**Candida albicans Sun41p, a Putative Glycosidase, Is Involved in Morphogenesis, Cell Wall Biogenesis, and Biofilm Formation**

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The SUN gene family has been defined in *Saccharomyces cerevisiae* and comprises a fungus-specific family of proteins which show high similarity in their C-terminal domains. Genes of this family are involved in different cellular processes, like DNA replication, aging, mitochondrial biogenesis, and cytokinesis. In *Candida albicans* the SUN family comprises two genes, *SUN41* and *SIM1*. We demonstrate that *C. albicans* mutants lacking *SUN41* show similar defects as found for *S. cerevisiae*, including defects in cytokinesis. In addition, the *SUN41* mutant showed a higher sensitivity towards the cell wall-disturbing agent Congo red, whereas no difference was observed in the presence of calcofluor white. Compared to the wild type, *SUN41* deletion strains exhibited a defect in biofilm formation, a reduced adherence on a CaCo-2 cell monolayer, and were unable to form hyphae on solid medium under the conditions tested. Interestingly, Sun41p was found to be secreted in the medium of cells growing as blastospores as well as those forming hyphae. Our results support a function of *SUN41* as a glycosidase involved in cytokinesis, cell wall biogenesis, adhesion to host tissue, and biofilm formation, indicating an important role in the host-pathogen interaction.

*Candida albicans* is the most frequent causative agent of candidiasis, which is among the most important nosocomial infections of humans. As a commensal organism, present in the gastrointestinal or urogenital tract of 30 to 60% of the population, this opportunistic pathogen is widely spread and a constant risk for immunocompromised patients (16). In recent years, some key mechanisms and factors critical for virulence were characterized, including polymorphic switches and secreted proteases and lipases, as well as proteins localized to the cell wall, like the adhesins (10, 11, 19). The cell wall and its components are prime targets for antifungal strategies, as they mediate the host-pathogen interaction. In addition, no similar structure exists in the host, facilitating targeted development of antifungals. The SUN gene family comprises a family of fungus-specific proteins which has been defined in *Saccharomyces cerevisiae* as a group of four proteins, *Sim1p*, *Uth1p*, *Nca3p*, and *Sun4p*, with highly similar C termini (3). This gene family is involved in different cellular processes, like DNA replication, aging, mitochondrial biogenesis, and cytokinesis. Glucosidase activity has been assigned to this family of proteins due to the characterization of the β-glycosidase *BglBp*, homologous to the SUN family in *C. albicans* (32). In *C. albicans*, the SUN family comprises only two genes—*SUN41*, the ortholog of *SUN4*, and *SIM1*, the ortholog of *UTH1*—which have not been characterized in detail. *SUN41* was previously found to be up-regulated in hyphae, as demonstrated by Sohn et al., indicating a role in morphogenesis (33). In addition, Sun41p has been mentioned as a putative substrate for *KE2*, a serine protease of the kexin superfamily, which participates in both the constitutive and regulated secretory pathways, indicating secretion (25). The ortholog *SUN4* in *S. cerevisiae* is involved in cell septation (21). The corresponding protein, Sun4p, was identified as a glycosylated soluble cell wall protein, extractable by reductive agents (4). The association of Sun4p to the cell wall was also demonstrated by Velour et al., who additionally were able to show a localization of Sun4p to mitochondria (35). Furthermore, genetic interactions of various degrees between the four SUN family proteins have been reported for *S. cerevisiae* (21, 22).

We were interested in the involvement of Sun41p from *C. albicans* in morphogenesis, cell wall biogenesis, and the host-pathogen interaction. Therefore, we constructed *SUN41* deletion mutants to test for phenotypic deviations from the wild-type strain under various conditions.

Our results indicate that *SUN41* has a function as a glucosidase and is involved in morphogenesis and cell wall biogenesis as well as biofilm formation. *SUN41* deletion mutants failed to grow as hyphae on solidified media and showed a decreased ability to adhere to these media. In addition, adherence to tissue was also negatively affected in strains lacking *SUN41*, as was the ability to form biofilms. Most interestingly, Sun41p was found to be secreted from blastospores as well as hyphae, pointing to the possibility of additional functions of Sun41p in *C. albicans*, e.g., in the formation of extracellular matrices.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *C. albicans* strains used in this study are listed in Table 1. The strains were routinely inoculated from overnight YPD cultures (10 g yeast extract, 20 g peptone, and 20 g glucose per liter) into fresh medium and grown at 30°C on a rotary shaker for 6 h. Hyphae were induced in YPD plus 10% heat-inactivated fetal calf serum at 37°C or in α-MEM plus 2% glucose at 37°C. Difco yeast nitrogen base (YNB; without amino acids; Becton Dickinson, Heidelberg, Germany) supplemented with 75 mM ammonium sulfate and 2% glucose was used as synthetic medium. Spider medium contained 1% nutrient broth, 0.2% K2HPO4, and 1% mannitol as a carbon source (18). For growth on plates, 1.5% agar was added to the medium.

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Deletion of SUN41 and reconstitution of mutant strains. SUN41 was deleted in C. albicans strain SC5314 by FLIP-mediated, site-specific recombination according to the methods of Reuß et al. (29). Briefly, one pair of sequences flanking the SUN41 coding sequence was amplified by PCR using the following primers: ortf.2071_FLR1a_Apa1 (5'-GCTGCTGGCCCAACCACTGTTGCAATGAA-3') and ortf.2071_FLR1a_Xho1 (5'-CCGCTAGCTGAATGGACTGACCATAAATTT-3') for FLR1; orf.2071_FLR2_Not1 (5'-ATAAGATCCGGCCGGGACCCCTTTTCTCTGCTCTTCT-3') and orf.2071_FLR2_Sac2 (5'-CTCCCGCGGACGAAAAGAATTTGCGACAGA-3') for FLR2. Unique cleavage sites were embedded in the primer sequences for directed ligation of the flanking regions into the plasmid pSFS2A (29). FLR1 was cloned into the vector after ApaI and XhoI digestion, and FLR2 was cloned after NotI and SacII digestion, resulting in the plasmid pSFS2A-sun1/2. Reconstituted mutants were produced in a similar way.

The SUN41 open reading frame, including 1,031 bp upstream, was PCR amplified with the primers ortf.2071_FLR1a_Apa1 and 2071_end_rev_Xho1 (5'-CCGCTAGCTGACCGAAAGAGAATTTGCGACAGA-3') and cloned together with FLR2 into pSFS1A (29) using the restriction sites ApaI/Xhol and NotI/SacI for FLR2. C. albicans strains were transformed by electroporation as described previously (29).

Deletion and reconstitution were confirmed by Southern blotting and real-time PCR.

Isolation of chromosomal DNA and Southern hybridization. Chromosomal DNA from C. albicans cells was isolated as described according to the method of Hoffman and Winston (9). Southern blot analysis was performed according to standard protocols using 25 μg of BglII-digested genomic DNA. To show the gene deletion, the blots were probed with a DNA fragment that was PCR amplified using primers for FLR2 (see above). The reconstitution was detected by a probe using primers for FLR2 (see above). The reconstitution was detected by a probe

Growth rate determinations. The doubling times of the C. albicans strains were determined by growing them in liquid YPD medium on a rotary shaker at 30°C. At 1-hour intervals, an aliquot was removed and sonicated briefly, followed by measurement of the optical density at 600 nm (OD600).

Cell size determination. Cells were grown under standard conditions in YPD and, after brief sonication, cells were collected by centrifugation. The cell pellet was incubated in Brilliant Blue staining solution to contrast cells followed by two washing steps in phosphate-buffered saline (PBS). Resuspended cells were imaged using light microscopy, and the contrast of cells to background was enhanced using Adobe Photoshop software. Afterward, the area of single cells was determined with the software Scion Image (Scion Corporation). The software Origin (OriginLab Corporation) was used to analyze the data. Two sample independent t tests were performed.

Light microscopy. C. albicans strains were grown under standard conditions to exponential phase and imaged using an Olympus BX 60 microscope (Olympus, Hamburg, Germany).

Scanning electron microscopy. C. albicans strains for scanning electron microscopy were grown under standard conditions to exponential phase. Cells were washed two times with H2O and dropped onto glass slides. After freezing, cells were dried by lyophilization and were sputtered according to standard protocols. Scanning electron microscopy was performed using the 1530 VP electron microscope (LEO).

Transmission electron microscopy. Samples for transmission electron microscopy were prepared according to the protocol described by Reinhard et al. (28). Briefly, 3 × 107 blastospores of each strain were collected by centrifugation at 1,600 × g after 6 h of growth in liquid YPD medium and fixed for 1 h by addition of glutaraldehyde at a final concentration of 2.5%. The samples were then washed with YPD and postfixed for 1 h with 1% osmium tetroxide at room temperature. The fixed cells were dehydrated through a graded series of acetone and embedded in Spurr's resin (Sigma-Aldrich). Thin sections were stained with uranyl acetate and lead citrate and then imaged with a Zeiss EM 10 electron microscope.

Plate assays. Cultures of C. albicans strains were inoculated from overnight cultures, grown to an optical density at 600 nm of 1, and gently sonicated. Series of 10-fold dilutions were prepared, and approximately 107, 106, 105, 104, and 103 cells were spotted onto YPD plates supplemented with 10% serum, YNB plates with Congo red (300 μg/ml), or α-MEM plates. As a control, cells were applied to YPD plates without any supplement. The plates were documented after incubation at 30°C or 37°C for 5 days using a digital camera (Easyshare Z7590; Kodak GmbH, Stuttgart, Germany) or a flatbed digital scanner (Epson Expression 1680 Pro).

Congo red. C. albicans strains were inoculated from overnight cultures and grown to exponential phase in liquid YPD medium which was supplemented with Congo red (300 μg/ml).

Gluconease treatment. Cells were treated with the recombinant β-1,3-glucanase Quantzyme Ylg (MP Biomedicals, Illkirch, France) according to the protocol of the manufacturer. The OD600 was measured before and every minute after addition of 500 U/ml enzyme. Data were plotted against time, and the time required for a 50% decrease in the OD was determined as the half-life for spheroplast lysis.

Cell culture. The human colorectal adenocarcinoma cell line Caco-2 (ATCC HTB-37) was grown in 75 cm² tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, and 1% gentamicin was used as the medium. Cells were cultivated at 37°C under 5% CO2. Just before reaching confluence, Caco-2 cell cultures were split 1:5 by standard methods.

Adhesion assay. Twenty-four-well polystyrene plates were employed to study the adhesion behavior of C. albicans on different surfaces as described previously (5). The quantitative data were based on four experiments.

Biofilm formation. Biofilms from different C. albicans strains were produced on sterilized, polystyrene, flat-bottom 96-well microtiter plates (Greiner Bio-One) as described previously (12) with some modifications. Briefly, 100 μl of a standardized cell suspension (3 × 107 cells/ml) was transferred into each well (eight replicates) of a microtiter plate and incubated for 1.5 h at 37°C to allow the yeast to adhere to the surfaces of the wells (15). As controls, eight wells of the microtiter plate were handled in an identical fashion, except that no Candida suspensions were added. Following the adhesion phase, the cell suspensions were aspirated and each well was washed twice with 150 μl of PBS to remove loosely adherent cells. A total of 100 μl of yeast nitrogen base medium was then transferred into each of the wells, and the plates were incubated at 37°C. The biofilms were allowed to develop for 48 h and then the yeasts were quantified by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay (see below).

XTT reduction assay. The XTT reduction assay used was a modification of the methods described previously (15). Briefly, XTT (Sigma-Aldrich) solution (1 mg/ml in PBS) and menadione (Sigma) solution (1 mM in acetone) were prepared immediately before each assay. XTT solution was mixed with the menadione solution at a ratio of 1,000:1 by volume. The biofilms were first washed two times with 200 μl of PBS, and then 100 μl of the XTT-menadione solution was added to each well. The plate was then incubated in the dark for 2 h at 37°C. Following

TABLE 1. C. albicans strains

| Strain name(s) | Description | Genotype* | Reference |
|---------------|-------------|-----------|-----------|
| SC5314        | Wild type   | Clinical isolate | 8         |
| Can417 and Can423 | Precursor of sun41Δ | sun41Δ::SAT1-FLP/SUN41 | This study |
| Can418 and Can424 | Precursor of sun41Δ | sun41Δ-Δ::FRT/SUN41 | This study |
| Can419 and Can425 | Precursor of sun41Δ | sun41Δ::FRT/sun41Δ::SAT1-FLP | This study |
| Can420 and Can426 | sun41Δ | sun41Δ::FRT/sun41Δ::FRT | This study |
| Can421 and Can427 | Precursor of sun41Δ-SUN41 | sun41Δ::FRT/SUN41::SAT1-FLP | This study |
| Can422 and Can428 | sun41Δ-SUN41 | sun41Δ::FRT/SUN41::FRT | This study |

* FRT, FLP recombinase target (20).
incubation, 80-μl aliquots of solution were transferred to new wells and the color change in the solution was measured at 490 nm with a microtiter plate reader (SpectraMax Plus microplate spectrophotometer; Molecular Devices, Ltd., Sunnyvale, CA). The absorbance values of the controls were then subtracted from the values of the test wells to eliminate spurious results due to background interference.

**RNA preparation.** C. albicans cells were grown in suspension cultures as described before. Cells were collected by centrifugation at 1,600 × g and dropped into liquid nitrogen for generating cell pellets. These cell pellets were stored at −80°C. In order to isolate total RNA, the cell pellets were broken mechanically by grinding in a precooled Retch mill (Retch, Haan, Germany), and RNA was further purified using the RNeasy mini kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. The quality of the isolated RNA was checked by agarose electrophoresis prior to analysis.

**Real-time PCR.** A selection of all enzymes annotated as modifying the major backbone of the cell wall was tested. Amounts of CDNA of selected genes in the sun41A mutant strain were compared to the wild type by using TDI3 (glyceraldehyde-3-phosphate dehydrogenase) as a reference and assuming an equal amount of transcript of this gene in the samples. Additionally, the transcript amounts of the genes of interest were compared to the amount of TDI3 transcript to get an idea about the abundance of the respective genes. One μg total RNA was transcribed to cDNA using the QuantTec reverse transcription kit (Qiagen). Two ng of this cDNA was used to perform real-time PCR in a LightCycler 480 (Roche, Mannheim, Germany) with the LightCycler 480 probes master kit according to the manufacturer's instructions on 96-multwell plates. Probes used (as indicated) originated from the human Universal ProbeLibrary (Roche), and gene-specific primers (TIB MOLBIOL, Berlin, Germany) were as follows: for BGL2 (orf19.4565), 5′-TGAAGCTTGCAGAGCAGGAAGG-3′ and probe 18; for SUN41 (orf19.3829), 5′-TCTCTTGGAATGCTCAATCCT-3′, 5′-GGTGCCTGCTGTTAGTATG-3′, and probe 6; for ACF2 (orf19.3417), 5′-TCAAAATCATGCAAACAACCAA-3′, 5′-TGTATGGTTGGGCAGATCTT-3′, and probe 89; for ENGI (orf19.3086), 5′-CACCAACAGTTCGAGAAGA-3′, 5′-TTGTTTACCGTTGTTGTCG-3′, and probe 18; for SCWI1 (orf19.3899), 5′-ACCACAAATACCTTCACCTAC-3′, 5′-TTGACTAGGTGTTGAG-3′, and probe 11; for XGL1 (orf19.2990), 5′-TGCTAATGGTGAAGTTCGTG-3′, 5′-GCTATTAGCTGTAACGCTCTCA-3′, and probe 82; for ACE2 (orf19.6124), 5′-TATCGGCAAGAACACATT-3′, 5′-GGATATCGTGCTGTTGTTT-3′, and probe 60; for SIM1 (orf19.5002), 5′-CCAGTGTGGTTGGGTGATCTG-3′, 5′-TGGGTGTCAGATCAATGACA-3′, and probe 131; for CBK1 (orf19.4909), 5′-AACCACAA AGCAAGCAAA-3′, 5′-GGTGCCTGCTGTTAGTATG-3′, and probe 5; for CHT3 (orf19.7580), 5′-GGTGCCTGCTGTTAGTATG-3′, 5′-CCCAAAGG TATGACGAAATTT-3′, and probe 50; for SUN41 (orf19.3642), 5′-CAAGTG ACTGAAATATGGTATTC-3′, 5′-TGATACAAACGCTGTTATGCAC GA-3′, and probe 10; for PHR2 (orf19.6801), 5′-GGTTCACTAGCGACCTCT TTTGC-3′, 5′-TTGTTATACGAAAGAAATTCGAGC-3′, and probe 9; for TDI3 (orf19.6814), 5′-GCCGTCGACGACTCATCTC-3′, 5′-AGAATGTATTTG AGAACAGG-3′, and probe 50. The LC480 software (Roche) was used to analyze the data.

**Isolation of secreted proteins.** C. albicans was inoculated after two washing steps in H2O in fresh synthetic medium YNB or α-MEM and grown for 7 h. Cells were removed from 10 ml of medium by three rounds of centrifugation (3,500 g, 3,500 rpm, and 12,000 × g). Cells were then resuspended in 1 ml of 100 μl of ammonium bicarbonate. Reduction and alkylation of disulfide chains were achieved by addition of 10 mM dithiothreitol and incubation at 65°C for 15 min, followed by adding iodoacetamide to a final concentration of 20 mM and incubation at room temperature in the dark for 15 min. Thereupon, 1 μg trypsin (sequencing grade, modified; Promega, Mannheim, Germany) was added, and the digest mixture was incubated for 5 h at 37°C before it was stopped by adding trifluoroacetic acid to a final concentration of 1%. Analysis of peptides was performed by tandem mass spectrometry (MS/MS) using matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) or liquid chromatography–coupled electrospray ionization (ESI).

MALDI-TOF MS. C. albicans was grown in the synthetic medium YNB or α-MEM for 7 h before collecting the supernatant. After precipitation of the proteins, they were resuspended and digested using trypsin. Peptides in a volume of 10 μl were purified by ZipTipC18 (Millipore, Billerica, MA) using a standard protocol and directly spotted on a prespotted AnchorChip target (Bruker Daltonik GmbH, Bremen, Germany). The monoisotopic molecular mass of the peptides was analyzed by MALDI-TOF MS using an Ultraflex II TOF/TOF 200 apparatus (Bruker). The mass spectrometer was set to scan over 700 to 4,000 Da, and the mass spectra were acquired in reflector mode and processed using FlexControl and FlexAnalysis software (Bruker). The most dominant peaks were further microsequenced using post-source decay analysis.

LC-MS/MS. Reverse-phase liquid chromatography (LC)–MS/MS was performed using a Surveyor LC system (Thermo Fisher Scientific, Ulm, Germany) which was coupled to a Finnigan LCQDECA mass spectrometer (Thermo) equipped with an electrospray ion source.

The peptide mixtures were autosampled in 0.1% aqueous trifluoroacetic acid and separated on the analytical column (Jupiter; C18, 3 mm [inner diameter] by 10 cm; Phenomenex) using a linear gradient of 7 to 100% acetonitrile in 0.1% (vol/vol) formic acid for 44 min at a flow rate of 50 μl/min and ionized by an applied voltage of 5 kV to the emitter. The mass spectrometer was operated in data-dependent acquisition mode to automatically switch between MS and MS/MS. Survey MS spectra were acquired for 1 s, and the most intense ions were isolated and sequentially fragmented for 1.5 s by low-energy collision-induced dissociation. The mass spectrometer was set to scan over 300 to 2,000 Da, and the mass spectra were acquired and processed using Xcalibur software (Thermo).

**Analysis of mass spectrometric data.** The tandem MS spectra were submitted to the database search program MASCOT (Matrix Science, Great Britain) in order to identify the proteins. Data files were searched against a C. albicans database. This database is based on assembly 19 of the translated open reading frames of the nucleotide sequence of the Stanford Genome Technology Center. The MASCOT search parameters were the following: allowing up to one missed cleavage, a tolerance of 1.5 Da for peptides and 1 Da for MS/MS (MALDI-TOF/TOF, 100 ppm for peptides and 0.5 Da for MS/MS). Probability-based MASCOT scores were used to evaluate protein identifications. Only peptides with P values of <0.05 for random occurrence were considered to be significant.

**RESULTS**

The switch from yeast to hyphae has been shown to be crucial for virulence of C. albicans. Genes showing up-regulated expression during this step are of special interest to understanding pathogenicity. In a study by Sohn et al. (33), SUN41 was found to be up-regulated during hyphal development. For further functional characterization, we constructed sun41A mutant strains by transformation and homologous recombination with the SAT1 flipper cassette (29). SUN41 was shown not to be essential in C. albicans. Homozygous sun41Δ mutants were reverted with the SUN41 gene under its native promoter (Table 1). Southern blot analyses confirmed the genotype, and real-time PCR confirmed the absence of SUN41 transcription in the homozygous sun41Δ mutant strains (not shown).

**Phenotypic analysis of sun41Δ cells.** (i) Deletion of SUN41 results in increased cell size and cytokinesis defects. Deletion of SUN41 did not affect growth rate during growth in the exponential phase in YPD (not shown). However, an increased flocculation could be observed in liquid cultures of the sun41Δ mutant compared to the wild type. This occurred during growth as blastospores in YPD medium (Fig. 1) as well as for hyphae developing in α-MEM at 37°C (not shown). Light microscopy of exponentially growing cells revealed that the sun41Δ blastospores appeared irregular, not separated after budding, in a tree-like structure (Fig. 1B). This phenotype was stable even after gentle sonication, which separates cells just sticking together. To get a more detailed view of the cells and the non-separated budding sites, electron microscopy was performed. Scanning electron microscopy confirmed that sun41Δ blastospores were still connected at the site of the bud/birth scar, even after completion of budding, indicated by the start of the next cell cycle of former mother and daughter cells (Fig. 1D and E). Using transmission electron microscopy to investigate the buds sites inside of the cells, we did not observe any significant differences in cell wall appearance between separat-
ing wild-type cells (Fig. 1F) and still-connected sun41Δ mutant cells (Fig. 1G). A thin contrasted line at the sun41Δ mutant’s budding site seemed to indicate the completion of the cell walls and septae from both sides, just missing the final cut (Fig. 1H).

SUN4 deletion mutants of S. cerevisiae, the orthologous gene to C. albicans SUN41, showed an increase in cell size. We investigated this phenotype also in C. albicans. Measurement of the cell size on light micrographs of ultrasonically separated cells showed that sun41Δ/H9004 mutants were enlarged during exponential growth at about 1.3 times that of the wild type (Fig. 2). This effect was reduced in the reconstituted strain sun41Δ/SUN41. The reason for this cell size enlargement remains unclear.

sun41Δ mutant cells grown in liquid culture under hyphae-inducing conditions, like α-MEM or YPD supplemented with 10% bovine calf serum at 37°C, revealed no difference in morphology compared to wild type. Only a slightly reduced acidification of the α-MEM and enhanced flocculation, as mentioned above, could be observed (not shown).

(ii) sun41Δ mutants show altered sensitivity to cell wall-modifying substances. The sensitivity towards cell wall-perturbing substances was tested by a survival test on plates containing Congo red or calcofluor white. Congo red interferes with the assembly of the glucan microfibrils (14), and calcofluor white disturbs chitin assembly (6). Mutant and wild-type cells were diluted in a serial manner and grown for up to 5 days on supplemented agar plates. sun41Δ cells showed a higher sensitivity towards Congo red, whereas no effect with calcofluor white was observed (Fig. 3B and data not shown). This indicates a variation in the glucan network, possibly resulting in a defect in cell wall structure. Therefore, the sensitivity of the cells against the cell wall-corrupting β-1,3-glucanase Quantzyme was analyzed. Cells harvested during exponential growth were tested for the half-life of spheroplast lysis (time required for a 50% decrease in the OD). sun41Δ mutants (42-min half-life) showed more resistance towards the wild type (21-min half-life) or reconstituted sun41Δ/SUN41 (28-min half-life), indicating compensatory mechanisms to strengthen the cell wall possibly in combination with a reduced amount of β-1,3 linkages in the cell wall.

Using calcofluor white in fluorescence microscopy to stain chitin of growing cells, no differences between the wild type and sun41Δ mutants were observed on blastospores and hyphae (not shown), indicating no severe changes in chitin structure.

Most interestingly, C. albicans wild-type strains incubated in liquid YPD medium supplemented with Congo red also showed an increased flocculation. Light microscopy of exponentially growing cells revealed that the blastospores did not
separate after budding, growing in a tree-like structure, similar to the phenotype observed with sun41Δ (Fig. 3C).

(iii) SUN41 is required for hypha formation on solid medium. Formation of hyphae is important for C. albicans to invade into the host tissue. In liquid medium under hypha-inducing conditions, sun41Δ mutants did not show differences in hyphae formation compared to the wild type (see above). Investigation of filamentation during growth on solid medium revealed a remarkable difference from this result. Exponentially grown C. albicans cells were diluted and cultivated on agar plates containing α-MEM, YPD with 10% bovine calf serum, or Spider medium for up to 5 days at 37°C. Whereas the wild type and reconstituted serum, or Spider medium for up to 5 days at 37°C. Whereas the

/H9251 agar plates containing partially grown revealed a remarkable difference from this result. Exponentially Sun41Δ/SUN41 showed peripheral hyphae and hyphal cells within the colony, this switch in growth form was barely detectable in the sun41Δ mutant, revealing an involvement of SUN41 in hypha formation on solidified medium (Fig. 4A and B). Most interestingly, addition of Congo red to sun41Δ strains in liquid medium (YPD plus serum, 37°C) blocked hyphae formation almost completely and resulted again in the formation of blastospore-like cells not separated from each other, as observed on solidified medium or in liquid YPD at 30°C. The hyphae formation of the wild type was affected only moderately by Congo red under the conditions tested (Fig. 3D).

(iv) sun41Δ shows defects in adhesion and reduced biofilm formation. Adhesion of sun41Δ cells was tested by several different assays: on agar plates, using an epithelial model of host-pathogen interaction, and in a biofilm assay. On agar plates, depending on the medium used, several distinct phenotypes were observed. On YPD plates after 24 h of growth, sun41Δ strains adhered more strongly than the wild type and the reconstituted strain in a plate washing assay (Fig. 4C). Microscopic observation of plate-grown sun41Δ strains revealed the same phenotype of mutant cells, a cytokinesis defect, as described for liquid YPD medium (see above). The increased adhesion observed may be due to the increased cell-cell interaction in the mutant strain as a result of the cytokinesis defect. To test whether the separation defect is the cause of increased adhesiveness on agar plates, we incubated the wild type and sun41Δ mutant and its reconstituted strain for 48 h on YPD plates containing Congo red, which induces a cytokinesis defect in these strains, as described above. Consistent with the idea that the defect in cytokinesis is responsible for the increased adhesiveness, an equal amount of cells from all strains persisted on the plates after washing (data not shown). Microscopy confirmed that cells of all strains showed the tree-like structure on solid media as observed in liquid media supplemented with Congo red (Fig. 3C).

On hypha-inducing media, however, sun41Δ colonies were found to be washed away much easier from the plates than hyphal wild-type cells, indicating that the tree-like structures also observed on hypha-inducing solid medium with sun41Δ mutant cells have a reduced potential to adhere and invade in the agar than hyphal filaments present in the wild type (Fig. 4C).

Adhesion of C. albicans on epithelia is a necessary precondition for penetration of tissues in order to mediate pathogenesis in the host. To test the differences in adhesion of wild-type and sun41Δ strains, we used an in vitro adhesion assay (5). For this purpose, Candida cells were incubated to adhere to a

Caco-2 cell monolayer. To determine whether adhesion of sun41Δ and the wild-type C. albicans strain occurs with different kinetics or efficiencies, the numbers of C. albicans cells adhering to the surface and those remaining in the supernatant were analyzed in a time-dependent manner (Fig. 5A). Adhesion of the wild type to Caco-2 cells was rapid and efficient, as indicated by an adherence of 67% after the first 30 min. A maximum of 93% was nearly reached after 90 min. After 30 min, 54% of sun41Δ C. albicans cells were found adhered to the surface, reaching their maximum of 81% after 120 min. In reconstituted sun41Δ-SUN41 cells, the same kinetics and efficiency of adhesion as the wild type were observed (Fig. 5A). Adhesion of sun41Δ to the plastic surface did not differ significantly from the wild type (not shown).

FIG. 4. Phenotype of different C. albicans strains growing on solidified media. (A) Approximately 100 cells of the indicated C. albicans strains were spotted onto agar plates containing α-MEM or YPD supplemented with 10% bovine calf serum. Plates were incubated for 5 days at 37°C. (B) The rim of the colonies grown on YPD supplemented with 10% bovine calf serum was imaged using light microscopy (upper three panels). Additionally, cells were scraped off the colonies and examined microscopically (lower three panels). (C) The ability to adhere on YPD agar was tested by washing layers formed by the indicated strains on plates with or without serum. Bars, 10 μm.
Both filamentation and adhesion of Candida are involved in the formation of biofilms. These biofilms are found on medical devices, like catheter surfaces (2), and have attracted attention because of their persistence and resistance to antifungals (13, 31). These characteristics of Candida biofilms are likely to contribute to both superficial (30) and systemic (26) candidiasis. We used an in vitro assay to investigate if the ability of the sun41Δ C. albicans to form biofilms was altered. Cells were added to 96-well plates for 2 days. The amount of biofilm-forming cells was measured in an XTT activity assay. Results represent means with standard deviations (error bars) from two independent experiments.

Deletion of SUN41 results in compensatory regulation in other glycosidases. Real-time PCR was applied to study the differential regulation on the transcriptional level and the amount of transcript of selected genes between wild type and sun41Δ. Testing ACE2, ACF2, BGL2, CBK1, CHT3, ENG1, PHR1, PHR2, SCW11, SIM1, and XOG1 during growth as blastospores, none of these genes was regulated to a factor less than 0.5 or more than 2.0, indicating that the loss of SUN41 had no effect to the regulation of these genes (not shown). This result is in contrast to hyphae, where we observed that PHR2 and ACF2 were up-regulated with a factor of ≥2.0, and the amount of SCW11 and CHT3 transcript in the sun41Δ mutant was less than half that of the wild type (Fig. 6). Also, ACE2, BGL2, and PHR1 seemed to be down-regulated but did not reach the 0.5× regulation factor. Determination of the transcription level revealed mostly a very low abundance, except for BGL2, PHR1, PHR2, and SUN41.

Localization of Sun41p. Our observation of the phenotypic effects of SUN41 deletion on blastospores indicated a function during the last step of cell division. In this context, it is remarkable that the Sun41p ortholog, Sun4p, in S. cerevisiae was found in the cell wall (4). Attempts to localize Sun41p using a C-terminal green fluorescent protein tag were not successful. As shown by Lee et al., the amino acid sequence of SUN41 contains with high probability an N-terminal secretory signal peptide, indicating entry in the secretory pathway (17; http://info.med.yale.edu/intmed/infdis/candida/). To test if Sun41p is secreted, we used mass spectrometry. Comparison of the tryptic peptide spectra of proteins in the supernatant of YNB medium gained from wild-type and sun41Δ C. albicans cells showed that very similar spectra could be observed. However, the sun41Δ MALDI-TOF spectrum lacked two dominant peaks of 1,048.629 Da and 3,794.181 Da (Fig. 7, peaks 1 and 5). Sequencing by analysis of post-source decay spectra showed that these peptides belong to Sun41p. Dominant peptides found in both spectra belonging to Sim1p, MP65p, and Tos1p were identified in an equal manner (Fig. 7, peaks 2, 3, and 4). To verify these results, the tryptic digests were analyzed in reverse-phase high-performance liquid chromatography (HPLC)-coupled ESI MS/MS. This resulted in the repeated identification of Sun41p in YNB as well as in α-MEM obtained from wild-type cells but not from sun41Δ. This indicates that...
Sun41p, at least partially, is secreted under these conditions. Several other proteins could be identified during this analysis as being secreted into the medium (Table 2). With the exception of Dsl1p (Table 2), the proteins contain a typical signal peptide characteristic for secreted proteins (17).

Following the detection of Sun41p in the supernatant, we tested whether the secreted protein could compensate for the blastospore separation defect of the \textit{sun41}/H9004 mutant cells by cultivating this strain together with the wild type. During incubation for 24 h, numbers of mutant and wild-type cells were determined. No significant change in separation of \textit{sun41}/H9004 cells was observed at any time point investigated (not shown).

\textbf{DISCUSSION}

We have investigated the role of \textit{SUN41} in cell wall biogenesis and morphogenesis in \textit{C. albicans}. \textit{SUN41} of \textit{C. albicans} was annotated as homologous to the \textit{SUN4} gene in \textit{S. cerevisiae}, sharing 57\% identity and 70\% similarity. In \textit{S. cerevisiae}, several functions have been assigned to \textit{SUN4}, including cell wall biogenesis, cell size, cytokinesis, and mitochondrial function (4, 35). In \textit{C. albicans} our previous work identified \textit{SUN41} as transcriptionally up-regulated in hyphae (33). To identify the functions of \textit{SUN41} in \textit{C. albicans}, we constructed strains deleted for \textit{SUN41} and compared them to the wild type under different conditions. Homozygous deletion mutants exhibited normal growth rates under the conditions tested, showing that \textit{SUN41} is not essential. However, \textit{SUN41} mutant strains showed several abnormalities with respect to cell wall biogenesis and morphogenesis. This included defects in cytokinesis, biofilm formation, and hyphae formation on solid substrates as well as defects in adhesion. In addition, the cell wall biogenesis-perturbing agent Congo red, but not calcofluor white, resulted in reduced survival of strains lacking Sun41p.

\textbf{Morphogenesis and cell wall biogenesis.} Microscopic observations of \textit{sun41Δ} blastospores revealed a cytokinesis defect producing a tree-like structure. Furthermore, our results indicate that \textit{sun41Δ} blastospores are slightly larger than the wild type. However, this phenotype is not restored completely in the reconstituted strain, allowing other interpretations for this effect. A cytokinesis defect and cell enlargement have also been reported for \textit{S. cerevisiae} \textit{sun4} (21). Cells were not able to separate after completion of cell division (Fig. IB and D). However, electron microscopy pictures revealed an intact cell wall and septum similar to wild-type cells (Fig. 1G). More-detailed electron micrographs focusing on differences at the budding site might reveal potential structural changes leading to this defect. The tree-like structure was observed for \textit{sun41Δ} mutant cells growing as blastospores in liquid media as well as on solid media.

Most interestingly, we found that the deletion of \textit{SUN41} did not affect hyphae formation in liquid media like YPD plus serum (37°C) or α-MEM (37°C). However, on the respective solid media, \textit{sun41Δ} mutant strains did not form hyphae, but a tree-like structure was observed for growth in liquid YPD media, mixed with single or budding cells. Hyphae/pseudohyphae-like structures were barely observed, and true hyphae could not be detected microscopically (Fig. 4B, middle lower panel). This shows that \textit{sun41Δ} mutant cells in general are able to form hyphae; however, the morphogenetic program required to induce hyphae formation seems to be perturbed in

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**FIG. 7.** Comparison of proteins found in supernatants of different \textit{C. albicans} strains growing in YNB. Different \textit{C. albicans} strains were grown exponentially in the liquid synthetic medium YNB. Cells were removed and media collected. Proteins were precipitated and digested with trypsin. Shown are the mass spectra of the resulting peptide mixtures from 700 to 4,000 Da from the MALDI-TOF MS. The sequences of the indicated peptides were identified by MS/MS and associated to proteins using the MASCOT software. Peaks: 1 and 5, Sun41p; 2, Sim1p; 3, MP65p; 4, Tos1p.
Comparing transcript levels of several putative glycosidases between the wild type and sun41/H9004 strains on solidified media. Comparing transcript levels of several putative glycosidases between the wild type and sun41/H9004 revealed a prominent up-regulation (factor of 2) of PHR2 in the deletion mutant if grown in liquid medium under hypha-inducing conditions (MEM, 37°C) (Fig. 6). Phr2p has been reported as a putative -1,3-glycosidase (7, 23). The compensatory up-regulation of Phr2p, at pH 7.4, in a sun41/H9004 strain would be consistent with a function of Sun41p as a -1,3-glycosidase. This up-regulation was not found in blastospores, where the cytokinesis defect was observed. In blastospores we observed a higher resistance to -1,3-glucanase in sun41/H9004 strains, which is in agreement with a function for Sun41p in cell wall biogenesis, potentially as a -1,3-glycosidase.

Although sun41Δ strains are more resistant to -1,3-glucanase, they show a general defect in cell wall biogenesis as observed by incubation on Congo red but not calcofluor white plates (Fig. 3B and data not shown). This is also in agreement with a putative function as a glycosidase, since Congo red has been shown to interfere with glucan in the S. cerevisiae cell wall and affects the assembly of the glucan microfibril biogenesis (14, 34). Interestingly, the addition of Congo red also resulted in a separation defect of C. albicans wild-type blastospores, similar to the phenotype observed for SUN41 deletion mutants (compare Fig. 1B to 3C). This effect of Congo red was also observed in S. cerevisiae (14, 34). In hypha-inducing liquid medium, Congo red only had a limited effect on the morphogenesis of the wild type; however, sun41Δ strains predominantly form a tree-like structure, as observed on the solid media tested. This indicates that the potential compensatory effect observed by induction of additional glycosidases in sun41Δ strains is blocked by Congo red. These results suggest that cell wall composition/alterations are sensed by C. albicans, resulting in different decisions for morphogenesis.

### TABLE 2. Proteins identified by MS/MS (MALDI-TOF and ESI) in media supernatants of wild-type C. albicans cells growing in α-MEM or YNB

| Medium and ORF no. | Protein name | Secretion predicted | Peptide sequence | P value |
|-------------------|-------------|---------------------|-----------------|--------|
| Grown in α-MEM    |             |                     |                 |        |
| orf19.2060        | Sod5p       | Yes                 | HGNIMGESYK      | 1.3E-06|
|                   |             |                     | GLPSDIFPPFY     | 5.6E-03|
|                   |             |                     | ATPAHEVGDAGK    | 1.0E-07|
|                   |             |                     | SGEYIFSGSK      | 2.8E-06|
|                   |             |                     | FGNLASYANADNSGSSTPVPLEETTIK | 6.4E-08|
| orf19.1690        | Tos1p       | Yes                 | YWGHYSN         | 2.0E-03|
|                   |             |                     | GDSNLAVPSK      | 4.7E-04|
|                   |             |                     | NVLITETGWPSK    | 1.1E-04|
|                   |             |                     | SNOQAAISSK      | 3.1E-04|
|                   |             |                     | SESQIAASEIAQLSGFDVIR* | 4.7E-15|
|                   |             |                     | NVLITETGWPSR*   | 1.3E-03|
| orf19.3642        | Sun41p      | Yes                 | SNYLCEWGVK      | 4.1E-05|
|                   |             |                     | LSETVAICR*      | 7.6E-06|
| orf19.3111        | Pra1p       | Yes                 | NGWAGYWR        | 1.6E-02|
|                   |             |                     | NGWAGYWR*       | 2.6E-05|
| orf19.2370        | Dsl1p       | No                  | MEGINGGDSSLQEK  | 2.7E-02|
| Grown in YNB      |             |                     |                 |        |
| orf19.1690        | Tos1p       | Yes                 | EAGSVATIGY      | 9.4E-03|
|                   |             |                     | SGEYIFSGSK      | 1.8E-05|
|                   |             |                     | GCSFEGGNYCSETK  | 8.2E-08|
|                   |             |                     | TGGCGLLPEVLSSGSNK | 5.2E-11|
|                   |             |                     | CTQOLISHFSLPLDEELSVHR* | 1.2E-13|
| orf19.3642        | Sun41p      | Yes                 | LSETVAICR      | 4.1E-07|
|                   |             |                     | SNYLCEWGVK      | 3.2E-04|
|                   |             |                     | TOWPEDQPSNGVSIQGLC | 7.0E-07|
|                   |             |                     | LSETVAICR*      | 9.0E-06|
| orf19.1779        | Mp65p       | Yes                 | YWGHYSN         | 2.0E-03|
|                   |             |                     | GDSNLAVPSK      | 3.5E-05|
|                   |             |                     | SNOQAAISSK      | 2.1E-07|
|                   |             |                     | NVLITETGWPSK    | 6.6E-04|
|                   |             |                     | AFFGDGHVAEANSGPWVLOQIOR | 3.7E-03|
|                   |             |                     | SESQIAASEIAQLSGFDVIR* | 7.7E-15|
| orf19.3618        | Ywp1p       | Yes                 | NLYGAGAVPFQFQVHLEK | 1.2E-06|
| orf19.5032        | Sim1p       | Yes                 | APCSVDGDTYFK    | 2.4E-05|
| orf19.5063        | Hypothetical protein | Yes | YSCLLDMVK | 3.8E-05|
| orf19.4688        | Dag7p       | Yes                 | SVGNPPHNPLCNEK | 2.0E-04|

- Number of the open reading frame (ORF) according to assembly 19.
- Predicted by Lee et al. (17).
- Sequences were identified using ESI MS/MS except for sequences with an asterisk, which were identified by MALDI-TOF MS/MS.
- Expectation value for the peptide match; values below 0.05 indicate identity or extensive homology.
Adhesion and biofilm formation. The observed phenotypes of sun41Δ strains also have significant impact on their adhesiveness. The inability to form hyphae on solid medium results in strongly reduced adhesion compared to the reconstituted strain or the wild type (Fig. 4C). A reduced adhesion of sun41Δ cells was also observed on an epithelial model consisting of Caco-2 cells (Fig. 5A). However, among cells on YPD plates grown for 24 h, the cytokinesis defect resulting in a tree-like structure of the mutant cells led to a stronger adhesion of sun41Δ cells (Fig. 4C). By adding Congo red to the YPD plates, both the wild type and the mutant now adhered equally strongly, presumably due to the induced cytokinesis defect (tree-like structure) present now in all strains (Fig. 3C and data not shown). The degree of adhesion observed on agar medium therefore directly reflects the degree of cellular networks, from single blastospores to cells adherent due to a defect in cytokinesis all the way to hyphae as a continuous network of cells.

We also observed a strong defect in biofilm formation (Fig. 5B). According to Nobile et al., three basic stages are necessary for biofilm formation in vitro: (i) attachment of yeast cells to the surface and (ii) growth and proliferation of yeast cells to form a basal layer, followed by (iii) extensive filamentation combined with the production of extracellular matrix (27). Attachment of blastospores to a plastic surface was not significantly affected by deletion of SUN41 (data not shown). Therefore, an inability to form hyphae on a solid support may be the main reason for the observed defect in biofilm formation in sun41Δ cells. In recent years, analysis of the biofilm matrix of C. albicans revealed glucan as a major component (1, 24). This indicates that the putative glycosidase Sun41p may have additional functions in the generation of the extracellular matrix in biofilms. This hypothesis is supported by our observation that Sun41p is a protein secreted into the medium (Fig. 7 and Table 2). We could not detect any effect of conditioned medium containing Sun41p on sun41Δ strains with regard to cytokinesis (data not shown), indicating potential additional functions of the secreted Sun41p. Further studies will have to verify this hypothesis.

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