Candida albicans Sfl1 Suppresses Flocculation and Filamentation[▽]

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Hyphal morphogenesis in *Candida albicans* is regulated by multiple pathways which act by either inducing or repressing filamentation. Most notably, Tup1, Nrg1, and Rfg1 are transcriptional repressors, while Efg1, Flo8, Cph1, and Czf1 can induce filamentation. Here, we present the functional analysis of Ca*SFL1*, which encodes the *C. albicans* homolog of the *Saccharomyces cerevisiae SFL1* (suppressor of flocculation) gene. Deletion of Ca*SFL1* results in flocculation (i.e., the formation of clumps) of yeast cells, which is most pronounced in minimal medium. The flocs contained hyphae already under noninducing conditions, and filamentation could be enhanced with hypha-inducing cues at 37°C. Expression of *SFL1* in a heterozygous mutant under the control of the Ca*MET3* promoter was shown to complement these defects and allowed switching between wild-type and mutant phenotypes. Interestingly, increased expression of *SFL1* using a *MET3prom-SFL1* construct prior to the induction of filamentation completely blocked germ tube formation. To localize Sfl1 in vivo, we generated a *SFL1-GFP* fusion. Sfl1-green fluorescent protein was found in the nucleus in both yeast cells and, to a lesser extent, hyphal cells. Using reverse transcription-PCR, we find an increased expression of *ALS1*, *ALS3*, *HWP1*, *ECE1*, and also *FLO8*. Our results suggest that Sfl1 functions in the repression of flocculation and filamentation and thus represents a novel negative regulator of *C. albicans* morphogenesis.

Among opportunistic human fungal pathogens, *Candida albicans* is one of the most frequently isolated species. In addition to superficial infections of the skin, vagina, or oral epithelia, *C. albicans* can cause systemic infections. Particularly in immunocompromised hosts, bloodstream infections pose a serious threat (3). *C. albicans* is a dimorphic fungus that can switch between yeast and filamentous vegetative growth modes. The ability to switch growth modes, in conjunction with the ability to induce stage-specific gene expression, is viewed to be important for its pathogenicity (34). Niche-specific responses may allow *C. albicans* to make the best use of its repertoire to survive as a commensal in the human host (2, 13).

Dimorphism in *C. albicans* is an attractive model to study morphogenetic events on the molecular level (3). In vitro-specific conditions can be used to maintain *C. albicans* cells either in the yeast form or by using external cues, such as serum or amino acids, to induce filament formation and mycelium development. On the molecular level, several regulators of hypha-specific gene expression have been identified. Two basic classes can be distinguished—proteins that act as inducers of hypha-specific gene expression, e.g., Efg1, Cph1, Flo8, Tec1, and Czf1, and proteins that act as negative regulators, particularly Tup1 and Nrg1 (3, 10). The cyclic AMP (cAMP) pathway appears to play a major role in regulating hyphal morphogenesis, since both Ras1 and the adenylate cyclase Cdc35 (in association with Cap1) are essential for hyphal

growth and virulence (1, 14, 29). Efg1 is a downstream target of the Ras1-cAMP signaling pathway, and *efg1/efg1* mutants are defective in filament formation in liquid media (32). Flo8 was shown to regulate the hypha-specific gene subset controlled by Efg1 and does so via direct interaction with Efg1. This finding is consistent with the evidence that deletion of *FLO8* blocks hyphal development in *C. albicans* (10).

CaNrg1 is a zinc finger DNA-binding protein that represses hypha-specific genes in a CaTup1-dependent manner. Expression of *NRG1* is downregulated upon hyphal induction, and deletion of either *TUP1* or *NRG1* enhances filamentous growth under noninducing conditions (8, 26). This indicates that relief of transcriptional repression is a major event in the dimorphic switching of *C. albicans* (22).

Hyphal induction media may include various additives that trigger filamentation at 37°C, e.g., serum, amino acids, or *N*-acetylglucosamine (GlcNAc). It has been observed that when serum is used as an inducer, cells readily aggregate and form clumps, while this is true to a much lesser extent when GlcNAc is used (4). The ability of *C. albicans* cells to adhere to other cells or to surfaces poses an important medical problem, particularly in biofilm formation on medical devices that allow these cells to gain access to the bloodstream of patients (25). On an industrial scale, the ability to form cell aggregates is used during beer fermentation by *Saccharomyces cerevisiae* to separate yeast cells from the beer. This process is termed flocculation and leads to a rapid sedimentation of lager yeast cells. Flocculation is dependent on the expression of *FLO* genes. There are two classes of *FLO* genes, which confer either cell-cell adhesion (e.g., Flo1, Flo5, Flo9, and Flo10) or cell-substrate adhesion (Flo11) (20). Different pathways are known to regulate the expression of, e.g., the *FLO11* gene in *S. cer*-

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
SC5314	*C. albicans* wild type	17
CAI-8	*ura3::λimm434/ura3::λimm434 ade2::hisG/ade2::hisG*	30
CAI-8+S*alexA*-lexOP	*RPS1/rps1::*S*alexA-RPS1-URA3 ade2::hisG/ade2::*OP-LacZ-*ADE2*	30
CAI8-S*alexA-NRG1*-lexOP	*RPS1/rps1::*Sl*LexA-NRG1-RPS1-URA3 ade2::hisG/ade2::*OP-LacZ-*ADE2*	30
CAI8-S*alexA-GCN4*-lexOP	*RPS1/rps1::*S*alexA-GCN4-RPS1-URA3 ade2::hisG/ade2::*OP-LacZ-*ADE2*	30
SN148	*ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG leu2::hisG/leu2::hisG*	27
CAJ025	SN148; *SFL1/sfl1::*Cm*LEU*	This study
CAJ027	SN148; *SFL1/sfl1::*Cd*HIS1*	This study
CAJ029	SN148; *sfl1::*Cm*LEU2/sfl1::*Cd*HIS1*	This study
CAJ035	SN148; *sfl1::*Cd*HIS1/sfl1::*Cm*LEU2*	This study
CAJ039	SN148; *sfl1::*Cm*LEU2/sfl1::*HIS1-MET3p-*SFL1*	This study
CAJ042	SN148; *sfl1::*Cm*LEU2/sfl1::SFL1*-GFP-Cd*HIS1*	This study
CAJ049	CAI-8; *RPS1/rps1::*S*alexA-SFL1-RPS1-URA3 ade2::hisG/ade2::*OP-LacZ-*ADE2*	This study
CAJ066	CAI-8; *RPS1/rps1::*S*alexA-SFL1-RPS1-URA3 ade2::hisG/ade2::*OP-LacZ-*ADE2*	This study
CAJ071	SN148; *sfl1::*Cm*LEU2/sfl1::URA3*	This study

evisiae, among them the Ras1-cAMP signaling pathway and the mitogen-activated protein kinase-dependent filamentous growth pathway, as well as the glucose repression pathway and the TOR pathway (33). Downstream of the cAMP-signaling pathway, Tpk2 activates Flo8 and represses Sfl1, which encodes a suppressor of flocculation (11, 15).

We were interested to determine whether flocculation in *C. albicans* is regulated by a similar mechanism as that for *S. cerevisiae* and whether flocculation and filamentation can be separated genetically. We identified a *SFL1* homolog in the *C. albicans* genome. The deletion of *SFL1* led to a flocculent phenotype best seen in minimal media. This flocculation was accompanied by filamentation under noninducing conditions. *SFL1-GFP* localizes to the nucleus in both yeast and hyphal cells. Overexpression of *SFL1*, on the other hand, led to a block in filamentation. This result suggests that Sfl1 is a negative regulator of both flocculation and filamentation in *C. albicans*. Consistently, hypha-specific genes, particularly *ALS1* and *ALS3*, were found to be expressed in a *sfl1* mutant under noninducing conditions.

MATERIALS AND METHODS

Strains and media. The *C. albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown either in rich medium (YPD; 2% peptone, 2% glucose, 1% yeast extract) or in minimal medium (CSM [2% glucose, 6.7 g/liter yeast nitrogen base with ammonium sulfate and without amino acids], 0.79 g/liter CSM, or SD [2% glucose, 6.7 g/liter yeast nitrogen base with ammonium sulfate and without amino acids]) at 30°C. Minimal media were supplemented with the strain-specific requirements for amino acids, adenine, and uridine. Hyphal induction was done at 37°C by adding 10% serum (calf serum; Sigma) or 0.5g/liter GlcNAc to the media.

Regulation of gene expression was done using the *MET3* promoter. Activation of *MET3p-SFL1* expression was achieved by growing cells in media lacking methionine and cysteine, and repression of the *MET3p* promoter could be done by adding 3.5 mM methionine and 3.5 mM cysteine to the medium. For plasmid construction and amplification, *Escherichia coli* DH5α served as a host.

Disruption of Ca*SFL1*. The *C. albicans* homolog of the *S. cerevisiae SFL1* was identified in the *C. albicans* genomic sequence (http://www.candidagenome.org) as orf19.454. Transformation was done by electroporation (24) followed by selective incubation on minimal media lacking the appropriate amino acids or uridine for 2 to 3 days at 30°C. Consecutive complete open reading frame (ORF) deletions of both alleles of Ca*SFL1* were achieved by using PCR-generated disruption cassettes from pFA plasmids amplified with primers S1-SFL1 and S2-SFL1 as described previously (31). All primers (Table 2) were obtained from

biomers.net GmbH (Ulm, Germany). For each new strain, at least two independent mutants were generated. Verifications of correct integration at the target locus and of the absence of the target ORF in homozygous null mutants were done by diagnostic PCR. The fusion of the *MET3* promoter to the *SFL1* ORF was done by PCR-based gene targeting using primers S1-SFL1 and S2-MET3p-SFL1. A 3′-end fusion of *SFL1* with green fluorescent protein (GFP) was generated by homologous recombination of a GFP-Cd*HIS1* cassette in a heterozygous *SFL1* mutant, thus tagging the sole allele of *SFL1* with GFP.

Promoter shutdown experiments. To deplete cells of Sfl1, shutdown experiments of *MET3* promoter-controlled *SFL1* were performed. To this end, overnight cultures of the strains were grown in SD with the appropriate amino acid supplements. Starting cultures were diluted to an optical density at 600 nm of 0.1 in the same type of fresh medium with or without the addition of 3.5 mM methionine and cysteine. These yeast cultures were then incubated for 4 h at 30°C and then used for imaging. In the case of hyphal induction, 0.5 g/liter GlcNAc was added to the cultures, which were incubated for 4 to 5 h at 37°C prior to photography.

Embedded growth. Growth conditions were as described previously (9). Cells of the strains were pregrown in YPD and then poured as dilutions thereof in YPS agar (containing 2% sucrose instead of glucose). These plates were incubated at room temperature for up to 5 days.

One-hybrid assay. The *SFL1* ORF was amplified using primers *CaSFL1*-OH1-*Mlu*I and *CaSFL1*-OH2-*Sph*I and cloned into Clp-lexA after digestion with MluI and SphI (New England BioLabs, Germany). This generated a SalexA-SFL1 fusion construct. This plasmid was linearized by StuI and transformed into CAI-8 and CAJ043. Control strains containing *NRG1* and *GCN4* fusion constructs were kindly provided by Al Brown and are described in reference 30. The strains were pregrown in selective media and then inoculated in YPD and grown for 4 to 6 h at 30°C. Cultures were then adjusted to an optical density at 600 nm of 0.5. For qualitative analysis of *lacZ* expression, 3 μl of each cell suspension was dropped onto CSM plates and incubated for 2 days at 30°C. Colonies were then subjected to an agarose overlay (0.5 M phosphate buffer [pH 7.0], 6% *N,N*-dimethylformamide, 0.5% agarose, 0.1% sodium dodecyl sulfate, 20 μl 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal; 0.1 mg/ml stock solution]). 2-Nitrophenyl-β-D-galactopyranoside (ONPG) assays were used to quantify β-galactosidase activities for different strains. A 2-ml volume of the cell suspension was pelleted and washed with H_2O, resuspended in 300 μl Z buffer (60 mM sodium phosphate monobasic dihydrate, 40 mM sodium phosphate dibasic, 10 mM potassium chloride, 1 mM magnesium sulfate), and divided into three aliquots of 100 μl. Cells were opened by using liquid nitrogen. The enzyme reaction was started by adding 160 μl ONPG-Z buffer solution (4 mg/ml ONPG in Z buffer) to each of the aliquots, followed by incubation at 37°C for 30 min. The reaction was stopped by adding 400 μl of 1 M sodium carbonate. Twofold dilutions were measured at 420 and 550 nm.

RNA isolation and RT-PCR. Total RNA was isolated using an RNAgents total RNA isolation kit (Promega). Reverse transcription (RT)-PCR was performed with an Enhanced Avian RT first-strand synthesis kit (Sigma, Germany) using Oligo(dT)$_{23}$ according to the manufacturer's protocol. Primers for RT-PCR were derived from the 3′ end of the genes tested (Table 2). As a control, *RHO3* was

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]
#600 U2	GTGTTACGAATCAATGGCACTACAGC
#599 U3	GGAGTTGGATTAGATGATAAAGGTGATGG
#601 H2	CAACGAAATGGCCTCCCCTACCACAG
#602 H3	GGACGAATTGAAGAAAGCTGGTGCAACCG
#1031 5′RHO3-cDNA	CCTTAAATATCATTAAAACATGCCTCTTTGTGG
#1430 L2	CGGTACCGACGTGATCACCTGGTA
#1431 L3	GTTGGTGACGCGATTGTCGAAGCTG
#1744 S1-SFL1	CATAACTCCAAATAAGAATAAGTTCATTTATCGCTTAAGGGAATAAGATCAAATTATATTTG CTATTGTTTTGCCAATATCACTTTAATTTTCCACCTGCgaagcttcgtacgctgcaggtc
#1745 S2-SFL1	CCTGCTAGTGAAATATTCAATTTCTTGGAAACAAACACCGCTACTCCTTATTATATTGTTTAT ATCAAAGAAGAATTAGAATCAAGTTATATAGAACCtctgatatcatcgatgaattcgag
#1746 G1-SFL1	CCAACGTTAAGTAGCCCAAGTTGC
#1747 G4-SFL1	CATTCATCGTGGTGAAGCAAGCTATGG
#1748 I1-SFL1	CCACCGACTTCTCATCCAATGGAATAC
#1749 I2-SFL1	GCAACGTTGAGGTTGGTTGTAGACG
#1852 S2-SFL1-MET3p	GTTTTGATTGTAGGGGGTGCTATGATTGGTATGTGGACTTCTTGAAGTTGGAGTAGCAGTC GTTGTCGTACCAAGTGAAGACAGTACCAAATGACTcatgtttctggggagggtatttac
#1901 G4-SFL1-MET3p	GATTCCAGATGGTCTCCTCTAGAAG
#1960 CaFLO8-I1	GATGAATGCTGTAGGTGGTGG
#1961 CaFLO8-I2	GACCGGCAAACATCATTCCATTACTC
#1982 S1-SFL1-GFP	GAGTTTGCCACCAATCAAATCAATCAAAGATAATGATAATAAAAACGATAACGGTAATAGT GATGACAATGGGAACGATCATAAAAAGAGAAAATTAGAAggtgctggcgcaggtgcttc
#3127 ALS1-I1	CACCACATCTCAACCTACAG
#3128 ALS1-I2	GTTGGTTGAAGGTGAGGATGAG
#3129 HWP1-I1	CTCCAGGTACTGATTCAACTCC
#3130 HWP1-I2	CAGATGGTTGCATGAGTGGAAC
#3131 ECE1-I1	CAACATGAAGAGAGATGTTGCTCC
#3132 ECE1-I2	GACTGTCTTTCAAAGCAGATTCAGC
#3191 G4-CaRHO3	GTGGTTCATTGTCATTGGCACC
#3204 ALS3-I1	CGGAAATGGTCCTTATGAATCACC
#3205 ALS3-I2	CAAACCACATAACCAAGTAGAATGTTG

[a] Capital letters correspond to genomic sequences, and lowercase letters correspond to annealing regions of pFA plasmids.

used and was amplified from plasmids containing either a genomic or a cDNA fragment.

Microscopy. Cells were grown to exponential phase either in minimal medium or in hypha-inducing medium for 4 to 6 h at 30°C or 37°C, respectively. Nuclear staining was done by adding 1 μl of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1 mg/ml, Sigma) to 100 μl of cell suspension. Microscopy was done by using a bifunctional confocal laser scanning and fluorescence microscope on a Zeiss AxioImager platform with the appropriate filter combinations for the acquisition of GFP or DAPI fluorescence. Image acquisition and processing were done using Metamorph software (Universal Imaging Corporation).

RESULTS

Sequence analysis of Sfl1. Based on sequence similarity, orf19.454 encodes the *C. albicans* homolog of the *S. cerevisiae* *SFL1* gene. *C. albicans* Sfl1 encodes one of the longest ascomycetous Sfl1 proteins, with a length of 805 amino acids. A protein alignment reveals a number of conserved features (Fig. 1). Most notable is a strongly conserved domain within the N terminus that shares sequence similarity with the heat shock factor (HSF) domain. There are two regions in the *C. albicans* Sfl1 that are shared with Sfl1 proteins of other species: *C. albicans* and *Aspergillus fumigatus* Sfl1 proteins share an extended N-terminal sequence, and *C. albicans* and *Kluyveromyces lactis* share an internal Q-rich region. With both AfSfl1p and KlSfl1p *C. albicans* shares the highest degrees of sequence identity (21.5% over the entire proteins), although sequence similarities in the C-terminal halves of the proteins are rather low.

Deletion of Ca*SFL1* results in flocculation and filamentation in the absence of inducers. We deleted both copies of

Ca*SFL1* in the SN148 background (27). To this end, we amplified disruption cassettes from pFA plasmids and generated independent heterozygous and homozygous *sfl1* mutant strains (Table 1). Mutant strains that were of the *sfl1*::Cd*HIS1*/*sfl1*::Cm*LEU2* or *sfl1*::Cm*LEU2*/*sfl1*::*URA3* genotype were phenotypically identical. Subsequently, we present the analysis of the Ura[+] *sfl1* mutant. To analyze cell growth and colony morphology phenotypes, we grew the mutant on solid medium and in liquid full medium at yeast and hyphal growth conditions (Fig. 2). Both the wild-type and the *sfl1* mutant strains formed yeast colonies at 30°C and showed filamentous growth at 37°C in the presence of serum. We noted an increased degree of clumping for the mutant in liquid culture, both in conditions promoting yeast growth and under serum-inducing conditions. We quantified this aggregation behavior during growth in YPD (Table 3). It became evident that in YPD, a large amount of *sfl1* mutant cells were found in aggregates. These aggregates appeared not to be generated due to cytokinesis defects as were observed previously, e.g., for *C. albicans rho4* and *cht3* mutants (12). We went on to analyze cell aggregation in SD (Fig. 3). This showed that *sfl1*/*sfl1* cells form macroscopic flocs consisting of large amounts of cells. Microscopic observations revealed that these flocs also contained true hyphal cells.

An analysis of hyphal induction was done to determine whether the onset of filamentation occurs more rapidly in the *sfl1* mutant. To this end, cells were induced to form filaments with serum. Aliquots of cells were taken in half-hourly intervals, and the average lengths of germ tubes were determined microscopically.

FIG. 1. Protein alignment of ascomycetous Sfl1 proteins. Protein sequences of Sfl1 proteins from *A. fumigatus* (Af; GenBank accession number, XP_001481550), *C. albicans* (Ca; XP_715888), *A. gossypii* (Ag; NP_985683), *K. lactis* (Kl; XP_454089), and *Candida glabrata* (Cg) were aligned using the Clustal W algorithm. Matching residues are shaded. The alignment shows the N-terminal part where sequence conservation is highest.

Over a time course of 4 h, we did not find significant differences in the average lengths of the generated filaments between the wild type and the *Sfl1* mutant (data not shown).

Embedded growth induces increased filamentation in a *sfl1/sfl1* strain. Several mutants in genes encoding proteins within the cAMP pathway were shown to exhibit enhanced filamentation under embedded growth conditions, e.g., the *efg1/efg1* and *flo8/flo8* mutants (10, 18). Interestingly, both of these mutant strains are defective in filamentation under all standard inducing conditions. Thus, we went on to examine the growth phenotype of the *sfl1/sfl1* mutant under embedded conditions in YPS agar at 25°C. *SFL1* strains, including the wild-type SC5314, the SN148, and the heterozygous mutant strains, started to show increased filamentation after more than 2 days under embedded conditions. In contrast, the *sfl1/sfl1* strain formed consistently more abundant filaments under these conditions (Fig. 4). This result suggests that Sfl1p acts as a repressor of filamentation also under microaerophilic conditions when cells are embedded in an agarose matrix and grown at room temperature.
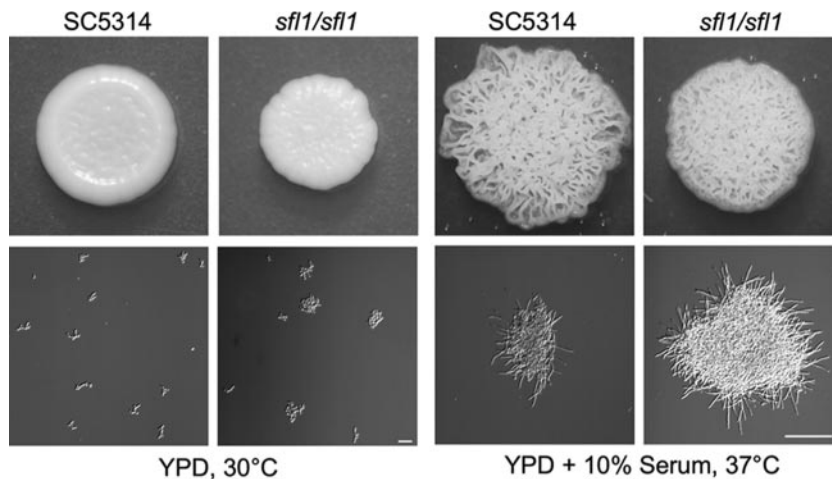

FIG. 2. Yeast and hyphal growth of the *sfl1/sfl1* mutant strain. The wild type and the *sfl1/sfl1* mutant were grown on YPD with or without 10% serum at 30°C or 37°C, respectively, in either liquid culture or on solid medium plates. Images were taken after an overnight incubation in liquid medium or after 4 days on solid medium plates. Bars, 30 μm (left) and 100 μm (right).

TABLE 3. Cell-cell aggregates in *C. albicans* strains

No. of cells in aggregates	No. of cells (%) of:		
	SC5314	SN148	*sfl1/sfl1*
1 to 2	166 (39)	155 (30)	60 (9)
3 to 4	134 (32)	204 (39)	76 (11)
5 to 10	96 (23)	162 (31)	98 (15)
>10	24 (6)	0 (0)	428 (65)
Total	420 (100)	521 (100)	662 (100)

Upregulated expression of *MET3p-SFL1* inhibits filamentation. To study the effect of depleting cells of Sfl1 or of the overexpression of *SFL1*, we placed the single copy of *SFL1* in a heterozygous *SFL1/sfl1* mutant under the control of the regulatable Ca*MET3* promoter. The use of minimal medium supplemented with methionine and cysteine led to transcriptional repression of *SFL1* and thus to the depletion of Sfl1 protein from the cells. In these cells, promoter shutdown resulted in the restoration of the *sfl1* mutant phenotypes of flocculation and increased filamentation (Fig. 5). The growth of cells in SD lacking methionine and cysteine resulted in increased expression of *MET3p-SFL1*, which reduced the clumping phenotype, and all cells remained in the yeast phase. We analyzed the strength of different promoters by using GFP as a reporter. Use of the *Ashbya gossypii TEF* promoter resulted in high-level expression. Induced expression via the *MET3* promoter
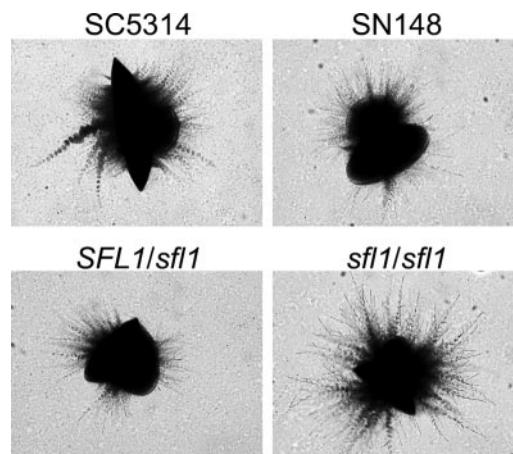

FIG. 4. A *sfl1/sfl1* mutant shows increased filamentation under embedded growth conditions. Cells of the SC5314 wild-type, SN148, heterozygous *SFL1/sfl1*, and homozygous *sfl1/sfl1* strains were plated submersed in molten YPS agar (2%) and grown for 56 h at 25°C. Bar, 50 μm.

reached half of that level while *SFL1* promoter-driven expression was only half of that of the *MET3* promoter. Therefore, induced expression of a *MET3p-SFL1* construct results in a twofold overexpression on the protein level compared to endogenous *SFL1* expression (data not shown).

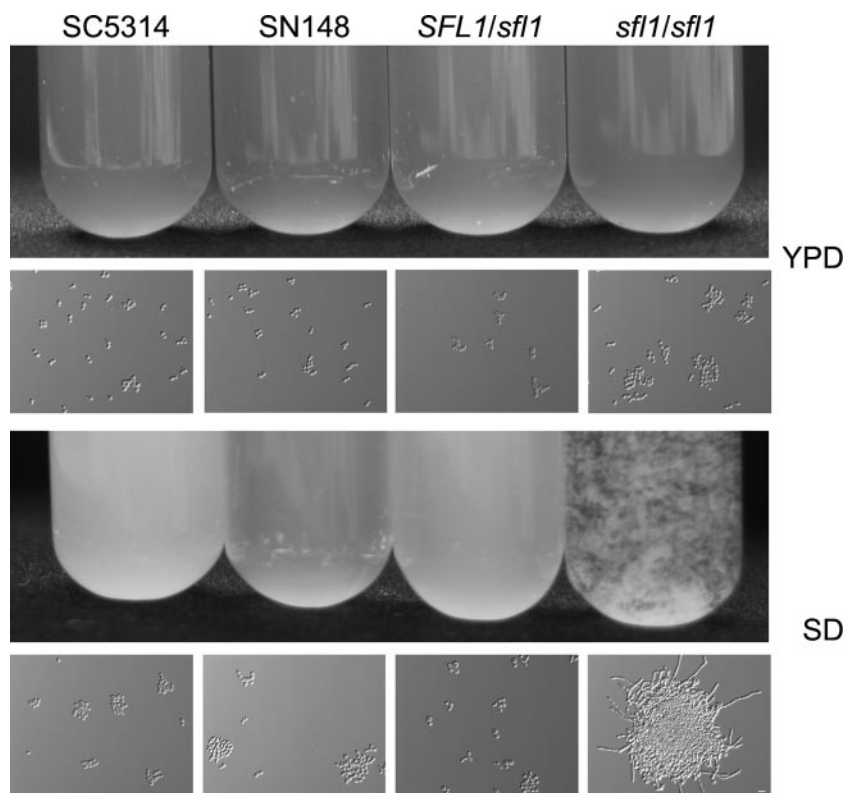The promoter shutdown and inducing experiments indicate

FIG. 3. Deletion of *SFL1* promotes flocculation in minimal medium. The indicated strains were grown overnight in liquid YPD (rich medium) or SD (minimal medium) at 30°C. Images of cell solutions in reagent tubes (upper panels) and microscopic images (lower panels) are shown. Bar, 10 μm.
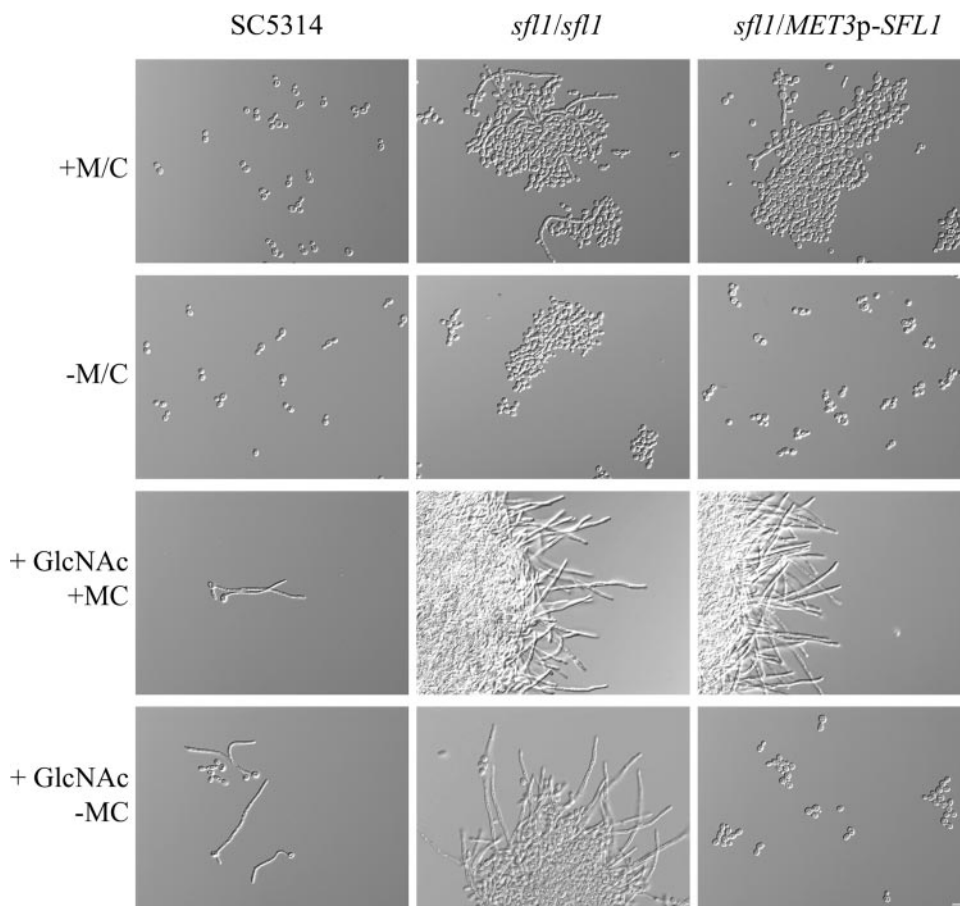
FIG. 5. *MET3* promoter-controlled expression of *SFL1*. The wild-type, *sfl1/sfl1*, and *MET3p-SFL1/sfl1* strains were pregrown in minimal media at 30°C with or without methionine/cysteine (+M/C and −M/C, respectively), diluted, and inoculated to the same cell density in fresh medium. Filamentation was induced with the addition of GlcNAc to the medium. Note that filamentation is blocked in cells overexpressing *SFL1* (bottom right panel). Bar, 10 μm.

that the mutant phenotypes observed with our *sfl1/sfl1* strains were due solely to the deletion of *SFL1*. Adding GlcNAc to minimal medium and increasing the temperature for incubation to 37°C induces *C. albicans* cells to form hyphal filaments. This became evident in the wild type, the *sfl1/sfl1* mutant strain, and the *MET3*p-controlled *SFL1* strain under repressing conditions. Surprisingly, however, if cells were pregrown in SD (without methionine and cysteine), which allows for full expression of *SFL1* via the *MET3* promoter, and were then transferred into SD plus GlcNAc medium, germ tube formation was entirely abolished. This block in filamentation required a continued high expression level of *SFL1* since cells started to filament when methionine or cysteine was present in the inducing medium. Our results, therefore, indicate that overexpression of *SFL1* can lead to a block in hyphal formation in *C. albicans* (Fig. 5).

Sfl1-GFP localizes to the nucleus. Consistent with the idea of a transcriptional regulator and DNA-binding protein should be a nuclear localization of the Sfl1 protein. Therefore, we wanted to determine the subcellular localization of Sfl1p during yeast and hyphal growth. To this end, we generated an *SFL1-GFP/sfl1* strain. This strain was phenotypically like the wild-type strain, suggesting that *SFL1-GFP* is functional. We

observed a Sfl1-GFP signal in the nuclei of yeast cells, which would be consistent with the idea of Sfl1 suppressing flocculation and filamentation under conditions that allow yeast growth. Upon filament induction, Sfl1-GFP could still be observed in the nuclei of hyphal cells but was found to be stronger in yeast or pseudohyphal cells (Fig. 6). This would be consistent with either a diminished expression of *SFL1* or a reduced amount of Sfl1p in the nuclei of hyphal cells, which is in line with the notion of a derepression of Sfl1-controlled genes under hypha-inducing conditions.

One-hybrid analysis with *SFL1*. The data presented suggested that Sfl1p fulfills a role of a transcriptional repressor in *C. albicans*. To gain further evidence for such a role, we employed a recently developed one-hybrid system with *C. albicans* (30). To this end, we fused *SFL1* to the *Staphylococcus aureus lexA* DNA-binding domain and integrated this fusion gene at the *RPS1* locus in CAI8. The readout of transcriptional regulation was done using a *lex* operator containing *Streptococcus thermophilius lacZ* reporter gene construct. As controls, we used strains provided by the Brown lab that assay the Nrg1 repressor and the Gcn4 activator activity (30). We assayed the level of reporter gene expression by using an X-Gal overlay assay and quantitative analysis of β-galactosidase activity
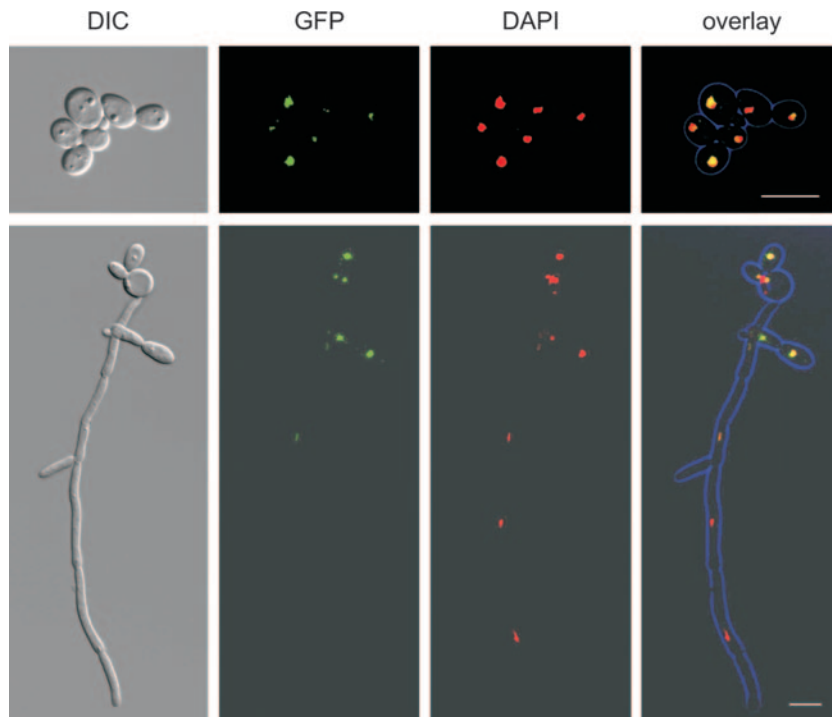
FIG. 6. Localization of Sfl1p-GFP in yeast and hyphal cells. Strain CAJ042 expresses *SFL1-GFP* from its endogenous promoter in a heterozygous strain. Fluorescence images were acquired from yeast cells and hyphal cells that were induced with GlcNAc for 4 h. Cells were fixed prior to the imaging and were costained by DAPI to visualize the nuclei. DIC, differential interference contrast. Bar, 10 μm.

(Fig. 7). This assay confirmed that Gcn4 acts as a transcriptional activator while Nrg1 functions as a repressor. In these assays, we found no activating activity of Sfl1.

A set of hypha-specific genes is derepressed in the *sfl1* mutant. The flocculation phenotype of the *sfl1/sfl1* mutant in minimal medium and the increase in filament formation provide some evidence for an altered transcriptional profile in the mutant. To gain first insight into which genes may be affected on the transcriptional level by the deletion of *SFL1*, we performed RT-PCR to analyze the expression of hypha-specific
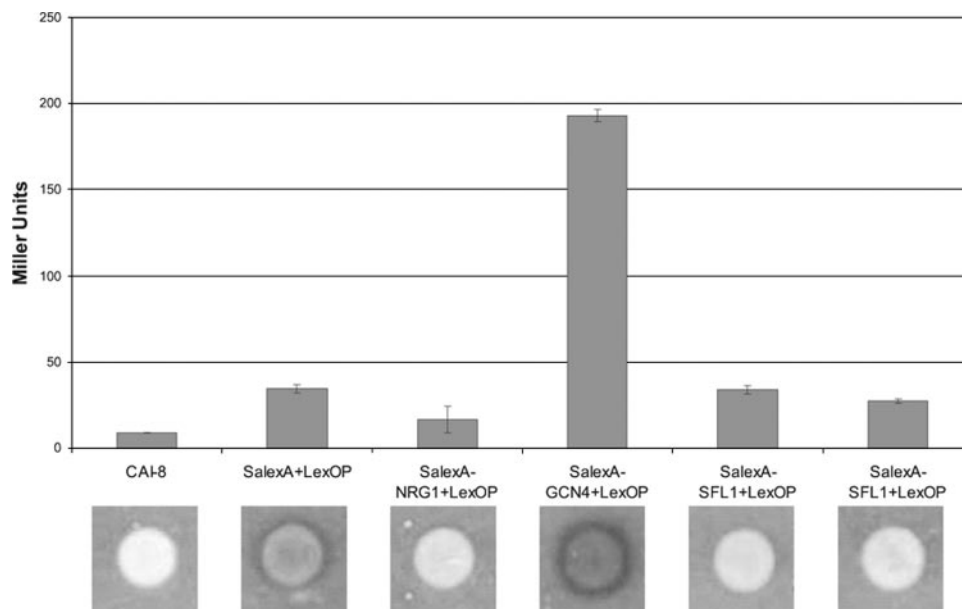

FIG. 7. One-hybrid assay with Sfl1. A S*alexA-SFL1* fusion construct was transformed into a LexOP-carrying strain in a manner similar to that done previously with *GCN4* and *NRG1* (30). The level of reporter gene expression was determined by the analysis of β-galactosidase activity and quantitated by the use of an X-Gal overlay assay as indicated.
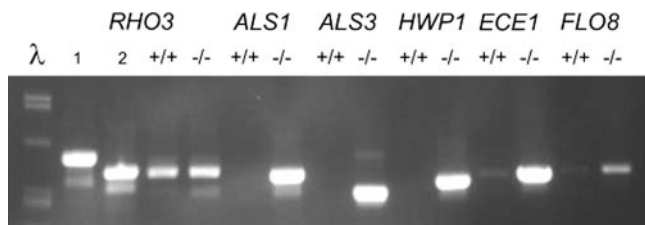
FIG. 8. Hypha-specific genes are upregulated in an *sfl1* mutant. Cells of the wild type (SC5314; +/+) and the *sfl1/sfl1* mutant (−/−) were grown in minimal medium for 6 h at RT and then collected for RNA preparation. The quality of the RNA was assessed by using the Ca*RHO3* gene as a control. Ca*RHO3* genomic DNA (lane 1) contains one intron that is missing in its corresponding cDNA (lane 2; PCR derived from a cloned cDNA fragment).

genes (*HWP1*, *ECE1*), agglutinin-like sequences (*ALS1*, *ALS3*), and also *FLO8*, which is a known positive regulator of hypha-specific gene expression in *C. albicans*. Also based on the regulation circuitry in *S. cerevisiae*, Sc*FLO8* is known to be regulated by ScSfl1. In this assay, we found increased expression of these genes in the *sfl1/sfl1* mutant (Fig. 8).

DISCUSSION

We have identified the *C. albicans* SFL1 gene by similarity to its *S. cerevisiae* homolog. Sfl1p contains an N-terminal domain with a high degree of similarity to the HSF DNA-binding domain (15). *C. albicans* Sfl1p shares an extended N terminus with *A. fumigatus* Sfl1p, the function of which, however, needs to be elucidated. Sfl1 is a conserved protein and known as a suppressor of flocculation in *S. cerevisiae*. In *S. cerevisiae*, Sfl1p acts as transcriptional repressor of flocculation (*FLO*) gene (15, 28). The deletion of Sc*SFL1* leads to floc formation and to enhanced pseudohyphal and invasive growth (28). We found that Ca*SFL1* regulates similar processes in *C. albicans*. The deletion of Ca*SFL1* resulted in strong flocculation in minimal medium accompanied by increased filamentation in the absence of inducers as well as enhanced filamentous growth under embedded conditions at 25°C. This result suggests that some of the transcriptional circuitry controlled by Sfl1p has been conserved between *S. cerevisiae* and *C. albicans*.

The prevailing view in the regulation of *C. albicans* hyphal morphogenesis is that transcriptional repression by Tup1, Nrg1, Rpg1, and Ssn6 and complexes thereof provide the cells with the means to repress specific gene sets (5–8, 21, 22, 26). The mechanistic difficulties in explaining the complex pattern of regulation of hypha-specific gene expression came from comparative transcript profiling experiments that indicated that in *nrg1*, *tup1*, and *ssn6* mutant strains, overlapping but extensively different subsets of genes were regulated (16). Our studies now present one additional player in this scheme, Sfl1p. It is noteworthy that deletion of *SFL1* does not induce filamentation to an extent seen, for example, in the *tup1* mutant. On the other hand, the flocculation phenotype seems to be rather specific for the *sfl1* mutant. Formation of cell clumps occurs in the *sfl1* mutant strain under different medium conditions but is most obvious in minimal media. As flocs also contain hyphal cells in minimal media, more pronounced floc-

culation might be the result of hypha-specific gene expression in these cells.

In *S. cerevisiae*, Sfl1p recruits the Ssn6-Tup1 complex via an Ssn6 interaction domain (11). This domain is only rather weakly conserved on the amino acid level between different fungal Sfl1 proteins. However, it leads to a testable hypothesis in *C. albicans*, namely, that Sfl1p might repress hypha- and/or flocculation-specific genes via a Sfl1-Ssn6-Tup1 complex. We analyzed the potential of CaSfl1p to act as a repressor of transcription using a recently developed one-hybrid system, which proved useful to identify Gcn4 as a transcriptional activator (30). In these assays, CaSfl1 was only weakly downregulating reporter gene transcription and certainly not activating it. Consistent with the idea of a repressor could be a differential localization of CaSfl1 in the nucleus of yeast cells but not of hyphal cells. Even though we found evidence to support this idea, we cannot rule out a nucleocytoplasmic shuttling of Sfl1 that could contribute to its specific activity. Such a shuttling mechanism is quite common and has been described, e.g., for the Msn2/4 global stress response regulator in *S. cerevisiae* (19).

In *S. cerevisiae*, Sfl1 is regulated via phosphorylation by Tpk2 (28). By analogy, this could place Sfl1 downstream of the cAMP-signaling pathway in concert with Flo8. Furthermore, in *S. cerevisiae*, Sfl1p regulates the expression of *FLO8* in a negative manner (23). A similar scenario seems likely for *C. albicans*, since overexpression of *SFL1* using the *MET3* promoter resulted in the inability of these cells to form hyphae under GlcNAC-induced filamentation. This result is consistent with the role of CaFlo8 as an inducer of hypha-specific genes, as the *flo8/flo8* mutant is blocked for hypha-specific gene expression and filamentation (10). First insights into the target genes of Sfl1 came from RT-PCR, which showed expression of the adhesins *ALS1* and *ALS3* as well as of other hypha-specific genes. *FLO8* appears to be upregulated in a *sfl1* mutant. Interestingly, the deletion of *SFL1* points toward a role of Sfl1 that is not exclusive for filamentation, but it reveals a novel role in flocculation. This function could be important for cell-cell adhesion or cell-surface adhesion, such as that seen in biofilm formation. Determining the Sfl1 regulon, particularly with respect to adhesins and hyphal specific genes, therefore, will be an essential task to understand the role of Sfl1.

In conclusion, our functional analysis of the *C. albicans* SFL1 gene provides evidence that Sfl1 is a conserved negative regulator of flocculation and filamentation in *C. albicans* that may regulate a specific gene set required for flocculation but may regulate hypha-specific genes in concert with other known repressors, particularly Tup1 and Nrg1.

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