

Rapid Hypothesis Testing with *Candida albicans* through Gene Disruption with Short Homology Regions

R. BRYCE WILSON, DANA DAVIS, AND AARON P. MITCHELL*

Department of Microbiology and Institute of Cancer Research, Columbia University, New York, New York 10032

Received 5 November 1998/Accepted 6 January 1999

Disruption of newly identified genes in the pathogen *Candida albicans* is a vital step in determination of gene function. Several gene disruption methods described previously employ long regions of homology flanking a selectable marker. Here, we describe disruption of *C. albicans* genes with PCR products that have 50 to 60 bp of homology to a genomic sequence on each end of a selectable marker. We used the method to disrupt two known genes, *ARG5* and *ADE2*, and two sequences newly identified through the *Candida* genome project, *HRM101* and *ENX3*. *HRM101* and *ENX3* are homologous to genes in the conserved *RIM101* (previously called *RIMI*) and *PacC* pathways of *Saccharomyces cerevisiae* and *Aspergillus nidulans*. We show that three independent *hrm101/hrm101* mutants and two independent *enx3/enx3* mutants are defective in filamentation on Spider medium. These observations argue that *HRM101* and *ENX3* sequences are indeed portions of genes and that the respective gene products have related functions.

Candida albicans is an opportunistic fungal pathogen. It exists as a benign commensal organism in healthy individuals but causes infections in susceptible individuals, such as those with diminished immune function (14). Molecular genetic analysis of *C. albicans* has permitted evaluation of antifungal drug targets and elucidation of requirements for infection and pathogenesis (16).

New *C. albicans* genes have been identified frequently through sequence homology to known genes or gene families. Gene discovery has been facilitated greatly by access to much of the *C. albicans* genomic sequence (11). Now, the rate-limiting step in analysis of gene function in this diploid organism is the creation of a homozygous disruption mutant. Gene disruption has been accomplished through successive transformations with insertion/deletion alleles that are constructed in vitro (2, 7, 12). These methods have thus far required isolation of substantial DNA segments, and yet new genes of interest are often identified through DNA sequences of 400 to 600 bp (3a). We report here a rapid method for disruption of *C. albicans* genes with PCR products that contain short regions of homology to the genome.

MATERIALS AND METHODS

Strains. The *C. albicans* strains used in this study are SC5314 (wild type [2]) and its derivatives CAI4 (*ura3Δ::λimm434/ura3Δ::λimm434* [2]) and RM1000 (*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG* [12]). Strain Arg-het1 (*ura3Δ::λimm434/ura3Δ::λimm434 arg5::hisG/ARG5*) was constructed through transformation of strain CAI4 with the *arg5::hisG-URA3-hisG* disruption fragment derived from plasmid pUC-ARG-U (12); uridine-prototrophic (Uri⁺) transformants were then purified and subjected to 5FOA selection for excision of *URA3*. Presence of the *hisG* cassette was verified by PCR detection with primers hisG-N and hisG-C (Table 1). Strain BWP17 (*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*) was constructed through two successive transformations of strain RM1000 with an *arg4::hisG-URA3-hisG* disruption fragment derived from plasmid pRS-ArgBlaster (see below); each transformation was followed by Uri⁻ counterselection on 5FOA medium. *Escherichia coli* DH5α was used for plasmid propagation.

Media. YPD+Uri medium was used for routine nonselective propagation of *C. albicans* strains; it contains, per liter, 20 g of dextrose, 20 g of Bacto Peptone, 10 g of Difco yeast extract, and 80 mg of uridine. Synthetic medium (SD)

contains, per liter, 20 g of dextrose and 6.7 g of Difco yeast nitrogen base without amino acids (6). SD was supplemented with necessary auxotrophic requirements as described previously (6), except that uridine was added at 80 mg/liter to supplement Uri⁻ strains. Solid Spider medium was prepared as described previously (9), except that it was supplemented with uridine for growth of Uri⁻ strains.

Plasmids. (i) **pGEM-URA3.** A 1.2-kbp *URA3* fragment was amplified by PCR from strain SC5314 template DNA with primers ca-ura-3 and ca-ura-5 (Table 1). The fragment was gel purified and ligated to vector pGEM-T (Promega) to yield plasmid pGEM-URA3 (Fig. 1A).

(ii) **pGEM-HIS1.** A 1.0-kbp *HIS1* fragment was amplified by PCR from strain SC5314 template DNA with primers ca-his-3 and ca-his-5 (Table 1). The fragment was gel purified and ligated into vector pGEM-T (Promega) to yield plasmid pGEM-HISX. This insert lacked a few 3' codons of *HIS1* and failed to complement a *Saccharomyces cerevisiae his1* mutant, so we used integrative transformation to retrieve additional *HIS1* sequences. The integrating plasmid pGEM-URA/HIS was constructed by ligation of the 1.0-kbp *NcoI* fragment of plasmid pGEM-HISX (which contains *HIS1* sequences) into *NcoI*-cut, phosphatase-treated plasmid pGEM-URA3. One resulting plasmid had the *HIS1* 3' end adjacent to *URA3* sequences. This plasmid, designated pGEM-URA/HIS, was digested within *HIS1* sequences with *NruI* and transformed into *C. albicans* CAI4. Genomic DNA purified from Uri⁺ transformants was digested with *SalI*, ligated, and transformed into *E. coli* DH5α. The resulting plasmid, pGEM-HIS, had a 2.1-kbp insert with a complete *HIS1* gene (Fig. 1B).

(iii) **pRS-ARG4 and derivatives.** A 2.3-kbp *ARG4* fragment was amplified by PCR from strain CAI4 template DNA with primers Arg4-N2K and Arg4-CS. The gel-purified PCR product was cloned into plasmid pRS314 after digestion of both PCR product and plasmid with *XnaI* and *Asp718*. The resulting plasmid was called pRS-ARG4. Sequences outside of *ARG4* were deleted by *SpeI* digestion of pRS-Arg and ligation to yield plasmid pRS-Arg4ΔSpeI (Fig. 1C).

(iv) **pRS-ArgBlaster.** Plasmid pRS-Arg4 was digested with *XbaI* and religated to yield plasmid pRS-Arg4ΔXbaI. Plasmid pRS-Arg4ΔXbaI was digested with *BglII* and *HindIII*, thus releasing a fragment with a 3' portion of *ARG4* and some downstream sequences. The digested plasmid was ligated together with a 4.1-kbp *BglII-HindIII* fragment from plasmid pMB7 (2) containing a *hisG-URA3-hisG* cassette. The resulting plasmid, pRS-ArgBlaster, was digested with *Asp718* and *SmaI* for transformation into *C. albicans*.

PCR amplification. For gene disruption, typical PCR mixtures contained 1 μl of quick-prep template DNA (plasmid pGEM-HIS1, pGEM-URA3, or pRS-Arg4ΔSpeI), 2 μl of a 5 μM stock of each (5DR and 3DR) primer, 10 μl of 10× PCR buffer (Boehringer), 10 μl of a mixture of 2.5 mM deoxynucleoside triphosphates (Boehringer), 2 μl of 0.1 M MgCl₂, 75 μl of water, and 0.5 μl of *Taq* DNA polymerase (Boehringer or Sigma) (1 U/μl). The mixture was overlaid with mineral oil and incubated at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 4 min. After a final extension at 72°C for 8 min, the reaction mixture was stored at either 4°C or -20°C before further use. Generally, 5 μl of the reaction mixture was examined on an agarose gel to confirm the presence of a product of expected size.

For analytical PCRs, 1 to 5 μl of *C. albicans* DNA, prepared by glass bead lysis (5), served as a template in a PCR. Primers 5-detect and 3-detect (Table 1) were used to detect vector sequences flanking a marker. Primers RIM101-5a and seq7

* Corresponding author. Mailing address: Department of Microbiology and Institute of Cancer Research, Columbia University, 701 West 168th St., New York, NY 10032. Phone: (212) 305-8251. Fax: (212) 305-1741. E-mail: apm4@columbia.edu.

TABLE 1. Primer sequences

Primer	Sequence ^a	Purpose ^b
ARG5-5DR	AAAGTGTTCCTTAGAGCAAAAACCTTGGCGTTTGGTGACAGCTTTGGGAAAAAATTTGGTGTTCACCGTTTTCACGAGCTT	Disruption of <i>ARG5</i>
ARG5-3DR	ATCTTGGCAAAATGTTGTAATTAATTCGAAATTTCCCTTGATCTTCAATTTAGTACCATATTTGGGAATTTGTGAGCGGATA	Disruption of <i>ARG5</i>
ADE2-5DR	GTCCATTATATGCTGAAAAATGGTGTCTTTCACCAAAAAGAAATTTGGTGTGTTTCCACGATCAGCAGCTT	Disruption of <i>ADE2</i>
ADE2-3DR	GGGTTGCCCTTATCACCCCAAGACATTCACATTAATAGCATTTGGTGTGAAATTTGGAAATTTGTGAGCGGATA	Disruption of <i>ADE2</i>
ARG4-N2K	ATTCGGATCCGGTACCCCGCTTTAGTAAAGATTTTTCAAAGAGTAG	<i>ARG4</i> cloning
ARG4-CS	ATTCTCGAGCCCGGGCAATGCTTTGAGGAGAAATCAGAACCGC	<i>ARG4</i> cloning
ca-ura-5	TTGGATGGTATAAACGGGAAACA	<i>URA3</i> cloning
ca-ura-3	TCTAGAAAGGACCACCTTTTGATTG	<i>URA3</i> cloning
ca-his-5	CCTGGAGGATGAGGAGACAG	<i>URA3</i> cloning
ca-his-3	CCAATATATCGGTTTGCACCA	<i>HIS1</i> cloning
5-delet	GTTTTCCCAGTCAAGGACGTTTGTAAAAACGAC	<i>HIS1</i> cloning
3-delet	TGTGGAAATTTGTGAGCGGATTAACAATTTTCAC	Detection of vector sequences
hisG-N	CGCGATACAGACCCGGTTTCAGACAGGA	Flanking disruption marker
hisG-C	TGGTCTTTACTCCATCAACAGGGTTCC	Flanking disruption marker
RIM101-5DR	ACGATCATTGTGTGACGACCATGTTGTGTAAGAAAAGTCTTTCGAAACAATTTGTCATTGACTTGTGTGGAATTTGTGAGCGGATA	Detection of vector sequences
RIM101-3DR	ACATGGACTCTCAAGTGAAGAAATGATGATCTCTTAACGTGATGTTGGCCACAATTTTTCACGATCAGCAGCTT	Detection of vector sequences
RIM101-5a	GGGGAATTCGTTGTAATCAATCTTAACACACACAGGCTCTGC	Detection of <i>HRM101</i>
seq7	GGTGAACCTCAGCCAGAACCTGGC	Detection of <i>HRM101</i> alleles
Pala-5DR	GCAAGCAAGAGTTAATTAAGAAAAGTATGATAAAAATGAAAACAATATTTTGTACAGGCTAACCAACGAGATGGGTGGAAATTTGTGAGCGGATA	Detection of <i>ENX3</i>
Pala-3DR	GGAACGAGTTACTAATAGCTAATTTTAAAGTCTCGACTCTCAACTCTTCGTATACAAATATATTCCTCACCATTCCACGATCAGCAGCTT	Detection of <i>ENX3</i>
Pala5'	ACTGATGATGACGACCAAGAG	Detection of <i>ENX3</i> alleles
Pala3'	CCAGGTTTACTAATATAGTCGG	Detection of <i>ENX3</i> alleles

^a Boldface sequences in 5DR and 3DR primers are segments that anneal to plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4ΔSpeI for amplification of disruption cassettes.
^b Relevant use of primer in this study.

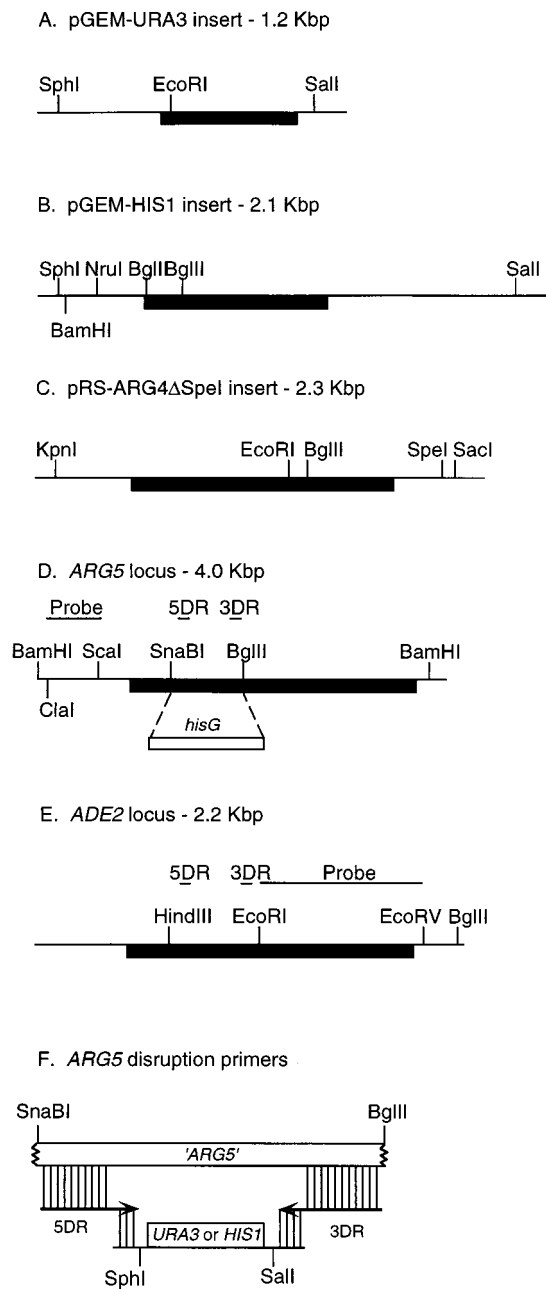


FIG. 1. Restriction maps. In panels A to E, thin horizontal lines represent DNA segments and thick lines represent the relevant open reading frame with 5' and 3' ends on the left and right, respectively. Panel D is drawn at a smaller scale than the other panels. Panels A to C represent the entire cassettes used for primer-directed gene disruption. (A) Insert of plasmid pGEM-URA3. The *URA3* PCR product lies between the *SphI* and *Sall* sites in vector pGEM-T. (B) Insert of plasmid pGEM-HIS1. The *HIS1* genomic fragment lies between the *SphI* and *Sall* sites in vector pGEM-T. (C) Insert of plasmid pRS-ARG4ΔSpeI. The *ARG4* PCR product lies between the *KpnI* and *SpeI* sites in vector pRS314. (D) Genomic *ARG5* locus. The open box represents the insertion/deletion allele *arg5::hisG* (12). Positions of the disruption primers and probe for Southern analysis are shown above the restriction map. (E) Genomic *ADE2* locus. Positions of the disruption primers and probe for Southern analysis are shown above the restriction map. (F) Homology relationships between *ARG5*, the *ARG5* disruption primers, and *URA3* and *HIS1* cassettes. The *ARG5*-5DR and *ARG5*-3DR primers are represented by arrows, which point to each primer's 3' end. Sets of parallel vertical lines indicate homology between the primers and *ARG5* sequences or disruption cassette templates. The 461-bp segment of *ARG5* shown above the primers is deleted from the *arg5::hisG* allele (12). This diagram is not to scale.

were used to detect integration at *HRM101*. Primers PalA5' and PalA3' were used to detect integration at *ENX3*.

C. *albicans* transformations. For most experiments, we used the method of Braun and Johnson (3). Overnight YPD+Uri cultures were inoculated at a 1/100 dilution into 50 ml of YPD+Uri and incubated at 30°C for two generations (around 4 h). Cells were pelleted, washed with 5 ml of LATE buffer (0.1 M lithium acetate, 10 mM Tris HCl [pH 7.5], 1 mM EDTA), and suspended in 0.5 ml of LATE buffer. Cell suspension (0.1 ml) was mixed with 5 μl of 10-mg/ml calf thymus DNA (Sigma) and transforming DNA and incubated at 30°C for 30 min. Then, 0.7 ml of PLATE buffer (40% polyethylene glycol 3350 in LATE buffer) was added and the tube was vortexed for 2 s. After overnight incubation at 30°C, the mixture was heat shocked for 1 h at 42°C. Cells were pelleted, washed with 1 ml of TE (10 mM Tris HCl [pH 7.5], 1 mM EDTA), suspended in 0.2 ml of TE, and plated on a selective medium. For transformations with PCR products, we used 80 μl of the PCR mixture per transformation. For transformations with linearized plasmids or restriction fragments, we used 2 to 10 μg of digested DNA per transformation.

For disruption of *ENX3*, we used a Frozen-EZ Yeast Transformation II kit (Zymo Research) and followed the supplier's instructions.

Identification of *HRM101* and *ENX3* sequences. *HRM101* sequences were identified through a tblastn search of *Candida* sequences (3b) with the *S. cerevisiae* Rim101p protein sequence (20) as a query. Two individual homologous sequences were aligned (Geneworks) to assemble a 627-bp segment. To identify *ENX3*, we scanned the *C. albicans* gene list (4a) for genes involved in pH regulation; *ENX3* corresponded to a 753-bp segment.

Southern analysis. Genomic DNA was prepared by glass bead lysis (5). Southern analysis was carried out as described previously (6) with randomly primed probes. The *ARG5* probe was a 700-bp *Clal*-*ScaI* fragment of plasmid pUC-ARG-U (12). The *ADE2* probe was an 800-bp *EcoRI*-*KpnI* fragment from plasmid pRS314-ADE, which contains a 1,587-bp *XbaI*-*EcoRV* *ADE2* fragment (18, 21), obtained by PCR amplification, in vector pRS314 (19).

RESULTS

PCR product-directed disruption of known genes in *C. albicans*. We first used the *ARG5* gene in assays for homologous integration in *C. albicans* of PCR products with short regions of homology. An *arg5* homozygous mutant has an Arg⁻ phenotype (12). Our strategy was to transform an *arg5::hisG/ARG5* heterozygote (strain Arg-het1) with a PCR product that was capable of homologous integration into the functional *ARG5* allele; homology to *ARG5* was specified only by synthetic primer sequences (primers ARG5-5DR and ARG5-3DR [Fig. 1F]). We assayed homologous integration through identification of Arg⁻ transformants. The PCR product included *URA3* sequences for selection and 60 bp of homology at each end to the *ARG5* allele; it had no homology to the *arg5::hisG* allele (Fig. 1D and F). Transformation of the PCR product into strain Arg-het1 yielded 18 Uri⁺ transformants (Table 2), two of which were Arg⁻. These results indicated that 60 bp of homology on each end of a PCR product was sufficient to direct homologous integration at *ARG5*.

To disrupt *ARG5* through the sole use of PCR products, we used a *ura3/ura3 his1/his1* double auxotroph (strain RM1000) in successive transformations with PCR products specifying *arg5::HIS1* and *arg5::URA3* (Fig. 2). PCR products were synthesized with a single pair of primers that anneal to plasmid sequences flanking the *HIS1* and *URA3* templates (Fig. 1F). Transformation of the *arg5::HIS1* PCR product into strain RM1000 yielded 24 His⁺ transformants (Table 2), four of which had incorporated the *HIS1* gene into one copy of *ARG5*, as indicated by two lines of evidence. First, Southern analysis of these four transformants showed that one copy of *ARG5* had a novel *BamHI* site, as expected from the *arg5::HIS1* disruption (as shown for one transformant in Fig. 3A by comparison of lanes 1 and 2). Second, PCR analysis of genomic DNA indicated that only these four transformants had retained vector sequences flanking the *HIS1* cassette (data not shown). The other His⁺ transformants may have replaced one *his1::hisG* allele with *HIS1* sequences and thus lost vector sequences. Transformation of one *arg5::HIS1/ARG5* heterozygote (strain Arg-het2) with the *arg5::URA3* PCR product yielded 27 Uri⁺

TABLE 2. Summary of PCR product transformation experiments

Expt	Recipient strain (genotype)	PCR product in transformation	Total no. of transformants ^a	No. of integrants at wild-type allele of targeted locus	Screening method ^b	Comments
ARG5 disruption	Arg-het1 (<i>arg5::hisG/ARG5</i>)	<i>arg5::URA3</i>	18	2	Arg ⁻ phenotype	
ARG5 disruption	RM1000 (<i>ARG5/ARG5</i>)	<i>arg5::HIS1</i>	24	4	Southern	21 transformants were His ⁻ and Arg ⁺
	Arg-het2 (<i>arg5::HIS1/ARG5</i>)	<i>arg5::URA3</i>	27	1	Arg ⁻ phenotype & Southern	
ADE2 disruption	RM1000 (<i>ADE2/ADE2</i>)	<i>ade2::HIS1</i>	19	3	Southern & PCR	12 transformants were His ⁻ and Ade ⁺
	Ade-het1 (<i>ade2::HIS1/ADE2</i>)	<i>ade2::URA3</i>	40	3	Ade ⁻ phenotype & Southern	
HRM101 disruption	BWP17 (<i>HRM101/HRM101</i>)	<i>hrm101::ARG4</i>	11	2	PCR	Arg ⁻ transformants were not screened Arg ⁻ transformants were not screened
	Hrm-het1 (<i>hrm101::ARG4/HRM101</i>)	<i>hrm101::URA3</i>	14	2	PCR	
	Hrm-het2 (<i>hrm101::ARG4/HRM101</i>)	<i>hrm101::URA3</i>	21	1	PCR	
ENX3 disruption	BWP17 (<i>ENX3/ENX3</i>)	<i>enx3::URA3</i>	5	2	PCR	Selection eliminated Uri ⁻ transformants
	Enx-het1 (<i>enx3::URA3/ENX3</i>)	<i>enx3::ARG4</i>	6	2	PCR	

^a Results from a single transformation are reported for each disruption.

^b Details of screening methods are described in Materials and Methods and in Results.

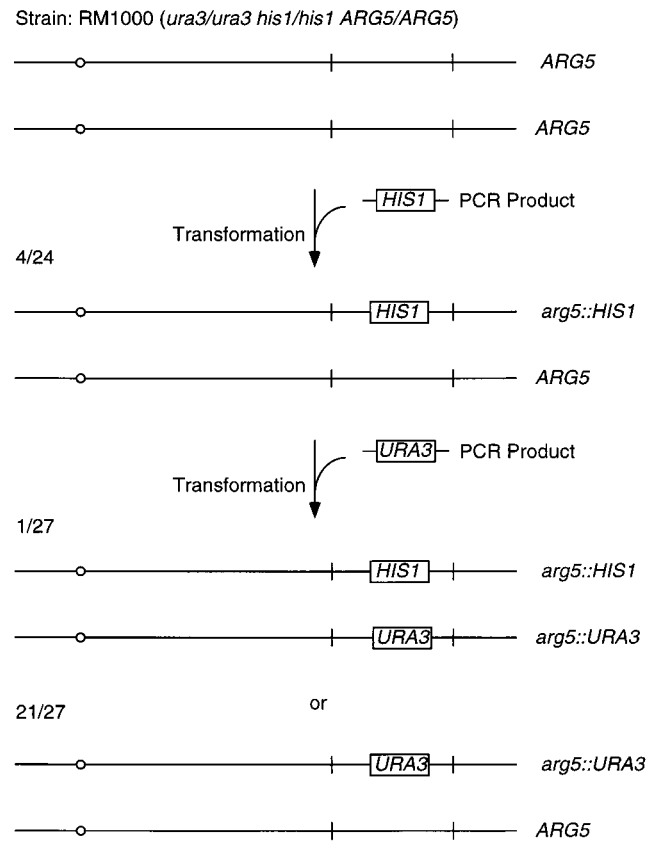


FIG. 2. Schematic diagram of de novo ARG5 disruption. Each pair of parallel lines represents the two ARG5 alleles. Successive transformations are indicated by arrows. Strain RM1000 was transformed with an *arg5::HIS1* PCR product; 4 of 24 transformants were *arg5::HIS1/ARG5* heterozygotes, as indicated by Southern analysis. One heterozygote was transformed with an *arg5::URA3* PCR product; 1 of 27 transformants was an *arg5::HIS1/arg5::URA3* homozygous mutant, as indicated by Southern analysis and its Arg⁻ phenotype. Twenty-one of the remaining transformants were *arg5::URA3/ARG5* heterozygotes, as indicated by their Arg⁺ His⁻ phenotype and, for a representative group, by Southern analysis.

transformants (Table 2 and Fig. 2), one of which was Arg⁻. Southern analysis confirmed that the Arg⁻ transformant carried one copy of *arg5::HIS1*, one copy of *arg5::URA3*, and no copies of *ARG5* (Fig. 3A, lane 3). Among the other Uri⁺ transformants, 21 remained Arg⁺ but had become His⁻. Southern analysis indicated that the *arg5::URA3* PCR product had replaced the *arg5::HIS1* allele in two of the transformants that we examined (Fig. 3A, lanes 4 and 5). Thus the majority of transformants had incorporated the *arg4::URA3* PCR product by homologous recombination. These results show that an *arg5/arg5* homozygous mutant was created through homologous integration directed by PCR primer sequences.

To see if this gene disruption strategy was applicable to other loci, we attempted to disrupt the *ADE2* locus with *ade2::HIS1* and *ade2::URA3* PCR products (Fig. 1E). In this case, each PCR product was flanked by 50 bp of homology to *ADE2*. Transformation of the *ade2::HIS1* PCR product into strain RM1000 yielded 19 His⁺ transformants (Table 2), 3 of which retained vector sequences flanking *HIS1* (data not shown). A Southern blot verified that one transformant, Ade-het1, was of genotype *ade2::HIS1/ADE2* (Fig. 3B; compare lanes 1 and 2). Transformation of the *ade2::URA3* PCR product into Ade-het1 yielded 40 Uri⁺ transformants (Table 2), 3

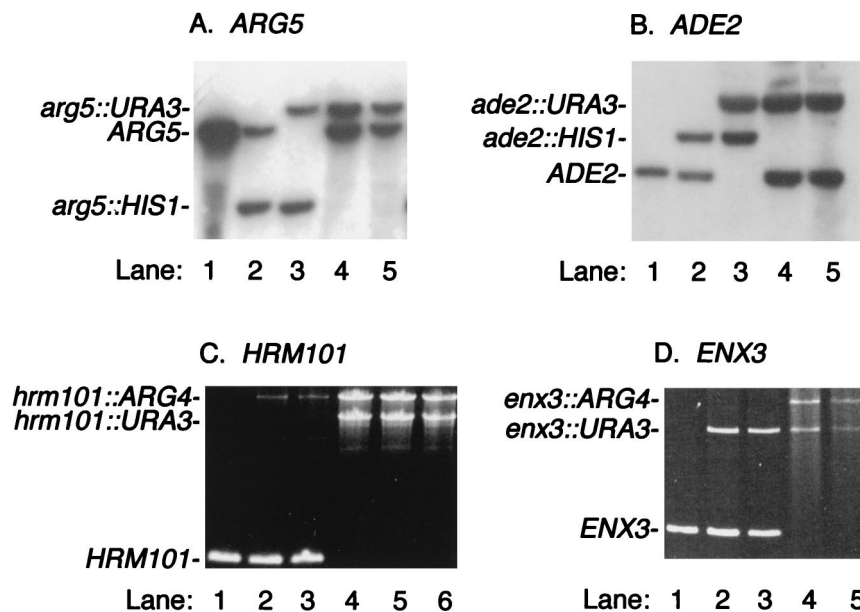


FIG. 3. Analysis of transformant strains. (A) Southern blot analysis of *ARG5* disruption experiment. Genomic DNA samples, digested with *Bam*HI, were prepared from strain RM1000 (*ARG5/ARG5* [lane 1]), *Arg*-het1 (*arg5::HIS1/ARG5* [lane 2]), a *Uri*⁺ *His*⁺ *Arg*⁻ transformant from *Arg*-het1 (*arg5::HIS1/arg5::URA3* [lane 3]), and two *Uri*⁺ *His*⁻ *Arg*⁺ transformants from *Arg*-het1 (*arg5::URA3/ARG5* [lanes 4 and 5]). The blot was probed with a 0.7-kbp *Cla*I-*Sca*I fragment of *ARG5*, as indicated in Fig. 1D. (B) Southern blot analysis of *ADE2* disruption experiment. Genomic DNA samples, digested with *Bgl*II and *Hind*III, were prepared from strain RM1000 (*ADE2/ADE2* [lane 1]), *Ade*-het1 (*ade2::HIS1/ADE2* [lane 2]), a *Uri*⁺ *His*⁺ *Ade*⁻ (red) transformant from *Ade*-het1 (*ade2::HIS1/ade2::URA3* [lane 3]), and two *Uri*⁺ *His*⁻ *Ade*⁺ transformants from *Ade*-het1 (*ade2::URA3/ADE2* [lanes 4 and 5]). The blot was probed with a 0.8-kbp *Eco*RI-*Eco*RV fragment of *ADE2*, as indicated in Fig. 1E. (C) PCR analysis of *HRM101* disruption experiment. PCR amplification with primers RIM101-5a and seq7 was carried out on genomic DNA samples from strain BWP17 (*HRM101/HRM101* [lane 1]), *Hrm*-het1 (*hrm1::ARG4/HRM101* [lane 2]), *Hrm*-het2 (*hrm1::ARG4/HRM101* [lane 3]), and *Uri*⁺ *Arg*⁺ transformants from *Hrm*-het1 and *Hrm*-het2 (*hrm1::ARG4/hrm101::URA3* [strains BWP29, BWP30, and BWP31] [lanes 4 to 6]). (D) PCR analysis of *ENX3* disruption experiment. PCR amplification with primers PalA5' and PalA3' was carried out on genomic DNA samples from strain BWP17 (*ENX3/ENX3* [lane 1]), *Enx*-het1 (*enx3::URA3/ENX3* [lane 2]), *Enx*-het2 (*enx3::URA3/ENX3* [lane 3]), and two *Uri*⁺ *Arg*⁺ transformants from *Enx*-het1 (*enx3::URA3/enx3::ARG4* [strains DAY23 and DAY24] [lanes 4 and 5]).

of which were *Ade*⁻. Colonies of the *Ade*⁻ transformants were red in color, as expected for *ade2/ade2* mutants (4). Southern analysis of one *Ade*⁻ transformant showed the presence of *ade2::HIS1* and *ade2::URA3* fragments and the absence of an *ADE2* fragment (Fig. 3B, lane 3). Twelve transformants remained *Ade*⁺ but became *His*⁻; Southern analysis of two confirmed that *ade2::URA3* had replaced the *ade2::HIS1* allele (Fig. 3B, lanes 4 and 5). Thus, 15 of 40 transformants had acquired the *ade2::URA3* PCR product through homologous recombination. These results show that *ade2/ade2* homozygous mutants were created through homologous integration directed by PCR primers.

Primer-directed disruption of newly identified sequences.

We set out to test the hypothesis that a possible *C. albicans* homolog of *RIM101* may have a role in filamentation. In *S. cerevisiae*, *RIM101* (previously called *RIM1*) specifies a zinc finger protein (20) that is required for haploid invasive growth (8), a form of filamentation (17). We identified a *C. albicans* sequence that may specify a Rim101p homolog; the predicted translation product showed over 50% identity to the Rim101p zinc finger region. We refer to this putative *C. albicans* gene as *HRM101* (for "homolog of *RIM101*"). The hypothesis that *HRM101* and *RIM101* are functional homologs predicts that *hrm101* homozygous mutants may be defective in filamentous growth.

The two genomic copies of *HRM101* were inactivated by primer-directed integration of *hrm101::ARG4* and *hrm101::URA3* PCR products in strain BWP17 (*ura3/ura3 his1/his1 arg4/arg4*). We first isolated 11 *Arg*⁺ transformants with an *hrm101::ARG4* PCR product. PCR amplification with flanking

HRM101 primers verified presence of the *hrm101::ARG4* insertion in two of these transformants (Fig. 3C; compare lanes 2 and 3 to lane 1). We used these two *hrm101::ARG4/HRM101* heterozygotes for transformation with a PCR product specifying *hrm101::URA3* and obtained 35 *Uri*⁺ transformants. PCR amplification of genomic DNA templates with flanking *HRM101* primers indicated that three transformants had only *hrm101::ARG4* and *hrm101::URA3* alleles and lacked the wild-type *HRM101* allele (Fig. 3C, lanes 4 to 6). Therefore, we had obtained three independent *hrm101::ARG4/hrm101::URA3* transformants.

To determine whether *HRM101* may be required for filamentation, we examined filamentous growth on Spider medium. We observed that *HRM101/HRM101* and *hrm101::ARG4/HRM101* strains produced abundant filaments at the edges of colonies (Fig. 4A to C) and that *hrm101::ARG4/hrm101::URA3* strains did not produce filaments (Fig. 4D to F). The filamentation defect was not simply a consequence of the auxotrophies in the transformants, because *Arg*⁺ *Uri*⁺ *His*⁻ derivatives of strain BWP17 that retained *HRM101* alleles were capable of filamentation (data not shown). These observations suggest that *HRM101* has a positive role in filamentation and that the *hrm101::ARG4* mutation is recessive.

In *Aspergillus nidulans*, the *RIM101* homolog (PacC) lies in a pathway with several *pal* gene products, including PalA (13, 15). The *C. albicans* sequence *ENX3* may specify a PalA homolog. If *ENX3* and *HRM101* lie in the same pathway, then *enx3* homozygous mutants should be defective in filamentous growth. We created two *enx3::URA3/ENX3* heterozygotes and, subsequently, two *enx3::URA3/enx3::ARG4* homozygotes by

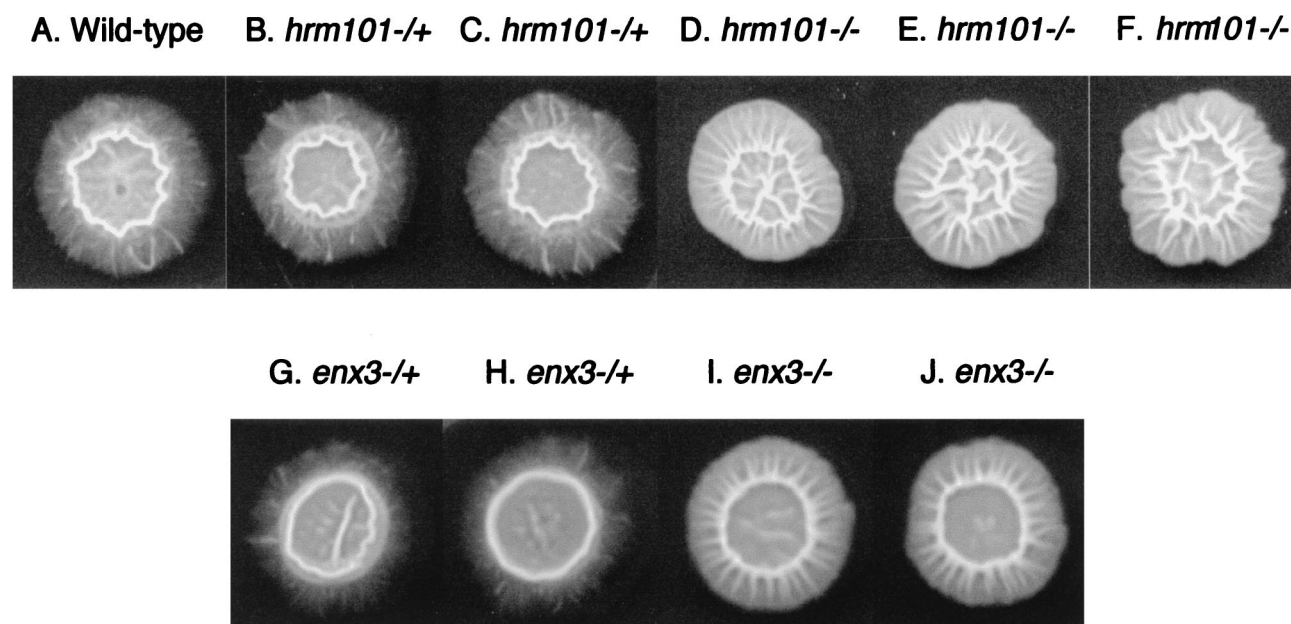


FIG. 4. Filamentation on Spider plates. Colonies were incubated for 6 days on Spider plates at 37°C. Strains were BWP17 (wild type [A]), Hrm-het1 and Hrm-het2 (both *hrm1::ARG4/HRM101* [B and C]), BWP29, BWP30, and BWP31 (all *hrm1::ARG4/hrm101::URA3* [D to F]), Enx-het1 and Enx-het2 (both *enx3::URA3/ENX3* [G and H]), and DAY23 and DAY24 (both *enx3::URA3/enx3::ARG4* [I and J]).

primer-directed integration (Table 2). Genotypes were confirmed by PCRs with flanking primers (Fig. 3D). We observed that the *enx3::URA3/enx3::ARG4* homozygotes were defective in filamentation on Spider medium (Fig. 4I and J); the *enx3::URA3/ENX3* heterozygotes were not (Fig. 4G and H). These findings indicate that both *HRM101* and *ENX3* have positive roles in filamentation.

DISCUSSION

We describe here a rapid method for disruption of *C. albicans* genes. The method is based upon PCR primer-directed gene disruption in *S. cerevisiae* (1, 10) and upon the idea of using two markers to create homozygous disruptions in *C. albicans* (7, 12). We were able to disrupt four different genes with PCR products, so it is likely that the method will be applicable to many other loci.

We had expected that the *ARG4* and *URA3* cassettes would integrate more efficiently than *HIS1* at targeted loci. The *URA3* cassette in plasmid pGEM-URA3 lacks sequences to direct integration at the *URA3* locus, because the entire *URA3* locus has been deleted in strain CAI4 (2). The *ARG4* cassette in plasmid pRS-ARG4 Δ SpeI also lacks sequences to direct integration at the *ARG4* locus, because it does not extend beyond one end of the *arg4::hisG* insertion/deletion allele in strain BWP17. On the other hand, the *HIS1* cassette in plasmid pGEM-HIS1 extends far beyond the *HIS1* coding region, so it should be capable of integration into the *his1::hisG* allele. However, targeted integration of *hrm101::ARG4* and *enx3::URA3* in strain BWP17 occurred with efficiency comparable to that of *arg5::HIS1* and *ade2::HIS1* in strain RM1000. These results are not directly comparable, because they involve different targeted loci and different transformation recipients. However, they suggest that all three markers may be equally useful for future gene disruption experiments.

The *ARG4* and *URA3* cassettes lack known sequences for homologous integration at each respective locus in strain

BWP17, yet we recovered transformants that did not carry the markers at targeted loci. We are uncertain whether the PCR products integrate into the genome or are maintained in an extrachromosomal state. However, the markers are much more stable than the ARS plasmid pRC2312 (4) in our hands (unpublished results), so we believe that they are integrated. These observations underscore the importance of verifying targeted integration through either Southern analysis or PCRs with outside primers.

We observed a significant allelic integration bias in the second of successive transformations. For example, transformation of the *arg5::HIS1/ARG5* strain with the *arg5::URA3* PCR product yielded more frequent integration into the *arg5::HIS1* allele than into the *ARG5* allele. A similar bias, though less severe, was observed during disruption of *ADE2*. The bias may reflect a greater recombination efficiency between molecules that have more extensive homology: the ends of the *arg5::URA3* PCR product have 160 and 210 bp of homology to the *arg5::HIS1* allele but 60 bp (at each end) to the *ARG5* allele. Also, our use of unpurified PCR primers for creation of disruption constructs may contribute to allelic integration bias: contaminating primers with 5' truncations will yield PCR products with little or no homology to a wild-type allele. In practice, this problem is not a significant impediment because undesired integrants may be eliminated by selection. Where cost is not a factor, the problem might be eliminated entirely through use of nonhomologous primers for amplification of each disruption cassette.

The main value of a rapid gene disruption method in *C. albicans* is to provide functional information about a sequence before investing significant effort in its characterization. In this study, we examined the function of two *C. albicans* genomic segments that were identified through Blast searches of the genomic sequence database. They were relatively short for traditional gene disruption strategies (*HRM101*, 627 bp; *ENX3*, 753 bp); in addition, both sequence records include uncertain nucleotides, so their isolation by PCR amplification might be

difficult. Both sequences are also too short to specify an entire protein. By primer-directed disruption, we created homozygous mutant strains within 4 weeks of sequence identification. Our hypothesis predicted that *hrm101* and *enx3* homozygotes would have similar phenotypes, and this prediction was verified. However, we might have found that *enx3* and *hrm101* homozygotes have different phenotypes, thus suggesting that the respective gene products have distinct physiological functions. We might have found that one or both homozygotes have no detectable phenotype, thus suggesting that the sequences may be pseudogenes, that they may have functional homologs elsewhere in the *C. albicans* genome, or that our phenotypic assays are too crude to detect their function. We might have been unable to isolate homozygous mutants, thus suggesting that the respective gene products may be essential for growth, essential for recovery from transformation, or perhaps essential for completion of recombination. Each of these possible outcomes would affect our priorities for further characterization of a possible *RIM101* pathway in *C. albicans*. Also, the outcomes might change our view of the *RIM101* pathway in *S. cerevisiae* (8). Thus it is extremely valuable to be able to characterize a homozygous mutant at the start of a research effort.

Our information about *HRM101* and *ENX3* function is preliminary, because we have not complemented or reverted the defects. It is formally possible that the phenotypes of mutant strains arise from coincident mutations that were inadvertently isolated during transformation. However, the finding that three independent *hrm101/hrm101* homozygotes have a filamentation defect supports the idea that the phenotype arises from the *hrm101* mutation, not from a coincidental secondary mutation. A similar argument applies to the two *enx3/enx3* homozygotes. These observations provide preliminary support for the idea that *HRM101* and *ENX3* sequence fragments are parts of genes, that they specify products, and that Hrm101p and Enx3p may have related functions.

ACKNOWLEDGMENTS

We thank Teresa Lamb for critical reading of the manuscript. We are grateful to William Fonzi and Jesus Pla for providing strains and plasmids and to Pete Magee and Jack Edwards for their interest and encouragement.

This work was supported by a Mycology Scholar Award from the Burroughs Wellcome Fund (to A.P.M.) and by training grant T32 AI07161-21 from the National Institutes of Health.

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