

# Strains and Strategies for Large-Scale Gene Deletion Studies of the Diploid Human Fungal Pathogen *Candida albicans*

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***Candida albicans* is the most common human fungal pathogen and causes significant morbidity and mortality worldwide. Nevertheless, the basic principles of *C. albicans* pathogenesis remain poorly understood. Of central importance to the study of this organism is the ability to generate homozygous knockout mutants and to analyze them in a mammalian model of pathogenesis. *C. albicans* is diploid, and current strategies for gene deletion typically involve repeated use of the *URA3* selectable marker. These procedures are often time-consuming and inefficient. Moreover, *URA3* expression levels—which are susceptible to chromosome position effects—can themselves affect virulence, thereby complicating analysis of strains constructed with *URA3* as a selectable marker. Here, we describe a set of newly developed reference strains (*leu2Δ/leu2Δ*, *his1Δ/his1Δ*; *arg4Δ/arg4Δ*, *his1Δ/his1Δ*; and *arg4Δ/arg4Δ*, *leu2Δ/leu2Δ*, *his1Δ/his1Δ*) that exhibit wild-type or nearly wild-type virulence in a mouse model. We also describe new disruption marker cassettes and a fusion PCR protocol that permit rapid and highly efficient generation of homozygous knockout mutations in the new *C. albicans* strains. We demonstrate these procedures for two well-studied genes, *TUPI* and *EFG1*, as well as a novel gene, *RBD1*. These tools should permit large-scale genetic analysis of this important human pathogen.**

*Candida albicans* is a major human pathogen that is especially virulent among people with compromised immune systems, such as premature infants, cancer chemotherapy patients, solid-organ transplant recipients, and patients coinfecting with human immunodeficiency virus. The direct cost of treating *Candida* bloodstream infections in the United States is between \$1 and \$2 billion annually (34, 51), and despite aggressive therapies, mortality approaches 50% (49, 18, 32, 3). Complicating efforts to treat disseminated candidiasis is a continued ignorance of basic principles of *Candida* pathogenesis. Unlike many bacterial pathogens, the pathways to virulence of most pathogenic fungi remain largely mysterious. In the case of *C. albicans*, this situation is largely attributable to its combination of a diploid genome and an incomplete sexual cycle, both of which have hindered forward genetic approaches to the study of virulence.

Several reagents are now available which, in principle, should make it possible to apply powerful genetic approaches to understanding *C. albicans* pathogenesis. The new tools include a fully sequenced genome (<http://www-sequence.stanford.edu/group/candida/>), molecular biological techniques, including protocols for gene disruption (17, 52), microarray technology for expression profiling (23, 26, 27, 5, 41), and mammalian animal model systems for both localized and disseminated infection (28, 31). The mouse model of intravenous infection via the tail vein has been particularly useful in assessing *C. albicans* behavior in disseminated infection and, at least grossly, resembles disseminated infection in the human host. As in the mouse model, clinically significant candidiasis of humans commonly originates intravenously via contaminated

catheters in hospitalized patients. As is true for the mouse (40), the brain and kidneys are prime targets of disseminated infection in humans. If the *C. albicans* genes mediating virulence in the mouse model overlap those responsible for pathogenesis in humans, this model will be key to elucidating new targets for antimicrobial therapy.

In light of the central role of the mouse model in studying *C. albicans* pathogenesis, a *C. albicans* genetic system should be compatible with it. For historical reasons, the majority of *C. albicans* knockout strains have been generated with the selectable auxotrophic marker *URA3*; however, *URA3* expression levels are now known to influence *C. albicans* virulence (8, 10, 22, 45) as well as morphogenesis and adhesion (4, 10). In some cases, the extent of *URA3* expression has been shown to vary with the chromosomal position it occupies (8, 10, 24). Use of *URA3* at heterologous loci to mark gene knockouts may therefore confound interpretation of observed virulence defects, and it has been suggested that as many as 30% of published virulence mutants have misattributed virulence defects linked to *URA3* to deletions of other genes (8). One proposed solution to this problem is to construct knockout mutants for virulence analysis so that *URA3* is restored to a single chromosomal location from which it is consistently well expressed (e.g., the *ENO1* or *RPS10* locus) (45). Another solution is to use a dominant selectable marker that can be recycled for each round of gene disruption (35). A third solution, which we propose below, is to use alternative auxotrophic markers that do not significantly influence virulence.

A second, more general problem with current *C. albicans* gene disruption methodology is the absence of reliable and efficient protocols. Because *C. albicans* is diploid, complete abolition of gene function requires disruption of both alleles of a given target gene. A typical gene disruption protocol (52) uses long PCR primers with 60 to 80 nucleotides of homology to genomic DNA flanking the target genes to amplify the

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*URA3* gene. The *URA3*-linked disruption fragment is transformed into a  $Ura^-$  *C. albicans* strain, and  $Ura^+$  transformants are screened for the absence of one allele of the target gene. To recycle the *URA3* marker, heterozygous disruption mutants are passaged on medium containing 5-fluoroorotic acid (1, 6), which selects intragenic recombinations that delete the *URA3* gene. Exposure of *C. albicans* to 5-fluoroorotic acid also generates unlinked chromosomal rearrangements and other genetic abnormalities (50a), making this step a potential source of unwanted genetic changes. Finally, the  $Ura^-$  heterozygous knockout strain is transformed with the same *URA3*-linked disruption fragment to disrupt the second allele of the target gene. Often the second gene disruption event is inefficient, with the majority of  $Ura^+$  transformants resulting from reinsertion of the disruption fragment into the first knockout locus or elsewhere in the genome. In some cases, it has been necessary to screen more than 100 transformants by PCR to obtain a single strain in which the second allele was properly disrupted (our unpublished results).

In this paper, we address these problems through the derivation of new *C. albicans* strains as well as supporting plasmids and protocols. We describe three newly constructed *C. albicans* strains that contain homozygous null mutations in *HIS1* and *LEU2* (strain SN87), *HIS1* and *ARG4* (strain SN95), or *HIS1*, *LEU2*, and *ARG4* (strain SN152). These strains have normal karyotypes, as determined by pulsed-field gel electrophoresis, and we show that disruption of *HIS1*, *LEU2*, or *ARG4* in these strains has minimal effect on *C. albicans* virulence in the mouse tail vein injection model, permitting use of these genes to mark deletions for virulence analysis. With a fusion PCR technique and homologous complementing *HIS1*, *LEU2*, and *ARG4* plasmids, we show that genes can be rapidly and efficiently knocked out, providing as specific examples two previously studied genes, *EFG1* and *TUP1*, and one previously uncharacterized gene, *RBD1*. An important component of the new method is the use of *HIS1*, *LEU2*, and *ARG4* from non-*C. albicans* *Candida* species as selectable markers for the gene disruption steps; the heterologous markers likely suppress undesirable recombination with the chromosomal marker loci. These strains and procedures permit the rapid creation and testing of *C. albicans* knockout mutants that are suitable for animal studies of *C. albicans* virulence.

## MATERIALS AND METHODS

**Construction of reference strains.** See Table 2 for the sequences of all PCR primers. Fusion PCR (see below) (50) was performed to create disruption fragments of *C. albicans* *LEU2* and *ARG4* coding sequences. For the first round of PCRs (Fig. 5B), genomic DNA from *C. albicans* strain CAF2-1 (17) and *LEU2* and *ARG4* primer pairs 1 and 3 and 4 and 6 were used to amplify the 5'- and 3'-flanking sequences, respectively, for the two genes. *URA3* flanked by 200-nucleotide repeats was amplified from pDDB57 (52) with pDDB57 primers 2 and 5. The remainder of the fusion PCR protocol was performed as described below.

The *LEU2* and *ARG4* disruption fragments were transformed into RM1000#2 by standard methods (39) and plated on medium without uracil.  $Ura^+$  transformants were tested by colony PCR for the presence of correct knockout junctions with the following primers: for the 5' ends, *LEU2* Upstream Check or *ARG4* Upstream Check plus *URA3* Left, and, for the 3' ends, *LEU2* Downstream Check or *ARG4* Downstream Check plus *URA3* Right. Positives from these diagnostic PCRs were confirmed by colony PCR with primers that flank the disrupted locus (i.e., Upstream and Downstream Check primers); positives in this test had two bands, one of normal genomic size and a second, knockout product that was longer because of the introduction of *URA3*.

PCR-confirmed heterozygous disruptants were streaked onto 5-fluoroorotic acid-containing medium to recycle the *URA3* marker as previously described (6, 1).  $Ura^-$  strains were transformed with the disruption fragments to knock out the second allele of *LEU2* or *ARG4*.  $Ura^+$  transformants were screened as above with the Upstream Check and Downstream Check PCR primers for loss of the shorter PCR product corresponding to the intact gene and persistence of the longer PCR product corresponding to the disrupted alleles. Candidates that passed this test were subjected to a final PCR to exclude the ectopic presence of *LEU2* or *ARG4* elsewhere in the genome, with primers that amplify sequences within the *LEU2* and *ARG4* genes, *LEU2* open reading frame (ORF) 5' and *LEU2* ORF 3' or *ARG4* ORF 5' and *ARG4* ORF 3'. Strains that were correct by PCR, that had expected growth on appropriate dropout media, and that had a normal karyotype by contour-clamped homogenous electric field gel analysis (below) were designated SN78 ( $Leu^- His^- Ura^-$ ) and SN76 ( $Arg^- His^- Ura^-$ ). Note that SN76 is similar to the previously published reference strain BWP17 (53), with the exception that SN76 has a full-length copy of chromosome 5.

Strain SN148 ( $Arg^- Leu^- His^- Ura^-$ ) was derived from SN76 by repetition of the *LEU2* gene disruption process, as described above.

*URA3* (and the adjacent gene, *IRO1*) were restored to their normal loci in SN78, SN76, and SN148 by transformation with the 4.9-kb PstI-BglII restriction fragment from pLUBP (33). Transformants were selected on medium without uracil, and integration at the proper locus was confirmed by colony PCR with a primer upstream of the integrated fragment (*URA3* Left Flank) and a primer internal to the restored sequence (*URA3* Right Flank). The new strains, now heterozygous for wild-type *URA3* at the genomic locus, were designated SN87 ( $Leu^- His^-$ ), SN95 ( $Arg^- His^-$ ), and SN152 ( $Arg^- Leu^- His^-$ ).

**CHEF gel analysis.** Chromosome plugs were prepared from strains SC5314 (19), RM1000 (29), RM1000#2 (B. B. Magee and P. T. Magee, unpublished), and SN78, SN76, SN87, SN95, SN148, and SN152 as described previously (11). Plugs were introduced into 1% agarose gels, and electrophoresis was performed with a Bio-Rad CHEF-DRIII system. The gels were run in 0.5x Tris-borate-EDTA buffer and maintained at 14°C throughout the procedure. Electrophoresis settings were as follows (adapted from those described on the National Research Council of Canada website for the Whiteway laboratory, <http://cbr-rbc.nrc-cnrc.gc.ca/biovis/candida>): field angle was 106° throughout; during the first 24 h, voltage was 4.2 V/cm with a switch time of 300 s; for the subsequent 96 h, voltage was 2.4 V/cm with a switch time of 1,200 s.

**Cloning of *C. maltosa* *LEU2*, *C. dubliniensis* *HIS1*, and *C. dubliniensis* *ARG4* genes.** *C. maltosa* *LEU2* was amplified by PCR with primers *C. maltosa* *LEU2* 5' and *C. maltosa* *LEU2* 3' as well as a template of *C. maltosa* genomic DNA (prepared from strain 38041, obtained from the American Type Culture Collection). *C. dubliniensis* *HIS1* (cloned before the *C. dubliniensis* genomic sequence was available) was amplified with primers based on *C. albicans* genomic sequence, *C. albicans* *HIS1* 5' and *C. albicans* *HIS1* 3', and *C. dubliniensis* genomic DNA (prepared from strain MYA-577, obtained from the American Type Culture Collection). *C. dubliniensis* *ARG4* was amplified with primers *C. dubliniensis* *ARG4* 5' and *C. dubliniensis* *ARG4* 3' and *C. dubliniensis* genomic DNA. The *C. dubliniensis* *ARG4* primers were designed with sequences provided by the Wellcome Trust Sanger Institute Pathogen Sequencing Unit, available online at [http://www.sanger.ac.uk/Projects/C\\_dubliniensis/](http://www.sanger.ac.uk/Projects/C_dubliniensis/).

The PCR products were ligated via blunt ends to the vector, pCR-BluntII-TOPO, with the Zero Blunt TOPO PCR cloning kit from Invitrogen, creating plasmids pSN40 (*C. maltosa* *LEU2*), pSN52 (*C. dubliniensis* *HIS1*), and pSN69 (*C. dubliniensis* *ARG4*). The PCR inserts pf pSN40 and pSN52 were sequenced, and the nucleotide sequences of *C. dubliniensis* *HIS1* and *C. maltosa* *LEU2* have been deposited in GenBank (accession numbers AY534141 and AY534142, respectively).

**Complementation analysis with *C. maltosa* *LEU2*.** For complementation analysis, the *C. maltosa* *LEU2* gene was subcloned into pBES116 (16), a plasmid that interposes the *URA3* gene and a polylinker sequence between genomic sequences that flank the *ADE2* gene. An EcoRI-EcoRI fragment from pSN40 was blunt ended with T4 DNA polymerase and ligated to EcoRV-cut pBES116. The resulting plasmid was digested with AscI to generate an integrating fragment that targets *C. maltosa* *LEU2* to the *C. albicans* *ADE2* gene locus.

The integrating fragment was transformed into SN78 ( $Leu^- Arg^-$ ), and transformants were selected on uracil dropout medium.  $Ura^+$  transformants were screened for the ability to grow on leucine dropout medium, as shown for one transformant in Fig. 3B (incubated at 30°C for 3 days).

**Virulence studies. (i) Homogeneous infections.** Strains SC5314, RM1000#2, SN100, SN87, SN95, and SN152 were inoculated from overnight cultures 1:30 into fresh YEPD medium and grown for 4 h at 30°C. Logarithmically growing cells were pelleted, washed in sterile normal saline, and counted with a hemacytometer;  $8 \times 10^5$  cells of each strain were inoculated into the tail veins of 8-

10-week-old BALB/c mice, seven mice per *C. albicans* strain. The mice were monitored daily and sacrificed when moribund (defined as hunched posture, minimal motor activity, and weight loss of >15% of starting body weight).

**(ii) Competition experiment** Strains RM1000#2, SN100, SN87, and SN95 were inoculated from saturated overnight cultures into fresh YEPD medium and washed as above. Equal numbers of cells from the four strains were mixed, and  $5 \times 10^5$  cells of the mixture were inoculated into the tail veins of five BALB/c mice. The mice were monitored daily and sacrificed when moribund. The infected kidneys were recovered, homogenized, diluted, and plated on Sabouraud agar with added ampicillin (50  $\mu$ g/ml) and gentamicin (15  $\mu$ g/ml). Recovered CFU were grown for 2 days at 30°C and replica plated to appropriate dropout media to determine the relative numbers of each of the four starting strains.

Statistical analysis of differences between growth curves was performed with the log-rank test.

**Fusion PCR and creation of *efg1*, *tup1*, and *rbd1* mutants.** The fusion PCR strategy (50) is outlined in Fig. 4; see Table 2 for primer sequences. The first round of reactions involves amplification of the flanking sequences of the target gene (with a template of genomic DNA and primers 1 and 3 or 4 and 6, in separate reactions) and the selectable marker (with cloned *HIS1*, *LEU2*, or *ARG4* and primers 2 and 5). The 5' tails of primers 2 and 3 are complementary, as are the 5' tails of primers 4 and 5. In the second or "fusion" round of PCR, all three products of the first round are combined, and a fusion product is amplified with primers 1 and 6.

*TUP1*, *EFG1*, and *RBD1* gene disruption fragments were created as follows. For the first round of PCRs, 50- $\mu$ l reactions were assembled with 0.5  $\mu$ l of KlenTaq LA DNA polymerase (BD Biosciences) in 1x KlenTaq buffer, 0.25 mM deoxynucleoside triphosphates, 0.2 uM each primer, and appropriate template DNA. The template for flanking sequences was CAF2-1 genomic DNA, and the template for *C. maltosa LEU2*, *C. dubliniensis HIS1*, and *C. dubliniensis ARG4* markers was pSN40, pSN52, and pSN69, respectively. The reactions were incubated as follows: 93°C for 5 min; then 35 cycles of 93°C for 30 s, 45°C for 45 s, and 72°C for 3 min; and finally 72°C for 10 min. The flanking sequence products were purified with the QIAquick PCR purification kit (Qiagen), whereas the marker products were gel purified on a 1% agarose gel prior to purification with the QIAquick gel extraction kit (Qiagen).

For the fusion reactions, 50- $\mu$ l reactions were assembled as above except that the template consisted of 1  $\mu$ l of each of the three preceding reaction products, and the primers were primers 1 and 6. Reaction temperatures were as follows: 93°C for 5 min; then 35 cycles of 93°C for 30 s, 50°C for 45 s, and 72°C for 4.5 min; and finally 72°C for 10 min. The fusion PCR product was purified with the QIAquick PCR purification kit prior to transformation into *C. albicans* strains SN87, SN95, and SN152.

The primers used for diagnosis of the three gene knockouts were, for the 5' junctions, *TUP1* Upstream Check, *EFG1* Upstream Check, or *RBD1* Upstream Check plus the Left primer for *HIS1*, *LEU2*, or *ARG4*, as appropriate; for the 3' junctions, *TUP1* Downstream Check, *EFG1* Downstream Check, or *RBD1* Downstream Check plus the Right primer for *HIS1*, *LEU2*, or *ARG4*; and, for trans-ORF PCR, the Upstream and Downstream checks as appropriate for each gene.

**Complementation analysis of *rbd1* and *tup1* mutants.** Vectors pSN75 and pSN78 were created to facilitate complementation analysis of homozygous disruption mutants by targeting a wild-type version of the appropriate gene to the *RPS10* locus, with *C. dubliniensis HIS1* or *C. maltosa LEU2*, respectively, as the selectable marker. One-kilobase regions just upstream and downstream of the *RPS10* gene were amplified by PCR with primers *RPS10* 5' HindIII and *RPS10* 5' SalI, and *RPS10* 3' EcoRV and *RPS10* 3' SacII, with CAF2-1 genomic DNA as the template. The primers included sites for the named restriction enzymes at their 5' ends. PCR products were cut with the appropriate restriction enzymes and subcloned into the vector backbone of pAG25 (20). To create pSN75, *C. dubliniensis HIS1* was subcloned from pSN52 as a BamHI-NotI (blunt ended at NotI with T4 DNA polymerase) fragment into the BamHI- and EcoRV-cut intermediate plasmid; this placed the selectable marker between the *RPS10* upstream and downstream sequences. To create pSN78, *C. maltosa LEU2* was subcloned from pSN40 as a SacI-NotI (blunt ended at both sites with T4 DNA polymerase) fragment into the same intermediate plasmid, this time cut with BamHI and EcoRV and blunt-ended with T4 DNA polymerase.

Wild-type *RBD1* was amplified by PCR with primers *RBD1* 5' XhoI and *RBD1* 3' XhoI, cut with XhoI, and subcloned into the XhoI site of pSN75 (between the *RPS10* upstream sequence and *C. dubliniensis HIS1*) to create plasmid pSN84. Wild-type *TUP1* was amplified with primers *TUP1* 5' SacI and *TUP1* 3' SacI, cut with SacI, blunt-ended with T4 DNA polymerase, and subcloned into pSN78 cut with SalI and blunt ended with T4 DNA polymerase to create plasmid pSN83.

The wild-type *RBD1* and *TUP1* complementation fragments were released

from pSN84 and pSN83 with HisI (*RBD1*) or NotI (*TUP1*) (both enzymes cut just upstream of the *RPS10* upstream flanking sequence) and SacI, which cuts just downstream of the *RPS10* downstream flanking sequence, purified with a QIAquick PCR purification kit (Qiagen), and transformed into the *rbd1* $\Delta$ /*rbd1* $\Delta$  and *tup1* $\Delta$ /*tup1* $\Delta$  strains, respectively, selecting on medium lacking leucine, arginine, and histidine.

Growth of the homozygous deletion mutants, addback strains, and CAF2-1 was assessed by plating dilutions of each strain onto Spider medium and incubating the plates at 30°C. Growth of single colonies was assessed after 6, 9, and 12 days.

**Nucleotide sequence accession numbers.** Sequence data from this article have been deposited with the EMBL and GenBank data libraries under accession numbers AY534141 and AY534142.

## RESULTS

### Construction of new *Candida albicans* reference strains.

RM1000 (*ura3* $\Delta$ /*ura3* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *iro11* $\Delta$ /*iro1* $\Delta$ ) is a strain derived from the clinical isolate SC5314 and is routinely used to make gene disruptions in *C. albicans* (29) (Table 1). Recent karyotyping of this strain by B. B. Magee and P. T. Magee and by our laboratory has revealed a large deletion in one copy of chromosome 5 (P. T. Magee, personal communication; A. E. Tsong and A. D. Johnson, unpublished observations). Shown in Fig. 1, lanes 1 and 2, are the contour-clamped homogeneous electric field gel results for chromosomes prepared from SC5314 and RM1000, respectively; the increased mobility of one copy of chromosome 5 can easily be observed.

With an independent isolate of RM1000 (called RM1000#2) provided by the Magee laboratory that is wild type for both copies of chromosome 5 (P. T. Magee, personal communication; Fig. 1, lane 3), we have derived three new strains for gene disruption and virulence analysis. The genotypes of the strains are presented in Table 1.

The rationale for strain design was as follows. For reasons outlined in the introduction, we wished to avoid use of *URA3* as a marker for gene disruption studies. Therefore we sought alternative auxotrophic markers that would be neutral for virulence in the mouse. *HIS1* and *LEU2* have previously been reported to have minimal effects on *C. albicans* virulence (2, 22), and *ARG4* was previously untested. With a fusion PCR technique (see below and Materials and Methods) and the vector pDDB57 (encoding a recyclable *URA3* gene flanked by 200-nucleotide direct repeats [52]), we created strains SN78 (*leu2* $\Delta$ /*leu2* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *ura3* $\Delta$ /*ura3* $\Delta$ ), SN76 (*arg4* $\Delta$ /*arg4* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *ura3* $\Delta$ /*ura3* $\Delta$ ), and SN148 (*arg4* $\Delta$ /*arg4* $\Delta$ , *leu2* $\Delta$ /*leu2* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *ura3* $\Delta$ /*ura3* $\Delta$ ). The disruption primers (Table 2) were designed to precisely delete the open reading frames of the auxotrophic markers while leaving promoter and termination sequences intact, so as to minimize disruptions of neighboring genes. To restore prototrophy for uracil biosynthesis to the three strains, a 4.9-kb BglII-PstI fragment from pLUBP (encoding wild-type *URA3* as well as *IRO1* [33]) was introduced into SN78, SN76, and SN148, creating strains SN87 (*leu2* $\Delta$ /*leu2* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *URA3*/*ura3* $\Delta$ ), SN95 (*arg4* $\Delta$ /*arg4* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *URA3*/*ura3* $\Delta$ ), and SN152 (*arg4* $\Delta$ /*arg4* $\Delta$ , *leu2* $\Delta$ /*leu2* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *URA3*/*ura3* $\Delta$ ), respectively. These strains all have one copy of *URA3* restored to its normal genetic locus.

At each step of strain construction, integration of the disruption construct at the correct genomic location was verified by PCR of the 5' and 3' ends of the fragment to genomic DNA to assess disruption junctions; PCR across the genomic locus to

TABLE 1. *C. albicans* strains used in this study

Strain	Phenotype	Relevant genotype <sup>a</sup>	Source or reference
SC5314	Prototroph	Wild-type clinical isolate	19
RM1000	His <sup>-</sup> Ura <sup>-</sup>	<i>his1Δ/his1Δ ura3Δ::imm<sup>434</sup>/ura3Δ::imm<sup>434</sup></i> <i>iro1Δ::imm<sup>434</sup>/iro1Δ::imm<sup>434</sup></i> heterozygous deletion in chromosome 5	29
RM1000#2	His <sup>-</sup> Ura <sup>-</sup>	<i>his1Δ/his1Δ ura3Δ::imm<sup>434</sup>/ura3Δ::imm<sup>434</sup></i> <i>iro1Δ::imm<sup>434</sup>/iro1Δ::imm<sup>434</sup></i>	B. B. Magee and P. T. Magee, unpublished data
CAF2-1	Prototroph	<i>ura3Δ::imm<sup>434</sup>/URA3 iro1Δ::imm<sup>434</sup>/IRO1</i>	17
SN49	His <sup>-</sup> Ura <sup>-</sup>	<i>leu2Δ::URA3/LEU2 ura3Δ/ura3Δhis1Δ/his1Δ</i>	This study
SN76	Arg <sup>-</sup> His <sup>-</sup> Ura <sup>-</sup>	<i>arg4Δ/arg4Δ his1Δ/his1Δ ura3Δ::imm<sup>434</sup>/ura3Δ::imm<sup>434</sup></i> <i>iro1Δ::imm<sup>434</sup>/iro1Δ::imm<sup>434</sup></i>	This study
SN78	Leu <sup>-</sup> His <sup>-</sup> Ura <sup>-</sup>	<i>leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm<sup>434</sup>/ura3Δ::imm<sup>434</sup></i> <i>iro1Δ::imm<sup>434</sup>/iro1Δ::imm<sup>434</sup></i>	This study
SN87	Leu <sup>-</sup> His <sup>-</sup>	<i>leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN95	Arg <sup>-</sup> His <sup>-</sup>	<i>arg4Δ/arg4Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN100	His <sup>-</sup>	<i>his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN148	Arg <sup>-</sup> Leu <sup>-</sup> His <sup>-</sup> Ura <sup>-</sup>	<i>arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm<sup>434</sup>/ura3Δ::imm<sup>434</sup></i> <i>iro1Δ::imm<sup>434</sup>/iro1Δ::imm<sup>434</sup></i>	This study
SN152	Arg <sup>-</sup> Leu <sup>-</sup> His <sup>-</sup>	<i>arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup></i> <i>IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN171	Leu <sup>-</sup> Tup1 <sup>-</sup>	<i>tup1Δ::C.d.HIS1/tup1Δ::C.d.ARG4 arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN181	Tup1 addback	<i>tup1Δ::C.d.HIS1/tup1Δ::C.d.ARG4 rps10Δ::TUP1-C.m.LEU2/RPS10 arg4Δ/arg4Δ, leu2Δ/leu2Δ his1Δ/his1Δ, URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN198	His <sup>-</sup> Rbd1 <sup>-</sup>	<i>rbd1Δ::C.m.LEU2/rbd1Δ::C.d.ARG4 arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study
SN203	Rbd1 addback	<i>rbd1Δ::C.m.LEU2/rbd1Δ::C.d.ARG4 rps10Δ::RBD1-C.d.HIS1/RPS10 arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm<sup>434</sup> IRO1/iro1Δ::imm<sup>434</sup></i>	This study

<sup>a</sup> C.d., *C. dubliniensis*; C.m., *C. maltosa*.

confirm alteration in the size of the disrupted gene; and (for homozygous disruption mutants) PCR with primers internal to the disrupted open reading frame (ORF) to confirm the absence of the ORF at any position in the genome (data not shown). To verify proper restoration of *URA3-IRO1* in strains SN87 and SN95, PCR was performed with one primer from within the restored sequence and another from flanking genomic DNA (data not shown).

**New strains possess the expected auxotrophies.** SN78, SN76, SN87, SN95, SN148, and SN152 behave as predicted on uracil, histidine, leucine, and arginine amino acid dropout plates (Fig. 2). In each case, the strains are unable to grow on medium lacking the amino acid whose synthesis gene was targeted by the disruption but are able to grow normally on YEPD as well as other dropout media. These growth defects independently confirm that the targeted gene disruptions inactivate both alleles of the auxotrophic marker genes.

**New strains possess full-length copies of chromosome 5.** Chromosomal DNAs prepared from SC5314 RM1000#2 and the six new strains were resolved by contour-clamped homogeneous electric field gel electrophoresis, shown in Fig. 1 (lanes 4 through 11). RM1000#2 DNA (Fig. 1, lanes 3 and 5) has two normal-length copies of chromosome 5 (which comigrate with those of SC5314; Fig. 1, lanes 1 and 3), as do the six new strains (Fig. 1, lanes 6 through 11). By this assay, all seven numbered chromosomes appear normal in the new strains; however, we cannot rule out small differences in chromosomes that would not be detected by this low-resolution method. Chromosome R—characterized by hundreds of repeats of the rRNA genes—has altered mobility in several strains; such variation in length

of chromosome R is commonly observed among clinical and laboratory isolates and is not associated with defective virulence (37).

**Unlike *URA3*, deletions of *HIS1*, *LEU2*, and *ARG4* do not strongly influence virulence of *C. albicans* in the mouse model.** To assess the effects of sequential deletion of *HIS1*, *LEU2*, and *ARG4* as well as *URA3* on *C. albicans* virulence in the mouse model, the new strains were compared to each other, to the RM1000#2 parental strain (Ura<sup>-</sup> His<sup>-</sup>), and to SN100 (RM1000#2 to which one copy of *URA3* has been restored); these results are presented in Fig. 3. Except for the indicated differences in auxotrophic markers, these strains are isogenic. To confirm that the derived strains retain wild-type virulence, SN100 was also compared to SC5314, the wild-type clinical isolate that is the ultimate progenitor of the other strains.

Figure 3A presents a plot of time to illness of seven immunocompetent BALB/c mice infected with  $8 \times 10^5$  CFU of SN100 (His<sup>-</sup>) or the Ura<sup>-</sup> but otherwise isogenic parental strain RM1000#2 (Ura<sup>-</sup> His<sup>-</sup>). Whereas all mice infected with SN100 became moribund within 1 week, those infected with RM1000#2 continued to be healthy and to gain weight until the experiment was terminated at 60 days (the results for only the first 20 days are depicted in Fig. 1a). This difference in virulence is highly statistically significant ( $P = 0.0004$ ) and echoes previous reports of the strong influence of *URA3* on virulence (19). In contrast, Fig. 3B depicts a comparison between mice infected with SN100 (His<sup>-</sup>), SN87 (His<sup>-</sup> Leu<sup>-</sup>), and SN95 (His<sup>-</sup> Arg<sup>-</sup>) under the same conditions. Here, the morbidity curves were quite similar and statistically indistinguishable.

TABLE 2. Primers used in this study

Sequence <sup>a</sup>	Name
AAGCCGAAGTCGACTATGTC	LEU2 primer 1
cacggcgcctagcagcggGATATTGGTTTTAAAAGAAAGG	LEU2 primer 3
gtcagggcgcctacccctgcACAGTATATACAGTAGTTAGC	LEU2 primer 4
TGACAAATGAATTCAGTCAG	LEU2 primer 6
GATGCCTTAGCTCATTCTTC	LEU2 Upstream Check
GACCAGACTCTATCCCAAAG	LEU2 Downstream Check
CTTGCCTGGTGACCATGTCCG	LEU2 ORF 5'
ACCGACTTCTGTAGTAGAGC	LEU2 ORF 3'
GGACAGAAAGTTATTGTACAG	ARG4 primer 1
cacggcgcctagcagcggGATTATTCTTGATAGCTGTTATG	ARG4 primer 3
gtcagggcgcctacccctgcGTCATATAATAATCACAGTATTG	ARG4 primer 4
TCAGACGATCTTTACAATGG	ARG4 primer 6
CAAGAGTAGTCTCAAATAAACC	ARG4 Upstream Check
CGTTTGAAGCTGTATATCG	ARG4 Downstream Check
CTGGTACTAAAGTATACACAC	ARG4 ORF 5'
CACTTTTAGCAGTACCACCA	ARG4 ORF 3'
ccgctgtaggcgcgcctgTGTGGAATTGTGAGCGGATA	pDDB57 (URA3) primer 2
gcaggatgcggcgcctgacTTTCCCAGTCACGACGTT	pDDB57 (URA3) primer 5
TATCGATAACCGTCGACCTCG	URA3 Right (near primer 2)
AATTCGCCCTATAGTGAGTC	URA3 Left (near primer 5)
GGAACTGATATTATCATTGTTGG	URA3 Internal (near primer 2)
TGTTTCCGTTTATACCATCC	URA3 Internal (near primer 5)
GATGTTCTTGAAGCGTAAAAC	URA3 left flank (near primer 2)
CACCGTCGATAGTTTTACGG	URA3 right flank (near primer 5)
GGATCCAATCATCACTGGTG	<i>C. maltosa</i> LEU2 5'
ACCTACCCATGTCTAGAAAG	<i>C. maltosa</i> LEU2 3'
GTTTGATACCATGGCAAGCATAC	<i>C. albicans</i> HIS1 5'
CTAGAAAACCGTACCAGGTGAAC	<i>C. albicans</i> HIS1 3'
AAGAACATTTCTGTACCGCA	<i>C. dubliniensis</i> ARG4 5'
ACAAAAGCTATTTGCATCGT	<i>C. dubliniensis</i> ARG4 3'
TTTTGTTGTGAAGATACACG	EFG1 primer 1
cacggcgcctagcagcggGTTATATTCTTGGTAGTCAAATAG	EFG1 primer 3
gtcagggcgcctacccctgcTATCATTCGTGTACATCACC	EFG1 primer 4
TGGAAAAGACAATGACTTAC	EFG1 primer 6
GGAAACATACATACACTAAC	EFG1 Upstream Check
CTTGTTTGAATATTTTAGATAC	EFG1 Downstream Check
TTGGGCAGTGACAGCTTTAC	TUP1 primer 1
cacggcgcctagcagcggGATGGAAGAAGTTGTGTTGT	TUP1 primer 3
gtcagggcgcctacccctgcTAGTATATATATATGTGAGAAA	TUP1 primer 4
GGAGTATCTAATGATTCCAA	TUP1 primer 6
CTTTTACAAAACACTCTGTG	TUP1 Upstream Check
TTCGATAGAGGCACGTAAC	TUP1 Downstream Check
ATATTTATTTATATACTTATTTAT	RBD1 primer 1
cacggcgcctagcagcggATATTGTTGAATCTTTTAGGGGAG	RBD1 primer 3
gtcagggcgcctacccctgcAGAAAAGAATTGATTAGAAATGTC	RBD1 primer 4
TGGATAGATCTTTGTTAATCTTTC	RBD1 primer 6
AGGAATCAAACATCCCACCTTTATT	RBD1 Upstream Check
TAAGCATTATAAAAGGGGTGGTTC	RBD1 Downstream Check
ccgctgtaggcgcgcctgACCAGTGTGATGGATATCTGC	Universal primer 2 (for <i>HIS1</i> , <i>LEU2</i> , and <i>ARG4</i> cassettes)
gcaggatgcggcgcctgacAGCTCGGATCCACTAGTAACG	Universal primer 5 (for <i>HIS1</i> , <i>LEU2</i> , and <i>ARG4</i> cassettes)
ATTAGATACGTTGGTGGTTC	<i>HIS1</i> Left (near primer 2)
AACACAACCTGCACAATCTGG	<i>HIS1</i> Right (near primer 5)
AGAATTCCCAACTTTGTCTG	<i>LEU2</i> Left (near primer 2)
AAACTTTGAACCCGGCTGCG	<i>LEU2</i> Right (near primer 5)
ACACAGAGATACCTTGTACT	<i>ARG4</i> Left (near primer 2)
ACGGATACCACATACGATG	<i>ARG4</i> Right (near primer 5)
TAATAAGCTTCCCACGTTAACAATTTTCATCAAG	<i>RPS10</i> 5' HindIII
TAATGTCGACGCCATGTTGTACTTGAGTTGGTG	<i>RPS10</i> 5' SalI
TAATGATATCGGATAATGATAACTGAAGAGAAG	<i>RPS10</i> 3' EcoRV
TAATCCGCGGCTCGACTATTACTCATTGATAAAGAC	<i>RPS10</i> 3' SacII
TAACTCGAGTATAACAAGCCAATGAAAGGGGT	<i>RBD1</i> 5' XhoI
TAACTCGAGGAAAATTTGGGCATATTTTGTTC	<i>RBD1</i> 3' XhoI
TAAAGAGCTCTTTGACATCAACGGCAATAC	<i>TUP1</i> 5' SacI
TAAAGAGCTCCTTGGGGCAACTCGTATAAC	<i>TUP1</i> 3' SacI

<sup>a</sup> Sequences in lowercase correspond to exogenous, complementary sequences introduced to primers 2, 3, 4, and 5 for mutually primed synthesis in the second round of fusion PCR.

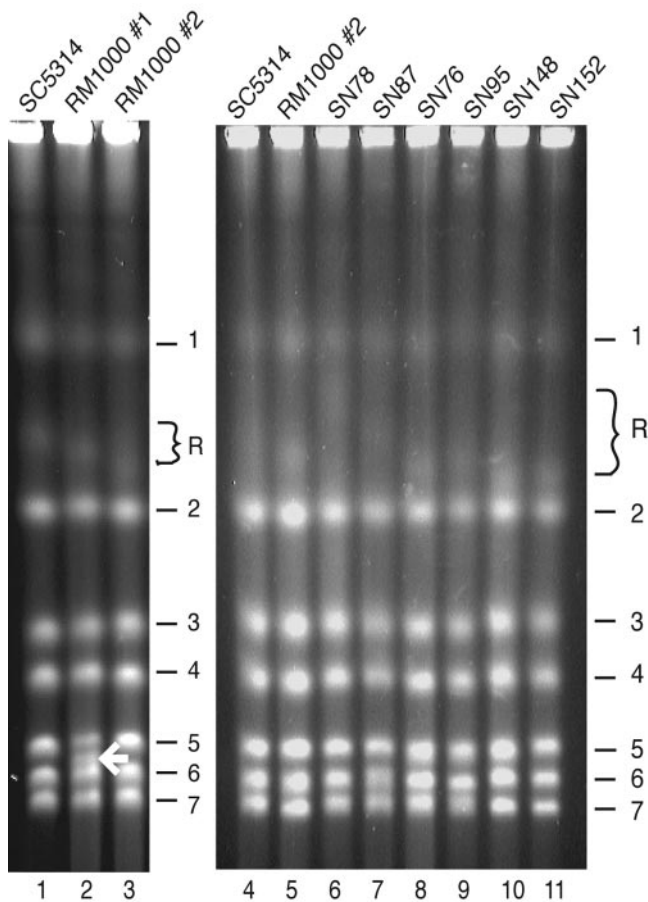


FIG. 1. Contour-clamped homogenous electric field gel analysis of *C. albicans* reference strains and SN78, SN87, SN76, and SN95. Chromosome plugs were prepared from SC5314, RM1000, RM1000#2, SN78, SN87, SN76, and SN95 and electrophoresed on a contour-clamped homogenous electric field gel as described in Materials and Methods. Chromosome identities are marked to the right of the gel. The fast-migrating copy of chromosome 5 in RM1000#1 (lane 2) is indicated with an arrow.

Figure 3C presents a final comparison between SN100 ( $\text{His}^-$ ), the triply auxotrophic strain SN152 ( $\text{His}^- \text{Leu}^- \text{Arg}^-$ ), and the wild-type clinical isolate SC5314. Here, SN152 appears to have attenuated virulence compared to SN100; for example, the time to sacrifice of half the mice is approximately 8 days for SN152 compared to only 4.5 days for SN100. This difference in virulence is statistically significant ( $P = 0.0112$  by the log-rank test), although of lesser magnitude than the difference between SN100 and RM1000#2. Finally, comparison between the virulence curves of SN100 and SC5314 confirms that SN100 has normal virulence and supports previous reports (2) that auxotrophy for histidine does not significantly affect virulence in the mouse model.

Strains RM1000#2, SN100, SN87, and SN95 were compared in a second set of experiments designed to detect even subtle differences in virulence. Here, the four strains were mixed in equal numbers, and the pooled inoculum was used to infect five mice. Each mouse was sacrificed as it became moribund (after 7 days, on average; data not shown), and viable *C.*

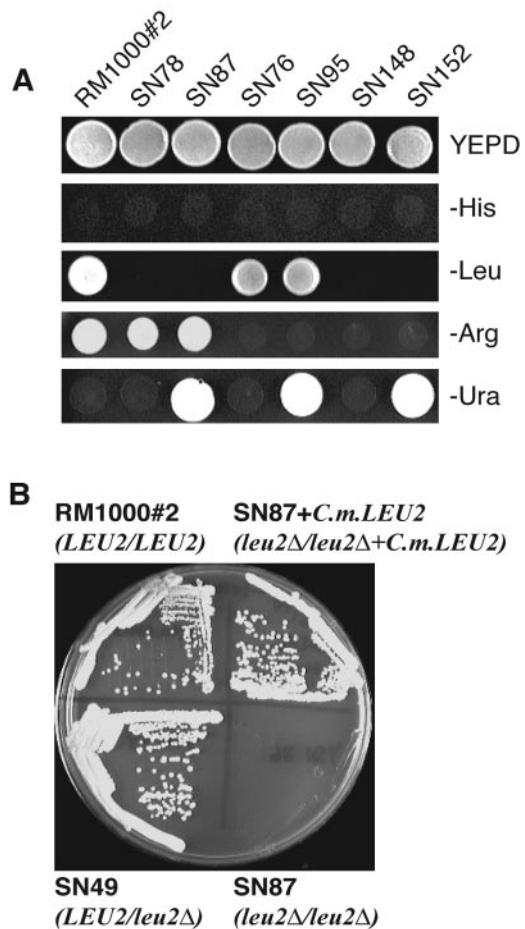


FIG. 2. Growth of new reference strains on dropout media and complementation of a *C. albicans* *leu2Δ/leu2Δ* strain by *C. maltosa* *LEU2*. (A) RM1000#2 and the new strains were compared for growth on YEPD lacking histidine, leucine, arginine, or uracil. (B) RM1000#2 was compared for growth on medium without leucine with SN49, which lacks one allele of *LEU2*; SN87, which lacks both alleles of *LEU2*; and a transformant of SN87 which has a single copy of the *C. maltosa* *LEU2* gene integrated at the *ADE2* locus.

*albicans* were recovered from mouse kidneys. The representation of each *C. albicans* strain in the infecting versus recovered pools was determined by growth of cells on amino acid dropout media. As shown in Fig. 3D, the four strains were represented equally in the inoculum; however, in the pool recovered after infection of the mice, RM1000#2 ( $\text{Ura}^- \text{His}^-$ ) was absent, whereas the remaining three strains were represented equally.

**HIS1, LEU2, and ARG4 from heterologous *Candida* species complement the auxotrophies of *C. albicans* *his1Δ/his1Δ*, *leu2Δ/leu2Δ*, and *arg4Δ/arg4Δ* strains.** SN87, SN95, and SN152 were constructed so that each allele of a target gene may be disrupted with a different genetic marker; SN152 has a third auxotrophic marker available for restoring a wild-type (or mutant) version of the target gene, if desired. Because the genomic disruptions of *LEU2* and *ARG4* delete only the coding sequence, leaving promoter and terminator sequences intact, we sought complementing genes from other species. The reasoning was that sequence heterogeneity between the promoter and terminator sequences of the complementing and

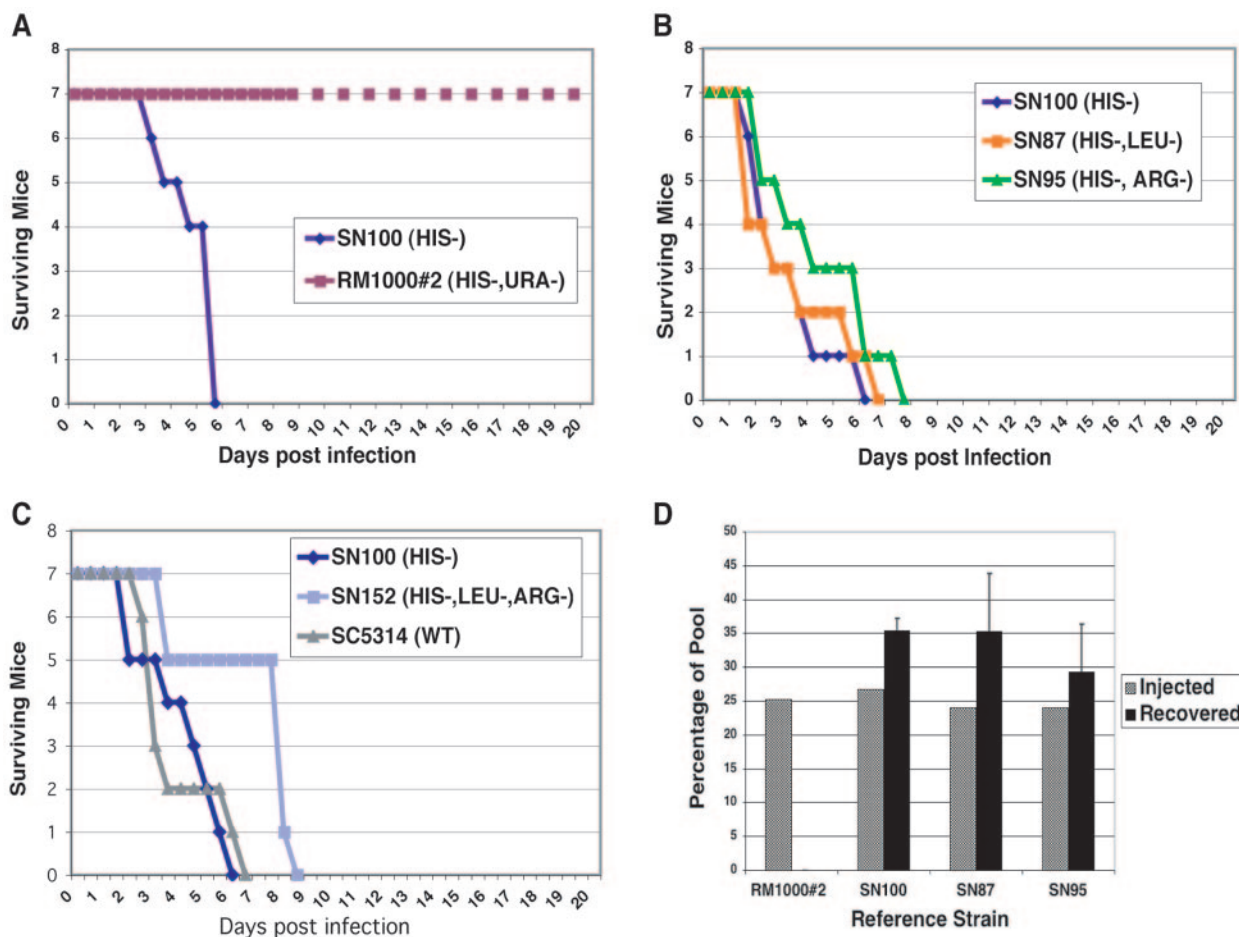


FIG. 3. New strains are virulent in the mouse. A) The virulence of the  $Ura^-$  strain RM1000#2 (*ura3Δ/ura3Δ*) was compared to that of the  $Ura^+$  strain SN100 (*ura3Δ/URA3*) in the mouse tail vein model of disseminated candidiasis, as described in the text and in Materials and Methods. B) Virulence of SN87 and SN95 compared to that of SN100. The time-to-illness curves for the  $His^- Leu^-$  strain SN87 and the  $His^- Arg^-$  strain SN95 are similar to that of the  $His^-$  strain SN100. C) The virulence of SN152 was compared to that of SN100 and SC5314. The triply auxotrophic strain SN152 ( $His^- Arg^- Leu^-$ ) has mildly attenuated virulence compared to SN100. SN100 is similar in virulence to SC5314, the original clinical isolate that is the progenitor of all the other strains. D) Four strains, RM1000#2, SN100, SN87, and SN95, were compared for virulence in a mixed infection in which all four strains were used as a mixed inoculum. The shaded bars represent the percentage of each of the four strains in the infecting inoculum. The black bars represent the percentage of each strain in the recovered *C. albicans* cells. Error bars for the recovered strains represent the standard deviation of the mean for each strain among five infected mice.

disrupted genes should minimize recombination at the corresponding *C. albicans* genomic loci.

Using the published genomic sequence of *Candida maltosa* *LEU2* (which retains the same noncanonical CTG codon usage as *C. albicans* [43, 46]), we synthesized primers to anneal approximately 1 kb upstream and 0.5 kb downstream from the *LEU2* coding sequence; the gene was then amplified by PCR with *C. maltosa* genomic DNA. DNA sequencing revealed some differences from the previously published sequence of *C. maltosa* *LEU2* (presumably due to strain differences) and 61% DNA sequence identity to *C. albicans* *LEU2* over the same region (the new *C. maltosa* *LEU2* sequence has been deposited in GenBank, accession number AY534142).

At the time this work was started, published sequences for *HIS1* from organisms with *C. albicans* CTG codon usage were not available, so we designed primers flanking this gene based on the *C. albicans* genomic sequence. Using primers that anneal approximately 1 kb upstream and 0.5 kb downstream of *C.*

*albicans* *HIS1*, we successfully amplified the cognate gene from *C. dubliniensis*, the *Candida* species most closely related to *C. albicans* (44). Over the region amplified, *C. dubliniensis* *HIS1* is 83% identical to *C. albicans* *HIS1* at the DNA level (deposited as GenBank accession number AY534141). *C. dubliniensis* *ARG4* was cloned with *C. dubliniensis*-specific primers after the provisional *C. dubliniensis* genomic sequence was published on the Internet by the Wellcome Trust Sanger Institute Pathogen Sequencing Unit (available online at [http://www.sanger.ac.uk/Projects/C\\_dubliniensis/](http://www.sanger.ac.uk/Projects/C_dubliniensis/)).

The heterologous *HIS1*, *LEU2*, and *ARG4* genes were tested for the ability to complement the new *C. albicans* strains. Each gene fragment was subcloned into pBES116 (16), a vector that directs integration into the *Candida* *ADE2* gene and is linked to a wild-type *URA3* gene. Transformants were selected on uracil dropout medium and then tested for growth on histidine, leucine, and arginine dropout plates, as appropriate. Shown in Fig. 2B is complementation of SN87 (*URA3Δ/ura3Δ*, *his1Δ/*

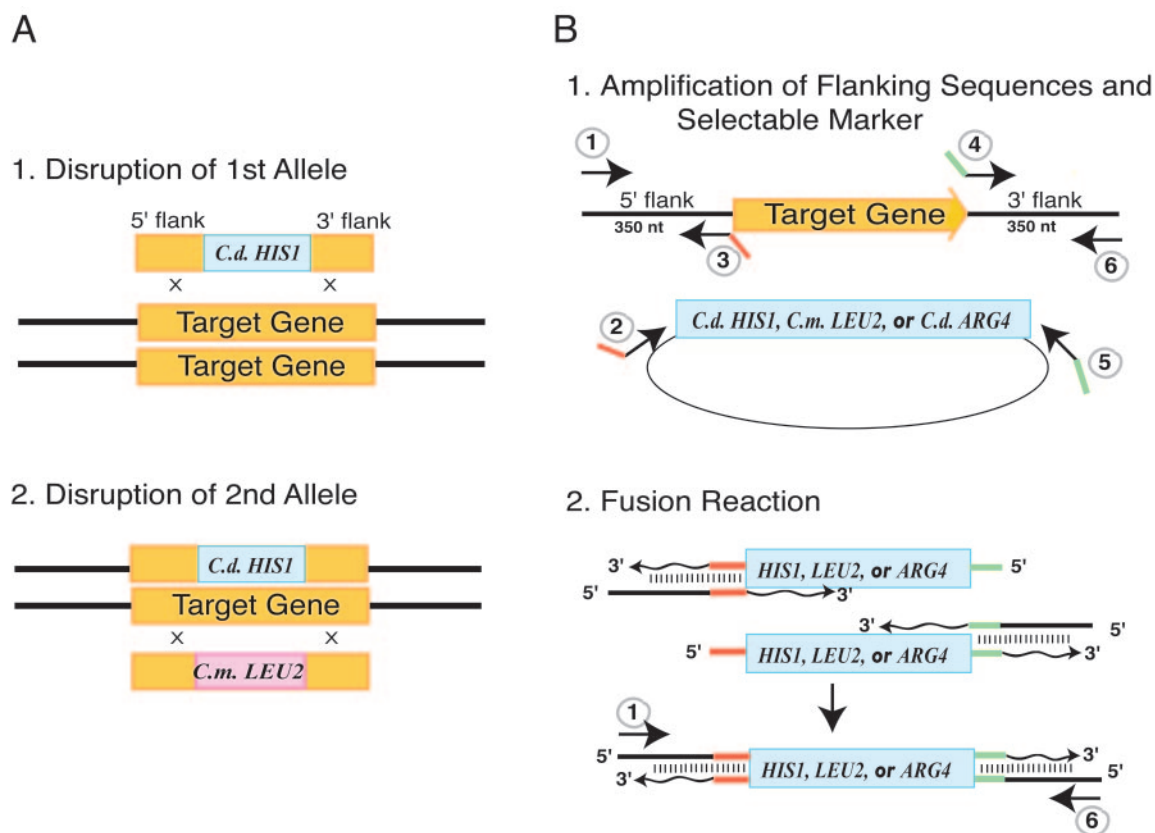


FIG. 4. New method of gene disruption in *Candida albicans* with fusion PCR and heterologous markers. (A) The new method of gene disruption is diagrammed in this schematic. For detailed explanations of the technique, please see Results and Materials and Methods. (B) Fusion PCR consists of two rounds of PCRs. In the first, primers 1 and 3 are used to amplify genomic DNA on the 5' side of the target gene; primers 4 and 6 are for the 3' side of the target; and primers 2 and 5 are used to amplify the selectable marker. The red (primers 2 and 3) and green (primers 4 and 5) primer tails represent exogenous, complementary sequences used for mutually primed synthesis in the second round of PCR.

*his1* $\Delta$ , *leu2* $\Delta$ /*leu2* $\Delta$ ) by *C. maltosa* *LEU2* on medium without leucine. *C. dubliniensis* *HIS1* similarly complements SN87 on medium without histidine, and *C. dubliniensis* *ARG4* fully complements SN95 (*URA3* $\Delta$ /*ura3* $\Delta$ , *his1* $\Delta$ /*his1* $\Delta$ , *arg4* $\Delta$ /*arg4* $\Delta$ ) on medium without arginine (data not shown).

**Use of fusion PCR and the new strains for reliable, efficient construction of double knockout mutants.** To achieve more efficient gene disruption, we modified a protocol for creating disruption fragments that has previously been used for *Saccharomyces cerevisiae* and *Cryptococcus neoformans* (12, 50; O. Liu and H. D. Madhani, personal communication). Our current method (Fig. 4A and B) uses a two-step fusion PCR protocol that results in a disruption fragment with relatively long (>350 nucleotides) regions of homology with the target locus. The first step consists of three PCRs, two to amplify the upstream and downstream regions of the target gene and one to amplify the selectable marker. The internal PCR primers have complementary tails that are used in the second round of PCRs for mutually primed synthesis. The result is a disruption fragment with long regions of homology to the upstream and downstream flanking sequences of the target gene (350 nucleotides in this example).

**Efficient disruptions of *TUP1* and *EFG1* in SN87, SN95, and SN152.** To test the new disruption technique and strains, prim-

ers were designed to disrupt *TUP1* and *EFG1* in SN87, SN95, and SN152. We chose *TUP1* and *EFG1* because they have been studied extensively, and the phenotypes of heterozygous and homozygous mutant strains have been well described. To create *tup1* mutants of SN87, SN95, and SN152, disruption constructs were prepared with all three heterologous selectable genes, *C. maltosa* *LEU2*, *C. dubliniensis* *HIS1*, and *C. dubliniensis* *ARG4*.

In independent experiments, the first round of gene disruption was carried out with one of the three selectable markers, and the second round was carried out with one of the two remaining markers. After selection of transformants on the appropriate single or double amino acid dropout medium, gene disruption candidates were screened by PCR for expected 5' and 3' junctions as well as the size of the disrupted gene (data not shown). The *tup1*/*tup1* double disruptant was therefore constructed six different ways and, in all cases, the phenotypes of independent mutants were identical. Regardless of the marker used, the first round of gene disruption was approximately 90% efficient; that is, of the transformants that grew on the appropriate amino acid dropout media, 90% carried a precisely disrupted allele, as judged by PCR. By these same criteria, the second round of gene disruption was approximately 70% efficient. The same procedures were repeated for



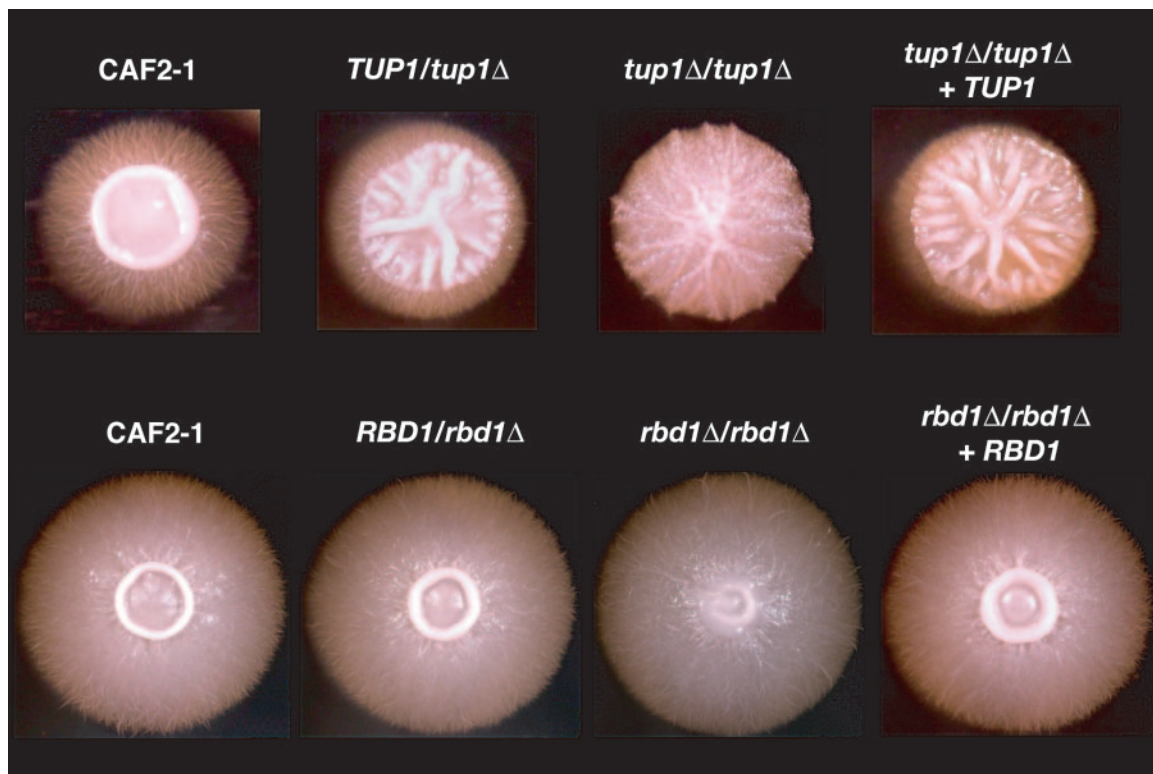


FIG. 5. Growth of CAF2-1, *tup1*, and *rbd1* mutants on Spider medium. Shown are individual colonies of CAF2-1 compared to *tup1* mutants (upper row) and *rbd1* mutants (lower row) after growth on Spider medium at 30°C. The colonies depicted were incubated for 6 days (upper row) or 9 days (lower row).

*EFG1*, and the efficiencies of the first and second gene disruption events were similar to those of *TUP1*. Again, the phenotypes of newly created *efg1/efg1* strains matched those of published *efg1* mutants (data not shown). These observations demonstrate that the gene disruption procedures described here are highly efficient and do not require a prescribed order of marker use for constructing double disruptants.

The advantage of using heterologous genetic markers from non-*C. albicans* yeasts was revealed by the following experiment. If *C. albicans ARG4* instead of *C. dubliniensis ARG4* was used to mark the first disrupted allele of either *TUP1* or *EFG1*, a significantly lower efficiency (approximately 25%) was observed. Use of *C. albicans ARG4* to select for disruption of the second allele was even lower, <1% (data not shown). These results indicate that use of selectable markers from non-*C. albicans Candida* species greatly improves the efficiency of gene disruption, probably by preventing undesired integration events at the endogenous locus of the auxotrophic marker.

The triply auxotrophic strain SN152 ( $\text{Arg}^- \text{His}^- \text{Leu}^-$ ) was created to permit disruption of both alleles of a given target gene with two auxotrophic markers; the third marker is available for introduction of another genetic element, such as a wild-type copy of the disrupted gene for complementation analysis. We performed such analysis for the *tup1/tup1* mutant. The *TUP1/tup1Δ::C. dubliniensis ARG4* heterozygous knockout and *tup1Δ::C. dubliniensis HIS1/tup1Δ::C. dubliniensis ARG4* homozygous knockout strains were created in the experiments described above. For complementation analysis,

wild-type *TUP1* was amplified by PCR and—along with *C. maltosa LEU2*—subcloned between 1-kb sequences flanking the *RPS10* gene; previous studies have established that introduction of exogenous genes to the *RPS10* locus is efficient and associated with high levels of gene expression (8). The homozygous disruption strain, *tup1Δ::C. dubliniensis HIS1/tup1Δ::C. dubliniensis ARG4*, was transformed with the linearized fragment containing wild-type *TUP1* and *C. maltosa LEU2* and selected on medium without arginine, histidine, or leucine. Transformants were screened by PCR for the presence of *TUP1*.

Shown in Fig. 5 is the growth of each mutant and the complemented strain versus that of CAF2-1 on Spider medium at 30°C, which weakly induces filamentation of wild-type *C. albicans*. Under these conditions, the heterozygous and homozygous *tup1* knockout strains recapitulate the previously published phenotype of exuberant hyperfilamentation (corresponding to increased numbers of pseudohyphae and seen here as increased wrinkling of the surface of the colony) (9) compared to CAF2-1. A second notable phenotype of *tup1* mutants is a progressive increase in the central part of the colony relative to peripheral hyphae, so that peripheral filaments are virtually undetectable in the double mutant. Restoration of one copy of *TUP1* partially suppresses both the hyperfilamentation phenotype and the loss of peripheral filaments, so that the complemented strain resembles the heterozygous knockout mutant. This is consistent with previous reports for heterozygous and homozygous *tup1* mutants (9) and similar to the haploinsuffi-

ciency phenotypes of other *C. albicans* mutations that affect cell morphology (47).

***RBD1* affects colony morphology on Spider medium.** To determine the utility of the new reagents and methods in constructing *C. albicans* knockout mutants on a larger scale for use in genetic screens, we constructed 144 mutants in a 96-well format and assessed their colony morphology on Spider medium at 30°C (data not shown). In the initial screen, one mutant, which we have named *rbd1/rbd1* (for rhomboid-like), had a phenotype almost opposite that of *tup1/tup1*, namely, a diminution of the central part of the colony so that almost the entire colony consisted of peripheral filaments. *RBD1* (whose two alleles correspond to ORF19.5234 and ORF19.12698 in the Stanford Genome Technology Center sequence) is a previously uncharacterized *C. albicans* open reading frame with significant homology (BLAST score of  $10^{-32}$ ) to a family of genes conserved from archaeobacteria to humans, which encode putative intramembrane serine proteases (48). For example, *Drosophila melanogaster* rhomboid protein P20350 cleaves the transforming growth factor alpha growth factor Spitz, allowing it to activate the epidermal growth factor receptor (38, 48). An *S. cerevisiae* rhomboid homolog, Rbd1/Pcp1, is localized in mitochondria, where it cleaves proteins localized in the intermembrane space (15, 21, 25).

We constructed a set of independent *rbd1/rbd1* homozygous deletion mutants in the SN152 (Arg<sup>-</sup> Leu<sup>-</sup> His<sup>-</sup>) background with *C. dubliniensis* *ARG4* and *C. maltosa* *LEU2* to mark the double disruptants. The phenotype was identical to that described above. Next, we restored one copy of wild-type *RBD1* to the *RPS10* locus, with *C. dubliniensis* *ARG4* as a selection. Shown in Fig. 5 are the colony morphologies of CAF2-1, *RBD1/rbd1*, *rbd1/rbd1*, and *rbd1/rbd1* with *RBD1* added back at the *RPS10* locus after growth on Spider medium at 30°C for 9 days. The phenotypes of the single- and double-disruption mutants are notable for a progressive decrease in area occupied by the central part of the colony, a defect that is partially complemented (back to the intermediate phenotype of the heterozygous disruption mutant) by restoration of one copy of wild-type *RBD1*. This phenotype was consistent among five independent double knockout mutants and was complemented in all five by restoration of wild-type *RBD1* (data not shown).

## DISCUSSION

In this paper, we describe three newly derived strains that are useful for gene disruption and subsequent virulence analysis of *C. albicans*. Unlike some strains currently used for these purposes, the new strains have a chromosomal complement similar to that of the original clinical isolate, SC5314. They also feature auxotrophic markers, *HIS1*, *LEU2*, and *ARG4*, that do not substantially affect virulence in the mouse tail vein injection model. *HIS1* and *LEU2* have previously been demonstrated to be neutral for virulence (2, 22), and we show in this paper that *ARG4* is also neutral. We also describe a new set of complementing plasmids with auxotrophic markers from heterologous *Candida* species, a fusion PCR technique for generating deletion constructs, and a protocol for sequential selection of deletions of the first and second alleles. Taken together, these materials and procedures greatly facilitate the

generation of homozygous knockout mutants that are suitable for virulence studies.

The efficiency of these approaches was demonstrated by creating de novo disruptions of two previously characterized genes, *TUP1* and *EFG1*, as well as a novel gene, *RBD1*. The well-described hyperfilamentation phenotype of the *tup1Δ/tup1Δ* mutant (9) and the well-documented hypofilamentation defect of the *efg1Δ/efg1Δ* strain (42) were recapitulated in the new strain backgrounds. Disruption of the previously uncharacterized open reading frame *RBD1* resulted in a novel colony morphology phenotype; this phenotype was partially suppressed by introduction of a wild-type version of the disrupted gene.

Using the new method to disrupt *TUP1* and *EFG1*, we observed a high frequency of targeted integration to the correct genomic locus, approximately 90% for the first allele and 70% for the second allele. We are not aware of the average efficiency of prevailing gene disruption methods, which are typically carried out with long PCR primers with 60 to 80 nucleotides of homology. In practice, however, it is common to observe a relatively low ratio (e.g., 1 in 100) of correct disruption products relative to other types of genetic changes, particularly in disruption of the second allele. There are several possible explanations for the improved efficiency of the new methods described here. First, 350 nucleotides of homology to regions flanking the target locus (as opposed to 60 to 80 nucleotides) likely facilitates homing to the correct locus, as has been reported for *Cryptococcus neoformans* (30). Second, since the two *C. albicans* alleles of a given gene are often heterologous in sequence (54), the opportunity for recombination at an exact sequence match between the disruption fragment and genomic DNA should be enhanced by inclusion of both allelic sequences in the disruption construct that is synthesized with genomic DNA as a template. Third, sequential gene disruption with different auxotrophic markers for each allele maintains selection for the first knockout at the time that the second is created, thereby avoiding selection of reintegrants into the first disrupted allele. Finally, the high efficiency of creating knockouts with selectable genes from related species instead of from *C. albicans* itself suggests that unwanted recombination events associated with standard *C. albicans* auxotrophic markers commonly involve recombination with the marker locus rather than the gene of interest.

Others have reported methods for creating large numbers of targeted gene mutations in *C. albicans*. For example, Roemer et al. presented the PCR-based GRACE (gene replacement and conditional expression) method, in which one allele of a target gene is replaced by standard methods and the promoter of the second allele is replaced with a regulatable promoter, with a dominant selectable marker (*sat1*) used to select the second event (36). This method has the advantage that expression of essential as well as nonessential genes may be disrupted; however, the efficiency of this method has not been reported. Likewise, Enloe et al. (14) described a method for creating homozygous gene disruptions in *C. albicans* with a single round of transformation followed by selection for homozygosity. This method relies on gene conversion or mitotic recombination (crossover) events to replace the second allele of the target gene with the disrupted copy and has the advantage of requiring only one round of transformation to disrupt

both copies of the target gene. A disadvantage is that the gene conversion or mitotic recombination event that results in homozygosity of the deletion mutation will also affect adjacent chromosomal DNA, potentially including the entire chromosome arm distal to the recombination site. Thus, mutants created with this strategy may differ from the parental strain not only by the targeted deletion mutation but also by loss of heterozygosity along the affected chromosome. While this procedure is extremely useful for rapidly determining whether a given gene is essential, it cannot be used routinely to create genetically matched strains.

The new strains and procedures that we describe also provide an advantage in virulence studies by avoiding the use of *URA3* as a selectable marker. As previously reported, *ura3Δ/ura3Δ* strains of *C. albicans* have striking virulence defects (Fig. 4A) (22). Because *URA3* expression can be subject to chromosome position effects, its use in genetic manipulations may produce unwanted effects on virulence. In contrast, as we show here, *LEU2*, *HIS1*, and *ARG4* individually do not affect virulence in the tail vein injection model. Only the triple mutant, SN152, displayed significantly attenuated virulence, and this defect is relatively mild compared to that conferred by deletion of *URA3* (Fig. 4). We conclude from these results that *LEU2*, *HIS1*, and *ARG4* may be used safely to mark disrupted genes intended for virulence analysis since even if expression of marker genes is incomplete at heterologous loci, the aggregate effect on virulence should be no greater than that caused by the absence of the markers altogether. The mild virulence defect of the SN152 triple mutant is in keeping with the observation that *GCN4*, a general regulator of amino acid levels, is not required for virulence in *C. albicans* (8); i.e., the upregulation of amino acid biosynthetic pathways does not seem to be necessary for normal virulence. It unclear why *URA3* is so critical for virulence, but a likely possibility is that nutritional components are differentially available in the context of the mouse host, with uridine being in particularly short supply.

Our recommendations for use of the new strains are as follows. SN152, with its three auxotrophic markers (*his1*, *leu2*, and *arg4*), offers a convenient background for most studies. The presence of a third auxotrophic marker is particularly useful for complementation analysis, in which a mutant phenotype can be reversed (at least partially) by addition of a wild-type copy of the deleted gene. Because SN152 has a mild virulence defect, however, it should probably not be used for assessing subtle virulence defects. For such uses, we recommend instead the fully virulent strains SN87 (*his1 leu2*) and SN95 (*his1 arg4*). In these strain backgrounds, linkage of a mutant phenotype can be correlated to deletion of the target gene either by examination of multiple independent knockout mutants or by adding back the wild-type version of the gene with a dominant selectable marker such as *sat1* (36, 35). For studies focused on mutants with substantial virulence defects, SN152 may still be used, since the potential for poor expression of *C. dubliniensis HIS1*, *C. dubliniensis ARG4*, and *C. maltosa LEU2* to attenuate virulence should be no greater than the mild defect of SN152 itself.

An advantage of an efficient gene disruption system is that, by reducing the time and effort required to make individual knockout mutants, large numbers of previously uncharacterized genes can be targeted. Screens of such mutants can yield

rapid and unexpected insights. As an example, we disrupted *RBD1*, a gene that had not previously been studied in *C. albicans*. Surprisingly, this mutant yielded a novel "loss-of-center" colony morphology phenotype on Spider medium, almost opposite that of the well-studied *tup1* mutant. Although we had no preconceived ideas that *RBD1* might affect filamentation and, perhaps, cell-cell communication in *C. albicans*, these are now both reasonable areas for future research.

The past 10 years have produced exponential increases in publicly available sequence information for organisms known to cause disease in humans. Genome-scale studies and bioinformatics have already produced key scientific insights into mechanisms of pathogenesis (7, 13). The development of essential experimental tools such as standardized strains and efficient, rapid disruption technologies is crucial to test ideas developed from such studies. We have already used the new strains, reagents, and techniques presented in this article to construct 144 homozygous deletion mutants of *C. albicans* within a few months' time (S. M. Noble and A. D. Johnson, unpublished data). Performed on a larger scale, it should be possible to create a *C. albicans* genomic disruption library (of nonessential genes) within a year or two. Such a genome-scale knockout collection for *C. albicans* would enable systematic, direct screens for virulence determinants of this important fungal pathogen.

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