Candida albicans Als3p is required for wild-type biofilm formation on silicone elastomer surfaces

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INTRODUCTION

Many different micro-organisms can form biofilms. Biofilms are a functional association of surface-attached microbial cells that become encased in extracellular matrix material (Costerton et al., 1987; Donlan & Costerton, 2002). Once committed to biofilm growth, microbial cells exhibit different phenotypic properties compared to free-floating, planktonic cells (Costerton et al., 1995). One clinically relevant consequence of biofilm growth is the development of resistance to antimicrobial compounds (Schwank et al., 1998; Wilson, 1996; Stewart et al., 2004; Rupp, 2005). Among the fungi, Candida albicans is the most common organism associated with biofilm formation on bioprosthetic materials (reviewed by Douglas, 2003; Ghannoum & O’Toole, 2004).

In the search to identify C. albicans genes that are important for biofilm formation, investigators have assessed the effect of deleting single genes (Kelly et al., 2004; Krueger et al., 2004; Granger et al., 2005; Kumamoto, 2005), or studied transcriptional profiles using microarrays (Garcia-Sanchez et al., 2004; Cao et al., 2005; Murillo et al., 2005; K. M. Yeater and others, unpublished). The importance of transcription factors in biofilm formation has been analysed individually for factors such as Efg1p (Ramage et al., 2002b) or by assaying sets of mutant strains (Nobile & Mitchell, 2005). Some of these studies suggested that hyphae are important in biofilm formation and indicated that hypha surface proteins may be important in
biofilm formation (Ramage et al., 2002b; Nobile & Mitchell, 2005; Richard et al., 2005).

During the course of our studies of the C. albicans ALS (agglutinin-like sequence) gene family, we noted that strains in which ALS3 was deleted showed an obvious defect in biofilm formation on silicone elastomer surfaces. ALS3 expression was first associated with germ tube/hypha formation using Northern blot analysis (Hoyer et al., 1998a). Since then, the idea that high-level ALS3 expression is associated with germ tubes and hyphae has been supported by other methods including flow cytometry analysis of a PALS3-GFP (green fluorescent protein) reporter strain 2185 (Zhao et al., 2004; Green et al., 2005b), and real-time RT-PCR quantification of transcript copy number (Green et al., 2005b). Flow cytometry analysis of strain 2185 also showed that ALS3 transcriptional activity increases markedly when germ tubes become visible microscopically, rather than being induced immediately upon inoculation into fresh growth medium as is ALS1 (Zhao et al., 2004; Green et al., 2005b). ALS3 expression in model biofilms was demonstrated by RT-PCR (Green et al., 2004).

The focus of this paper is the role of Als3p in biofilm formation using the catheter biofilm model described by Kuhn et al. (2002). Here, we demonstrate the importance of Als3p in catheter biofilm formation and demonstrate how the Als3p functional domains important for biofilm formation may differ from those important for adhesion to human epithelial cells. We also address the relationship between biofilm formation and filamentous growth by demonstrating that overexpression of ALS3 in the biofilm- and filamentation-defective efg1Δ/efg1Δ strain confers wild-type biofilm mass without the production of hyphae. These results separate C. albicans morphology from biofilm formation and further substantiate the importance of Als3p in biofilm formation.

METHODS

Biofilm growth and measurement. Model catheter biofilms were grown on silicone elastomer discs according to the method of Kuhn et al. (2002). Biofilm mass was measured by dry weight analysis. Prior to incubation in fetal bovine serum, the autoclaved silicone elastomer discs were baked overnight in an 80 °C oven to remove residual moisture. Each disc was preweighed using sterile technique. After 48 h growth of the model biofilm, the medium in each well was removed, and the catheter discs were transferred onto Whatman chromatography paper, dried overnight at 80 °C and weighed immediately after removal from the oven. Biofilm dry weight was calculated by subtracting the preweight of each disc from the disc weight following biofilm growth and baking. Biofilms were grown in triplicate in three separate experiments. Means and SEM were calculated using the LSMEANS option in the MIXED procedure in the SAS/STAT software package (version 8; SAS Institute).

Two-photon laser scanning microscopy. Biofilms were fixed with 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline without calcium or magnesium (DPBS; Cambrex catalogue no. 17-512Q) for 1 h at room temperature. Yeast-enhanced GFP fluorescence was detected using a Bio-Rad Radiance 2100MP multiphoton laser scanning system (Bio-Rad Microscopy) attached to a TE2000E microscope (Nikon USA) with a 60 × 1.2 NA plan-apochromat water immersion lens (Nikon). GFP was excited at 920 nm by a Mai-Tai titanium sapphire tunable laser system (Spectra-Physics). Biofilms grown from strains that did not encode GFP were stained with Calcofluor White M2R (Molecular Probes). Calcofluor was excited at 818 nm. Images were acquired using a non-descanned external direct detector PMT (Bio-Rad) and image stacks were captured using LaserSharp software (Bio-Rad). All images were processed using Adobe Photoshop software.

Creating serum immunoglobulin (Ig) preparations enriched for Als3p specifically. The Als5p N-terminal domain (Als5p18–329) was produced in Pichia pastoris and purified as described by Hoyer & Hecht (2001). Five hundred micrograms of Als5p18–329 was suspended in TiterMax adjuvant and injected subcutaneously into a New Zealand White Rabbit (Myrtle’s Rabbitry, Thompson Station, TN, USA). Subsequent inoculations consisted of 250 μg of the Als5p18–329 protein in Freund’s incomplete adjuvant and were administered 3 weeks after the previous injection. The rabbit was bled 7 days after every even-numbered inoculation and the anti-serum titre assessed by Western blotting against the Als5p18–329 protein. A total of eight protein inoculations were administered. A final inoculation was administered and the rabbit exsanguinated 7 days afterwards. Serum was collected and stored at −80 °C. The resulting antiserum titre was greater than 1:60 000 as measured by Western blotting. Lipoproteins were removed from the antiserum by adding sodium dextran sulfate to 0.25% (v/v) and then calcium chloride to 0.40% (v/v). Following overnight incubation on ice and centrifugation, the supernatant was recovered and the gammaglobulin fraction precipitated by addition of an equal volume of saturated ammonium sulfate solution. Following overnight on ice and centrifugation, the pellet was resuspended in and dialysed exhaustively against DPBS. Total protein was measured by the Bradford dye-binding procedure (Bio-Rad). Aliquots of the gammaglobulin fraction were stored at −80 °C.

Enrichment of the Ig preparation for reactivity against Als3p was accomplished by absorption against the als3Δ/als3Δ strain 1843 (Table 1; Zhao et al., 2004). Strain 1843 was grown under conditions known to cause high-level transcription of other ALS genes, such as ALS1, which shares considerable sequence identity with ALS3 (Hoyer et al., 1998a; Green et al., 2005b). Conditions included overnight growth in YPD (yeast forms) and 1 h growth in YPD (yeast forms) or RPMI 1640 (RPMI; germ tubes). All incubations were at 37 °C and 200 r.p.m. shaking. All cells from each culture were combined, collected by centrifugation and washed in DPBS. Cells were resuspended in Complete, Mini Protease Inhibitor Cocktail (Roche) to which 1 μM pepstatin (Roche) was added. Three separate rounds of absorption were completed, each with approximately one-third of the cell preparation and end-over-end mixing. Two absorptions were completed at 4 °C for 1 h and the last at room temperature for 1 h. Cells were removed by centrifugation and the protein concentration measured. Aliquots of the anti-Als3p Ig preparation were frozen at −80 °C.

Indirect immunofluorescence. Indirect immunofluorescent detection of Als proteins on RPMI-grown germ tubes was described previously (Hoyer et al., 1998b). The anti-Als3p (1843-adsorbed) Ig preparation (described above) and normal rabbit IgG (ICN catalogue no. 641471; negative control) were used at 150 μg ml⁻¹ concentration. Fluorescence was detected using a Nikon Eclipse E600 microscope fitted with a Spot camera (Diagnostic Instruments). Images of representative cells were collected using Metamorph software (Universal Imaging Corporation) and processed with Adobe Photoshop. The staining procedure was conducted on three different days with similar results on each day.
Flow cytometry analysis followed a published method (Zhao et al., 2004). Strains CAI12, 1843 and 2327 (see below) were combined with antibody following 30 min incubation in RPMI medium, which was sufficient to form small germ tubes. The anti-Als3p (1843-adsorbed) Ig preparation or normal rabbit IgG, diluted to 60 μg ml⁻¹, was used. Incubations were conducted at either room temperature (normal goat serum block) or 4 °C (primary antibody and FITC-labelled secondary antibody) on an end-over-end mixer. Washing steps used DPBS. Fluorescence was detected by flow cytometry, using a Beckman Coulter EPICS XL, equipped with an argon laser with an excitation wavelength of 488 nm. Flow cytometry analysis of strains HLC52 and 2296 used yeast cells that were grown for 16 h in YPD at 37 °C and 200 r.p.m. shaking.

**Reintegration of a wild-type ALS3 allele into an als3Δ/als3Δ strain.** Construction and phenotypic evaluation of the als3Δ/als3Δ mutant strain 1843 (Table 1) were described previously (Zhao et al., 2004). In previous work, reintegration of a wild-type ALS3 allele into strain 1843 was complicated because removing the disruption cassette from the ALS3 large allele (ALS3LA) locus also removed several kb of sequence downstream of ALS3. Subsequent efforts finally yielded a strain (named 2311) with the correct construction (Table 1). The ALS3 reintegration cassette was constructed in plasmid pUL, which can be used to make constructs for reintegration of any of the ALS genes (Zhao et al., 2004). ALS3 downstream sequence was amplified from SC5314 genomic DNA using primers ALS3dnF and ALS3dnR (Table 2) and Pfu Turbo polymerase (Stratagene). The amplified fragment was cloned into the SstI/ NcoI sites of pUL, generating plasmid 2303. Full-length ALS3 from strain SC5314 [large allele, 12 tandem repeat copies, ALS3LA or ALS3(12), GenBank accession no. AY225552; Zhao et al., 2004; Oh et al., 2005] with 400 bp of upstream sequence was amplified from genomic DNA using the primer pair ALS3upF and ALS3R. This fragment was digested with AavII and cloned into AvarI–Xhol cut plasmid 2303. The AvarI–NcoI fragment, consisting of ALS3 upstream sequence-ALS3LA coding region-URA3-ALS3 downstream sequence, was transformed into strain 2311 and transformants selected as described previously (Zhao et al., 2004). The correct transformant was identified by Southern blotting and named 2322 (Table 1; Fig. 1).

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

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<th>Strain</th>
<th>Parent</th>
<th>Genotype*</th>
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<td>SC5314</td>
<td>wild-type</td>
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<td>CAI4</td>
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<td>Fonz &amp; Irwin (1993)</td>
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<td>This study</td>
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<tr>
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<td>This study</td>
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<tr>
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<td>HLC67</td>
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<td>CAI4</td>
<td>CAI4 (RP10::1105-PTRPI1-GFP-URA3)</td>
<td>Green et al. (2005a)</td>
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*ALS3 alleles from strain SC5314 are marked with ‘LA’ or ‘SA’ to designate the large allele (GenBank accession no. AY225552) and small allele (AY223551), respectively.

**Table 2. Oligonucleotide primers used in this work**

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<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
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</tr>
<tr>
<td>ALS3dnR</td>
<td>CCC GCC GCC GTT TGG TAA TTA ACA CAT ATT GC</td>
</tr>
<tr>
<td>ALS3upF</td>
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</tr>
<tr>
<td>ALS3R</td>
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<tr>
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<td>AG21XhoR</td>
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<tr>
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</tr>
<tr>
<td>RTAG21R</td>
<td>TCC CTG AGA TGA GAG TGC TGT</td>
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Construction of a *C. albicans* strain that produces an Als3p–Agr1p fusion protein on its germ tube surface. An in-frame fusion between the 5′ end of ALS3 and the 3′ end of AG1 (Lipke et al., 1989; Hauser & Tanner, 1989) was constructed in the *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen). Primers ALS3Spf and ALS3Snl290 (Table 2) were used to amplify a fragment encoding the N-terminal 430 aa of Als3p. The fragment encoding the C-terminal 323 aa of Agr1p was amplified from *S. cerevisiae* genomic DNA using primers AG1Xba and AG1Int980 (Table 2). The C-terminal half of Agr1p does not contain the adhesive domain (Cappellaro et al., 1994; Chen et al., 1995; de Nobel et al., 1996; Zhao et al., 2001) and shares similar cellular localization signals and amino acid composition with the C-terminal domains of Als proteins. The C-terminal half of Agr1p, therefore, provides a cell-surface-localized, highly glycosylated protein stalk that can display the Als3p N-terminal domain on the cell surface. The ALS3 PCR product was digested with SpHl/KpnI and the AG1 PCR product was digested with KpnI/Xbol. These fragments were ligated into SpHl/Xbol-cut pYES2 DNA and transformed into *Escherichia coli* TOP10F’ (Invitrogen) to form plasmid 881. Restriction mapping and DNA sequencing verified the construct. The ALS3–AG1 fusion was amplified from plasmid 881 using primers 3cdHindIII and AG1XhoI (Table 2). The PCR product was digested with HindIII/Xbol and ligated into similarly digested plasmid 2303, generating plasmid 2326, which contained (5′–3′) the ALS3–AG1 fusion–URA3–ALS3 downstream sequence between the HindIII and SalI sites. Plasmid 2326 was digested with HindIII/SalI and the fragment transformed into strain 1926 (iro1-ura3Δ::immm34/iro1-ura3Δ::immm34 ALS3LA/als3SA-ura3), from which the ALS3 small allele (ALS3SA) had been deleted previously.

Integration of the fusion construct into the ALS3 large allele (ALS3LA) locus replaced the ALS3LA coding sequence, creating *C. albicans* strain 2327 (Table 1), which did not produce Als3p, but instead produced the Als3p–Agr1p fusion under control of the ALS3 promoter (Fig. 2). Southern blotting verified construction of clone 2327 (Fig. 2). The AG1 probe was amplified by PCR using primers RTAG1F and RTAG1R (Table 2). The ALS3 downstream sequence probe was synthesized using primers ALS3dnF and ALS3dnR (Table 2). Growth rate of strain 2327 in YPD medium and germ tube formation in RPMI 1640 were measured according to published methods (Zhao et al., 2004) and were not significantly different from the wild-type strain CAI12. The presence of the Als3p N-terminal domain on the surface of strain 2327 germ tubes was verified by indirect immunolabelling using the anti-Als3p Ig preparation (see below) and flow cytometry (Green et al., 2005b). Flow cytometry readings for strain 2327 showed that production of the fusion protein increased surface fluorescence of the als3Δ/als3Δ strain to the level observed for the CAI12 wild-type control.

Epithelial cell adhesion assays. The method for assaying *C. albicans* adhesion to pooled, fresh human buccal epithelial cells (BECs) was described previously (Zhao et al., 2004). Briefly, *C. albicans* cells were incubated in RPMI medium at 37°C for 1 h to form germ tubes, buccal epithelial cells were added to the incubation flask and the incubation continued for an additional 30 min. Non-adherent fungal cells were separated from the BECs by filtration and washing over a 12 μm pore-size filter. Results were evaluated by counting the number of fungal cells adhered to each of 50 randomly selected BECs. Results were reported as the mean ± SEM for each treatment.
To assess the effects of the anti-Als3p Ig preparation on C. albicans adhesion to the BECs, 150 μg Ig (37 ± 5 μg ml⁻¹ final concentration in the flask) were added to the RPMI medium 30 min after C. albicans germ tubes of wild-type strain CAI12 started to form. After an additional 30 min, the BECs were added to the culture flask and the assay completed as described above. Normal rabbit IgG was used as a negative control. The assay was conducted on three separate days.

Construction of C. albicans strains that overexpress ALS3.

ALS3 overexpression used plasmid 1105 (Green et al., 2005a), which is a modified version of Clp10 (Murad et al., 2000). Plasmid 1105 encodes the C. albicans TPI1 promoter and terminator sequences, separated by a polylinker that includes restriction sites (5'–3' XhoI–SmaI–NotI–BglII). The XhoI–BglII sites allow cloning for overexpression of any full-length ALS gene due to lack of these restriction sites in any of the ALS gene coding regions. These sites are used in various vectors in the laboratory and allow interchangeable cloning of ALS genes between the different constructs. ALS3LA (GenBank accession no. AY223552) from strain SC5314 was excised from previously built constructs and ligated into XhoI–BglII-cut plasmid 1105. The P_TPI1-ALS3 overexpression construct was linearized with HindIII, which cuts once within the 3' end of ALS3 to direct integration of the plasmid to the ALS3 locus in the efg1D/efg1D strain HLC52. The resulting strain, 2296, was verified by Southern blotting and showed integration of the plasmid at the ALS3LA locus (Fig. 3). The growth rate of strain 2296 was the same as the wild-type control, CAI12. Real-time RT-PCR analysis of ALS3 expression (Green et al., 2005b) in C. albicans yeast cells grown for 16 h in YPD at 37°C showed that strain 2296 produced 15-fold overexpression of ALS3.

Fig. 2. Construction and verification of a C. albicans als3Δ/als3Δ strain that produces an Als3p–Agα1p fusion protein under control of the ALS3 promoter. (a) ALS3LA locus in strains derived from SC5314. (b) Transformation fragment that includes the fused ALS3 and AGα1 coding regions and the URA3 selectable marker. The ALS3 downstream probe fragment is shown. (c) Integration of the fusion construct into the ALS3LA locus. (d, e) Southern blots of BglII-digested genomic DNA hybridized with the ALS3 downstream probe (d) or an AGα1-specific fragment (e). Molecular size (in kb) is indicated at the left of each image.

Fig. 3. Construction and verification of a C. albicans efg1D/efg1D strain that overproduces Als3p under control of the constitutive TPI1 promoter. (a) The ALS3LA locus in strains derived from SC5314, such as the efg1Δ/efg1Δ strain HLC52 (Lo et al., 1997). (b) The ALS3LA locus with the overexpression cassette integrated. (c) Southern blot of EcoRV-digested genomic DNA, hybridized with the ALS3 downstream probe. Molecular size (in kb) is indicated at the left of the image. (d) Relative fluorescence (FITC) intensities of immunostained strains HLC52 and 2296. Overexpression of ALS3 in strain HLC52 increased the surface fluorescence, consistent with increased production and correct localization of Als3p on the C. albicans cell surface.
more ALS3 transcript than strain HLC52. Flow cytometry analysis of immunolabelled yeast cells of strains HLC52 and 2296 indicated a shifted fluorescence peak consistent with greater surface expression of Als3p epitopes (Fig. 3).

Evaluation of C. albicans cellular morphology. C. albicans yeast forms were grown to stationary phase in YPD, washed in DPBS and inoculated into YPD + 10 % fetal calf serum or RPMI at a density of 5 × 10^6 cells ml⁻¹. After 2 h at 37 °C, aliquots of each culture were fixed with 1 % (v/v) glutaraldehyde and photographed as described above.

RESULTS

ALS3 is expressed in model catheter biofilms

Construction and characterization of the C. albicans PALS3-GFP reporter strain, 2185, was reported previously (Zhao et al., 2004). In strain 2185, GFP is transcribed under control of the ALS3 promoter and translated into soluble protein that is localized within the cytoplasm. Growth of strain 2185 in a model catheter biofilm showed fluorescent hyphae when visualized by two-photon laser scanning microscopy, suggesting that GFP is produced following transcription from the ALS3 promoter, which is active under these growth conditions (Fig. 4a). Fluorescence of hyphae from strain 2185 was evident throughout the biofilm, suggesting that ALS3 expression is not limited to a particular area of the biofilm. A control strain, 1143 (Green et al., 2005a), in which GFP is transcribed under control of the constitutive TPI1 promoter, was also tested (Fig. 4b). As expected, biofilm growth of strain 1143 showed fluorescence of both hyphae and yeast throughout the biofilm. Dry weight analysis of biofilms formed from strain 2185 showed a similar mass to those formed by wild-type control strain CAI12 (1.50 ± 0.12 mg vs 1.52 ± 0.12 mg; P = 0.92).

Als3p is localized diffusely on the germ tube surface

Although Als3p is cross-linked to β-1,6-glucan in the C. albicans cell wall (Kapteyn et al., 2000), its distribution on the cell surface has not been reported. To determine the cell-surface distribution of Als3p, serum Ig enriched for Als3p specificity was prepared by absorption of a polyclonal anti-Als serum with various morphological forms of the als3Δ/als3Δ strain 1843 (Zhao et al., 2004; see Methods). Immunostaining using the anti-Als3p Ig preparation showed diffuse fluorescence that covered nearly the entire length of the germ tube (Fig. 5a). Immunostaining with normal rabbit IgG (negative control) showed very faint fluorescence of the mother yeast and germ tube (Fig. 5c). Immunofluorescence was not detectable when the als3Δ/als3Δ strain was stained with the anti-Als3p Ig preparation (Fig. 5e).

ALS3 deletion results in a disorganized and weakened biofilm structure

Growth of the als3Δ/als3Δ strain 1843 in the catheter biofilm model resulted in a weakened structure that was obvious immediately when viewing the biofilms in the 12-well plate in which they were grown. The mature biofilm was dislodged easily from the catheter disc and readily crumbled into many pieces when the 12-well plate was moved from the incubator. The dry weight of the mutant 48 h biofilm was significantly less than that for the wild-type control (1.15 ± 0.12 mg vs 1.52 ± 0.12 mg; P = 0.04). Microscopic imaging of calcofluor-stained biofilms showed parallel hyphae in the mutant biofilm (Fig. 6a) that contrasted in appearance with the intertwining hyphae present in the wild-type biofilm structure (Fig. 6b). This difference in biofilm architecture is unlikely to be due to differences in filamentation or growth rate between the two strains since their phenotypes were indistinguishable when tested in various growth media (Zhao et al., 2004). Construction of a strain in which a copy of ALS3LA from strain SC5314 was reintegrated into strain 1843 was described in Methods (Fig. 1). The resulting strain (2322) restored wild-type dry weight of the model catheter biofilm (1.55 ± 0.12 mg vs 1.52 ± 0.12 mg; P = 0.84) and restored wild-type biofilm structure. These observations suggest that ALS3 deletion caused the reduced biofilm mass and structural instability.

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**Fig. 4.** Two-photon laser scanning micrograph of the model catheter biofilms grown using the PALS3-GFP strain 2185 (a) or TPI1-GFP strain 1143 (b). Fluorescence is detectable only in hyphae for strain 2185, but in hyphae and yeast forms for strain 1143. Bar, 5 μm.
Evaluation of the Als3p N-terminal domain function in biofilm formation

Als proteins are composed of three domains (Hoyer, 2001). The N-terminal domain contains approximately 433 aa and shares sequence similarities with the N-terminal half of *S. cerevisiae* alpha-agglutinin (Agα1p; Lipke et al., 1989; Hauser & Tanner, 1989), which contains residues critical for adhesive function (de Nobel et al., 1996). Therefore, it is reasonable to expect that adhesive function resides within the Als protein N-terminal domain. To investigate the role of the Als3p N-terminal domain in biofilm formation, and to probe the relationship between adhesion and biofilm formation, *C. albicans* strain 2327 was created (Fig. 2). Strain 2327, from which both ALS3 coding regions were deleted, produced a cell-surface fusion of the N-terminal 430 aa of Als3p to the C-terminal 323 aa of *S. cerevisiae* alpha-agglutinin under control of the native ALS3 promoter. The fusion protein displayed the Als3p N-terminal domain on a stalk-like portion of Agα1p that is targeted to the cell wall and heavily glycosylated with N- and O-linked carbohydrate, but does not contain any sequences involved in the Agα1p adhesive interactions (Cappellaro et al., 1994; Chen et al., 1995; de Nobel et al., 1996; Zhao et al., 2001). Growth of strain 2327 in the catheter biofilm model showed intertwined hyphae that were more characteristic of a wild-type biofilm (Fig. 6c), but production of the fusion protein did not restore wild-type biofilm mass (1·05 ± 0·12 vs 1·52 ± 0·12 mg; \( P = 0·01 \)).

This result prompted additional experimentation to characterize the phenotype of the Als3p–Agα1p-producing strain. Most work to define Als protein function has been conducted from the standpoint of adhesion. Previously
published data showed that the als3Δ/als3Δ mutant strain 1843 has a significantly decreased ability to adhere to human BECs (Zhao et al., 2004). Strain 2327 was tested for BEC adhesion to place it into the context of this knowledge. Consistent with the previous data (Zhao et al., 2004), strain 1843 was significantly less adherent to BECs than was wild-type CAI12 (Fig. 7a). Production of the Als3p–Agz1p fusion protein on the surface of strain 2327 significantly increased C. albicans adhesion to BECs compared to the als3Δ/als3Δ mutant, but did not fully restore wild-type adhesion levels. This intermediate adhesion level may be due to the length of the Als3p–Agz1p fusion protein. Previous studies showed that longer ALS3 alleles (more tandem repeat copies) produce proteins with greater adhesive function than shorter ALS3 alleles (Oh et al., 2005). It is possible that the greater number of tandem repeat copies in the longer alleles displays the N-terminal domain at a greater distance from the cell surface, making it more accessible to binding interactions. However, a role for the tandem repeat sequences in binding interactions has not been ruled out. The Als3p–Agz1p fusion protein in the current study was only 753 aa, which was considerably shorter than the smaller Als3p (1047 aa) and larger Als3p (1155 aa) tested by Oh et al. (2005).

These data are consistent with the conclusion that Als3p adhesive function is found predominantly within the N-terminal domain. To further evaluate this idea, we blocked adhesion of C. albicans to human BECs using the anti-Als3p Ig preparation. Wild-type CAI12 cells were grown in RPMI 1640 medium to form germ tubes, which were then pre-incubated with the anti-Als3p Ig preparation, or with a normal rabbit IgG control, before incubating them with BECs. Compared to cells to which no antibody was added, pre-incubation with normal rabbit IgG significantly

**Fig. 6.** Two-photon laser scanning micrograph of calcofluor-stained model catheter biofilms grown using the als3Δ/als3Δ strain 1843 (a) the wild-type control CAI12 (b), or strain 2327, which lacks ALS3 coding sequences, but produces a cell-surface Als3p–Agz1p fusion protein under control of the ALS3 promoter. Compared to hyphae in the wild-type biofilm (b), hyphae in the als3Δ/als3Δ biofilm (a) are parallel and less densely packed. Production of the Als3p–Agz1p fusion protein (c) restores intertwined hyphae that are characteristic of the wild-type biofilm (b), but does not restore wild-type biofilm dry weight. Bar = 5 μm.

**Fig. 7.** BEC adhesion assay data. (a) Wild-type CAI12, als3Δ/als3Δ mutant 1843, and the Als3p–Agz1p fusion protein-producing strain 2327 were tested for adhesion to BECs. Loss of ALS3 significantly reduced adhesion of C. albicans to BECs (**P < 0.0001). Production of the Als3p–Agz1p fusion protein on the surface of strain 2327 significantly increased C. albicans adhesion to BECs compared to the als3Δ/als3Δ mutant (*P = 0.01), but did not fully restore wild-type adhesion levels. (b) CAI12 germ tubes were pre-incubated without antibody (CAI12), with normal rabbit IgG (IgG) or with the anti-Als3p Ig preparation prior to their addition to a BEC adhesion assay. Pre-incubation with normal rabbit IgG significantly decreased adherence of C. albicans to BECs (*P = 0.01). Pre-incubation with the anti-Als3p Ig preparation significantly reduced adhesion compared to the rabbit IgG control (**P = 0.01). Collectively, these results suggest that Als3p is involved in C. albicans adhesion to human BECs and that the N-terminal domain of the protein mediates this interaction.
decreased adherence of wild-type *C. albicans* to BECs (Fig. 7b). Pre-incubation with the anti-Als3p Ig preparation significantly reduced adhesion compared to the rabbit IgG control (Fig. 7b). These results further support the role of Als3p in *C. albicans* adhesion to human BECs and the role of the N-terminal domain in this interaction.

**Wild-type biofilm mass is restored to a *C. albicans efg1Δ/efg1Δ* strain by overexpression of ALS3**

Leng *et al.* (2001) showed that *EFG1* is required for activation of *ALS8*, and more recent work demonstrated that *ALS3* and *ALS8* are the same locus (Zhao *et al.*, 2004). Deletion of *EFG1* destroyed the ability of *C. albicans* to form a biofilm; the *efg1Δ/efg1Δ* strain grew as a sparse monolayer of cells (Ramage *et al.*, 2002b). Integration of a wild-type *EFG1* copy restored biofilm formation to the mutant strain (Ramage *et al.*, 2002b). These results suggest the importance of hyphae in *C. albicans* biofilm formation. In the context of its role in hypha formation, Efg1p regulates expression of many *C. albicans* genes (Liu, 2002). Therefore, many different proteins could be responsible for deficient biofilm formation by the mutant strain. The importance of hypha-associated cell-surface proteins in biofilm formation has also been suggested by subsequent studies (Nobile & Mitchell, 2005; Richard *et al.*, 2005). The significance of *ALS3* and *EFG1* in wild-type biofilm formation, as well as the established regulatory connection between the two genes, suggested that *ALS3* might reverse the biofilm-deficient phenotype of an *efg1Δ/efg1Δ* strain. To test this idea, *ALS3* was overexpressed in strain HLC52 under control of the constitutive *TPI1* promoter (Fig. 3). In a previous study, constitutive *ALS1* overexpression in the *efg1Δ/efg1Δ* strain HLC52 resulted in formation of elongated morphologies under growth conditions that produce hyphae in wild-type cells (Fu *et al.*, 2002). Because of these observations, we examined the cellular morphology of our *efg1Δ/efg1Δ ALS3* overexpression strain, 2296. Wild-type strain CAI12 and the *efg1Δ/efg1Δ* strain were included as controls (Fig. 8). When grown in conditions that did not promote filamentation of the wild-type strain (YPD medium), strain 2296 formed elongated cells. Incubation in YPD + serum or in RPMI 1640 medium resulted in germ-tube-like structures for strain 2296, although these cells exhibited an obvious altered morphology compared to the wild-type control (Fig. 8). Growth of strain 2296 in the catheter model produced a biofilm with wild-type dry weight although the biofilm lacked hyphae and, instead, was composed mainly of yeast forms or short, elongated cells (Fig. 9). Therefore, although wild-type hypha formation was not restored in strain 2296, overproduction of Als3p was able to produce a biofilm of wild-type mass. These results dissect the effects of biofilm formation from those of cellular morphology (specifically hypha growth) and demonstrate a specific role for a hypha-associated surface protein in biofilm formation.

![Fig. 8](http://mic.sgmjournals.org)

*Fig. 8.* Light micrographs of the cellular morphology of the *ALS3* overexpression and control strains. Strains CAI12, HLC52 (*efg1Δ/efg1Δ*) and 2296 (*efg1Δ/efg1Δ* *TPI1*-ALS3) were grown in YPD overnight at 37 °C, washed, counted and transferred to YPD, YPD + serum or RPMI 1640 for 2 h at 37 °C. Images shown are representative of the morphologies present for each culture.
DISCUSSION

Previous analysis suggested that high-level ALS3 expression is associated with germ tubes and hyphae, and that Als3p is a strong adhesin that interacts with both epithelial and endothelial surfaces (Hoyer et al., 1998b; Zhao et al., 2004). ALS3 expression was previously demonstrated within model biofilms (Green et al., 2004) and in this work ALS3 expression was detected in hyphae throughout a model catheter biofilm using two-photon laser scanning microscopy of a PALS3–GFP reporter strain. Indirect immunofluorescence of germ tubes grown in vitro showed that Als3p is localized diffusely across the cell surface. In the absence of Als3p, model catheter biofilms are weakened and disorganized and lack the mass associated with a wild-type biofilm. Although the Als3p N-terminal domain is responsible for adhesion of C. albicans to BECs, display of the Als3p N-terminal domain on the C. albicans surface does not restore biofilm mass, but restores the intertwining of hyphae observed in a wild-type biofilm. Overproduction of Als3p is sufficient to restore wild-type biofilm mass to a C. albicans strain lacking EFG1, even though the strain does not form hyphae. This result uncouples the effects of a hypha-associated protein from cellular morphology and suggests a major role for Als3p in biofilm formation.

Results from this work begin to define Als3p function in biofilm formation. The most straightforward explanation to consider is that Als3p is involved in adhesion of C. albicans to silicone elastomer. However, because the adhesion phase of biofilm growth uses yeast forms that do not appear to express ALS3, this explanation is unlikely to account for the importance of Als3p in biofilm formation. It is more likely that Als3p becomes important at a later stage of biofilm development when germ tubes and hyphae are present. The ability of a C. albicans strain displaying the Als3p N-terminal domain to confer greater association between hyphae, but not wild-type biofilm mass, suggests that Als3p may have multiple roles in biofilm formation. The lack of association between hyphae in the mutant biofilm suggests that, in the absence of Als3p, C. albicans cells are not attracted to, or are repelled by, each other. These observations suggest that Als3p may mediate cellular aggregation by either homotypic or heterotypic interactions, alter the surface charge or hydrophobic properties of hyphae, or alter interactions between the extracellular matrix and cellular components of the biofilm. The requirement for Als3p to achieve wild-type biofilm mass suggests that the entire protein is either needed for cellular proliferation within the biofilm, or is a key component of the extracellular matrix. Previous work suggested that Als proteins are shed from the C. albicans surface at various stages of culture growth (Hoyer et al., 1998b). Current data suggest that the extracellular matrix is composed primarily of molecules that are present in planktonic C. albicans cells, rather than being made from biofilm-specific components (Vediyappan & Chaffin, 2006; D. A. Coleman, M. S. Kuhlenkamst & L. L. Hoyer, unpublished observations). During biofilm development, C. albicans grows in adherent microcolonies (Kuhn et al., 2002) that could trap Als3p shed from the cell surface. The large, mucin-like Als3p glycoprotein could act as glue that holds together the biofilm structure. Since the Als3p tandem repeat and C-terminal domains are predicted to be the most heavily glycosylated portions of the mature protein, these domains might have greater significance in accumulation of biofilm mass. Perhaps wild-type biofilm mass is not restored for strains producing the Als3p–Ag1p fusion protein because the non-native C-terminal sequences in the fusion construct do not permit shedding from the C. albicans surface. Additional experimentation is required to test these ideas and to further define the role of Als3p in biofilm formation.

Questions regarding Als3p function were placed into the context of C. albicans–host cell adhesion since this direction has dominated Als protein functional analysis. Production of the Als3p–Ag1p fusion protein in an als2Δ/als3Δ C. albicans strain demonstrated function of the Als3p N-terminal domain in BEC adhesion. The idea that the Als N-terminal domain is involved in adhesion came from comparisons to S. cerevisiae alpha-agglutinin, in which adhesive function resides within the N-terminal half of the protein (Cappellar et al., 1994; Chen et al., 1995; de Nobel et al., 1996; Zhao et al., 2001). Previous studies addressed adhesive function of the Als N-terminal domain. Adhesion of a C. albicans ALS1 overexpression strain to vascular endothelial cells was blocked by a monoclonal antibody that was raised against the Als1p N-terminal domain (Fu et al.,
S. cerevisiae, expressing ALS1 with mutations within the N-terminal domain-encoding sequences, showed reduced adhesion compared to a S. cerevisiae strain expressing the wild-type allele (Loza et al., 2004). Another study claimed to demonstrate adhesive specificity within the N-terminal domain of Als5p and Als6p using a domain-swapping approach (Sheppard et al., 2004). However, rather than swapping the N-terminal domain of Als5p onto the tandem repeat and C-terminal domain of Als6p (and vice versa), the investigators included the tandem repeat sequences in the swap. In addition, the sequences of the C-terminal domain of Als5p and Als6p are over 90% identical with conservative replacements for many of the mismatched amino acids. Therefore, the experiment essentially recreated native Als5p and Als6p, rather than testing the effects of placing the Als N-terminal domain onto a non-native C-terminal sequence. In our Als3p domain-swapping experiment, only the N-terminal domain of Als3p was fused to sequences of alpha-agglutinin that are C-terminal to the adhesive domain. The ability of the fusion protein to restore epithelial adhesion to the als3Δ/als3Δ mutant C. albicans strain supports the conclusion that the Als3p N-terminal domain has adhesive function.

Data presented here also address the relationship between C. albicans cellular morphology and biofilm formation. Several lines of evidence demonstrated the importance of filamentous growth in biofilm formation (reviewed by Lopez-Ribot, 2005). Hyphae are essential for the structural stability of mature biofilms (Baillie & Douglas, 1999). C. albicans strains with impaired hypha formation due to mutation in genes encoding the transcription factors EFG1 (Ramage et al., 2002b) or TEC1 (Nobile & Mitchell, 2005) cannot form wild-type biofilms. Also, blocking of hypha formation with farnesol inhibits biofilm formation (Ramage et al., 2002a). In our work, we restored wild-type biofilm mass by overexpression of ALS3 in a strain lacking Efg1p. Previous work showed that EFG1 is required for ALS3 expression (Leng et al., 2001) and ALS3 is now recognized as part of the ‘hyphal regulon’ (Kumamoto & Vinces, 2005). Although ALS3 overexpression caused the efg1Δ/efg1Δ strain to grow in a somewhat elongated form under hypha-inducing conditions, biofilms formed from this strain showed mainly yeast forms (Fig. 9). These results dissociate biofilm formation from hypha formation and suggest that the need for hyphae in biofilm growth is due, at least in part, to the role of hypha-associated proteins. Nobile & Mitchell (2005) also reached this conclusion by testing C. albicans transcription factor mutants for deficiencies in biofilm development. In their work, disruption of the zinc finger transcription factor Bcr1p did not affect the ability of planktonic cells to form hyphae, but resulted in a strain that was deficient in biofilm formation. Microarray analysis showed that Bcr1p regulates expression of several genes that encode hypha cell-surface proteins, including ALS3.

Nobile & Mitchell (2005) assayed ALS gene expression in the bcr1Δ/bcr1Δ strain in their catheter biofilm model and found that, in addition to the 16-fold reduction in ALS3 expression, ALS1 and ALS9 expression were reduced twofold in the mutant strain. Expression of other ALS genes, including ALS2, was not affected by Bcr1p loss. These results contrast with data from systematic testing of C. albicans strains with ALS gene mutations that showed reduced ALS2 expression caused a significant loss of biofilm mass in the catheter biofilm model (Zhao et al., 2005). In both studies, a silicone elastomer surface was used for biofilm formation, although growth media were different. Using our model, the contribution of Als3p to biofilm formation is clearly superior to that of Als2p. These results suggest the potential for model-specific effects on biofilm formation, but clearly support the importance of ALS3 in both cases. Additional comparisons will reveal the potential role of other Als proteins in biofilm formation.

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