, we argue here, in *C. albicans*. A second example is Cln3p, a G1 cyclin that appears to be essential in *C. albicans* (our results) but nonessential in *S. cerevisiae* (FUTCHER 1996). Cln3p acts in parallel with Bck2p to stimulate expression of G1 cyclin genes *CLN1* and *CLN2* in *S. cerevisiae* (EPSTEIN and CROSS 1994; WIJNEN and FUTCHER 1999). No Bck2p homolog is specified in the *C. albicans* genomic sequence, so it seems reasonable that Cln3p would be essential for *C. albicans* viability. Therefore, our determination that these genes are essential is provisional, but it is consistent with their known biological functions.

Screening of viable homozygous mutants offers a method for identifying genetic determinants of *C. albicans*'s unique biological features. Our approach complements overexpression and heterologous expression methods by yielding mutations that result mainly in a loss of function of the affected gene product. This property will accelerate conversion of the *C. albicans* genomic sequence into information vital for scientific and therapeutic applications.

These insertion mutants have allowed us to define a new pH-response regulator. External pH governs growth, differentiation, and pathogenicity of *C. albicans*, but our understanding of how cells sense and respond to this signal is limited. Work of Arst and colleagues has defined the one well-characterized fungal pH-response system, composed of PacC/Rim101p transcription factors and their processing pathways (DENISON 2000; PENALVA and ARST 2002). This pathway is conserved in *C. albicans*, where it is required for many transcriptional and morphological responses to alkaline pH (PORTA *et al.* 1999; RAMON *et al.* 1999; DAVIS *et al.* 2000b; EL BARKANI *et al.* 2000). However, our previous observations implied existence of a second, Rim101p-independent pH-response pathway (DAVIS *et al.* 2000b), which we sought to identify here through insertional mutagenesis.

Mds3p is required for normal responses to alkaline pH, on the basis of three observations. First, *mds3/mds3* mutants are defective in filamentation in response to alkaline pH but not in response to serum or GlcNAc. Second, *mds3/mds3* mutants are defective in expression of the filamentation-associated genes *HWP1* and *ECE1* at alkaline pH. Third, *mds3/mds3* mutants have a growth defect in YPD at pH 9; these are conditions that do not induce filamentation, so the mutant defect is not restricted to morphogenesis. These defects are similar to those of *rim101/rim101* mutants. However, several observations support the conclusion that Mds3p and Rim101p act in independent regulatory pathways. First, *mds3/mds3* mutants have more restricted gene expression, virulence, and alkaline pH-growth defects than do

rim101/rim101 mutants, thus arguing that Rim101p is not an effector or downstream target for Mds3p. This conclusion is consistent with the failure of activated Rim101-405p to suppress the *mds3/mds3* filamentation defect. Second, *mds3/mds3 rim101/rim101* double mutants have more severe defects in alkaline pH growth, lithium sensitivity, and virulence than does either single mutant. Such an observation, for null mutations, argues that the two gene products act in independent pathways. Prior studies in *S. cerevisiae* showing that Mds3p and Pmd1p inhibit meiosis (Benni and Neigeborn 1997), whereas Rim101p stimulates meiosis (Su and Mitchell 1993; Li and Mitchell 1997), are consistent with the hypothesis that Mds3p and Rim101p act independently in that organism as well.

The molecular function of Mds3p is presently uncertain. *S. cerevisiae* Pmd1p interacts with the mitogen-activated protein (MAP) kinase cascade scaffold Ste5p (Caponigro *et al.* 1998), so it seems possible that *C. albicans* Mds3p promotes filamentation by stimulating the homologous MAP kinase pathway. However, unlike *mds3/mds3* mutants, a MAP kinase pathway mutant (*cph1/cph1*) grows as well as wild-type strains in YPD at pH 9 (V. M. Bruno and A. P. Mitchell, unpublished observations). A clue about Mds3p function may come from the identities of Rim101p-independent pH-response genes in *S. cerevisiae*, which include phosphate-limitation genes and iron/copper-limitation genes (Lamb *et al.* 2001). So, *C. albicans* Mds3p may promote gene expression through a phosphate or iron/copper response pathway that is activated in alkaline media. Analysis of Mds3p function in both *C. albicans* and *S. cerevisiae* should identify other gene products that act in this newly defined pH-response pathway.

This study was supported by Public Health Services grant R01 AI50931. S. G. Filler and A. P. Mitchell were also supported by Burroughs Wellcome Fund awards in molecular pathogenic mycology.

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Communicating editor: J. J. Loros