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

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Abstract

Candida albicans ALS3 encodes a large cell-surface glycoprotein that has adhesive properties. Immunostaining of cultured *C.* albicans germ tubes showed that Als3p is distributed diffusely across the germ tube surface. Two-photon laser scanning microscopy of model catheter biofilms grown using a *PALS3*-green fluorescent protein (GFP) reporter strain showed GFP production in hyphae throughout the biofilm structure while biofilms grown using a *PTPI1*-GFP reporter strain showed GFP in both hyphae and yeast-form cells. Model catheter biofilms formed by an *als3 / als3* strain were weakened structurally and had approximately half the biomass of a wild-type biofilm. Reintegration of a wild-type *ALS3* allele restored biofilm mass and wild-type biofilm structure. Production of an Als3p-Agα1p fusion protein under control of the *ALS3* promoter in the *als3 /als3* strain restored some of the wild-type biofilm structural features, but not the wild-type biofilm mass. Despite its inability to restore wild-type biofilm mass, the Als3p-Agα1p fusion protein mediated adhesion of the als3 /als3 *C.* albicans strain to human buccal epithelial cells (BECs). The adhesive role of the Als3p N-terminal domain was further demonstrated by blocking adhesion of *C.* albicans to BECs with immunoglobulin reactive against the Als3p N-terminal sequences. Together, these data suggest that portions of Als3p that are important for biofilm formation may be different from those that are important in BEC adhesion, and that Als3p may have multiple functions in biofilm formation. Overexpression of *ALS3* in an *efg1 /efg1* strain that was deficient for filamentous growth and biofilm formation resulted in growth of elongated *C.* albicans cells, even under culture conditions that do not favour filamentation. In the catheter biofilm model, the ALS3 overexpression strain formed biofilm with a mass similar to that of a wild-type control. However, *C.* albicans cells in the biofilm had yeast-like morphology. This result uncouples the effect of cellular morphology from biofilm formation and underscores the importance of Als3p in biofilm development on silicone elastomer surfaces.

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

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(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), conducted systematic searches using collections of mutants (Richard *et al.*, 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 inbiofilm 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 *efg1 /efg1* 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 Microscience) attached to a TE2000E microscope (Nikon USA) with a $60 \times 1 \cdot 2$ 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 with LaserSharp software (Bio-Rad). All images were processed using Adobe Photoshop software.

Creating serum immunoglobulin (Ig) preparations enriched for Als3p specificity

The Als5p N-terminal domain ($Als5p_{18-329}$) was produced in *Pichia* pastoris and purified as described by Hoyer & Hecht (2001). Five hundred micrograms of $Als5p_{18-329}$ 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 $Als5p_{18-329}$ 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 $Als5p_{18-329}$ 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 *als3 /als3* 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 at 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 *als3 /als3* strain

Construction and phenotypic evaluation of the *als3 /als3* 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 *SstII/Ngo* MIV 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. AY223552; 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 *AvrIIB* and *XhoI* and cloned into *AvrII-XhoI*-cut plasmid 2303. The *AvrII-Ngo* MIV 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).

Construction of a *C. albicans* strain that produces an Als3p-Agα1p fusion protein on its germ tube surface

An inframe fusion between the 5′ end of *ALS3* and the 3′ end of AGα1 (Lipke *et al.*, 1989; Hauser & Tanner, 1989) was constructed in the *Saccharomyces cerevisiae* expression vector pYES2 (Invitrogen). Primers ALS3Sph and ALS3nt1290 (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 Agα1p was amplified from S. *cerevisiae* genomic DNA using primers AGα1Xba and AGα1nt980 (Table 2). The C-terminal half of Agα1p 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 Agα1p, therefore, provides a cell-surface-localized, highly glycosylated protein stalk that can display the Als3p N-terminal domain on the *C. albicans* cell surface. The *ALS3* PCR product was digested with *SphI/KpnI* and the *AGα1* PCR product was digested with *KpnI/XbaI.* These fragments were ligated into *SphI/XbaI*-cut pYES2 DNA and transformed into *Escherichia coli* TOP10F′ (Invitrogen) to form plasmid 881. Restriction mapping and DNA sequencing verified the construct. The *ALS3-AGα1* fusion was amplified from plasmid 881 using primers 3cd*Hind* IIIF and AGα1XhoR (Table 2). The PCR product was digested with *Hind III/XhoI* and ligated into similarly digested plas-mid 2303, generating plasmid 2326, which contained (5′-3′) the *ALS3-AGα1* fusion-URA3-ALS3 downstream sequence between the *HindIII* and *SstI* sites. Plasmid 2326 was digested with *Hind*III/SstI and the fragment transformed into strain 1926 (*iro1-ura3 :: λimm^{434}/iro1-ura3 :: λimm^{434} 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-Agα1p fusion under control of the *ALS3* promoter (Fig. 2). Southern blotting verified construction of clone 2327 (Fig. 2). The *AGα1* probe was amplified by PCR using primers RTAGα1F and RTAGα1R (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 *als3 /als3* 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 μgml^{-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 CIp10 (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 PTPI1-ALS3 overexpression construct was linearized with *Hind*III, which cuts once within the 3′ end of *ALS3* to direct integration of the plasmid to the *ALS3* locus in the *efg1 /efg1* 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-foldmore 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 ($1\cdot50\pm0\cdot12$ vs $1\cdot52\pm0\cdot12$ 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 *als3 /als3* 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 *als3 /als3* strain was stained with the anti-Als3p Ig preparation (Fig. 5e).

ALS3 deletion results in a disorganized and weakened biofilm structure

Growth of the *als3 /als3* 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 ($1\cdot15\pm0\cdot12$ mg vs $1\cdot52\pm0\cdot12$ 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.

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. Previouslypublished data showed that the *als3/als3* 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-Agα1p fusion protein on the surface of strain 2327 significantly increased *C. albicans* adhesion to BECs compared to the *als3/als3* mutant, but did not fully restore wild-type adhesion levels. This intermediate adhesion level may be due to the length of the Als3p-Agα1p 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-Agα1p 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 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 efg1 /efg1* 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 *efg1 /efg1* 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 *efg1 /efg1* 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 *efg1 /efg1* 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 *efg1 /efg1 ALS3* overexpression strain, 2296. Wild-type strain CAI12 and the *efg1 /efg1* 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.

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. Kuhlenschmidt & 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-Agα1p 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-Agα1p fusion protein in an als3 /als3   *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 (Cappellaro *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.*, 2002). 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 *als3 /als3*   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 *efg1 /efg1*   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 *bcr1 /bcr1* 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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Abbreviations

ALS	agglutinin-like sequence
BEC	buccal epithelial cell
GFP	green fluorescent protein
Ig	immunoglobulin

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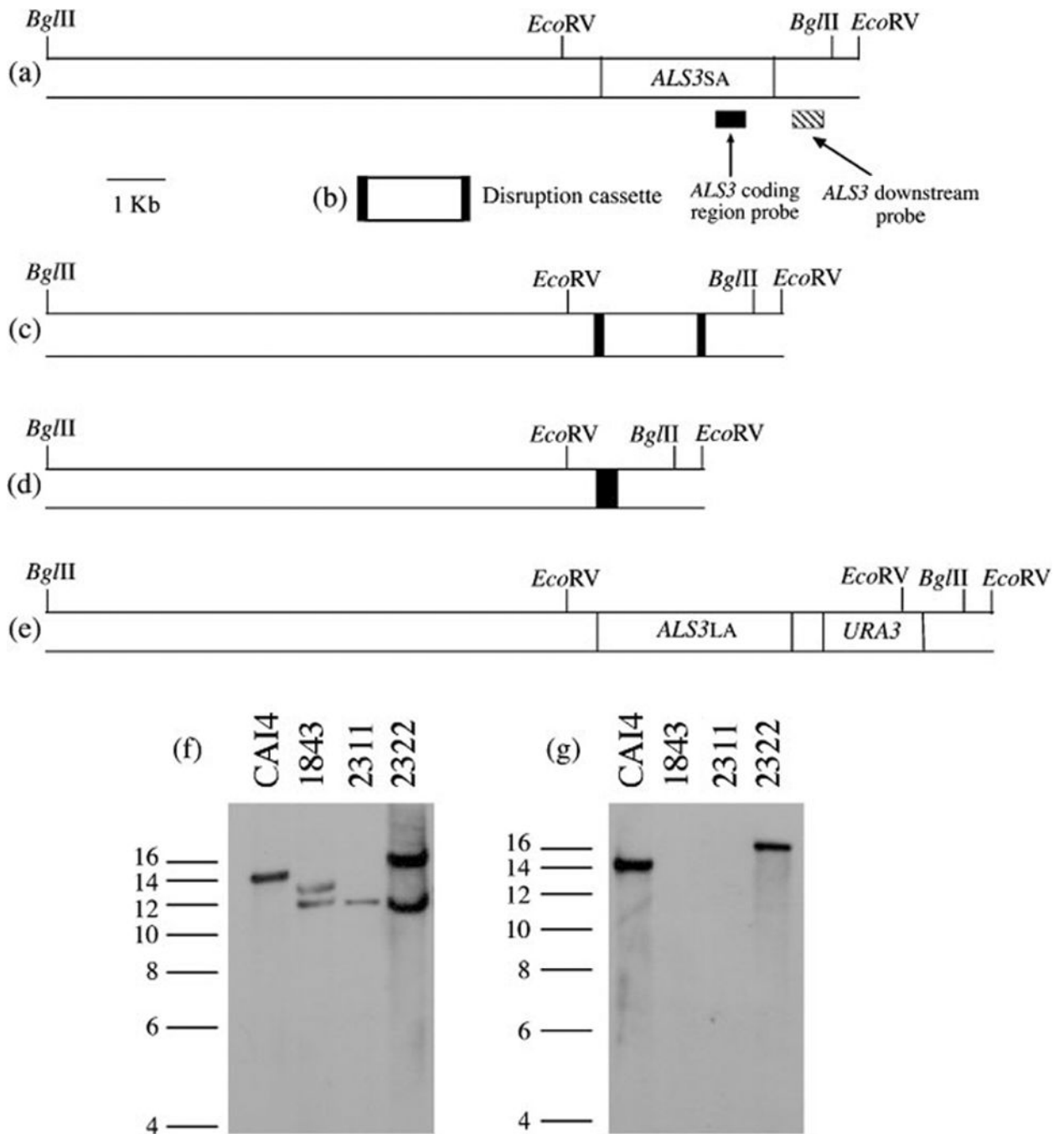
Fig. 1.
Reintegration of a wild-type *ALS3* allele into a *C. albicans als3 /als3* strain. The large allele of *ALS3* [12 tandem repeat copies, called *ALS3LA* or *ALS3(12)*, Gen-Bank accession no. AY223552; Zhao *et al.*, 2004; Oh *et al.*, 2005] was reintegrated into the *C. albicans* als3 /als3 mutant strain 1843 (Table 1). (a) Diagram of the *ALS3* small allele [9 tandem repeat copies, called *ALS3SA* or *ALS3(9)*, GenBank accession no. AY223551; Zhao *et al.*, 2004; Oh *et al.*, 2005] locus in strains derived from SC5314. Probes to detect the *ALS3* coding region (Hoyer *et al.*, 1998a) and the *ALS3* downstream region (Zhao *et al.*, 2004) are

shown. (b) The PCR-amplified disruption cassette used to delete *ALS3* alleles in strain 1843 (Zhao *et al.*, 2004). (c) *ALS3SA* locus with the disruption cassette integrated as in strain 1843. (d) *ALS3* small allele locus after selection of strain 1843 on 5-FOA to identify strain 2311. (e) *ALS3SA* locus with reintegrated copy of *ALS3LA* as in strain 2322. (f, g) Southern blots of *BglII*-digested genomic DNA from the strains indicated, probed with the *ALS3* downstream fragment (f) or the *ALS3* coding-region fragment (g). Molecular size (in kb) is indicated at the left of each image.
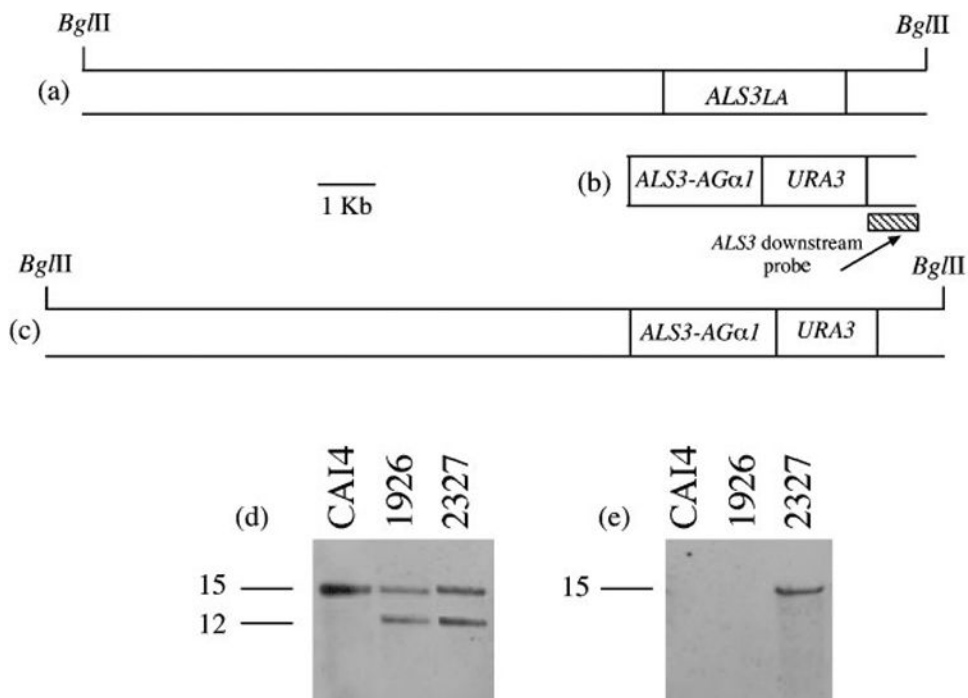
Fig. 2.
Construction and verification of a *C. albicans* als3 /als3 strain that produces an Als3p-Agα1p fusion protein under control of the *ALS3* promoter. (a) *ALS3LA* locus in strains derived from SC5314. (b) Transformation fragment that includes the fused *ALS3* and *AGa1* coding regions and the URA3 selectable marker. The *ALS3* down-stream 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.
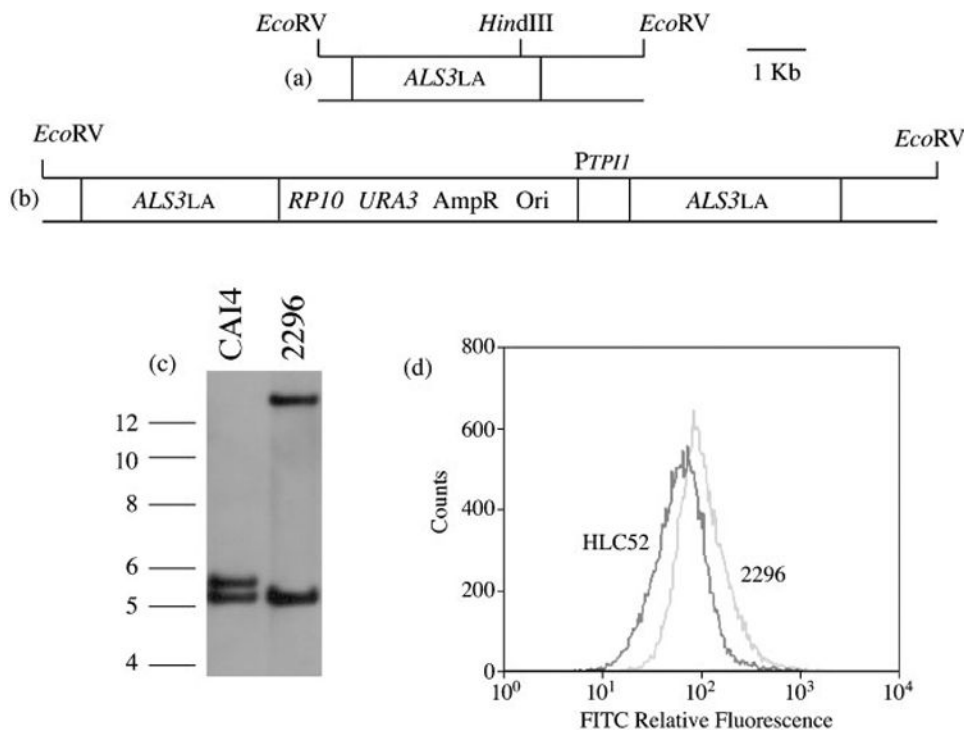
Fig. 3.
Construction and verification of a *C. albicans efg1*/*efg1* strain that overproduces Als3p under control of the constitutive TPI1 promoter. (a) The *ALS3LA* locus in strains derived from SC5314, such as the *efg1*/*efg1* strain HLC52 (Lo *et al.*, 1997). (b) The ALS3LA locus with the overexpression 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.
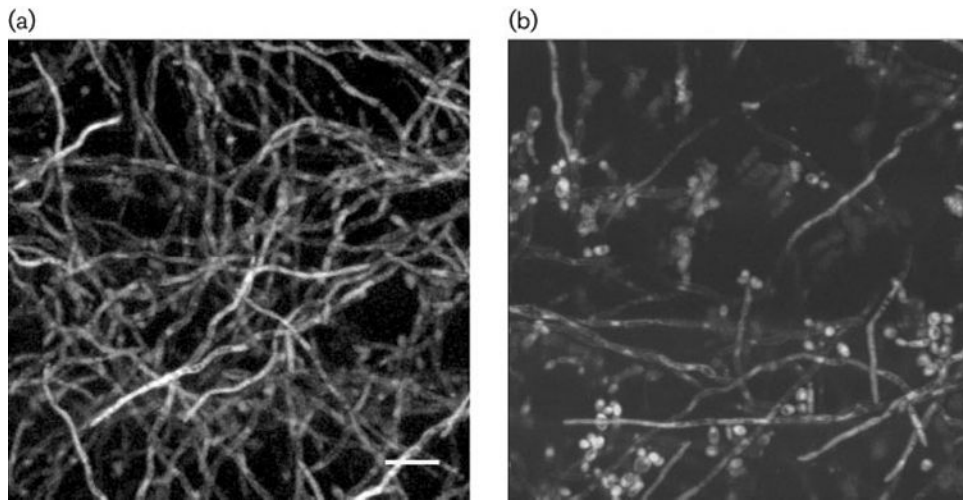
Fig. 4.
Two-photon laser scanning micrograph of the model catheter biofilms grown using the *PALS3*-GFP strain 2185 (a) or *PTPI1-GFP* strain 1143 (b). Fluorescence is detectable only in hyphae for strain 2185, but in hyphae and yeast forms for strain 1143. Bar, 5 μm.
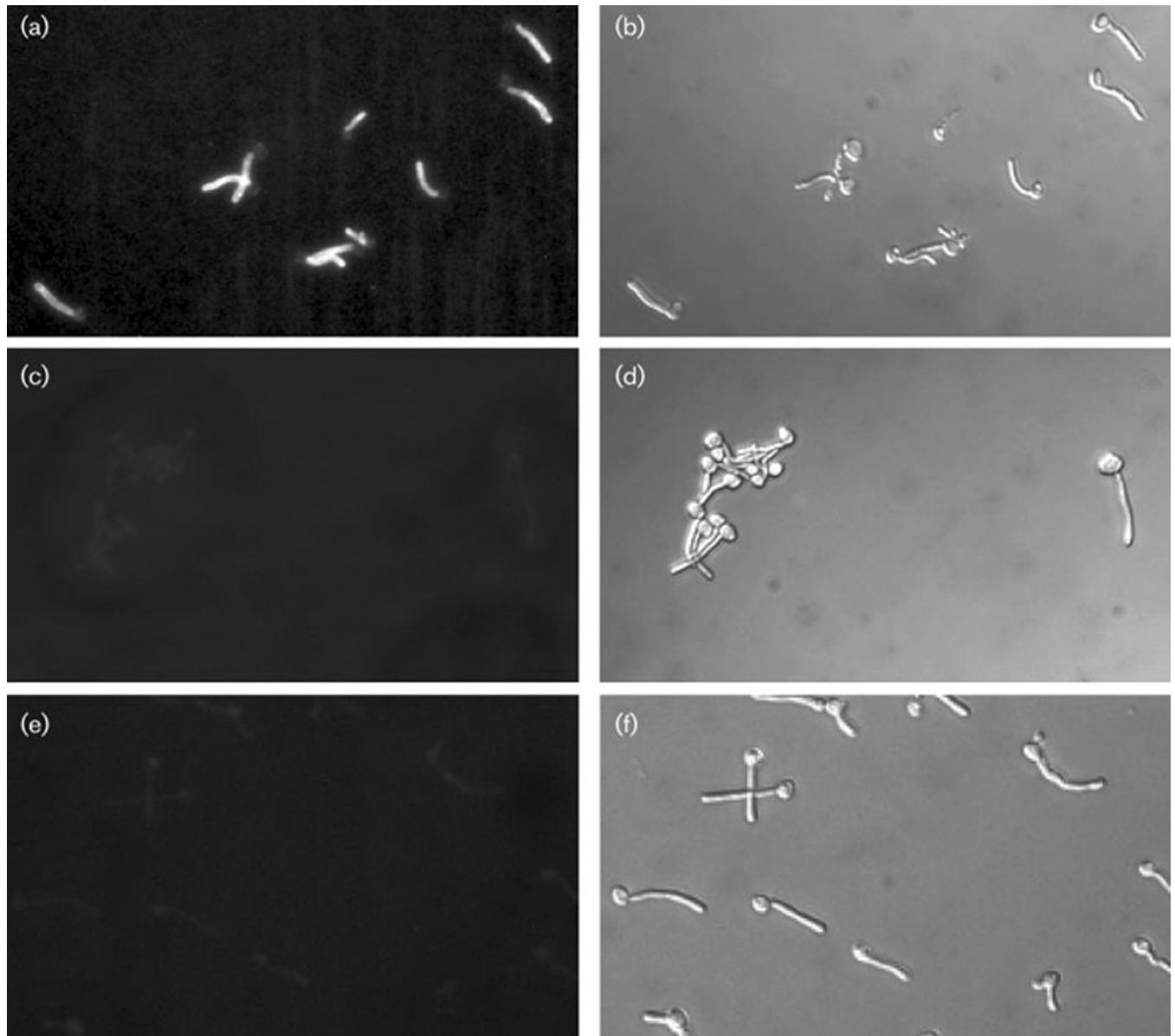
Fig. 5.
Fluorescence micrographs of *C. albicans* CAI12 germ tubes that were immunostained with
either the anti-Als3p Ig preparation (a) or normal rabbit control IgG (c) and a secondary
anti-rabbit FITC-labelled antibody. (e) Immunostaining of the als3 /als3 strain 1843 with
the anti-Als3p Ig preparation and the FITC-labelled secondary antibody. Light micrographs
(b, d, f) are matched with each fluorescence image. Anti-Als3p Ig produced a diffuse
staining pattern on nearly the entire germ tube length. The control rabbit IgG produced a
very faint background signal on both the mother yeast and germ tube. Because the antiserum
used for preparation of the anti-Als3p Ig was raised against the N-terminal domain of Als5p,
the anti-Als3p Ig cross-reacts with Als5p. This cross-reactivity is undetectable in the
experiments presented here, presumably because of the low *ALS5* transcriptional activity
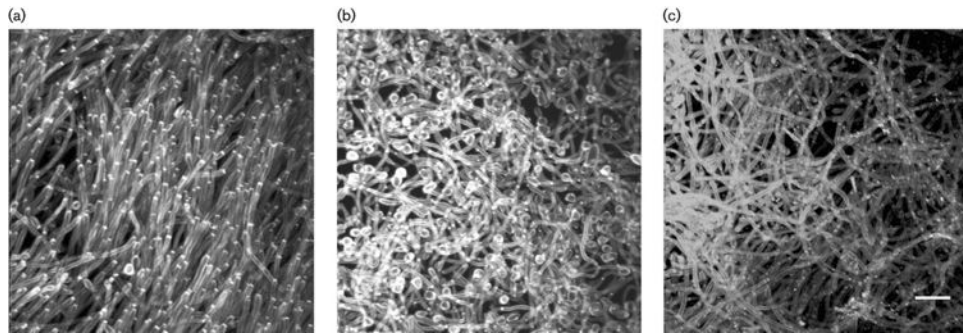under these growth conditions (Green *et al.*, 2005b).

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-Agα1p 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-Agα1p 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.
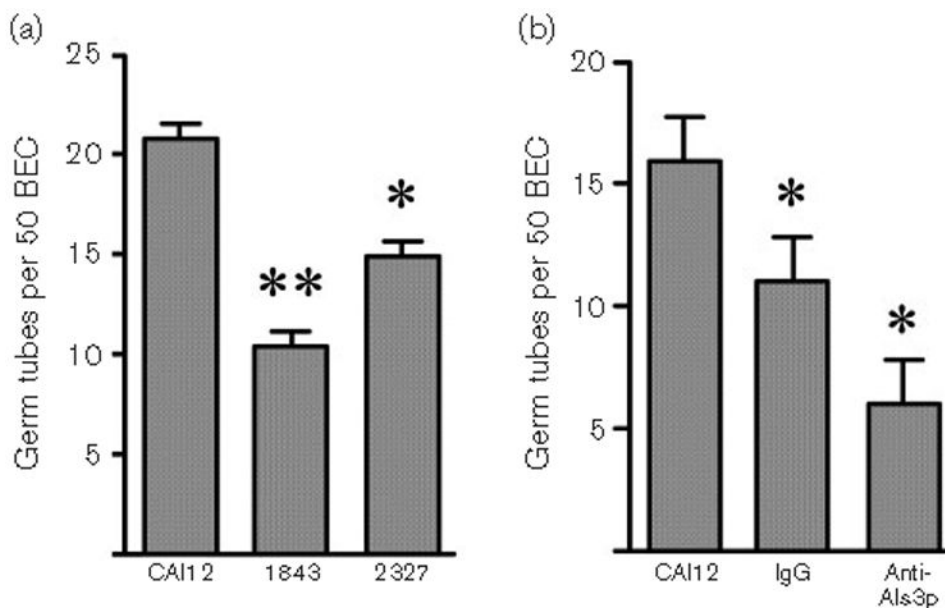
Fig. 7.

BEC adhesion assay data. (a) Wild-type CAI12, *als3 /als3* mutant 1843, and the Als3p-Agα1p 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-Agα1p fusion protein on the surface of strain 2327 significantly increased *C. albicans* adhesion to BECs compared to the *als3 /als3* 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.
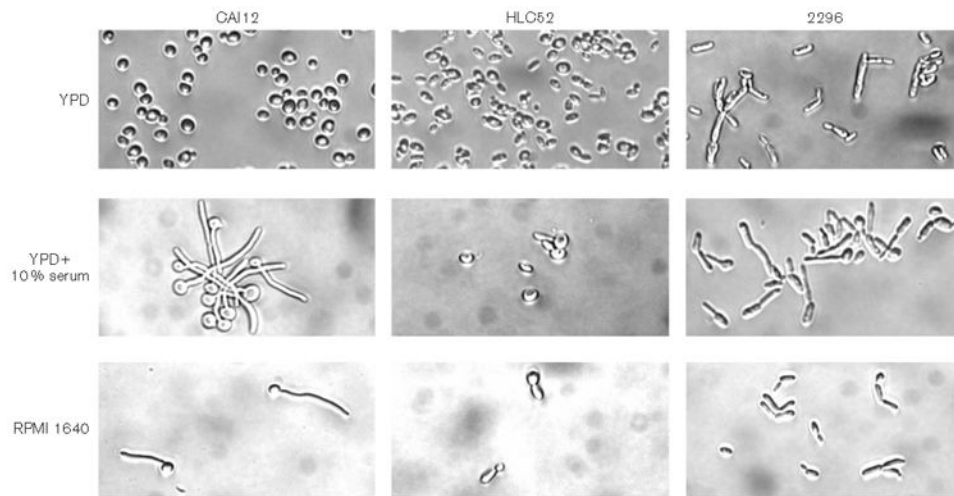
Fig. 8.
Light micrographs of the cellular morphology of the *ALS3* overexpression and control strains. Strains CAI12, HLC52 (*efg1 /efg1*) and 2296 (*efg1 /efg1 PTPI1-ALS3*) were grown in YPD overnight at 37 6C, washed, counted and transferred to YPD, YPD+serum or RPMI 1640 for 2 h at 37 6C. Images shown are representative of the morphologies present for each culture.
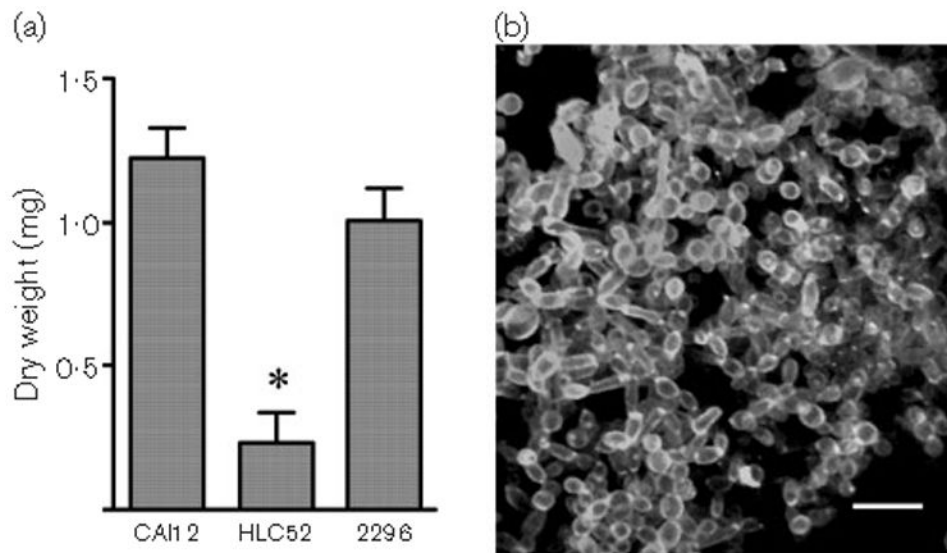
Fig. 9.
(a) Histogram comparing dry weights for biofilms formed from strains CAI12, HLC52 or 2296. Mass of the biofilm formed by the *efg1 /efg1* mutant strain (HLC52) was significantly reduced compared to a CAI12 biofilm (*P=0·0002). The inability of HLC52 to form biofilm was demonstrated previously (Ramage *et al.*, 2002b). Under biofilm-forming conditions, HLC52 grows as a sparse cellular monolayer. (b) Two-photon laser scanning micrograph of a calcofluor-stained model catheter biofilm grown using strain 2296, in which ALS3 is overexpressed in the HLC52 background. Overexpression of *ALS3* in strain HLC52 restores wild-type biofilm mass (a). In contrast to the morphology of a wild-type biofilm (Fig. 6b), the *ALS3* overexpression strain does not form hyphae (b). Bar, 5 µm.

Table 1

C. albicans strains used in this study

Strain	Parent	Genotype[*]	Source
SC5314		Wild-type	Gillum *et al.* (1984)
CAI4	SC5314	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434	Fonzi & Irwin (1993)
CAI12	CAI4	*iro1-ura3* ∷ λ*imm*^434/*IRO1 URA3*	Porta *et al.* (1999)
1843	1780	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *als3la /als3sa -URA3*	Zhao *et al.* (2004)
2311	1843	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *als3la /als3sa -ura3*	This study
2322	2311	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *als3la /als3sa -ALS3LA-URA3*	This study
1926	1892	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *ALS3LA/als3sa -ura3*	This study
2327	1926	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *ALS3-AGa1-URA3/als3sa -ura3*	This study
HLC52		*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *efg1* ∷ *hisG/efg1* ∷ *hisG-URA3-hisG*	Lo *et al.* (1997)
HLC67	HLC52	*iro1-ura3* ∷ λ*imm*^434/*iro1-ura3* ∷ λ*imm*^434 *efg1* ∷ *hisG/efg1* ∷ *hisG*	Lo *et al.* (1997)
2296	HLC67	HLC67 *ALS3SA/ALS3LA* ∷ 1105-P*TPI1-ALS3LA-URA3*	This study
2185	CAI4	CAI4 *ALS3LA/als3sa -PALS3SA-GFP-URA3*)	Zhao *et al.* (2004)
1143	CAI4	CAI4 (*RP10* ∷ 1105-P*TPI1-GFP-URA3*)	Green *et al.* (2005a)

[*] ALS3 alleles from strain SC5314 are marked with `LA' or `SA' to designate the large allele (GenBank accession no. AY223552) and small allele (AY223551), respectively.

Table 2

Oligonucleotide primers used in this work

Primer	Sequence (5′-3′)
ALS3dnF	CCC CCG CGG AAG AGC CTG CGA CTA TGA ATT G
ALS3dnR	CCC GCC GGC GTT TGG TAA TTA ACA CAT ATT GC
ALS3upF	CCC CCT AGG CAG TGA ATT GCA AAT CCT TAT GG
ALS3R	CCC CTC GAG CAA CAT TTT CCT TGG ACC TAC TAC
ALS3Sph	CCC CGC ATG CAT GCT ACA ACA ATA TAC ATT GTT ACT C
ALS3nt1290	TTG GCA GTG GTA CCT TGT ACA ATG ACA GTG
AGα1Xba	CCC TCT AGA TTA GAA TAG CAG GTA CGA CAA AAG CAG
AGα1nt980	CCT CGG TAC CGC TAG CGC CAA AAG CTC
3cdHindIIIF	CCC AAG CTT ATG CTA CAA CAA TAT ACA TTG TTA CTC
AGα1XhoR	CCC CTC GAG TTA GAA TAG CAG GTA CGA CAA AAG CAG
RTAGα1F	CGT ATC GTC CCT CTC CGT A
RTAGα1R	TCC CTG AGA TGA GAG TGC TGT