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Requirement for *Candida albicans* Sun41 in Biofilm Formation and Virulence[▽]

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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, *MSB2*, 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 *sun41*Δ/*sun41*Δ deletion mutants and *sun41*Δ/*sun41*Δ+p*SUN41*-complemented strains, we verified that Sun41 is required for biofilm formation and normal caspofungin tolerance. The *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ 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

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TABLE 1. Strains used in this study

Strain	Genotype	Reference
BWP17	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG*	52
DAY185	*ura3*Δ::λ*imm434* *HIS1*::*his1*::*hisG* *ARG4*::*URA3*::*arg4*::*hisG* *ura3*Δ::λ*imm434* *his1*::*hisG* *arg4*::*hisG*	8
CTN41	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *sun41*::*ARG4* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *sun41*::*URA3*	This study
CTN46	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG*::*pHIS1* *sun41*::*ARG4* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *sun41*::*URA3*	This study
CTN56	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG*::*pHIS1-SUN41* *sun41*::*ARG4* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *sun41*::*URA3*	This study
DAY286	*ura3*Δ::λ*imm434* *ARG4*:*URA3*::*arg4*::*hisG* *his1*::*hisG* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG*	9
CJN702	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG*::*pHIS1* *bcr1*::*ARG4* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *bcr1*::*URA3*	30
CJN432	*ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *cas5*::*Tn7-UAU1* *ura3*Δ::λ*imm434* *arg4*::*hisG* *his1*::*hisG* *cas5*::*Tn7-URA3*	30

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 oropharyngeal 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*/*ORF* heterozygotes (see below), we used SC-Arg-Ura Beefy 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), inositol (2.0 g), isoleucine (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).

Construction of the insertion mutant strains (Table 2) followed the basic procedure of Davis et al. (9), using a library of Tn*7*-*UAU1* insertions in *C. albicans* CAI4 DNA that were sequenced from one end. Details of the library construction will be published separately. The insertion sites for each clone are listed at http://www.tigr.org/tigr-scripts/e2k1/qzhao/page.cgi?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 Tn*7*-*UAU1* insertion, was excised from the plasmid backbone through digestion with NotI, and the entire digest was transformed into strain BWP17. Arg^+ transformants (presumably heterozygous for the insertion) were selected on SC-Arg+Uri 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-Arg-Ura Beefy plates to select for Arg^+ Ura^+ recombinants. Based on our past studies, some of these are homozygous insertion mutants (genotype *orf*::Tn*7*-*UAU1*/*orf*::Tn*7*-*URA3*) and others are allelic triplication derivative mutants (genotype *ORF*/*orf*::Tn*7*-*UAU1*/*orf*::Tn*7*-*URA3*). Following approximately 5 days of growth, one colony from each quarter was purified by streaking on SC-Arg-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*::Tn*7*-*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*::Tn*7*-*UAU1* product, which is too large to amplify reliably with the flanking primers, was amplified by adding a third primer to the mix (Arg4detect primer, sequence GGAATTGATCAATTATCTTTTGAAC).

Strain CTN46, the prototrophic *sun41*Δ::*ARG4*/*sun41*Δ::*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 SUN41-5DR (5′-GTTTCTTTTAGTCGTTCCTTTTTTTATAATTC ACTTGTTTGTCATATAGTCTCAACTGTACATTCGTTTTTCAACTACTGTTC ATTTATTTTAATTATCATTTCCCAGTCACGACGTT-3′) and SUN41-3DR (5′-T TAAAAAAACACTAACTTGAAAACAAACACTATTCTTTTTAAAAAAAACA ATATAAAGGAAGAAGAGAAAAAGGGGTATCTTGTACTTTTCTCAATCTC AGTGGAATTGTGAGCGGATA-3′). We transformed strain BWP17 with the *URA3* construct, selected for Ura^+ transformants, and screened by whole-cell PCR for the presence of a *sun41*Δ::*URA3* allele and a wild-type allele. Several of these heterozygotes were transformed with the *ARG4* construct and plated on SC-Arg-Ura medium. Ura^+ Arg^+ isolates were screened by whole-cell PCR for the presence of *sun41*Δ::*URA3* and *sun41*Δ::*ARG4* alleles and the absence of wild-type alleles. We used one His^- *sun41*Δ::*ARG4*/*sun41*Δ::*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 Esp3I site. In genotypic designations, pRYS2 is listed as p*HIS1*. The prototrophic *sun41*Δ::*ARG4*/*sun41*Δ::*URA3* mutant is designated CTN46.

To complement the *sun41* deletion, we used BWP17 genomic DNA as a

TABLE 2. *C. albicans* insertion mutant summary

ORF[a]	Gene[a]	Clone name[b]	Gene length (nt)	Insertion site[c] (nt)	*S. cerevisiae* ortholog (best match)[d]	No. screened[e]	No. recovered[f]	Mutant strain name	Biofilm formation	Caspfungin growth	Description
Orf19.10938	Orf19.10938	CAGEN68	2,969	1,781	(YGR067C)	12	2	CAGEN68-4	+	+	Predicted ORF
		CAGGG92	2,969	1,141	(YGR067C)	12	3	CAGGG92-1	+	+	
Orf19.1277	Orf19.1277	CAGD531	1,178	885	None	12	3	CAGD531-1	+	+	Predicted ORF
Orf19.1490	MSB2	CAGCO56	4,229	4,192	MSB2	12	5	CAGCO56-4	+	−	Protein of unknown function
		CAGFU32	4,229	2,852	MSB2	12	6	CAGFU32-1	+	−	
		CAGGD61	4,229	775	MSB2	12	3	CAGGD61-1	+	−	
Orf19.1563	ECM3	CAGDL37	1,640	896	ECM3	12	7	CAGDL37-2	+	+	Predicted ORF
		CAGG758	1,640	95	ECM3	12	9	CAGG758-4	+	+	
		CAGGW32	1,640	620	ECM3	12	7	CAGGW32-1	+	+	
Orf19.1714	PGA44	CAGAJ18	923	254	None	12	0				Putative GPI-anchored protein of unknown function
		CAGAU60	923	378	None	12	9	CAGAU60-1	+	+	
Orf19.2476	Orf19.2476	CAGCK11	5,054	3,610	ECM5	12	2	CAGCK11-2	+	+	Predicted ORF
		CAGCV89	5,054	285	ECM5	12	6	CAGCV89-3	+	+	
		CAGE838	5,054	3,961	ECM5	12	6	CAGE838-3	+	+	
Orf19.2613	ECM4	CAGDQ11	1,085	1,039	ECM4	12	8	CAGDQ11-3	+	+	Protein similar to *S. cerevisiae* Ecm4
		CAGES55	1,085	172	ECM4	12	7	CAGES55-1	+	+	
		CAGH736	1,085	64	ECM4	12	8	CAGH736-1	+	+	
Orf19.2623	ZCF12	CAGFB35	2,582	2,466	ECM22	12	4	CAGFB35-1	+	+	Putative transcription factor with zinc cluster DNA-binding motif
		CAGG709	2,582	2,067	ECM22	12	2	CAGG709-2	+	+	
Orf19.299	ECM14	CAGBY54	1,412	1,829	ECM14	12	6	CAGBY54-5	+	+	Predicted ORF
		CAGGG42	1,412	1,206	ECM14	12	6	CAGGG42-1	+	+	
Orf19.3010.1	ECM33	CAGBU26	1,238	730	ECM33	12	8	CAGBU26-1	+	+/+	Cell wall protein; putative GPI anchor
		CAGCB64	1,238	223	ECM33	12	5	CAGCB64-2	+	+/+	
		CAGEU08	1,238	1,465	ECM33	12	6	CAGEU08-4	+	+	
Orf19.310	Orf19.310	CAGFI61	1,520	1,274	ECM18	12	0				Predicted ORF
Orf19.3642	SUN41	CAGCV53	1,256	507	SUN4	12	3	CAGCV53-7	−	−	Putative cell wall protein
Orf19.3869	Orf19.3869	CAGBT32	1,754	496	None	3	2	CAGBT32-2	+	−[g]	Predicted ORF
		CAGCJ26	1,754	702	None	12	6	CAGCJ26-2	+	+	
		CAGCR48	1,754	1,542	None	12	6	CAGCR48-1	+	+	
Orf19.3893	SCW11	CAGA730	974	275	SCW11	12	0				Cell wall protein
		CAGCB65	974	30	SCW11	12	0				
		CAGEQ04	974	421	SCW11	4	0				
Orf19.3966	CRH12	CAGB642	1,514	56	CRH1	12	8	CAGB642-3	+	+	Putative cell wall protein
		CAGCT09	1,514	430	CRH1	12	5	CAGCT09-1	+	+	
		CAGGG86	1,514	463	CRH1	12	0				
		CAGGY57	1,514	570	CRH1	12	0				
Orf19.4884	WOR1	CAGAI54	2,357	504	YEL007W	12	6	CAGAI54-1	+	+	Transcriptional regulator of white-opaque switching
		CAGES09	2,357	407	YEL007W	12	6	CAGES09-4	+	+	
Orf19.4887	ECM21	CAGC007	2,189	1,152	CSR2	12	9	CAGC007-1	+	+	Protein not essential for viability
		CAGFA52	2,189	1,346	CSR2	12	8	CAGFA52-1	+	+	
Orf19.4981	Orf19.4981	CAGEI30	2,114	1,278	YDL206W	12	3	CAGEI30-2	+	+	Predicted ORF
		CAGFQ65	2,114	1,811	YDL206W	12	8	CAGFQ65-1	+	+	
		CAGHF63	2,114	1,167	YDL206W	12	5	CAGHF63-1	+	+	
Orf19.532	RBR2	CAGBL17	506	264	TIP1	12	7	CAGBL17-1	+	+	Cell wall protein
		CAGD166	506	483	TIP1	12	9	CAGD166-1	+	+	
Orf19.5412	Orf19.5412	CAGEI52	1,085	123	ECM9	12	3	CAGEI52-1	−	−	Predicted ORF
Orf19.5674	PGA10	CAGGO69	752	1,197	None	12	6	CAGGO69-1	+	+	Plasma membrane protein involved in heme-iron utilization; RBT51
Orf19.5861	KRE9	CAGFA83	815	61	KRE9	12	0				Protein of β-1,6-glucan biosynthesis
Orf19.5867	WSC1	CAGD120	1,076	150	WSC3	6	4	CAGD120-1	+	−	Protein described as a cell wall component
		CAGEO32	1,076	709	WSC3	12	12	CAGEO32-2	+	−	

Orf19.6958	ECM18	CAGFI61	1,520	1,274	ECM18	12	0			Predicted ORF
Orf19.893	PGA17	CAGE592	1,673	1,282	None	12	7	CAGE592-1	+	Putative GPI-anchored protein of unknown function

[a] ORF assignments and gene designations are taken from the Candida Genome Database (http://www.candidagenome.org/).
[b] The clone name refers to the insertion clone used to make each mutant (see Materials and Methods).
[c] The insertion site is the distance from the ATG initiator of the ORF to the transposon insertion site.
[d] *S. cerevisiae* orthologs or closest homologs (in parentheses) were taken from the Candida Genome Database (http://www.candidagenome.org/).
[e] The number screened refers to the number of independent transformants from which Arg^+ Ura^+ segregants were derived to screen for homozygotes.
[f] The number recovered is the number of independent homozygotes identified among the Arg^+ Ura^+ segregants screened.
[g] Only one isolate with this insertion allele was caspofungin hypersensitive; additional isolates behaved similarly to control strain DAY286.

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′-CATGTCATCAACAACTGTACTC-3′) and SUN41comp3′ (5′-TTGTTGTTTGGTGGATTATG-3′). The amplicon was ligated into the pGEMT-Easy vector (Promega) and then released by digestion with SapI and NgoMIV. This fragment was inserted into EcoRI- and NotI-digested pRYS2 by in vivo recombination in *Saccharomyces cerevisiae* to yield the pHIS1-SUN41 plasmid pCTN16. The complemented strain, CTN56, which contains the *SUN41* open reading frame (ORF), was constructed by transforming strain CTN41 (*sun41*Δ::*ARG4*/*sun41*Δ::*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 (OD_{600}) 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 adhesion 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 OD_{600} of 3 in 1 ml of double-distilled H_2O and then serially diluted fivefold to an OD_{600} of 0.6, 0.12, 0.024, 4.8×10^{-3}, or 9.6×10^{-4}. 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 2 days of growth.

RNA extraction and real-time PCR. Cells growing in 50 ml YPD were harvested by vacuum filtration at an OD_{600} 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 from 2.5 μg of RNA using the AffinityScript multiple temperature 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′-AACCCTTTCCCTTCCATCTG-3′, and SUN41RT R, 5′-ACCAGAACCAGAACCACCAG-3′; for *DDR48*, JRB212, 5′-TTTCGGTTTCGGTAAAGACG-3′, and JRB213, 5′-CTGTTGGAGGAACCGTAGGA-3′; for *PHR1*, JRB214, 5′-GATTGCTCGGCTATTTCTGC-3′, and JRB215, 5′-TGATTGAAGCACTGCCTTTG-3′; for *STP4*, JRB244, 5′-TCCTTTCAAGAACATCGATTCA-3′, and JRB245, 5′-TTATGCATCCAATCATCGACA-3′; for *CHT2*, CHT2 FWD PR, 5′-ACAAATGTGTTGCCACTCCA-3′, and CHT2 REV PR, 5′-GGCTTTTGGTTTTTGAGCAG-3′; for *TDH3*, TDH3 fwd, 5′-ATCCCACAAGGACTGGAGA-3′, and TDH3 rev, 5′-GCAGAAGCTTTAGCAACGTG-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 detection 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 ΔC_T 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 2×10^5 blastospores of DAY185, *sun41*, or *sun41*+p*SUN41* 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 10^6 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*+p*SUN41* 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 oral epithelial cells and endothelial cells were compared by an analysis of variance.

RESULTS

Identification of biofilm-defective and caspofungin-hypersensitive 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-hypersensitive 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 *SUN41* and Orf19.5412 (Table 2). Caspofungin sensitivity was tested in a spot dilution assay on solid agar (5). Several strains were hypersensitive to caspofungin, representing insertions in Orf19.1277, *MSB2*, *SUN41*, 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.

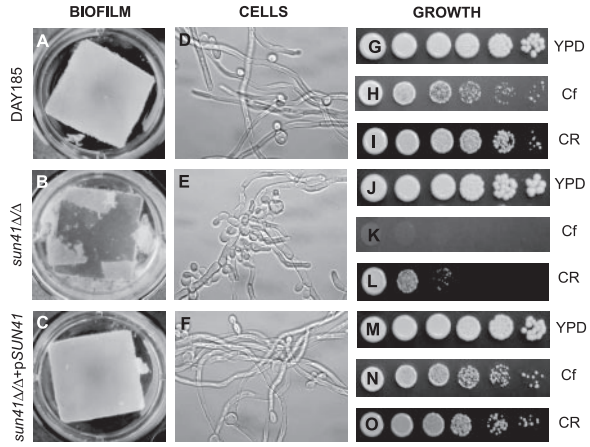

FIG. 1. Biofilm formation and cell wall inhibitor sensitivity of wild-type, mutant, and complemented strains. Biofilm formation assays were conducted with reference strain DAY185 (A and D), the *sun41*Δ/*sun41*Δ mutant strain (B and E), or the *sun41*Δ/*sun41*Δ+p*SUN41*-complemented strain (C and F) and were photographed after incubation for 60 h with 150-rpm agitation (A to C). Cells suspended from each biofilm incubation were visualized by light microscopy at ×100 magnification (D to F). Caspofungin and Congo red sensitivity assays employed serial dilutions of DAY185 (G, H, and I), *sun41*Δ/*sun41*Δ (J, K, and L), and *sun41*Δ/*sun41*Δ+p*SUN41* (M, N, and O) overnight cultures that were spotted onto YPD (G, J, and M), YPD plus 125 ng/ml caspofungin (H, K, and N), or YPD plus 200 μg/ml Congo red (I, L, and O) medium. Growth was visualized after 3 days of incubation at 30°C.

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 (S*IM1*, U*TH1*, and N*CA3*), 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 *sun41*Δ/*sun41*Δ deletion mutant and *sun41*Δ/*sun41*Δ+p*SUN41*-complemented strain for phenotypic analysis. Both strains were prototrophic (strains CTN46 and CTN56; see Materials and Methods). The deletion homozygote was defective in biofilm formation and hypersensitive 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 *sun41*Δ/*sun41*Δ 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

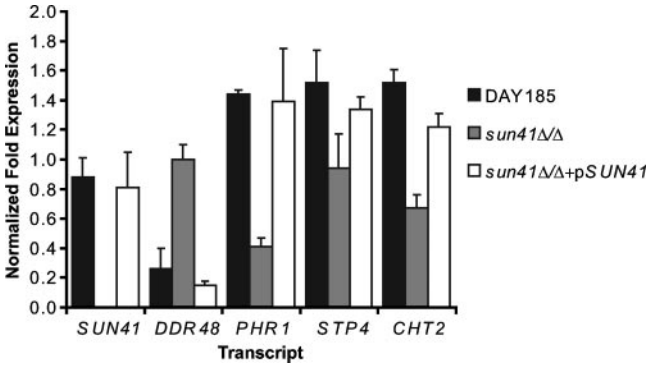

FIG. 2. Gene expression in wild-type, mutant, and complemented strains. Real-time PCR assays were conducted on RNA samples from reference strain DAY185, the *sun41*Δ/*sun41*Δ mutant strain, and the *sun41*Δ/*sun41*Δ+p*SUN41*-complemented strain. Reactions assayed RNA levels for *SUN41*, *DDR48*, *PHR1*, *STP4*, and *CHT2*. Levels of each RNA were normalized to *TDH3* RNA measurements carried out simultaneously. Results are the means of three determinations.

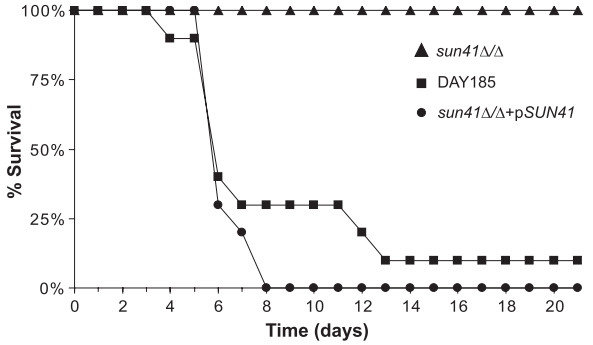

FIG. 3. *SUN41* requirement for virulence during hematogenously disseminated candidiasis. Mice were infected via the tail vein with 2×10^5 vegetative cells of DAY185 ($n = 10$), *sun41*Δ/*sun41*Δ ($n = 10$), or *sun41*Δ/*sun41*Δ+p*SUN41* ($n = 10$) and monitored for survival over 21 days. Median durations of survival were 6, 21, and 6 days, respectively.

construct 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 *sun41*Δ/*sun41*Δ mutant defect, we examined cells from biofilm cultures by light microscopy (Fig. 1). Cells from the reference strain and *sun41*Δ/*sun41*Δ+p*SUN41* cultures had abundant elongated hyphae with characteristic parallel cell walls (Fig. 1D and F). The *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ mutant expressed normal levels of Als3 on their surface, as measured through flow cytometry with anti-Als3 antiserum (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 *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ mutant is sensitive to a second cell wall inhibitor, Congo red (Fig. 1). Second, we reasoned that if the *sun41*Δ/*sun41*Δ 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). There was altered expression of all four genes in the *sun41*Δ/*sun41*Δ mutant relative to the reference strain and *sun41*Δ/*sun41*Δ+p*SUN41*-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 *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ mutant survived until the experiment was terminated at 21 days. Rescue of wild-type virulence was observed in mice infected with the *sun41*Δ/*sun41*Δ+p*SUN41* strain, as their median survival was 6 days. These results indicate that Sun41 is required for disseminated infection.

Candidemia 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, *sun41*Δ/*sun41*Δ, and *sun41*Δ/*sun41*Δ+p*SUN41* cells through the tail vein at days 1 and 4 postinfection and examined the kidney histopathology. The kidney fungal burden of mice infected with *sun41*Δ/*sun41*Δ cells was significantly less than those of mice infected with DAY185 and *sun41*Δ/*sun41*Δ+p*SUN41* cells ($P \leq 0.001$) on both days 1 and 4 (Fig. 4A). The difference between *sun41*Δ/*sun41*Δ and *sun41*Δ/*sun41*Δ+p*SUN41* fungal burden values increased from 10-fold to 1,000-fold over time. The attenuated virulence of *sun41*Δ/*sun41*Δ cells was also apparent from histopathological examination. Kidneys harvested from mice infected with DAY185 and *sun41*Δ/*sun41*Δ+p*SUN41* cells on day 1 showed numerous foci of infection that contained

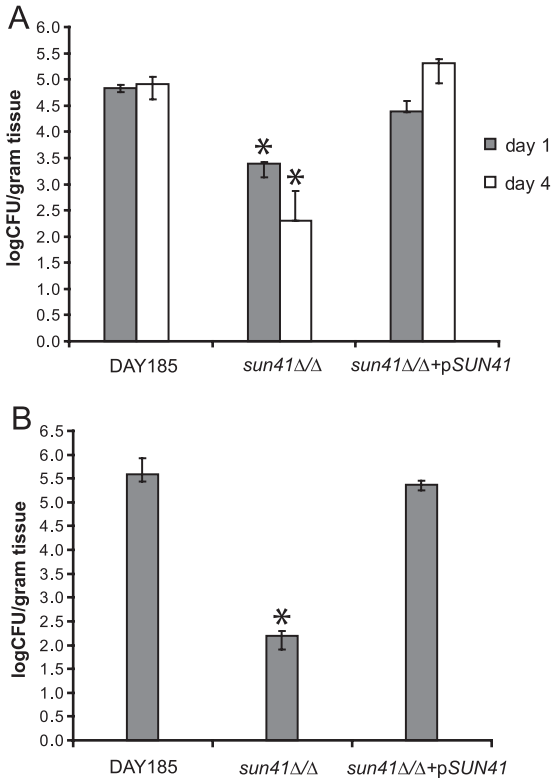

FIG. 4. *SUN41* requirement for fungal burden during disseminated and oropharyngeal candidiasis. (A) Mice were infected as described in the Fig. 3 legend, and the log CFU per gram of kidney tissue was measured in five animals per strain after day 1 and day 4 of infection. Results are presented as the median ± the interquartile range. (B) Three groups of seven mice were immunosuppressed with cortisone acetate and orally inoculated with swabs saturated in 10^6 vegetative cells per ml of DAY185, *sun41*Δ/*sun41*Δ, or *sun41*Δ/*sun41*Δ+p*SUN41* cells for 75 min. Tongues were excised after 5 days of infection, and the log CFU per gram of tongue tissue was measured. Results are presented as the median ± the interquartile range. An asterisk indicates a *P* value of ≤0.001 compared to either the DAY185 reference strain or the *sun41*Δ/*sun41*Δ+p*SUN41*-reconstituted strain.

many fungal cells (Fig. 5A and C). In contrast, the kidneys from mice infected with the *sun41*Δ/*sun41*Δ strain had rare lesions that contained few fungal cells (Fig. 5B). By day 4, the kidneys of mice infected with DAY185 and *sun41*Δ/*sun41*Δ+p*SUN41* cells had multiple microabscesses containing many organisms with extensive filamentation (Fig. 5D and F). At this time point, the kidneys of mice infected with *sun41*Δ/*sun41*Δ cells had a few lesions that contained a paucity of short filaments (Fig. 5E). These findings indicate that Sun41 is required for normal kidney infection in this disseminated infection model.

In the oropharyngeal candidiasis model, we investigated the contribution of Sun41 through measurement of fungal burden of tongue tissue and assessments of histopathology after 5 days of infection. The fungal burden of tongue tissue in mice infected with *sun41*Δ/*sun41*Δ cells was over 1,000-fold less than that of mice inoculated with DAY185 or *sun41*Δ/*sun41*Δ+p*SUN41* cells ($P < 0.001$) (Fig. 4B). Mice inoculated with DAY185 and *sun41*Δ/*sun41*Δ+p*SUN41* cells presented with ulcerative lesions infiltrated by extensive fungal filaments (Fig. 5G and I). However, in mice infected with *sun41*Δ/*sun41*Δ cells, the epithelium of the tongue 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, *sun41*Δ/*sun41*Δ, and *sun41*Δ/*sun41*Δ+p*SUN41* 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 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 *crh12*Δ/*crh12*Δ 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-

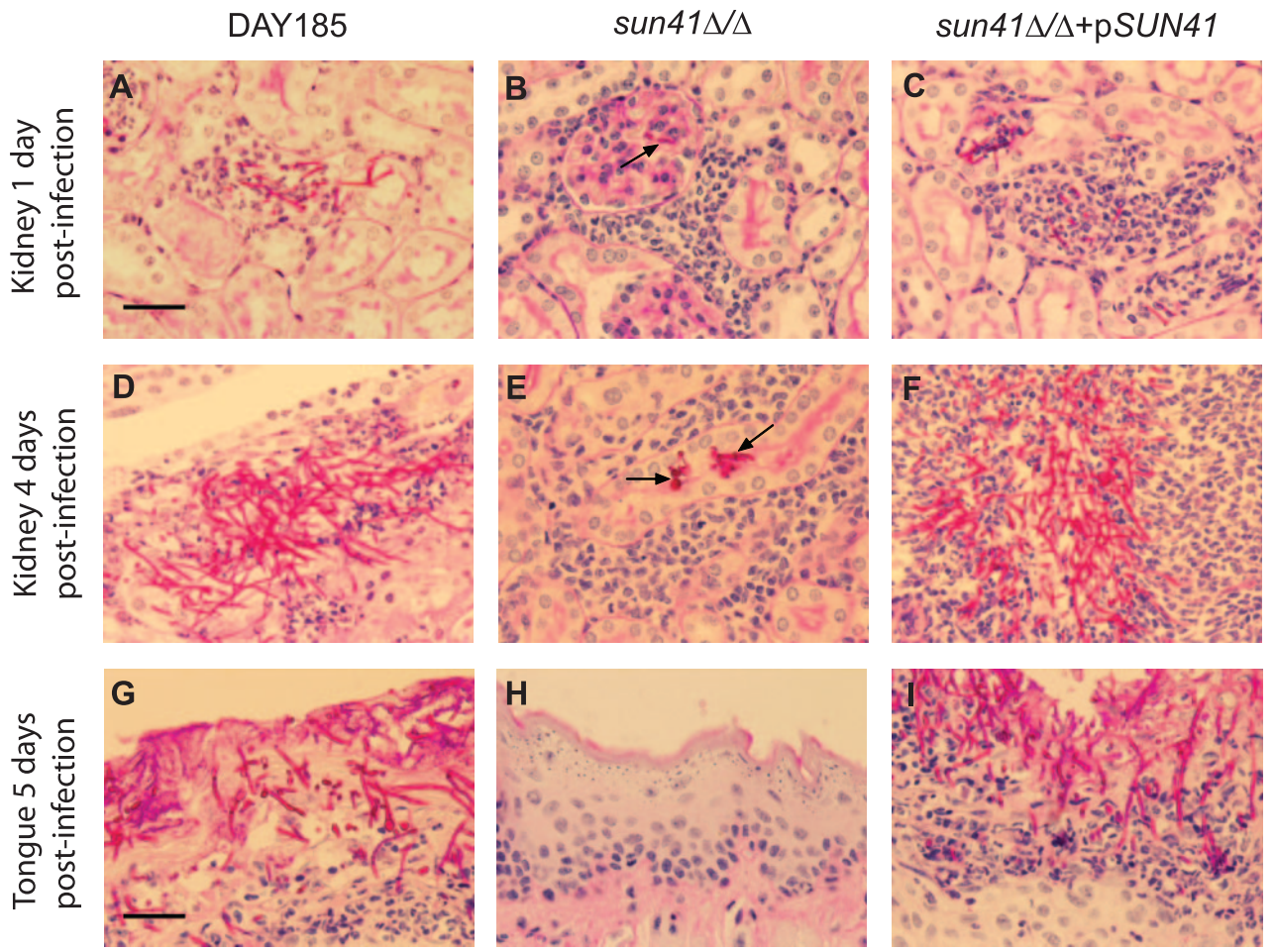

FIG. 5. *SUN41* requirement for tissue infiltration during disseminated and oropharyngeal candidiasis. (A to F) Mice were infected as described in the Fig. 3 legend, and kidneys were harvested after day 1 (A to C) and day 4 (D to F) of infection. Thin sections of the kidneys were stained with periodic acid-Schiff stain. Arrows in panels B and E point to a rare infiltration by the *sun41*Δ/*sun41*Δ mutant. (G to I) Three groups of seven mice were immunosuppressed with cortisone acetate and orally inoculated with swabs saturated in 10^6 vegetative cells per ml of DAY185, *sun41*Δ/*sun41*Δ, or *sun41*Δ/*sun41*Δ+p*SUN41* cells for 75 min. Tongues were excised after 5 days of infection, and thin sections were stained with periodic acid-Schiff stain. All sections in these panels were visualized at ×40 and are presented at the same magnification. Bars (A and G), 30 μM.

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 an essential gene assignment 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 *sun41*Δ/*sun41*Δ mutants have a clear biofilm defect. The defect is distinct from what we have seen with a *bcr1*Δ/*bcr1*Δ deletion mutant or a *tec1*$^{-/-}$ insertion mutant, in which the bulk of cells grow in suspension in fairly small clumps (30). The *sun41* 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 *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ mutant expresses Als3 on its surface, but perhaps it is defective in biogenesis of a different adhesin. A second possibility is that the *sun41*Δ/*sun41*Δ 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, *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ mutants and characterized hypha-defective mutants. For example, the *sun41*Δ/*sun41*Δ mutant expressed Als3, while many hypha-defective mutants do not (1). Second, the *sun41*Δ/*sun41*Δ mutant had no defect in endothelial cell damage, while all other hypha-defective mutants tested have such a defect (41). Third, the *sun41*Δ/*sun41*Δ mutant did not persist in asymptomatic infected mice, while two other attenuated hypha-defective mutants do persist (21, 43). These points lead us to believe that the *sun41*Δ/*sun41*Δ virulence defect may have a different mechanistic basis from other hypha-defective mutants.

We suggest that the *sun41*Δ/*sun41*Δ 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 *sun41*Δ/*sun41*Δ 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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REFERENCES

1. **Argimon, S., J. A. Wishart, R. Leng, S. Macaskill, A. Mavor, T. Alexandris, S. Nicholls, A. W. Knight, B. Enjalbert, R. Walmsley, F. C. Odds, N. A. Gow, and A. J. Brown.** 2007. Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. Eukaryot. Cell **6:**682–692.
2. **Ashman, R. B., A. Fulurija, and J. M. Papadimitriou.** 1996. Strain-dependent differences in host response to *Candida albicans* infection in mice are related to organ susceptibility and infectious load. Infect. Immun. **64:**1866–1869.
3. **Berman, J., and P. E. Sudbery.** 2002. Candida albicans: a molecular revolution built on lessons from budding yeast. Nat. Rev. Genet. **3:**918–930.
4. **Blankenship, J. R., and A. P. Mitchell.** 2006. How to build a biofilm: a fungal perspective. Curr. Opin. Microbiol. **9:**588–594.
5. **Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous, and A. P. Mitchell.** 2006. Control of the C. albicans cell wall damage response by transcriptional regulator Cas5. PLoS Pathog. **2:**e21.
6. **Castillo, L., A. I. Martinez, A. Garcera, J. Garcia-Martinez, J. Ruiz-Herrera, E. Valentin, and R. Sentandreu.** 2006. Genomic response programs of Candida albicans following protoplasting and regeneration. Fungal Genet. Biol. **43:**124–134.
7. **Chiang, L. Y., D. C. Sheppard, V. M. Bruno, A. P. Mitchell, J. E. Edwards, Jr., and S. G. Filler.** 2007. Candida albicans protein kinase CK2 governs virulence during oropharyngeal candidiasis. Cell. Microbiol. **9:**233–245.
8. **Davis, D., J. E. Edwards, Jr., A. P. Mitchell, and A. S. Ibrahim.** 2000. *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. Infect. Immun. **68:**5953–5959.
9. **Davis, D. A., V. M. Bruno, L. Loza, S. G. Filler, and A. P. Mitchell.** 2002. Candida albicans Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. Genetics **162:**1573–1581.
10. **de Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. Eukaryot. Cell **3:**955–965.
11. **Enloe, B., A. Diamond, and A. P. Mitchell.** 2000. A single-transformation gene function test in diploid *Candida albicans*. J. Bacteriol. **182:**5730–5736.
12. **Fonzi, W. A.** 1999. PHR1 and PHR2 of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans. J. Bacteriol. **181:**7070–7079.
13. **Fu, Y., G. Rieg, W. A. Fonzi, P. H. Belanger, J. E. Edwards, Jr., and S. G. Filler.** 1998. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. Infect. Immun. **66:**1783–1786.
14. **Kauffman, C. A.** 2006. Fungal infections. Proc. Am. Thorac. Soc. **3:**35–40.
15. **Kumamoto, C. A., and M. D. Vinces.** 2005. Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence. Cell. Microbiol. **7:**1546–1554.
16. **Lai, L. C., A. L. Kosorukoff, P. V. Burke, and K. E. Kwast.** 2006. Metabolic-state-dependent remodeling of the transcriptome in response to anoxia and subsequent reoxygenation in *Saccharomyces cerevisiae*. Eukaryot. Cell **5:**1468–1489.
17. **Letscher-Bru, V., and R. Herbrecht.** 2003. Caspofungin: the first representative of a new antifungal class. J. Antimicrob. Chemother. **51:**513–521.
18. **Li, F., and S. P. Palecek.** 2003. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. Eukaryot. Cell **2:**1266–1273.
19. **Li, F., M. J. Svarovsky, A. J. Karlsson, J. P. Wagner, K. Marchillo, P. Oshel, D. Andes, and S. P. Palecek.** 2007. Eap1p, an adhesin that mediates *Candida albicans* biofilm formation in vitro and in vivo. Eukaryot. Cell **6:**931–939.
20. **Liu, T. T., R. E. Lee, K. S. Barker, R. E. Lee, L. Wei, R. Homayouni, and P. D. Rogers.** 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. Antimicrob. Agents Chemother. **49:**2226–2236.
21. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous C. albicans mutants are avirulent. Cell **90:**939–949.
22. **Martinez-Lopez, R., L. Monteoliva, R. Diez-Orejas, C. Nombela, and C. Gil.** 2004. The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in Candida albicans. Microbiology **150:**3341–3354.
23. **Martinez-Lopez, R., H. Park, C. L. Myers, C. Gil, and S. G. Filler.** 2006. *Candida albicans* Ecm33p is important for normal cell wall architecture and interactions with host cells. Eukaryot. Cell **5:**140–147.
24. **Masuoka, J.** 2004. Surface glycans of *Candida albicans* and other pathogenic

fungi: physiological roles, clinical uses, and experimental challenges. Clin. Microbiol. Rev. **17:**281–310.
25. **McCreath, K. J., C. A. Specht, and P. W. Robbins.** 1995. Molecular cloning and characterization of chitinase genes from Candida albicans. Proc. Natl. Acad. Sci. USA **92:**2544–2548.
26. **Mitrovich, Q. M., B. B. Tuch, C. Guthrie, and A. D. Johnson.** 2007. Computational and experimental approaches double the number of known introns in the pathogenic yeast Candida albicans. Genome Res. **17:**492–502.
27. **Mouassite, M., N. Camougrand, E. Schwob, G. Demaison, M. Laclau, and M. Guerin.** 2000. The SUN family: yeast SUN4/SCW3 is involved in cell septation. Yeast **16:**905–919.
28. **Mouassite, M., M. G. Guerin, and N. M. Camougrand.** 2000. The SUN family of Saccharomyces cerevisiae: the double knock-out of UTH1 and SIM1 promotes defects in nucleus migration and increased drug sensitivity. FEMS Microbiol. Lett. **182:**137–141.
29. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. **2:**e63.
30. **Nobile, C. J., and A. P. Mitchell.** 2005. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr. Biol. **15:**1150–1155.
31. **Nobile, C. J., J. E. Nett, D. R. Andes, and A. P. Mitchell.** 2006. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. Eukaryot. Cell **5:**1604–1610.
32. **Noble, S. M., and A. D. Johnson.** 5 July 2007, posting date. Genetics of Candida albicans, a diploid human fungal pathogen. Annu. Rev. Genet. [Epub ahead of print.] doi:10.1146/annurev.genet.41.042007.170146.
33. **Pardini, G., P. W. De Groot, A. T. Coste, M. Karababa, F. M. Klis, C. G. de Koster, and D. Sanglard.** 2006. The CRH family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of Candida albicans. J. Biol. Chem. **281:**40399–40411.
34. **Park, H., C. L. Myers, D. C. Sheppard, Q. T. Phan, A. A. Sanchez, E. E. J., and S. G. Filler.** 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell. Microbiol. **7:**499–510.
35. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. **20:**133–163.
36. **Ramage, G., K. VandeWalle, J. L. Lopez-Ribot, and B. L. Wickes.** 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in Candida albicans. FEMS Microbiol. Lett. **214:**95–100.
37. **Richard, M. L., C. J. Nobile, V. M. Bruno, and A. P. Mitchell.** 2005. *Candida albican*s biofilm-defective mutants. Eukaryot. Cell **4:**1493–1502.
38. **Richard, M. L., and A. Plaine.** 2007. Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. Eukaryot. Cell **6:**119–133.
39. **Ruiz-Herrera, J., M. V. Elorza, E. Valentin, and R. Sentandreu.** 2006. Molecular organization of the cell wall of Candida albicans and its relation to pathogenicity. FEMS Yeast Res. **6:**14–29.
40. **Rupp, S.** 2004. Proteomics on its way to study host-pathogen interaction in Candida albicans. Curr. Opin. Microbiol. **7:**330–335.
41. **Sanchez, A. A., D. A. Johnston, C. Myers, J. E. Edwards, Jr., A. P. Mitchell, and S. G. Filler.** 2004. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. Infect. Immun. **72:**598–601.
42. **Sandini, S., R. La Valle, F. De Bernardis, C. Macri, and A. Cassone.** 2007. The 65 kDa mannoprotein gene of Candida albicans encodes a putative beta-glucanase adhesin required for hyphal morphogenesis and experimental pathogenicity. Cell. Microbiol. **9:**1223–1238.
43. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot. Cell **2:**1053–1060.
44. **Selmecki, A., A. Forche, and J. Berman.** 2006. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science **313:**367–370.
45. **Setiadi, E. R., T. Doedt, F. Cottier, C. Noffz, and J. F. Ernst.** 2006. Transcriptional response of Candida albicans to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. J. Mol. Biol. **361:**399–411.
46. **Sohn, K., J. Schwenk, C. Urban, J. Lechner, M. Schweikert, and S. Rupp.** 2006. Getting in touch with Candida albicans: the cell wall of a fungal pathogen. Curr. Drug Targets **7:**505–512.
47. **Spreghini, E., D. A. Davis, R. Subaran, M. Kim, and A. P. Mitchell.** 2003. Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. Eukaryot. Cell **2:**746–755.
48. **Sundstrom, P.** 2002. Adhesion in Candida spp. Cell. Microbiol. **4:**461–469.
49. **Sundstrom, P., E. Balish, and C. M. Allen.** 2002. Essential role of the Candida albicans transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. J. Infect. Dis. **185:**521–530.
50. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Reevaluation of the role of HWP1 in systemic candidiasis by use of *Candida albicans* strains with selectable marker URA3 targeted to the ENO1 locus. Infect. Immun. **70:**3281–3283.
51. **Velours, G., C. Boucheron, S. Manon, and N. Camougrand.** 2002. Dual cell wall/mitochondria localization of the SUN family proteins. FEMS Microbiol. Lett. **207:**165–172.
52. **Wilson, R. B., D. Davis, and A. P. Mitchell.** 1999. Rapid hypothesis testing with *Candida albican*s through gene disruption with short homology regions. J. Bacteriol. **181:**1868–1874.
53. **Zhao, X., K. J. Daniels, S. H. Oh, C. B. Green, K. M. Yeater, D. R. Soll, and L. L. Hoyer.** 2006. Candida albicans Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology **152:**2287–2299.