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

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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 RIM1) 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 genome 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::XmnI/344::ura3Δ::XmnI/344 [2]) and RM1000 (ura3Δ::XmnI/344::ura3Δ::XmnI/344 his1::his3/2::his1::his3G [12]). Strain Arg4-1 (ura3Δ::XmnI/344::ura3Δ::XmnI/344 arg5::arg5::ARG5) was constructed through transformation of strain CAI4 with the arg5::arg5::URA3-hisG disruption fragment derived from plasmid pUC-ARG-U (12); uridine-prototrophic (Uri) transformants were then purified and subjected to SPOA 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 BW2P17 (ura3Δ::XmnI/344::ura3Δ::XmnI/344 his1::his3/2::his1::his3G [12]) Strain Arg4-1 (ura3Δ::XmnI/344::ura3Δ::XmnI/344 arg5::arg5::ARG5) was constructed through transformation of strain CAI4 with the arg5::arg5::URA3-hisG disruption fragment derived from plasmid pUC-ARG-U (12); uridine-prototrophic (Uri) transformants were then purified and subjected to SPOA 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 Arg4-1 (ura3Δ::XmnI/344::ura3Δ::XmnI/344 arg5::arg5::ARG5) was constructed through transformation of strain CAI4 with the arg5::arg5::URA3-hisG disruption fragment derived from plasmid pUC-ARG-U (12); uridine-prototrophic (Uri) transformants were then purified and subjected to SPOA selection for excision of URA3. Presence of the hisG cassette was verified by PCR detection with primers hisG-N and hisG-C (Table 1).

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-ura3 and ca-ura5 (12). The fragment was gel purified and ligated into 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-his3 and ca-his5 (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 XhoI 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-Arg was digested with XhoI and religated to yield plasmid pRS-Arg4XhoI. Plasmid pRS-Arg4XhoI 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 (5′DR and 3′DR) 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
<table>
<thead>
<tr>
<th>Purpose</th>
<th>Primer Sequence</th>
</tr>
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<tr>
<td>Disruption of <strong>ARG5</strong></td>
<td><code>ARG5-5DR AAGTGTTTCTTAGAGCAAAACTTGCGTTTGGTGACAGCTTTGGAAAAAATTGGTGTTCAC</code></td>
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<tr>
<td>Disruption of <strong>ARG5</strong></td>
<td><code>ARG5-3DR ATCTTGGCAAATGTTGTAATAAATCGTGAATTTCCTTGATCTTCAATTTAGTACCATATT</code></td>
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<tr>
<td>Disruption of <strong>ADE2</strong></td>
<td><code>ADE2-5DR GTCCATTATATGCTGAAAAATGGTGTCCTTTCACCAAAGAATTGGCTGTG</code></td>
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<tr>
<td>Disruption of <strong>ADE2</strong></td>
<td><code>ADE2-3DR GGGTTGCCTTATCACCCAAGACATTCAACATAATAGCATTGGTGATGGAA</code></td>
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<tr>
<td><strong>ARG4</strong> cloning</td>
<td><code>ARG4-N2K ATTCGGATCCGGTACCCCCCTTTAGTAAGATTTTTCAAGAGTAG</code></td>
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<td><strong>ARG4</strong> cloning</td>
<td><code>ARG4-CS ATTCTCGAGCCCGGGCAATGCTTGAGGAGAAGAATCAGAACGC</code></td>
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<tr>
<td><strong>URA3</strong> cloning</td>
<td><code>ca-ura-5 TTGGATGGTATAAACGGAAACA</code></td>
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<td><code>ca-ura-3 TCTAGAAGGACCACCTTTGATTG</code></td>
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<td><strong>HIS1</strong> cloning</td>
<td><code>ca-his-5 CCTGGAGGATGAGGAGACAG</code></td>
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<tr>
<td><strong>HIS1</strong> cloning</td>
<td><code>ca-his-3 CCAATATATCGGTTGCACCA</code></td>
</tr>
<tr>
<td>Detection of vector sequences</td>
<td><code>5-detect GTTTTCCCAGTCACGACGTTGTAAAACGAC</code></td>
</tr>
<tr>
<td>Detection of vector sequences</td>
<td><code>3-detect TGTGGAATTGTGAGCGGATAACAATTTCAC</code></td>
</tr>
<tr>
<td>Detection of <strong>hisG</strong> sequences</td>
<td><code>hisG-N CGCGATACAGACCGGTTCAGACAGGADetection of **hisG** sequences</code></td>
</tr>
<tr>
<td>Detection of <strong>hisG</strong> sequences</td>
<td><code>hisG-C TGGTCTTTACTCCATCACAGGGTTCC</code></td>
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<tr>
<td>Disruption of <strong>HRM101</strong></td>
<td><code>RIM101-5DR ACGATCATTGTGTGACGACCATGTTGGTAGAAAGTCTTCGAACAATTTGTCATTGACTTG</code></td>
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<tr>
<td>Disruption of <strong>HRM101</strong></td>
<td><code>RIM101-3DR ACATGGACTCTCAAGTGAGAAGTAATGTGATCTCTCTAACTGTAGTTGTGCCACAATTTT</code></td>
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<tr>
<td>Detection of <strong>HRM101</strong> alleles</td>
<td><code>RIM101-5a GGGGAATTCGTGCTAATCAATCTAACACCACAGCTCTGCDetection of **HRM101** alleles</code></td>
</tr>
<tr>
<td>Detection of <strong>HRM101</strong> alleles</td>
<td><code>seq7 GGTGAACTCAGCCAGAACCTGCGDetection of **HRM101** alleles</code></td>
</tr>
<tr>
<td>Disruption of <strong>ENX3</strong></td>
<td><code>PalA-5DR GCAGCACAAGAGTTAATTAAGAAAGTAGATAAAATGAAACAATATTTGTTACAGGCTAACAACGGAGATGG</code></td>
</tr>
<tr>
<td>Disruption of <strong>ENX3</strong></td>
<td><code>PalA-3DR GGAACGAGTTACTAATAGCTAATTCTAAGTCTCGACTCTCAACTCTTCGTCTATACAAATATTCCTCAC</code></td>
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<tr>
<td>Detection of <strong>ENX3</strong> alleles</td>
<td><code>PalA-5 9 ACTGATGATGCAGCACAAGAGDetection of **ENX3** alleles</code></td>
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<tr>
<td>Detection of <strong>ENX3</strong> alleles</td>
<td><code>PalA3 9 CCAGGTTTACTAATAGTCGGDetection of **ENX3** alleles</code></td>
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</tbody>
</table>

*Boldface sequences in 5DR and 3DR primers are segments that anneal to plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4 and produce vector sequences flanking disruption cassettes.*

*Relevant use of primer in this study.*
**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− transformants.
transformants (Table 2 and Fig. 2), one of which was Arg$^2$. Southern analysis confirmed that the Arg$^2$ transformant carried one copy of arg$^5$:HIS1, one copy of arg$^5$:URA3, and no copies of ARG5 (Fig. 3A, lane 3). Among the other Uri$^+$ transformants, 21 remained Arg$^1$ but had become His$^2$. Southern analysis indicated that the arg$^5$:URA3 PCR product had replaced the arg$^5$:HIS1 allele in two of the transformants that we examined (Fig. 3A, lanes 4 and 5). Thus the majority of transformants had incorporated the arg$^4$:URA3 PCR product by homologous recombination. These results show that an arg$^5$/arg$^5$ 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 ade$^2$:HIS1 and ade$^2$:URA3 PCR products (Fig. 1E). In this case, each PCR product was flanked by 50 bp of homology to ADE2. Transformation of the ade$^2$: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 ade$^2$:HIS1/ade$^2$:HIS1 (Fig. 3B; compare lanes 1 and 2). Transformation of the ade$^2$:URA3 PCR product into Ade-het1 yielded 40 Uri$^+$ transformants (Table 2), 3

### TABLE 2. Summary of PCR product transformation experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Recipient strain (genotype)</th>
<th>PCR product in transformation</th>
<th>Total no. of transformants</th>
<th>No. of integrants at wild-type allele of targeted locus</th>
<th>Screening method(a)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG5 disruption</td>
<td>Arg-het1 (arg$^5$:HIS1/ARG5)</td>
<td>arg$^5$:URA3</td>
<td>18</td>
<td>2</td>
<td>Arg$^2$ phenotype</td>
<td>Arg$^2$ transformants</td>
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<tr>
<td>ADE2 disruption</td>
<td>RM1000 (ade$^2$:HIS1/ade$^2$:URA3)</td>
<td>ade$^2$:URA3</td>
<td>24</td>
<td>3</td>
<td>PCR, Southern &amp; PCR &amp; Southern</td>
<td>Arg$^+$ transformants were not screened</td>
</tr>
<tr>
<td>HRM101 disruption</td>
<td>Adc-het1 (ade$^2$:HIS1/HRM101)</td>
<td>HRM101::URA3</td>
<td>10</td>
<td>2</td>
<td>PCR, PCR, PCR</td>
<td>Arg$^+$ transformants were not screened</td>
</tr>
<tr>
<td>ERY3 disruption</td>
<td>Ena-het1 (ena$^3$:URA3/ENA3)</td>
<td>ena$^3$:URA3</td>
<td>10</td>
<td>2</td>
<td>PCR, PCR, PCR</td>
<td>Arg$^+$ transformants were not screened</td>
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</table>

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\(a\) Results from a single transformation are reported for each disruption. Details of screening methods are described in Materials and Methods and in Results.
of which were Ade<sup>−</sup>. Colonies of the Ade<sup>−</sup> transformants were red in color, as expected for ade2/ade2 mutants (4). Southern analysis of one Ade<sup>−</sup> 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<sup>−</sup> but became His<sup>+</sup>; 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 <i>C. albicans</i> homolog of <i>RIM101</i> may have a role in filamentation. In <i>S. cerevisiae</i>, <i>RIM101</i> (previously called <i>RIM1</i>) specifies a zinc finger protein (20) that is required for haploid invasive growth (8), a form of filamentation (17). We identified a <i>C. albicans</i> sequence that may specify a Rim101p homolog (PacC) lies in a pathway of which were Ade<sup>−</sup>. Colonies of the Ade<sup>−</sup> transformants were red in color, as expected for ade2/ade2 mutants (4). Southern analysis of one Ade<sup>−</sup> 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<sup>−</sup> but became His<sup>+</sup>; 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.

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The two genomic copies of <i>HRM101</i> were inactivated by primer-directed integration of <i>hrm101::ARG4</i> and <i>hrm101::URA3</i> PCR products in strain BWP17 (ura3/ura3 his1/his1 arg4/arg4). We first isolated 11 Arg<sup>+</sup> transformants with an <i>hrm101::ARG4</i> PCR product. PCR amplification with flanking <i>HRM101</i> primers verified presence of the <i>hrm101::ARG4</i> insertion in two of these transformants (Fig. 3C; compare lanes 3 and 2 to lane 1). We used these two <i>hrm101::ARG4/HRM101</i> heterozygotes for transformation with a PCR product specifying <i>hrm101::URA3</i> and obtained 35 Uri<sup>−</sup> transformants. PCR amplification of genomic DNA templates with flanking <i>HRM101</i> primers indicated that three transformants had only <i>hrm101::ARG4</i> and <i>hrm101::URA3</i> alleles and lacked the wild-type <i>HRM101</i> allele (Fig. 3C, lanes 4 to 6). Therefore, we had obtained three independent <i>hrm101::ARG4/HRM101::URA3</i> transformants.

To determine whether <i>HRM101</i> may be required for filamentation, we examined filamentous growth on Spider medium. We observed that <i>HRM101::HRM101</i> and <i>hrm101::ARG4/HRM101</i> strains produced abundant filaments at the edges of colonies (Fig. 4A to C) and that <i>hrm101::ARG4/HRM101</i> 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<sup>−</sup> Uri<sup>−</sup> derivatives of strain BWP17 that retained <i>HRM101</i> alleles were capable of filamentation (data not shown). These observations suggest that <i>HRM101</i> has a positive role in filamentation and that the <i>hrm101::ARG4</i> mutation is recessive.

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

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**FIG. 3. Analysis of transformant strains.** (A) Southern blot analysis of <i>ARG5</i> disruption experiment. Genomic DNA samples, digested with BamHI, were prepared from strain RM1000 (<i>ARG5</i>/<i>ARG5</i> [lane 1]), Arg-het1 (<i>arg5::HIS1/ARG5</i> [lane 2]), a Uri<sup>−</sup> His<sup>+</sup> Arg<sup>−</sup> transformant from Arg-het1 (<i>arg5::HIS1/arg5::URA3</i> [lane 3]), and two Uri<sup>−</sup> His<sup>−</sup> Arg<sup>−</sup> transformants from Arg-het1 (<i>arg5::URA5/ARG5</i> [lanes 4 and 5]). The blot was probed with a 0.7-kbp EcoRI-EcoRV fragment of <i>ARG5</i>, as indicated in Fig. 1D. (B) Southern blot analysis of <i>ADE2</i> disruption experiment. Genomic DNA samples, digested with BglII and HindIII, were prepared from strain RM1000 (<i>ADE2/ADE2</i> [lane 1]), Ade-het1 (<i>ade2::HIS1/ADE2</i> [lane 2]), a Uri<sup>−</sup> His<sup>−</sup> Ade<sup>−</sup> (red) transformant from Ade-het1 (<i>ade2::HIS1/ade2::URA4</i> [lane 3]), and two Uri<sup>−</sup> His<sup>−</sup> Ade<sup>−</sup> transformants from Ade-het1 (<i>ade2::URA3/ADE2</i> [lanes 4 and 5]). The blot was probed with a 0.8-kbp EcoRI-EcoRV fragment of <i>ADE2</i>, as indicated in Fig. 1E. (C) PCR analysis of <i>HRM101</i> disruption experiment. PCR amplification with primers <i>RIM101-5a</i> and <i>seq7</i> was carried out on genomic DNA templates from strain BWP17 (Fig. 3B, lane 1). Hrm-het1 (hrm1::ARG4/HRM101 [lane 1]), Hrm101 (hrm1::ARG4/HRM101 [lane 1]), and Uri<sup>−</sup> Arg<sup>−</sup> transformants from Hrm-het1 and Hrm-het2 (hrm1::ARG4/hrm1::URA3 [strains BWP29, BWP30, and BWP31]) (lanes 4 to 6). Therefore, we had obtained three independent <i>hrm101::ARG4/hrm101::URA3</i> transformants.
and H), and DAY23 and DAY24 (both enx3::URA3/enx3::ARG4 homozygotes were defective in filamentation on Spider medium (Fig. 4I and J)). 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-ARG4ΔSpeI 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 hem101 and enx3 homozygotes would have similar phenotypes, and this prediction was verified. However, we might have found that enx3 and hmr101 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 hmr101/hmr101 homozygotes have a filamentation defect supports the idea that the phenotype arises from the hmr101 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 Hmr101p and Enx3p may have related functions.

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