Requirement for *Candida albicans* Sun41 in Biofilm Formation and Virulence\(^7\)

Carmelle T. Norice,\(^1\) Frank J. Smith, Jr.,\(^1\) Norma Solis,\(^2\) Scott G. Filler,\(^2,3\) and Aaron P. Mitchell\(^1\)*

Department of Microbiology, Columbia University, New York, New York; Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California; and The David Geffen School of Medicine at UCLA, Los Angeles, California

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The cell wall of *Candida albicans* lies at the crossroads of pathogenicity and therapeutics. It contributes to pathogenicity through adherence and invasion; it is the target of both chemical and immunological antifungal strategies. We have initiated a dissection of cell wall function through targeted insertional mutagenesis of cell wall-related genes. Among 25 such genes, we were unable to generate homozygous mutations in 4, and they may be essential for viability. We created homozygous mutations in the remaining 21 genes. Insertion mutations in *SUN41*, *Orf19.5412*, *Orf19.1277*, *MSR2*, *Orf19.3869*, and *WSC1* caused hypersensitivity to the cell wall inhibitor caspofungin, while two different *ecm33* insertions caused mild caspofungin resistance. Insertion mutations in *SUN41* and *Orf19.5412* caused biofilm defects. Through analysis of homozygous *sun41Delta/sun41Delta* deletion mutants and *sun41Delta/sun41Delta+pSUN41*-complemented strains, we verified that *Sun41* is required for biofilm formation and normal caspofungin tolerance. The *sun41Delta/sun41Delta* mutant had altered expression of four cell wall damage response genes, thus suggesting that it suffers a cell wall structural defect. *Sun41* is required for inducing disease, because the mutant was severely attenuated in mouse models of disseminated and oropharyngeal candidiasis. Although the mutant produced aberrant hyphae, it had no defect in damaging endothelial or epithelial cells, unlike many other hypha-defective mutants. We suggest that the *sun41Delta/sun41Delta* cell wall defect is the primary cause of its attenuated virulence. As a small fungal surface protein with predicted glucosidase activity, *Sun41* represents a promising therapeutic target.

The pathogen cell surface plays critical roles in infection and disease because it mediates interactions with host cells, including adherence, invasion, and effector transfer. Surface molecules also play pivotal roles in attachment to abiotic materials, leading to biofilm formation and device-associated infection. Surface features contribute not only to pathogenicity but to defense and therapeutics as well. Many cell surface features often permit recognition and attack by host defense systems. In addition, pathogen-specific features of cell wall biosynthesis provide useful drug targets, particularly because they are not present in mammalian cells and the target molecules may be directly accessible from the surrounding aqueous environment.

Our focus is on *Candida albicans*, the major invasive fungal pathogen of humans. *C. albicans* is a natural commensal that causes mucosal or disseminated infection in susceptible individuals (14, 35). Risk factors for infection include defective local or systemic immune function and presence of an implanted medical device. Surface proteins play prominent roles in infection. For example, the *C. albicans* cell wall protein Hwp1 is required for full virulence in both a disseminated infection model and an oral mucosal infection model (49, 50). Hwp1 is also required for biofilm formation both in vitro and in vivo; much evidence indicates that it functions as an adhesin (31). The cell wall protein Ecm33 is also required for full virulence in a disseminated infection model, and this virulence defect correlates with decreased adhesion to and damage of epithelial and endothelial cells (22, 23). The altered sensitivity of an *ecm33* mutant to cell wall-perturbing agents, along with its slow growth, suggests that its virulence defect may arise from a defect in general cell wall structure (22, 23). These examples illustrate both that cell wall proteins may have diverse functions and that these functions are relevant to infection.

The *C. albicans* cell wall has proven to be an excellent drug target as well. The echinocandin class of drugs, such as caspofungin, acts through inhibition of synthesis of β-1,3-glucan, the major cell wall structural component (17). Disruption of cell wall synthesis with caspofungin induces a large number of genes that we call cell wall damage response genes (5, 20). Many of these genes specify predicted cell wall proteins or cell wall biosynthesis and modification enzymes, and so it seems likely that the response reflects a homeostatic mechanism to maintain cell wall integrity. In keeping with this idea, many caspofungin-induced genes are also induced in regenerating protoplasts (6) and may respond to an array of signals related to cell wall structure or integrity.

Thus far, functional analysis of *C. albicans* cell wall-related genes has been carried out largely on a candidate gene basis, in which a single gene is chosen for study based upon gene expression, gene product antigenicity, or known ortholog properties (48). Heterologous expression strategies have also proven valuable in functional screens, but the net yield of interesting genes has been low to date (13, 18). In part this gene-by-gene approach reflects the technical limitations of *C. albicans* gene disruption strategies, in which a null mutant is created through two successive transformations (3, 32). We have developed an insertional mutagenesis strategy that makes
it practical to disrupt larger numbers of genes and screen the mutants for phenotypes of interest (9). We have applied this basic strategy to both random genes and specifically to transcription factor genes (5, 7, 9, 30). Here, we have used this approach to analyze functions of several cell wall-related genes. Our detailed analysis of one gene, SUN41, shows that it plays major roles in biofilm formation, cell wall integrity, and virulence in both orpharyngeal and disseminated candidiasis. Previous studies have identified only two other cell wall proteins, Hwp1 and Mp65, that are required for virulence in both infection models (42, 49, 50). Thus, Sun41 has several properties of a useful therapeutic target. While Sun41 belongs to a conserved fungal protein family, no ortholog has been implicated previously in biofilm formation, cell wall integrity, or virulence. Thus, this postgenomic forward-genetics approach holds promise to reveal unique biological functions of both novel and conserved C. albicans genes.

MATERIALS AND METHODS

Media and chemicals. C. albicans strains were routinely passaged in YPD with or without uridine broth (2% dextrose, 2% Bacto Peptone, 1% yeast extract; 80 μg/ml uridine) at 30°C with 220-rpm agitation or on YPD solid medium at 30°C.

The selection of transformants was accomplished on synthetic dextrose medium (2% dextrose, 6.7% yeast nitrogen base plus ammonium sulfate), to which was added the necessary auxotrophic supplements. For Arg+ Ura+ selection from orf:UAU1/ORB heterozygotes (see below), we used SC-Arc-Ura Brefly plates, in which 1 liter of synthetic dextrose medium was supplemented with 10 mg of histidine HCl as well as 1.7 g of a supplement mixture comprising adenine sulfate (0.5 g), alanine (2.0 g), sodium aspartate (2.0 g), cysteine (2.0 g), glutamine (2.0 g), glycine (2.0 g), histidine HCl (2.0 g), histidine (2.0 g), histidine HCl (2.0 g), histidine (2.0 g), leucine (10 g), lysine (2.0 g), methionine (2.0 g), phenylalanine (2.0 g), proline (2.0 g), serine (2.0 g), threonine (2.0 g), tryptophan (2.0 g), tyrosine (2.0 g), and valine (2.0 g). Congo red was obtained from Sigma, and caspofungin acetate was a generous gift from Merck.

Strains and DNA manipulations. The strains used in this study are listed in Tables 1 and 2. All strains used were derived from BWP17 (52).

The strains used in this study are listed in Table 1. Strains used in this study

| Strain       | Genotype                     | Reference |
|--------------|------------------------------|-----------|
| BWP17        | ura3 Δ::imm3434 arg4::hisG his1::hisG | 52        |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG |           |
|             | DAY185 ura3 Δ::imm3434 HIS1::his1::hisG ARG4::URA3::arg4::hisG | 8         |
|             | ura3 Δ::imm3434 his1::hisG arg4::hisG |           |
| CTN41        | ura3 Δ::imm3434 arg4::hisG his1::hisG sun41::ARG4 | This study |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG sun41::URA3 |         |
| CTN46        | ura3 Δ::imm3434 arg4::hisG his1::hisG pHIS1 sun41::ARG4 | This study |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG sun41::URA3 |           |
| CTN56        | ura3 Δ::imm3434 arg4::hisG his1::hisG pHIS1-SUN41 sun41::ARG4 | This study |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG sun41::URA3 |           |
| DAY286       | ura3 Δ::imm3434 ARG4::URA3::arg4::hisG his1::hisG | 9         |
|             | his1::hisG |           |
| CJN702       | ura3 Δ::imm3434 arg4::hisG his1::hisG pHIS1 ber1::ARG4 | 30        |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG |           |
|             | ber1::URA3 |           |
| CJN432       | ura3 Δ::imm3434 arg4::hisG his1::hisG cas5::Tn7-UAU1 | 30        |
|             | ura3 Δ::imm3434 arg4::hisG his1::hisG cas5::Tn7-URA3 |           |

The insertion sites for each clone are listed at http://www.tigr.org/omim/cgi-bin/insertion.pl?num=1, and the specific sequence from the end of each insertion may be found through the Seq_ID link. Each cloned DNA insert, including the Tn7-UAU1 insertion, was excised from the plasmid backbone through digestion with NotI, and the entire insert was transformed into strain BWP17. Arg+ transfectants (presumably heterozygous for the insertion) were selected on SC-Arc-Ura plates. Twelve independent transformants were patched onto YPD plates (one-quarter plate patches), and after 2 days of growth at 30°C they were replica plated onto SC-Arc-Ura Brefly plates to select for Arg+ Ura+ recombinants. Based on our past studies, some of these are homozygous insertion mutants (genotype orf:UAU1/UAU1) and others are allelic triplication derivative mutants (genotype ORF::UAU1/UAU1). Following approximately 5 days of growth, one colony from each quarter was purified by streaking on SC-Arc-Ura plates and then screened by colony PCR to ensure absence of a PCR product from the wild-type allele and presence of a PCR product from the orf::UAU1 allele. For reference, the wild-type allele was amplified from BWP17 using primers flanking the insertion site and designed to yield a 1,000- to 1,600-bp product. The orf::UAU1 product, which is too large to amplify reliably with the flanking primers, was amplified by adding a third primer to the mix (Arg/UAU1/1 probe sequence GGAATTCGATACCAGTTTTAGAA).

Strain CTN46, the prototrophic sun41::ARG4::sun41::URA3 homozygous deletion mutant, was created by PCR-directed gene deletion according to previously described methods (52). URA3 and ARG4 constructs with flanking homology for SUN41 disruption were amplified from pGEM-URA3 and pRS-ARG4, respectively, using 120-mer oligonucleotides SUN41-SDR3 (5' GTTTCTTTTAGGTCTGGTCTTTTTTTTTTTAATTC ACTTTGTTGCTATATAGTCTCAAGTTGACATTCGGTTCAG3) and SUN41-3DR (5'T TAAAAACAAAACTAACTTGGAAAAACAAAACACTTGTTCCTAGGTTTACTTTCATCTATCAGTTGATTA5). We transformed strain BWP17 with the URA3 construct, selected for Ura+ transfectants, and screened by whole-cell PCR for the presence of a sun41::URA3 allele and a wild-type allele. Several of these heterozygotes were transformed with the ARG4 construct and plated on SC-Arc-Ura medium. Ura+ Arg+ isolates were screened by whole-cell PCR for the presence of sun41::URA3 and sun41::ARG4 alleles and the absence of wild-type alleles. We used one His+ strain sun41::ARG4::sun41::URA3 strain, CTN41, for all subsequent sun41 mutant strain constructions described in this report. To make the mutant His+ strain CTN41 was transformed with pRYS2, a derivative of the HIS1 pDDB78 vector (47) in which we substituted the NruI site with an SrfI site and introduced an EspIII site. In genotypic designations, pRYS2 is listed as pHIS1. The prototrophic sun41::ARG4::sun41::URA3 mutant is designated CTN46.

To complement the sun41 deletion, we used BWP17 genomic DNA as a
| ORF\(^ab\) | Gene\(^a\) | Clone name\(^b\) | Gene length (nt) | Insertion site (nt) | S. cerevisiae ortholog (best match)\(^c\) | No. screened\(^d\) | No. recovered\(^d\) | Mutant strain name | Biofilm formation | Caspfungin growth | Description |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Orf19.10938 | Orf19.10938 | CAGEN68 | 2,969 | 1,781 | (YGR067C) | 12 | 2 | CAGEN68-4 | + | + | Predicted ORF |
| Orf19.1277 | Orf19.1277 | CAGGO92 | 2,969 | 1,141 | (YGR067C) | 12 | 3 | CAGGO92-1 | + | + | Predicted ORF |
| Orf19.1490 | MSB2 | CAGD531 | 1,178 | 429 | MSB2 | 12 | 5 | CAGD531-2 | + | + | Protein of unknown function |
| Orf19.1563 | ECM3 | CAGCO56 | 1,640 | 896 | ECM3 | 12 | 7 | CAGDL37-1 | + | + | Predicted ORF |
| Orf19.1714 | PGA44 | CAGD578 | 1,640 | 230 | ECM3 | 12 | 7 | CAGGW32-1 | + | + | Putative GPI-anchored protein of unknown function |
| Orf19.2476 | Orf19.2476 | CAGD578 | 1,640 | 230 | ECM3 | 12 | 7 | CAGGW32-1 | + | + | Putative GPI-anchored protein of unknown function |
| Orf19.2613 | ECM4 | CAGD578 | 1,640 | 230 | ECM3 | 12 | 7 | CAGGW32-1 | + | + | Putative GPI-anchored protein of unknown function |
| Orf19.299 | ECM14 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative transcription factor with zinc cluster DNA-binding motif |
| Orf19.301 | ECM33 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative cell wall protein |
| Orf19.310 | SCW11 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative cell wall protein |
| Orf19.3893 | SCW11 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative cell wall protein |
| Orf19.3966 | CRH12 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative cell wall protein |
| Orf19.3897 | CRH12 | CAGE55 | 1,238 | 730 | ECM3 | 12 | 8 | CAGBU26-1 | + | + | Putative cell wall protein |
| Orf19.4887 | ECM21 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.4981 | CRH12 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.532 | RBR2 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.5412 | CRH12 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.563 | PGA10 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.5861 | KRE9 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |
| Orf19.5867 | WSC1 | CAGD95 | 2,114 | 1,054 | CRH1 | 12 | 8 | CAGF30-2 | - | - | Predicted ORF |

\(^a\) ORF = Open Reading Frame

\(^b\) Gene = Gene name

\(^c\) Clone name = Clone name

\(^d\) Gene length = Gene length

\(^e\) Insertion site = Insertion site

\(^f\) S. cerevisiae ortholog (best match) = S. cerevisiae ortholog

\(^g\) No. screened = Number of screened samples

\(^h\) No. recovered = Number of recovered samples

\(^i\) Mutant strain name = Mutant strain name

\(^j\) Biofilm formation = Biofilm formation

\(^k\) Caspfungin growth = Caspfungin growth

\(^l\) Description = Description
template to PCR amplify a fragment for SUN41 (Orf19.3642) reconstitution from 2,011 bp upstream of the ATG to 501 bp downstream of the stop codon. We used primers SUN41compL (5'-CATGTCATACAACTGAATGCT-3') and SUN41compR (5'-TTGTTGTTGTTGGAATTATG-3'). The amplicon was ligated into pGEMT-Easy vector (Promega) and then released by digestion with SapI and NcoI. This fragment was inserted into EcoRI- and NotI-digested pYRS2 by in vivo recombination in Saccharomyces cerevisiae to yield the pHS1-SUN41 plasmid pCTN16. The complemented strain, CTN56, which contains the SUN41 open reading frame (ORF), was constructed by transforming strain CTN41 (sun41Δ::ARG4::sun41::URA3) with SrfI-digested plasmid pCTN16. Ura+ Arg+ His+ isolates were screened by whole-cell PCR for the presence of a SUN41 allele.

In vitro biofilm assays. We used the biofilm assay method described by Nobile and Mitchell (30). Briefly, single colonies were inoculated into 3 ml of YPD and grown overnight at 30°C. Cultures were diluted to an optical density at 600 nm (OD600) of 0.5 in 2 ml of supplemented Spider medium in sterile 12-well plates containing silicone square substrates pretreated with bovine serum (Sigma). Inoculated plates were incubated for 90 min with 150-rpm agitation at 37°C for adherence to occur. The squares were transferred to 2 ml of phosphate-buffered saline (PBS) to wash away unadhered cells and then placed in 2 ml of fresh Spider medium and allowed to incubate for 60 h with 150-rpm agitation at 37°C.

Caspofungin and Congo red susceptibility assays. We tested for drug sensitivity as described by Bruno et al. (5). Single colonies were inoculated into 3 ml of YPD and grown overnight at 30°C. Cultures were diluted to an OD600 of 3 in 1 ml of double-distilled H2O and then serially diluted fivefold to an OD600 of 0.6, 0.12, 0.024, 4.8 × 10⁻², or 9.6 × 10⁻³. Cells were spotted onto YPD, YPD plus 125 ng/ml caspofungin, and YPD plus 200 µg/ml Congo red plates, allowed to dry, and incubated at 30°C. The plates were photographed after 24 h.

RNA extraction and real-time PCR. Cells growing in 50 ml of YPD were harvested by vacuum filtration at an OD600 of 1 and immediately frozen at −80°C. Cells were resuspended in 15 ml of chilled AE buffer (50 mM Na acetate pH 5.2, 10 mM EDTA) brought to 1% sodium dodecyl sulfate, and 17 ml of acid phenol was added. The mixtures were incubated at 65°C with shaking for 10 min, the aqueous phase was separated, and total RNA was precipitated. Samples were treated with the DNA-free kit (Ambion), followed by first-strand cDNA synthesis kit (Stratagene). In a control set of sample mixtures, reverse transcriptase was omitted from the reaction mixture so that the absence of DNA contamination could be verified.

Primer3 software (http://frodo.wi.mit.edu/) was used to design primers to measure expression of five target genes, SUN41, DDR48, PHR1, STP4, and CHT2, and the reference gene, TDH3. The primers were as follows: for SUN41, SUN41RT L, 5'-AACCCTTTCTTCCATCCTG-3', and SUN41RT R, 5'-AC CAGAACCAGAACCACAG-3'; for DDR48, JRB212, 5'-TTTGGTCGCTTG AAAAGACG-3'; and JRB213, 5'-CTTGTTGAGGAACCGTAGGA-3'; for PHR1, JRB214, 5'-GATTGCTCGGCTATTTCTGC-3', and JRB215, 5'-TGAT TGAACACGTCCGTTCG-3'; for STP4, JRB244, 5'-TCTTTCAGAACAC TCTTCTTT-3', and JRB245, 5'-CTGTTGGAGGAACCGTAGGA-3'; for CHT2, CHT2 FWD PR, 5'-AAACAGCTTGTCCACTACCA-3', and CHT2 REV PR, 5'-GGCTTTTGTGTGAGGACCAG-3'; for TDH3, TDH3 fwd, 5'-ATCCCAACA AGGACTGAGGA-3', and TDH3 rev, 5'-GCGAAGCCTTGAAGCCTG-3'. In a total volume of 50 µl, iQ SYBR Green Supermix (Bio-Rad), 2 µl of first-strand cDNA reaction mixture, and 0.5 µM of primers were mixed. Real-time PCR of samples in triplicate was carried out using the iCycler iQ real-time PCR system (Bio-Rad), with a program comprising 95°C for 5 min and then 40 cycles of 95°C for 45 s, followed by 58°C for 30 s. Amplification products were detected with SYBR Green, and the specificity of the amplification was confirmed by melting curve analysis. Bio-Rad iQ5 software was used to calculate normalized gene expression values by the ΔΔCT method, with TDH3 as the reference gene. The expression of each gene relative to TDH3 expression is presented.

Disseminated candidiasis model. The virulence of the various strains was tested in the mouse model of hematogenously disseminated candidiasis as described previously (41). To determine the role of Sun41 on survival, 10 male, BALB/c mice (20 g body weight; National Cancer Institute, Bethesda, MD) were infected via the tail vein with 2 × 10⁸ blastospores of DAY185, sun41Δ, or sun41Δ::pSUN41 suspended in 500 µl of PBS. All inocula were confirmed by colony counting. The mice were monitored at least three times daily, and moribund mice were euthanized. To determine the role of Sun41 on kidney fungal burden, 10 mice were inoculated with each strain as in the survival experiments. After 1 and 4 days of infection, five mice were randomly selected from each group and euthanized. Both kidneys were harvested. One kidney was processed for tissue fungal burden and the other for histopathological analysis. For tissue
fungal burden, the kidneys were homogenized in PBS and quantitatively cultured on Sabouraud dextrose agar containing 10 µg/ml chloramphenicol. For histopathological analysis, kidneys were fixed in zinc-buffered formalin followed by 70% ethanol and then embedded in paraffin. Thin sections were stained with periodic acid-Schiff stain.

**Oropharyngeal candidiasis model.** The virulence of the different strains was also tested in our previously described mouse model of oropharyngeal candidiasis (34). Briefly, male BALB/c mice were immunosuppressed with subcutaneous injections of cortisone acetate (225 mg/kg; Sigma-Aldrich) administered at days −1, 1, and 3 relative to infection. The mice were inoculated by sedating them with ketamine and xylazine (both from Phoenix Pharmaceuticals) and then placing calcium alginate swabs saturated with 10⁶ blastospores/ml of the various strains of C. albicans sublingually for 75 min. After 5 days of infection, the mice were euthanized, after which the tongue and adjacent hypoglossal tissue were excised for determination of tissue fungal burden and histopathological analysis as described above. All experiments were approved by the Institutional Animal Care and Use Committee and followed the National Institutes of Health guidelines for the ethical treatment of animals.

**Adherence, phagocytosis, and damage assays.** The capacities of DAY185, sun41, and sun41+pSUN41 to adhere to, invade, and damage the FaDu oral epithelial cell line (American Type Culture Collection) and primary human umbilical vein endothelial cells were determined exactly as described previously (7).

**Statistical analysis.** Differences in survival among mice infected with the various strains were analyzed using the log-rank test. The tissue fungal burden data were analyzed using the Wilcoxon rank-sum test. Interactions of strains with various strains were analyzed using the log-rank test. The tissue fungal burden (7).

**RESULTS**

**Identification of biofilm-defective and caspofungin-hyposensitive mutants.** The C. albicans cell wall plays critical roles in diverse processes related to infection and virulence. With that in mind, we attempted to create homozygous insertion mutations in 25 genes whose products are predicted to have functions related to the cell wall (Table 2). Some are known or predicted cell wall proteins; others participate in cell wall structure through biosynthetic or regulatory roles. We failed to recover homozygous mutations in four genes, ECM18, KRE9, SCW11, and Orf19.310, a possible indication that these genes may be essential for growth under our selection conditions. For SCW11 and Orf19.310, we had several different cloned insertion alleles that did not yield homozygous mutants, thus strengthening the argument that these genes may be required for growth or viability.

Viable mutants were recovered that were homozygous for insertions in 21 genes (Table 2). These mutants were screened for altered biofilm formation and caspofungin sensitivity, two phenotypes related to known cell wall functions. In many cases, we tested phenotypes associated with two or more different insertion alleles (MSB2, ECM3, Orf19.2476, ECM4, ZCF12, ECM14, ECM33, Orf19.3869, CRH12, WOR1, ECM21, Orf19.4981, RBR2, and WSC1). All mutants were Arg” Ura” His” and were compared in these screens to Arg” Ura” His” reference strain DAY286, biofilm-defective mutant CJN702, and caspofungin-hyposensitive mutant CJN432 (Table 1). Competence for biofilm formation was assessed by visual inspection of biofilm integrity in Spider medium (30). We found two biofilm-defective strains, representing insertions in SUN44 and Orf19.5412 (Table 2). Caspofungin sensitivity was tested in a spot dilution assay on solid agar (5). Several strains were hyposensitive to caspofungin, representing insertions in Orf19.1277, MSB2, SUN44, Orf19.3869, Orf19.5412, and WSC1 (Table 2). For MSB2 and WSC1, several insertion alleles gave the same phenotype. Two insertions in the ECM33 coding region caused mild resistance to caspofungin; an insertion in the ECM33 3′ untranslated region (UTR; CAGEU08) did not, thus serving as a fortuitous control (Table 2). These results suggest that Orf19.1277, Msb2, Orf19.3869, Wsc1, and Ecm33 are required for normal cell wall structure or integrity and that Sun41 and Orf19.5412 are required for both cell wall integrity/structure and biofilm formation.

**Assay of Sun41 biological function in vitro.** Sun41 is a putative cell wall protein, based on its homology to S. cerevisiae Sun4 and the presence of a predicted signal sequence. SUN4 belongs to the yeast SUN gene family (SIMI, UTH1, and NCA3), whose members share a 258-amino-acid glucosidase-like domain near the C terminus and have roles in DNA replication, aging, mitochondrial biogenesis, and septation (28). No member of this family is known to function in cell wall integrity or biofilm formation. In fact, a deletion of the S. cerevisiae ortholog SUN4 does not affect sensitivity to cell wall inhibitors (51). Thus, it seemed possible that Sun41 may have a unique biological function in C. albicans. To verify that Sun41 is required for these processes, we created a homozygous sun41Δ/sun41Δ deletion mutant and sun41Δ/sun41Δ+pSUN41-complemented strain for phenotypic analysis. Both strains were prototrophic (strains CTN46 and CTN56; see Materials and Methods). The deletion homoygote was defective in biofilm formation and hyposensitive to caspofungin (Fig. 1); the severity of the defects was similar to those of the insertion mutants. Similar results were obtained with a second independent sun41Δ/sun41Δ strain. To confirm that the sun41 deletion was the cause of these phenotypes, we created complemented strains by introducing a vector carrying the predicted SUN41 coding region, 500 bp of the 3′ UTR, and 2,000 bp of the 5′ UTR. (The complementing
constitute includes all of the recently discovered SUN41 5'-1,021-bp intron [26].) The complemented strain was similar to the wild-type reference strain DAY185 in the ability to form biofilms and sensitivity to caspofungin (Fig. 1). We verified that SUN41 expression was comparable in the reference strain and complemented strain (Fig. 2). These results establish that Sun41 is required for biofilm formation and cell wall integrity.

Many biofilm-defective mutants have aberrant hyphal morphogenesis (4, 30, 36, 37). To explore this possible explanation for the sun41Δ/sun41Δ mutant defect, we examined cells from biofilm cultures by light microscopy (Fig. 1). Cells from the reference strain and sun41Δ/sun41Δ+pSUN41 cultures had abundant elongated hyphae with characteristic parallel cell walls (Fig. 1D and F). The sun41Δ/sun41Δ culture also had many elongated cells, but the cell walls were not uniformly parallel (Fig. 1E). In addition, constrictions between cells were often apparent, giving an appearance intermediate between hyphae and pseudohyphae. Prior studies have shown that the biofilm defect of two hypha-defective mutants correlates with reduced ALS3 expression (29, 53). However, hyphae of the sun41Δ/sun41Δ mutant expressed normal levels of Als3 on their surface, as measured through flow cytometry with anti-Als3 antisera (data not shown). These results indicate that Sun41 is required for normal hyphal morphogenesis but suggest that this may not be the sole reason for the sun41Δ/sun41Δ mutant biofilm defect.

We considered the possibility that a general cell wall defect may contribute to a biofilm formation defect. This hypothesis is based on our finding that the two biofilm-defective mutants identified in our insertion mutant screen were also hypersensitive to caspofungin (Table 2). Two additional observations support the idea that Sun41 is required for cell wall integrity. First, we observed that the sun41Δ/sun41Δ mutant is sensitive to a second cell wall inhibitor, Congo red (Fig. 1). Second, we reasoned that if the sun41Δ/sun41Δ mutant had a defective cell wall, then the mutant might express cell wall damage response genes in the absence of exogenous cell wall-perturbing agents. We assayed expression of four cell wall damage response genes: DDR48, PHR1, STP4, and CHT2 (Fig. 2). We found altered expression of all four genes in the sun41Δ/sun41Δ mutant relative to the reference strain and sun41Δ/sun41Δ+pSUN41-complemented strain. Two of these genes, PHR1 and CHT2, specify proteins with catalytic roles in cell wall biogenesis (12, 25). These findings argue that Sun41 is required for general cell wall structure. The sun41 mutation may have direct effects on the cell wall, given that Sun41 is a predicted cell wall protein, as well as indirect effects through altered expression of cell wall biogenesis genes.

**Requirement for Sun41 in mouse models of infection.** To determine whether Sun41 may have a role in infection, we studied the virulence of the sun41Δ/sun41Δ mutant in murine models of disseminated and oropharyngeal candidiasis. In the disseminated candidiasis model, the median survival of mice infected with reference strain DAY185 was 6 days (Fig. 3). In contrast, mice infected with the sun41Δ/sun41Δ mutant survived until the experiment was terminated at 21 days. Rescue of wild-type virulence was observed in mice infected with the sun41Δ/sun41Δ+pSUN41 strain, as their median survival was 6 days. These results indicate that Sun41 is required for disseminated infection.

Candida can progress to acute hematogenously disseminated candidiasis and result in the infection of various organs. The murine tail vein infection model mimics this course of pathogenesis, as fungal cells introduced into the bloodstream disseminate and cause disease in organs such as the liver and kidneys. Acute infection of the kidneys primarily accounts for mortality in this model (2). Therefore, we measured the kidney fungal burden of mice inoculated with DAY185, sun41Δ/sun41Δ, and sun41Δ/sun41Δ+pSUN41 cells through the tail vein at days 1 and 4 postinfection and examined the kidney histopathology. The kidney fungal burden of mice infected with sun41Δ/sun41Δ cells was significantly less than those of mice infected with DAY185 and sun41Δ/sun41Δ+pSUN41 cells (P < 0.001) on both days 1 and 4 (Fig. 4A). The difference between sun41Δ/sun41Δ and sun41Δ/sun41Δ+pSUN41 fungal burden values increased from 10-fold to 1,000-fold over time. The attenuated virulence of sun41Δ/sun41Δ cells was also apparent from histopathological examination. Kidneys harvested from mice infected with DAY185 and sun41Δ/sun41Δ+pSUN41 cells on day 1 showed numerous foci of infection that contained...
was intact, and tissue invasion and inflammation were not observed (Fig. 5H). Therefore, Sun41 is required for maximal growth and invasion in this oral infection model.

Taken together, data from murine models of infection underscore a critical role for Sun41 in C. albicans pathogenicity. We hypothesized that Sun41 may be required for adherence, endocytosis, or damage to endothelial or epithelial cells. We assayed DAY185, sun41Δ/sun41Δ, and sun41Δ/sun41Δ+pSUN41 strains for adherence, endocytosis, and damage to cultured endothelial and epithelial cells. No significant differences in these interactions were observed (data not shown). These results suggest that Sun41 may not be required for the interactions of C. albicans with these host cells but perhaps with the capacity of the organism to resist being killed by professional phagocytes.

DISCUSSION

The C. albicans cell wall has long been of interest for its diverse biological roles. New approaches based on bioinformatics, expression profiling, and proteomics provide the opportunity to identify major cell wall proteins and infer possible functional activities (10, 38, 40, 46). Here we have begun to complement those strategies through creation of a panel of insertion mutants affecting cell wall-related genes. Our identification and analysis of Sun41 as a protein required for several traits related to pathogenicity illustrate the utility of this approach and highlight Sun41 itself as a prospective therapeutic target.

Gene discovery through insertional mutagenesis. We have extended our C. albicans insertional mutagenesis approach (9, 30) to understand the biological roles of cell wall-related genes. One unique aspect of the present study is that we have tested multiple insertion alleles of 18 genes. The results are of interest for two reasons. First, some insertion mutations may not abolish gene function completely, and so one might expect to see some phenotypic differences among alleles. For this set of strains, we saw viable mutants with phenotypic variation in only one case, Orf19.3869. In this case, one insertion allele was associated with caspofungin hypersensitivity and others were not (strain CAGBT32-2) (Table 2). However, additional isolates homozygous for the CAGBT32 insertion were not caspofungin hypersensitive, thus arguing that a secondary mutation caused the caspofungin hypersensitivity of strain CAGBT32-2. Therefore, we did not observe significant phenotypic variation in our screens of these strains. The second issue of interest was unexpected: we found for Orf19.1714/PGA44 and Orf19.3966/CRH12 that some insertion alleles allowed us to recover homozygous mutants have been made not to see some phenotypic differences among alleles. For this set of strains, we saw viable mutants with phenotypic variation in only one case, Orf19.3869. In this case, one insertion allele was associated with caspofungin hypersensitivity and others were not (strain CAGBT32-2) (Table 2). However, additional isolates homozygous for the CAGBT32 insertion were not caspofungin hypersensitive, thus arguing that a secondary mutation caused the caspofungin hypersensitivity of strain CAGBT32-2. Therefore, we did not observe significant phenotypic variation in our screens of these strains. The second issue of interest was unexpected: we found for Orf19.1714/PGA44 and Orf19.3966/CRH12 that some insertion alleles allowed us to recover homozygous mutants and others did not (Table 2). One might think that these genes are essential and that suppressor mutations arose in some rare cases during selection to permit survival of homozygotes. However, the alleles that yielded viable homozygous mutants did so at a high frequency. In addition, viable crh12Δ/crh12Δ homozygous mutants have been made previously by others (33). Thus, we believe that these genes are not essential for viability. One explanation is that our transformation recipient occasionally has a preexisting triplication of the targeted locus. In support of this explanation, we note that PGA44 and CRH12 lie on chromosomes 3 and 5, respectively, the two most frequently aneuploid chromosomes among non-azole-resistant C. albicans isolates (44). Thus, these chro-
mosomes may naturally be more unstable than others. A second possibility is that certain truncated protein fragments created by insertions are toxic. The toxicity may be augmented in a homozygous mutant due to an increased dosage of the toxic allele. Whether these or more complex explanations are correct, the data we have provided here emphasize that essential gene assignments by this method is tentative (9, 11). Generally, then, our findings underscore that essential gene assignments should be based upon independent transformations, preferably with different alleles.

**Function of Sun41 in biofilm formation.** We found that sun41Δ/sun41Δ mutants have a clear biofilm defect. The defect is distinct from what we have seen with a bcr1Δ/bcr1Δ deletion mutant or a tec1Δ insertion mutant, in which the bulk of cells grow in suspension in fairly small clumps (30). The sun41Δ insertion and deletion homozygotes produced a sheet of cells that detached readily from the substrate. Sometimes the sheet was further fragmented by agitation in the assay system; other times it folded over on itself. Thus, we believe that Sun41 functions to augment attachment of the biofilm to the substrate. The regulation of SUN41 expression is interesting in this regard: Ernst and colleagues found that SUN41 is induced under low-oxygen conditions (45). A similar regulatory response is seen in *S. cerevisiae* for the ortholog SUN4 (16). We can assemble these observations into a simple hypothesis: oxygen limitation at the base of a biofilm may induce SUN41 expression, which in turn modifies the cell wall to improve substrate adherence.

Although SUN41 is induced by hypoxic conditions, it is also expressed under aerobic conditions. This point is illustrated by our detection of SUN41 RNA from aerobic cultures and by the cell wall integrity defects of sun41Δ/sun41Δ mutants under aerobic conditions.

Other cell wall proteins that are required for biofilm formation—Als3, Hwp1, and Eap1—seem to function as adhesins (18, 19, 29, 31, 53). We doubt that Sun41 has such a role, because expression of several adhesins from the TEF1 promoter in a biofilm-defective bcr1 mutant restored biofilm formation (29), whereas TEF1-SUN41 and TDH3-SUN41 did not (C. T. Norice, unpublished results). One simple possibility is
that Sun41 is required for biogenesis of an adhesin. We know from flow cytometry that the sun41Δ/sun41Δ mutant expresses Als3 on its surface, but perhaps it is defective in biogenesis of a different adhesin. A second possibility is that the sun41Δ/sun41Δ mutant has a global defect in cell wall integrity that compromises adhesin function. For example, Sun41 may strengthen association of adhesin molecules with the cell wall, or it may be required for a suitable surface array of adhesin functional sites that yields increased binding affinity (19). The fact that the mutant is hypersensitive to caspofungin and Congo red supports the idea that it has a general cell wall defect. This idea is further supported by the altered expression of cell wall damage response genes in the sun41 mutant. C. albicans Sun41 and S. cerevisiae Sun4 have over 50% amino acid identity over their C-terminal regions, a putative glucohydrolase domain (27, 28). It seems reasonable that Sun41 may have a catalytic role in modification of cell wall carbohydrate to promote normal cell wall structure.

We note that overexpressed S. cerevisiae Sun4 has been found peripherally associated with mitochondria, based on biochemical fractionation (51). We do not know whether overexpressed C. albicans Sun41 would behave similarly. This may be an interesting avenue for future study.

Role of Sun41 in virulence. SUN41 is among the few C. albicans genes known thus far to be required in both mucosal and deep tissue infection models. In both infection models, sun41Δ/sun41Δ mutant cells fail to invade host tissue efficiently. Among the most well-established C. albicans requirements for virulence is hyphal formation (15), and so a simple model is that the aberrant hyphae produced by the sun41 mutant are the basis for its virulence defect. We cannot rule that explanation out, but there are several differences between the behavior of sun41Δ/sun41Δ mutants and characterized hypha-defective mutants. For example, the sun41Δ/sun41Δ mutant expressed Als3, while many hypha-defective mutants do not (1). Second, the sun41Δ/sun41Δ mutant had no defect in endothelial cell damage, while all other hypha-defective mutants tested have such a defect (41). Third, the sun41Δ/sun41Δ mutant did not persist in asymptomatic infected mice, while two other hypha-defective hypha-defective mutants do persist (21, 43). These points lead us to believe that the sun41Δ/sun41Δ virulence defect may have a different mechanistic basis from other hypha-defective mutants.

We suggest that the sun41Δ/sun41Δ cell wall defect is the major cause of its virulence defect. It is well established that cell wall integrity is required for virulence (24, 39). It is possible that the defects in cell wall integrity of the sun41Δ/sun41Δ mutant may render it highly susceptible to being killed by professional phagocytic cells. What makes Sun41 particularly interesting is the possibility that it may be a therapeutic target. It is well conserved in many ascomycetes, and its similarity to glucohydrolases raises the possibility that it acts catalytically. Thus, it might be susceptible to small-molecule inhibitors or may serve as a vaccine target.

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