ALS3 and ALS8 represent a single locus that encodes a Candida albicans adhesin; functional comparisons between Als3p and Als1p

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The ALS (agglutinin-like sequence) gene family of Candida albicans encodes eight cell-surface glycoproteins, some of which are involved in adherence to host surfaces. A mutational analysis of each ALS gene is currently being performed to deduce the functions of the encoded proteins and to better understand the role of these proteins in C. albicans biology and pathogenesis. This paper describes construction of an als3/als3 mutant and comparison of its phenotype to an als1/als1 strain. Efforts to disrupt ALS3 indicated that the gene could be deleted in two transformation steps, suggesting that the gene is encoded by a single locus and that the ALS3-like locus, ALS8, does not exist. Strains lacking ALS3 or ALS1 did not exhibit a defect in germ tube formation when grown in RPMI 1640 medium, but the als1/als1 mutant formed significantly fewer germ tubes in Lee medium. Analysis of ALS3 and ALS1 promoter activity using green fluorescent protein (GFP) reporter strains and flow cytometry showed that when cells are placed into medium that promotes germ tube formation, ALS1 is transcribed prior to ALS3. Comparison of the mutant strains in adhesion assays showed that the als3/als3 strain was defective in adhesion to both human umbilical vein endothelial cells (HUVEC) and buccal epithelial cells (BEC), but not to fibronectin-coated plastic plates. In contrast, the als1/als1 strain showed decreased adherence to HUVEC, but adherence to BEC and fibronectin were the same as wild-type controls. Inoculation of the buccal reconstituted human epithelium (RHE) model of oral candidiasis with the mutant strains showed nearly a total lack of adhesion and epithelial destruction by the als3/als3 mutant while the als1/als1 strain showed only a slightly reduced degree of epithelial destruction compared to the wild-type control. Adhesion data presented here suggest that, in the assays performed, loss of Als3p affects C. albicans adhesion more than loss of Als1p. Collectively, these results demonstrate functional similarities and differences between Als1p and Als3p, and suggest the potential for more complex interrelationships between the ALS genes and their encoded proteins.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that causes oral and vaginal mucosal infections as well as systemic disease (Odds, 1988). C. albicans has several gene families that encode proteins involved in pathogenesis (Hube et al., 2000; De Bernardis et al., 2001; Hube & Naglik, 2001; Monod & Borg-von Zepelin, 2002). Among these is the ALS (agglutinin-like sequence) family that encodes cell-wall glycoproteins (Hoyer, 2001). Some Als proteins function in adhesion to host surfaces and cellular components. Adhesive function was demonstrated in C. albicans for Als1p (Fu et al., 2002) and by heterologous expression in Saccharomyces cerevisiae for Als5p (Gaur & Klotz, 1997). The potential for Als proteins to function as adhesins was first suggested by the similarity of the N-terminal domain of the various proteins to that of α-agglutinin, a cell-surface

Abbreviations: ALS, agglutinin-like sequence; BEC, buccal epithelial cell; 5-FOA, 5-fluorooorotic acid; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; RHE, reconstituted human epithelium.

The GenBank accession numbers for the sequences reported in this paper are AY223551 (ALS3 small allele) and AY223552 (ALS3 large allele).
adhesin of \textit{S. cerevisiae} (Lipke \textit{et al.}, 1989; Hauser & Tanner, 1989). Als proteins have three different domains (Hoyer, 2001). The first is an N-terminal domain of approximately 433 amino acids that, by comparison to the structure of \textit{S. cerevisiae} z-agglutinin (Chen \textit{et al.}, 1995), is likely to possess adhesive function. The central Als domain consists entirely of tandem direct repeats of a 36 amino acid sequence. The C-terminal domain is variable in length and sequence, but in all Als proteins this region and the central domain are heavily glycosylated with N- and O-linked carbohydrates (Kapteyn \textit{et al.}, 2000). One likely model for the Als proteins is a functional N-terminal domain that is displayed on the extracellular surface by the heavily glycosylated central and C-terminal domains. The same model has been proposed for other cell-surface adhesion glycoproteins in pathogenic fungi (Frieman \textit{et al.}, 2002).

One main goal of our research efforts is to understand the function of the various Als proteins and the role each plays in \textit{C. albicans} biology and pathogenesis. The emergence of initial functional data for Als1p and Als5p is important because they have the most similar N-terminal domain sequences (87\% amino acid identity) within the ALS family (Hoyer \textit{et al.}, 1995; Gaur & Klotz, 1997; Hoyer & Hecht, 2000; Fu \textit{et al.}, 2002). The sequence of Als3p is also closely related to Als1p (84\% amino acid identity) and Als5p (81\% amino acid identity) within the N-terminal domain (Hoyer \textit{et al.}, 1998). Similarities in expression pattern have also been noted between \textit{ALS1} and \textit{ALS3}, with expression of each gene being strongly upregulated under certain growth conditions such as inoculation into RPMI 1640 medium (Hoyer \textit{et al.}, 1998). Comparisons between closely related Als proteins will begin to address functional similarities and differences within the Als family.

\textit{ALS3} was initially described as a hypha-specific gene in the ALS family (Hoyer \textit{et al.}, 1998). Previous unpublished work suggested the presence of a second \textit{ALS3}-like locus in \textit{C. albicans}, which was called \textit{ALS8}. The \textit{ALS8} gene was isolated as a false positive in a screen for \textit{C. albicans} isolates as a false positive in a screen for \textit{C. albicans} cell-surface adhesion glycoproteins in pathogenic fungi (Frieman \textit{et al.}, 1996). The same model has been proposed for other cell-surface adhesion glycoproteins in pathogenic fungi (Frieman \textit{et al.}, 1996).

\textbf{METHODS}

\textbf{Construction of \textit{ALS} gene deletion and replacement strains.}

The \textit{C. albicans} strains used in this study are listed in Table 1. \textit{ALS} genes and fragments for DNA sequencing were amplified using proofreading polymerases. When cloned, fragments were ligated into pCRBlunt (Invitrogen). DNA sequencing was done by EliGen Biopharmaceuticals. DNA sequence comparisons used Wisconsin Package Version 9.1 (Genetics Computer Group). Strains lacking ALS alleles were created using the PCR product-directed method of Wilson \textit{et al.} (2000). Plasmid pDD57 was a gift from Aaron Mitchell, Columbia University. Plasmid pDD57 contains a PCR-amplifiable copy of a deletion cassette that encodes the \textit{URA3} selectable marker and direct-repeat flanking sequences to cause excision of the cassette following growth on medium containing 5-fluoroorotic acid (5-FOA; Boeke \textit{et al.}, 1984). PCR reactions utilized \textit{Pfu} polymerase (Stratagene) and followed the manufacturer’s protocol. The deletion cassette for \textit{ALS3} was produced using primers ALS3-5DR and ALS3-3DR (Table 2). The deletion cassette for \textit{ALS1} was produced using primers ALS1-5DR and ALS1-3DR (Table 2). PCR-produced deletion cassettes were purified on agarose gels and extracted with a GeneClean kit (Qbiogene). \textit{C. albicans} strain CA14 (Table 1; Fonzi & Irwin, 1993), which is derived from the wild-type strain SC5314 (Gillum \textit{et al.}, 1984), was provided by William Fonzi, Georgetown University, and used as the parent for mutant construction. Strains for transformation were converted to spheroplasts using a method developed for \textit{Pichia pastoris} (Invitrogen). Transformants were selected on plates of synthetic complete medium without uridine (SC–\textit{U}; Hicks & Herskowitz, 1976) containing 1 M sorbitol. Transformants were grown in liquid SC–\textit{U} for DNA extraction. Genomic DNA was extracted from \textit{C. albicans} using the MasterPure Yeast DNA Purification Kit (Epicentre). Correct transformants were confirmed by PCR and Southern blotting. A typical PCR reaction contained 200 ng genomic DNA, 1-5 mM MgCl\(_2\), 2 \mu M of each primer, 0-2 mM of each dNTP and 2-5 units Taq polymerase (Invitrogen). Southern blotting utilized Genius reagents and chemiluminescent detection (Roche).

A plasmid that can be used for constructing a replacement cassette for any ALS gene was made from plasmid pMB7 (Fonzi & Irwin, 1993). The plasmid was first modified by the addition of polylinkers 5’ and 3’ of the URA-blaster cassette. Oligonucleotides L1 and L2 were annealed and ligated into HindIII–\textit{SphI}-digested pMB7, adding restriction sites for \textit{AvrII} and \textit{XhoI} 5’ of the URA-blaster cassette. Oligonucleotides L3 and L4 were annealed and ligated into the \textit{KpnI}–\textit{SstI}-digested growing construct to add restriction sites \textit{SstII} and \textit{NgoMIV} downstream of the \textit{hisG}–\textit{URA3}–\textit{hisG} cassette. The resulting plasmid, named pHUL, was digested with \textit{SphI} and \textit{KpnI} to remove the \textit{hisG}–\textit{URA3}–\textit{hisG} cassette and a PCR-amplified copy of \textit{URA3} was cloned in its place. The \textit{URA3} fragment was amplified from pHUL plasmid DNA using \textit{Pfu} Turbo polymerase and the \textit{URA}–\textit{URAR} primer pair (Table 2). The resulting plasmid was named pUL. For \textit{ALS1} reintroduction, the large allele of \textit{ALS1} from strain SC5314 along with 501 bp of upstream sequence and 488 bp of downstream sequence was amplified from genomic DNA using \textit{Pfu} Turbo polymerase and the \textit{ALS1up}–\textit{ALS1IR} primer pair (Table 2). The fragment was digested with \textit{AvrII} and \textit{XhoI} and cloned into plasmid pUL, which was digested with the same enzymes. Downstream flanking sequence of \textit{ALS1} was amplified from SC5314 genomic DNA using the \textit{ALS1dn}–\textit{ALS1dn} primer pair, digested with \textit{SstII}–\textit{NgoMIV} and cloned into the growing plasmid cut with the same enzymes. Digestion
of the final construct with *AvrII*–*NgoMIV* produced a fragment containing the upstream region of *ALS1* followed by the *ALS1* coding region, full-length *URA3* and its promoter and the downstream flanking region of *ALS1*. This fragment was transformed into the *als1/als1* mutant strain. Transformants were selected by growth on SC–Uri and verified by Southern blotting.

**Growth rate measurements.** Cells were grown overnight in YPD (per litre: 10 g yeast extract, 20 g peptone, 20 g glucose) at 37 °C with 200 r.p.m. shaking. Cells were counted in duplicate and inoculated into 20 ml fresh YPD at a density of 1 x 10⁶ cells ml⁻¹. Cultures were incubated at 37 °C with 200 r.p.m. shaking, and spectrophotometric readings were taken in triplicate every hour. Growth rates were measured on three different days from separate starting cultures. Rate of growth and doubling time were calculated from the linear portion of the growth curve using the exponential growth equation in nonlinear regression in GraphPad Prism (GraphPad Software). A mixed model analysis in two-way ANOVA was used to assess the statistical differences in the growth rates of the *C. albicans* strains. Statistical comparison of growth rates was completed using PROC MIXED in SAS (SAS Institute).

**Evaluation of germ tube formation and cellular aggregation.** *C. albicans* strains were grown overnight in YPD at 37 °C with 200 r.p.m. shaking. Cells were washed in Dulbecco’s phosphate-buffered saline without Ca²⁺ or Mg²⁺ (DPBS; Cambrex catalogue no. 17-513Q) and counted. An inoculum of 5 x 10⁶ cells ml⁻¹ was added to 10 ml prewarmed RPMI 1640 without l-glutamine (RPMI; Invitrogen catalogue no. 11875-085) or to Lee medium (Lee et al., 1975) and incubated at 37 °C with 200 r.p.m. shaking. Cell growth was stopped by the addition of glutaraldehyde to a final concentration of 1% and the culture kept on ice. A negative reading was assigned to cells that had a germ tube equal to or longer than one diameter of the mother yeast cell. A positive reading was assigned to cells that had a shorter germ tube or none at all. For each culture, 100 cells were evaluated and the number of positive cells was expressed as a percentage of the total. Cellular aggregation was also evaluated using the same cultures. The number of cells in the first 100 aggregates viewed was recorded. Cells that appeared individually were assigned a value of 1; other values represented the number of cells in the aggregate. Both assays were run in duplicates on 3 different days. The mean of these values was calculated and a mixed model analysis of variance (PROC MIXED in SAS) was used to assess differences in germ tube formation and cellular aggregation among the *C. albicans* strains.

**Construction of green fluorescent protein (GFP) reporter constructs.** Reporter strains were constructed using the method of Gerami-Nejad et al. (2001). Plasmid pGFP-URA3 was used as template for a PCR reaction with primers specific for the *ALS1* and *ALS3* locus, respectively. The PALSI–GFP cassette was amplified using primers ALS1F and ALS1R (Table 2). Primers ALS3F and ALS3R were used to amplify an ALS3-specific cassette (Table 2). PCR amplification reactions included 400 ng plasmid DNA as template. The PCR products were extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. DNA was resuspended in sterile water and used to transform spheroplasts of *C. albicans* strain CAI4 (Fonzi & Irwin, 1993). Transformants were selected on SC–Uri agar plates with 1 M sorbitol. Correct transformants were verified by Southern blotting of BglII-digested genomic DNA for the *ALS1* construct and EcoRV-digested DNA for the *ALS3* construct. Probes upstream of *ALS1* (produced by PCR using primers ALS1GFPPF and ALS1GFPR) and ALS3 (produced using primers 3upABKpm and 3upABXho) were used. A GFP probe fragment amplified from pGFP-URA3 with primers GFPXhoI and GFPBglII was also used to verify constructs. Fluorescence of correct clones was monitored microscopically following growth in media conditions shown previously by Northern blotting to increase transcription from the *ALS1* and *ALS3* promoters (Hoyer et al., 1995, 1998). Expression patterns matching previous Northern blot results confirmed that the clones displayed GFP production under control of the correct promoter.

**Flow cytometry analysis.** Strains CAI12 (control), 2185 (PALS3–GFP) and 2225 (PALS1–GFP) were grown to stationary phase in YPD. Cells were washed twice and then resuspended in DPBS. Cell stocks were counted and then inoculated at a density of 5 x 10⁶ cells ml⁻¹ into prewarmed RPMI medium. Cultures were
incubated at 37°C with 200 r.p.m. shaking for 1 h, taking samples at 15 min intervals. Flow cytometry was performed, using a Beckman Coulter EPICS XL machine. This instrument is equipped with an argon laser with an excitation wavelength of 488 nm. For fluorescence analysis, a region was set on a histogram, which represented side-angle light scatter versus forward-angle light scatter for a population of CAI12 yeast cells. The fluorescence was then gated on this region. Ten thousand events were collected at a laser power of 15 mW at medium flow rate. Fluorescence was measured on the FL1 channel with a 525 nm bandpass filter. Geometric mean fluorescence values for each time point were calculated using WinList software (Verity) and graphs were generated using the Summit 3.1 analysis software (Cytomation).

Table 2. Oligonucleotide primers used in this study

| Primer name          | Label* | Primer sequence (5’→3’)
|---------------------|--------|--------------------------
| ALS3-5DR            | (1)    | GAC GAA AGA GAT GCA TTT GCT AGA CTT TCA TGA ATG TAT ATA AAT GAG GCT TCC CCC CTC GTT TTC CCA GTC AGC AGG TT
| ALS3-3DR            | (2)    | ATA CCG AAA ATA GCA AAA TAT CAA TAA ACA CGA AGA AAA ACC AAA AAA TAT ATT TGT GGA ATT GTG AGC GGA TA
| ALS1-5DR            | (3)    | GCT TCA TAA ATG TAT ATA AAA GAG GCT CTG TCC TCC TCC CAA TTG AAA TGT GAA AGA TGG GTT TGC CCA GTC AGC AGG TT
| ALS1-3DR            | (4)    | ACA CGA AGA AAA GAT AAA TGT GAA CTA CAT GAA GCC AAA AAG GTG ATC ATA ACA ATA TAG TGT GGA ATT GTG AGC GGA TA
| L1                  | (5)    | AGC TTT TTC CTA GGT TTC TCG AGT TGT CAT G
| L2                  | (6)    | CAA ACT CGA GAA ACC TAG GAA AA
| L3                  | (7)    | CTT TCC GCG GTT TGC CGG CTT TGA GCT
| L4                  | (8)    | CAA AGC CGG CAA ACC CGC GAA AGG TAC
| URAF                | (9)    | CCC GGT ACC AGT GGC TAC AAG TAG CGA AG
| URAR                | (10)   | CCC GCA TGC ATA GGA ATT GTG CAT GG
| ALS1upF             | (11)   | CCC CTT AGG GCT AAT CAT CTG TGG AGA TAT CG
| ALS1R               | (12)   | CCC CTC GAG AAT TCC GTG TGT CTA AAA GTT GTC
| ALS1dnF             | (13)   | CCC CCG CGG GTA AGA TTC TTG TAG ACC ATA G
| ALS1dnR             | (14)   | GCC AAG GGA TTA GCA AAA ATT G
| ALS1-FPF            | (15)   | GTC TTT TCG TTT TAC TCC TCC TCC TCC GTA AGT TTG CTA TAA ACA ACT ACC AAC TGA TAT CAG ATG TAA TGC AGG CAA ATA CTT CAA TAA AAC CAC TCC TAA TGA GGC CAT ACC ACC ACC
| ALS1-FPR            | (16)   | CGA AGA AAA GAT AAA TGT GAA CTA CAT GAA GCC AAA AAG GTG ATC ATA ACA ATA TAG TGT AGT AAG ACC ACC ACC CCT TAT CAT CAG ATG TAA TGA GGC CAT ACC ACC ACC
| ALS3-FPF            | (17)   | AGT TTA TTT TAT CTT TTT ATG ATG GTA TAA ACA ACT ACC AAC TGC TCT TAA TAT TAG TGT CTA AAA GGC GAA TAA TGA TTT GTG CTC GCC GAT GTA TAT ACC TAA TGA GGC CTG CAT ACC AGC
| ALS3-FPR            | (18)   | AAA TAA AAA ACA TAA CAA AAA AAG TTA AAA CTA AAA AAC TCT TAA AAG GCG ACT ATG AGT GTA TCA TCC TCC TTA AGA ACC ACC TCC TGT CAT G
| ALS1 GFPF           | (19)   | CCC GGT ACC ATC ACT CIT CAA TAA TCC TCC CAT ACT
| ALS1 GFPR           | (20)   | CCC CTC GAG CTG ATA TTA ACA TAT GTG AG
| 3upABKpn            | (21)   | CCC GTA CGG TAA TAA GAC AAA AAT AAA AAG
| 3upABXho            | (22)   | CCC CTC GAG CAT GTA TAA GCA GAT GGT GGT AGC
| GFPXhoI             | (23)   | CCC TCG AGT ATT AAA ATG TCT AAA GGT GAA GAA TTA TGT ACT
| GFPBglII           | (24)   | CCC AGA CTG TTA TTT GAA CAT TCA TCA ACC ATG GGC
| ALS3dnF             | (25)   | CCC CGG CGG AGG AGC CTG CGA GTA TGA ATT G
| ALS3dnR2            | (26)   | TTT GTG CTC GCC GAT TCA GTA GAG
| ALS3upF             | (27)   | CCC CTT AGG CGA TGA ATT GCA AAT CCT TAT GG
| URAF2               | (28)   | CGA ATC AAT GGC ACT ACA GCA AC
| URAR2               | (29)   | CAA TCA AAG GTG GTC CTT CTA GA
| ALS3dn              | (30)   | ACT GTA CAA GCG ATG CAT AAC CTC

*Numbers are used to indicate primer locations in figures.

RNA analysis. RT-PCR analysis was conducted as described previously (Green et al., 2004). C. albicans strains were grown overnight in YPD at 37°C with 200 r.p.m. shaking. Cells were washed twice in sterile water and counted. An inoculum of 1 × 10⁷ cells ml⁻¹ was added to 100 ml RPMI medium that was prewarmed to 37°C. The culture was incubated at 37°C with 200 r.p.m. shaking for 90 min. Cells were collected by filtration, flash frozen in dry ice/ethanol and stored at −80°C until RNA was extracted. RNA extraction used the method of Collart & Oliviero (1993); RNA was stored in ethanol at −80°C. RT-PCR primers specific for ALS1 and ALS3 have been described previously (Green et al., 2004). PCR products were run on 8% acrylamide/TBE (Tris/borate/EDTA) gels and visualized by staining with ethidium bromide.
Endothelial cell and fibronectin adhesion assays. These assays were conducted in a six-well plate format using modifications of the method described by Ibrahim et al. (1995). Endothelial cells were purchased from Cambrex and grown according to the distributor’s instructions in EGM-2 medium without addition of antimicrobials. Cells formed confluent monolayers in a six-well tissue culture-treated polystyrene plate (Fisher; catalogue no. 07-200-83). The growth medium of the endothelial monolayer was changed with a fresh medium 1 day prior to the adhesion assay. C. albicans strains were inoculated from the stock plate into 10 ml liquid YPD and grown for 16 h at 37°C with 200 r.p.m. shaking. Cells were washed twice with DPBS and counted. In order to induce germ tube formation, 10^6 C. albicans cells were inoculated into 10 ml prewarmed RPMI in a 37°C incubator. After 1 h, each endothelial monolayer was rinsed twice with 37°C RPMI and 1 ml RPMI containing 10°C C. albicans germ tubes was added to at least six replicate wells. The inoculated plate was placed in the 37°C/5% CO2 incubator for 30 min to allow yeast cells to adhere to endothelial cells. To remove unattached C. albicans cells, the plate was tilted at a 20° angle and 5 ml DPBS was gently allowed to run across the cell monolayer with simultaneous aspiration from the bottom of the well. The plate was then placed at 180° and another 5 ml DPBS was applied to each well as described above and the well was covered with 4 ml YPD top agar. Viability of C. albicans was verified by plating 100 μl C. albicans inoculum on YPD plates in triplicate. The six-well plate and the YPD plates were incubated overnight at 37°C and c.f.u. were counted. The percentage adherence of each investigated C. albicans strain was calculated as (mean adherent c.f.u./mean total c.f.u.) x 100.

Adhesion to fibronectin was tested in a similar manner using fibronectin-coated six-well plates (BD Biosciences; catalogue no. 354402). C. albicans cells were grown and prepared in the same way as for the endothelial adhesion assay. After counting, 5 x 10^3 cells were inoculated in 20 ml prewarmed RPMI medium and incubated for 1 h at 37°C for germ tube formation. The fibronectin-coated plates were prewarmed at 37°C and 2 ml RPMI medium containing 10^8 yeast cells was added into six replicate coated wells. After 30 min incubation at 37°C/5% CO2, the initial RPMI solution was aspirated and 2 ml DPBS was applied to each well. To remove nonadherent C. albicans, the plate was gently shaken manually and DPBS was aspirated. The same washing was repeated one more time and then 4 ml YPD top agar was added to each well. The viability of C. albicans was evaluated by plating 200 μl inoculum on a YPD plate in triplicate. The percentage adherence of each C. albicans strain was calculated as described previously. All adhesion assays were performed in triplicate and only minor sequence differences in the remainder of the coding region. Allelic size differences due to variation in the number of copies of the 108 bp tandemly repeated motif than the smaller allele and only minor sequence differences in the remainder of the coding region. Allelic size differences due to variation in the number of copies of the 108 bp sequence are common for ALS genes (Hoyer, 2001). The PCR-mediated disruption method of Wilson et al. (2000) was used to make a strain lacking ALS3. In the first round of transformation with the ALS3-targeted disruption cassette (Fig. 1), clones in which the small ALS3 allele was removed were recovered

RESULTS

ALS3 and ALS8 are encoded by a single locus

Cloning and DNA sequencing of ALS3 alleles from SC5314 (GenBank accession nos YJ233551 and YJ233552) showed that the larger allele contained three more copies of the 108 bp tandemly repeated motif than the smaller allele and only minor sequence differences in the remainder of the coding region. Allelic size differences due to variation in the number of copies of the 108 bp sequence are common for ALS genes (Hoyer, 2001). The PCR-mediated disruption method of Wilson et al. (2000) was used to make a strain lacking ALS3. In the first round of transformation with the ALS3-targeted disruption cassette (Fig. 1), clones in which the small ALS3 allele was removed were recovered

Buccal epithelial cell (BEC) adhesion assays. BEC were collected from five human donors and pooled. Each donor provided written consent for participation in the study and collection procedures followed the guidelines of the University of Illinois Institutional Review Board. Cells were washed twice with DPBS and counted. Cells were resuspended at a concentration of 8 x 10^3 cells ml^-1 and kept on ice. C. albicans strains were inoculated from a stock YPD plate into 10 ml liquid YPD and grown for 16 h at 37°C with 200 r.p.m. shaking. Cells were counted and 2 x 10^6 fungal cells were inoculated into 4 ml RPMI in a 25 ml sterile Erlenmeyer flask. Cultures were incubated at 37°C with 200 r.p.m. shaking for 1 h to allow germ tube formation. At that time, 250 μl DPBS containing 2 x 10^9 BEC was added to each flask. Adhesion progressed for 30 min at 37°C with 200 r.p.m. shaking. Cell mixtures were vacuum filtered across 12 μm pore size Nuclepore polycarbonate filters (Corning; catalogue no. 111116). Filters were washed dropwise with 25 ml DPBS to remove nonadherent C. albicans cells. Filters were removed from the vacuum filtration device, inverted onto glass microscope slides and dried. Following removal of the filter from the slide, slides were heat fixed, stained with crystal violet, washed with tap water, dried and examined microscopically. The number of germ tubes adhering to the first 50 BEC observed on the centre of each slide was recorded. Replicates for each strain were run on three separate days using a different pool of BEC on each day. Results are expressed as the mean number of C. albicans germ tubes that adhere to each BEC. A mixed model analysis of variance was used to study the differences in adherence to BEC. The mean number of adherent germ tubes for each replicate within a strain per day was analysed using PROC MIXED in SAS. Separation of means was performed with the LSMEANS option.

Reconstituted human epithelium model. Reconstituted human epithelium (RHE) is a product of SkinEthic Laboratories (Nice, France). The product consists of human epithelial cell lines cultured on polycarbonate filters in vitro at the air–liquid interface in a serum-free chemically defined medium. The experiments here used oral RHE (derived from the TR146 cell line) in maintenance medium without antimicrobials. The maintenance medium is based on the MCDB-153 of Clonetics and contains 5 μg insulin ml^-1. Preparation of the inoculum strains followed the protocol described by Green et al. (2004). An inoculum of 50 μl C. albicans yeasts/PBS suspension (2 x 10^6 cells total) was added onto the surface of each RHE sample, which was incubated at 37°C, 5% CO2 and saturated humidity for either 1, 4 or 8 h. Replica samples were collected at the two later time points. Upon harvesting the specimens, each plastic well containing RHE was placed into 1 ml Karnovsky’s fixative in a 12-well plate and the RHE layer was covered by another 1 ml of fixative. Samples were fixed for 1 h at room temperature and stored at 4°C until processed. Prior to processing for microscopy, the Karnovsky’s fixative was removed from the surface of each RHE specimen and the specimen was washed twice with 1 ml DPBS per wash. Washing was intended to remove nonadherent organisms. Processing for microscopy followed the methods of Green et al. (2004). Tissue blocks were cut into four longitudinal fragments and sections taken from each of these points in the specimen. Five sections from each of these locations in the sample were evaluated for RHE damage, the number of C. albicans cells adherent to the RHE layer and percentage germination of C. albicans cells present. Percentage germination of the C. albicans strains in RHE maintenance medium was observed following the methods for evaluation of germ tube formation described above. Specimens were examined using a Nikon Eclipse E600 microscope fitted with a Spot camera (Diagnostic Instruments). Images of representative sections were collected using Metamorph software (Universal Imaging Corporation) and processed with Adobe Photoshop.
as well as clones in which the large allele was removed. 

EcoRV-digested genomic DNA was hybridized with an ALS3 downstream fragment generated by PCR with primers ALS3dnF and ALS3dnR2 (Fig. 1, Table 2). Results were confirmed by additional Southern blots hybridized with a fragment specific for the ALS3 coding region and a URA3 probe (data not shown).

Clones 1702 and 1704 were selected for further analysis. Clones were plated on 5-FOA agar to direct excision of the disruption cassette. The cassette was easily removed from clone 1704, while repeated efforts to remove the disruption cassette from clone 1702 proved problematic. Screening of approximately 100 clones from three separate 5-FOA platings of 1702 failed to identify a strain from which the disruption cassette was correctly excised. In the strains derived from 1702, a large stretch of sequence downstream of ALS3 was deleted with the ALS3 coding region during attempts to excise the disruption cassette (data not shown). Because of this problem, strain 1779 (als3D::dpl200/ALS3) was derived from strain 1704 and transformed with the same ALS3 disruption cassette as described above to replace the second ALS3 allele. Southern blotting of transformants identified strain 1843 as an als3/als3 mutant (Fig. 1). Plating of strain 1843 on 5-FOA medium resulted in the same observations as for strain 1702 and further highlighted problems with excising the disruption cassette from the large allele locus. Despite this problem, we demonstrated that only two rounds of disruption were required to delete all ALS3-like sequences in the C. albicans genome. To verify that these events were repeatable, the mutant construction process was repeated in its entirety, starting with strain

Fig. 1. Construction and verification of C. albicans als3/als3 strains. In all drawings, the locations of primers from Table 2 are shown by numbers in parentheses. Numbers placed above the line drawings denote forward primers; reverse primers are shown below the line drawings. (a) Cassette derived from pDDB57 used for disruption of ALS3. Filled ends depict ALS3 sequences to direct integration of the cassette. Checkerboard patterns show the direct repeats for excision of the cassette from cells plated on 5-FOA. The URA3 coding region is noted. (b) The ALS3 small allele from strain SC5314. Restriction sites and probes used in Southern blot analyses are shown. All locations are identical for the ALS3 large allele except that it is 324 bp longer in the tandem repeat domain. The EcoRV site downstream of ALS3 is 2·2 kb 3′ of the stop codon. (c) The ALS3 locus with the disruption cassette integrated. (d) Southern blot of EcoRV-digested genomic DNA from wild-type and als3/als3 strains (Table 1) hybridized with the ALS3 downstream probe from (b). Molecular sizes are shown on the left.

CAI4. This process resulted in construction of strain 1954, which has a genotype identical to strain 1843 (Fig. 1). In addition to verification by Southern blotting, mutant strains were tested using PCR amplification. Primers ALS3upF and URRAF2 amplified an 877 bp fragment that contained the 5' boundary of the deletion site and primers URAR2 and ALS3dn amplified a 441 bp fragment that contained the 3' boundary (Fig. 1). DNA sequencing of these PCR fragments verified that, as expected, the ALS3 alleles in each mutant strain were excised from 76 bp upstream of the ALS3 start codon to 125 bp downstream of the stop codon.

**Construction of an als1/als1 mutant strain**

We constructed an als1/als1 strain in which the entire coding region of the gene was deleted. The resulting strain, 1467, was validated using Southern blots (Fig. 2) and PCR. PCR primers ALS1upF and ALS1dn were used to amplify

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**Fig. 2.** Construction and verification of als1/als1 mutant and ALS1 replacement strains. In all drawings, the locations of primers from Table 2 are shown by numbers in parentheses. Numbers placed above the line drawings denote forward primers; reverse primers are shown below the line drawings. (a) Restriction map of the ALS1 small allele locus from strain SC5314. (b) Allelic restriction maps from strain 1467. The disruption cassette was integrated into the small allele in the first disruption step and the large allele in the subsequent step. The disruption cassette is identical to that shown in Fig. 1 except it was amplified with ALS1-specific primers. (c) Restriction map of the ALS1 large allele locus in strain 2151 with a copy of the ALS1 large allele replaced. The replacement cassette included ALS1 upstream and coding region sequences followed by 488 bp of downstream sequence. URA3 and 500 bp of ALS1 downstream sequence are 3' in the construct. (d) Southern blot of BglII-digested genomic DNA from wild-type (CAI4), als1/als1 mutant (1467) and ALS1 replacement (2151) strains. The blot was hybridized with the ALS1 coding region fragment [XbaI–HindIII; (a)]. (e) The same Southern blot from (d) hybridized with the ALS1 upstream probe from (a). Molecular sizes are shown on the left.
genomic DNA from strain 1467. DNA sequencing of the resulting PCR product verified that the ALS1 coding region was deleted from 56 bp upstream of the ALS1 start codon to 3 bp downstream of the stop codon (data not shown). A replacement strain, 2151, was constructed in the 1467 background using the large ALS1 allele from strain SC5314, which contains 20 copies of the 108 bp tandem repeat sequence in the central domain (Fig. 2). These strains were used in functional analyses described below for comparisons to the als3/als3 mutants and provided the first direct comparison of Als protein function in C. albicans.

Expression of ALS3 and ALS1 in PALS–GFP reporter and als/als mutant C. albicans strains

In order to validate growth conditions for the comparison of Als1p and Als3p function, we constructed reporter strains where production of yeast-enhanced GFP (Cormack et al.,

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**Fig. 3.** Flow-cytometry analysis of PALS–GFP reporter constructs. (a) Time course of GFP expression in PALS3–GFP (strain 2185; marked with vertical lines) and PALS1–GFP (strain 2225; marked with diagonal lines) reporter strains. Flow-cytometry histograms and the geometric mean channel fluorescence values are shown for analysis of the ALS3 and ALS1 reporter strains at 15 min intervals following inoculation into RPMI medium at 37 °C. (b) Light micrographs of cells from each culture and from a wild-type control (CAI12) culture to show the morphology of each strain at the indicated time points. Background fluorescence from YPD-grown cells was nearly undetectable (data not shown). Experiments were repeated in duplicate and yielded similar fluorescence values with the exception of the ALS1 reporter strain at 60 min, which did not decrease in the second experiment. (c) Histogram of geometric mean fluorescence values for the ALS1 reporter strain sampled at 1 min intervals to better visualize the temporal nature of GFP production within the first 15 min of incubation in RPMI medium. Means and standard deviations of replicate observations are shown.
1997; Gerami-Nejad et al., 2001) was under control of either the ALS1 or the ALS3 promoter. Previous studies using Northern blot analysis showed that the transcription of ALS1 and ALS3 greatly increased when C. albicans wild-type cells were inoculated into RPMI medium (Hoyer et al., 1998). These results were supported by flow-cytometry analysis of GFP production using the reporter strains (Fig. 3a), which also showed strong ALS1 transcription before germ tubes were visible, and increased ALS3 transcription approximately 30 min later in germ tube development. Since ALS1 transcription was already strong at 15 min after PALS1–GFP cells were inoculated into RPMI medium, the flow-cytometry analysis was repeated at 1 min intervals to determine when the increase began (Fig. 3b). Results showed that in 8 min, the mean fluorescence of the PALS1–GFP strain was similar to that of the PALS3–GFP strain at 30 min of incubation. In addition to highlighting the temporal differences in ALS1 and ALS3 expression, these results supported the choice of a 1 h time point for studying these proteins in C. albicans germ tubes.

RT-PCR using primer pairs specific for ALS1 and ALS3 (Green et al., 2004) was used to demonstrate lack of gene expression in the C. albicans als/als mutant strains (Fig. 4). Analysis of total RNA extracted from C. albicans strains 1843 and 1954 that were grown in RPMI medium for 1 h showed an absence of the ALS3-specific message, but detection of an ALS1-specific transcript. Strain 1467 lacked an ALS1-specific transcript, but produced an ALS3 transcript. The ALS1 transcript was detected in strain 2151, where the large allele of ALS1 from SC5314 was reintroduced into the mutant 1467 background. As expected from the flow-cytometry results above, the control strain, CAI12, showed the presence of both ALS1 and ALS3 transcripts. These data supported the conclusion that the mutant strains did not express the deleted genes and also that a functional copy of ALS1 was returned to the als1/als1 mutant. Lack of an ALS3 signal in strains 1843 and 1954 further supported our earlier conclusion that there was only one ALS3 locus in strain SC5314 and that ALS8 did not exist.

**Germ tube formation and aggregation assays**

The als/als mutant strains were further validated phenotypically to evaluate their ability to form germ tubes and the level of cellular aggregation since these parameters could bias adhesion assay results. Germ tube formation was tested by incubating the various C. albicans strains in RPMI medium at 37 °C. After 1 h incubation, cells were fixed with glutaraldehyde and the extent of germ tube formation was evaluated microscopically. In RPMI medium, none of the mutant strains or the ALS1 replacement strain differed significantly from the wild-type for germ tube formation or extent of cellular aggregation (Table 3). Germ tube formation was reported to differ for an als1/als1 mutant grown in Lee medium in a previous study (Fu et al., 2002) and was shown to catch up to the wild-type strain as incubation time progressed. To test this effect for our als1/als1 mutant, and also for the strains lacking ALS3, we grew them in Lee medium at 37 °C, fixed and observed cells from 45 and 90 min time points (Table 3). Similar to the previous result, strain 1467 was significantly reduced in the number of cells that formed germ tubes (P=0.001). As time progressed, a difference from the wild-type was detected for one of the als3/als3 strains, but not for the other. The difference may be due to the slightly slower growth rate of strain 1954 compared to 1843 (Table 1). Failure of one als3/als3 strain to exhibit slowed germ tube formation in Lee medium suggested that the effect was strain specific and not attributable to the loss of functional Als3p. There was no difference in degree of cellular aggregation for any of the strains in Lee medium, with most strains growing predominantly as single cells. Results from these assays showed that growth of cells in RPMI medium for subsequent adhesion assays provided a population of comparable cells across the various strains. The results also showed that the defect in germ tube formation for the

**Fig. 4.** RT-PCR products from analysis of total RNA from RPMI-grown C. albicans strains. Both ALS1 and ALS3 transcripts were present in RNA from the wild-type control (CAI12). Analysis of als3/als3 strains (1843, 1954) showed absence of ALS3 transcript. The ALS1 transcript was absent in the als1/als1 mutant (1467) and restored in the replacement strain (2151). Control reactions (–RT) where a reverse transcriptase step was not performed are included to demonstrate the lack of amplifiable genomic DNA in each reaction. An equal quantity of RNA was added to the experimental and control reactions. PCR products were separated on a polyacrylamide gel and stained with ethidium bromide. Molecular sizes are shown on the left.
als1/als1 strain was growth-medium-specific. Finally, the data also indicated that loss of Als1p or Als3p did not alter the cellular aggregation properties of *C. albicans* compared to wild-type under either growth condition.

**Als3p is an adhesin**

Because of the similarities in protein sequence between Als1p and Als3p, it was reasonable to assume that Als3p was also an adhesin. Here, we tested this hypothesis and also compared the results of these adhesion assays to results for our als1/als1 mutant. The various mutant and replacement strains were compared to CAI12 (wild-type) for their ability to adhere to endothelial and epithelial cells, and fibronectin-coated plastic wells (Fig. 5). Results from the adhesion assays showed that loss of Als3p from *C. albicans* germ tubes resulted in a 42% (strain 1843) to 63% (strain 1954) reduction in wild-type adherence to endothelial cells and approximately a 60% reduction in adherence to BEC. Loss of Als1p resulted in a 20% loss of adherence to endothelial cells, similar to previously reported data (Fu et al., 2002). The difference in adhesion to BEC between the als1/als1 mutant and the wild-type control was not statistically significant. Neither Als3p nor Als1p appeared to contribute to fibronectin adhesion in our assays (Fig. 5). Although assays for each set of strains were run on different days, the comparable results for the wild-type controls suggested a high degree of assay repeatability. Variation in the wild-type controls was greatest for the BEC adhesion assay, but this result was expected since the pool of BECs varied by donor and also by day. Collectively, these results suggested that loss of Als3p resulted in a greater reduction of adhesion to HUVEC and BEC than did loss of Als1p.

**Phenotype of als/als strains in the RHE model of oral candidiasis**

Lack of adherence of the als3/als3 strain to BEC suggested that the mutant would not adhere to buccal RHE. We previously used the buccal RHE model to study ALS gene expression using an RT-PCR assay (Green et al., 2004). Both ALS1 and ALS3 expression were detected in this model. RHE was inoculated with strains CAI12, 1467, 2151, 1843 and 1954; replicate specimens were examined at 1, 4 and 8 h time points. Focusing on early time points allowed evaluation of differences in adherence to the epithelial cells, hyphal formation in association with the epithelial cells and RHE destruction. Adherence was evaluated for the 1 h RHE specimens by counting the number of cells/BEC.

![Fig. 5. Adhesion assay data. Histograms showing the adherence of wild-type (CAI12), als3/als3 (1843, 1954), als1/als1 (1467) and ALS1 replacement (2151) strains to vascular endothelial cell monolayers, BEC and fibronectin-coated plastic plates. Significant differences from wild-type adhesion (P<0.05) are indicated by asterisks.](image-url)
C. albicans cells attached to the RHE surface over 250 μm of tissue on each of 15 different sections cut from different locations in the specimen. The mean number of cells for CAI12 was 5.5 ± 1.1, compared to 6.2 ± 0.8 for 1467, 6.2 ± 0.6 for 2151, 0.1 ± 0.1 for 1843 and 0.2 ± 0.1 for 1954. Adhesion for the als3/als3 strains was significantly different from the others (P < 0.0001). Strain 1467 also had a reduced ability to form germ tubes in the RHE model, having approximately one-tenth as many germ tubes as the wild-type strain. Incubation of strains CAI12, 1467 and 2151 in RHE maintenance medium alone also showed impaired germ tube formation of the mutant strain compared to the others (data not shown). RHE damage at 8 h by the various C. albicans strains is shown in Fig. 6. Compared to the wild-type control, the als1/als1 mutant showed less destruction of RHE, but the most marked observation was the near total lack of epithelial cell destruction by the als3/als3 strains. A less extensive analysis was conducted as a replicate to verify these results. Similar patterns of hyphal formation and RHE destruction were observed in the replicate experiment (data not shown).

**DISCUSSION**

The ability to reproducibly generate disruptions in each ALS3 allele as a result of a single transformation step and to complete the mutant construction in two transformation steps suggests that ALS3 is encoded by a single locus in strain SC5314. These data further suggest that the apparent need for four rounds of disruption to delete ALS3 and ALS8 locus was an artifact. This artifact was most likely caused by gene duplication events that generated extra copies of the ALS3 sequence. Since the first allele disrupted in those experiments was the large ALS3 allele, it is possible that attempts to remove the disruption cassette from this locus were similarly problematic to the ones described here and that, as a result, a segregant with additional copies of the gene was selected. Duplication of C. albicans genes during gene disruption is observed frequently (Enloe et al., 2000; P. Dennison & A. J. P. Brown, unpublished data) and has been observed while constructing mutants of other genes in the ALS family (X. Zhao & L. L. Hoyer, unpublished data). Current nomenclature for the ALS family includes genes ALS1 through to ALS9 (Hoyer, 2001). Data suggesting that

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**Fig. 6.** Light micrographs of buccal RHE inoculated with various C. albicans strains for 8 h. Destruction of the epithelial layer is most evident for the wild-type strain (a) and the ALS1 replacement construct (2151; c). Epithelial damage was less for the als1/als1 mutant (1467; b) and nearly absent for the als3/als3 strain (1843; d). Results for strain 1954 were comparable to those for 1843. A small number of sections of RHE exhibited damage following inoculation with either 1843 or 1954. An example of the maximal damage observed is shown in (e).
ALS8 is an artifact raise the possibility that the ALS genes should be renumbered. However, since ALS8 has been mentioned several times in the literature as an ALS3-like gene (Hoyer & Hecht, 2000; Leng et al., 2001; Murad et al., 2001), renaming the current ALS9 is likely to cause confusion. This confusion would be heightened since there is relatively less sequence similarity between ALS3 and the current ALS9 (Hoyer, 2001; Zhao et al., 2003). The work described here is consistent with our decision to recognize eight different ALS genes, to eliminate ALS8 from the family nomenclature, and to maintain the current name of ALS9.

Previous work showed an increase in the transcriptional activity of ALS3 and ALS1 as yeasts form germ tubes, particularly in RPMI medium (Hoyer et al., 1995, 1998; Fu et al., 2002). These results are confirmed here using GFP reporter constructs. With these constructs, we were able to separate transcription of the two genes temporally, showing a large increase in transcription from the ALS1 promoter within minutes after the cells are transferred to RPMI medium. In contrast, ALS3 transcription does not increase until germ tubes are visible. These observations suggest a role for Als1p in very early events during the change in cellular morphology while the function of Als3p is likely to be associated with a more mature germ tube. Understanding the relationship between transcriptional activity of these two genes and their connection with changes in cellular morphology will allow more appropriate design of microarray analyses to better understand the effects of als/als mutation on C. albicans. These experiments are likely to identify cellular pathways in which the ALS genes function.

Because previous publications have suggested that Als proteins might serve in aggregation of C. albicans cells (Fu et al., 2002; Gaur & Klotz, 1997) and cellular aggregation can potentially have a large effect on adhesion assays, we included experiments to ascertain whether removal of Als proteins creates a phenotype different from wild-type controls. Data presented here showed that cellular aggregation was statistically the same as wild-type for als3/als3 or als1/als1 strains forming germ tubes in RPMI or Lee medium. Mutation of ALS3 also did not affect the ability of the strain to form germ tubes in either medium. In contrast, the als1/als1 strain showed a slowed germ tube formation in Lee medium, consistent with previous reports (Fu et al., 2002). This defect was not apparent in RPMI medium, indicating that the defect is growth-medium-specific. The als1/als1 filamentation defect was also noted in vivo at early time points in a murine model of disseminated candidiasis (Fu et al., 2002), but not in a murine model of oropharyngeal candidiasis (Kamai et al., 2002). Collectively, these results suggest that local environment plays a large role in the function of Als1p.

In the work presented here, the RHE model of oral candidiasis was used to assess the effects of the als1/als1 and als3/als3 strains on epithelial adhesion and destruction. This model has been used previously to assess adhesion and early events in epithelial damage using strains lacking the two-component histidine kinase, CHK1, or a response regulator, CSSK1 (Li et al., 2002). Inoculation of RHE with the als1/als1 mutant strain resulted in a slight decrease in epithelial damage compared to the wild-type and replacement strain controls. In contrast, inoculation with the als3/als3 strain resulted in a marked decrease in epithelial damage. These conclusions paralleled results from in vitro BEC adhesion assays, which showed a slight change in adhesion for the als1/als1 mutant and a large decrease in adhesion for the als3/als3 strain (Fig. 5). Two previous reports address the ability of Als1p to adhere to epithelial cells. In the first report, ALS1 was overexpressed in S. cerevisiae, converting the normally nonadherent organism to one that adhered to epithelial cells (Fu et al., 1998). Although use of the S. cerevisiae model system may be attractive for isolating and characterizing the function of C. albicans adhesins, results need to be interpreted carefully because of differences in codon usage (Santos & Tuite, 1995) and glycosylation (Herrero et al., 2002) that exist between the organisms. An adhesive phenotype might be created by the stickiness of denatured or improperly glycosylated protein, or by disruption of normal cell-wall structure by overproduction of a large, heavily glycosylated protein. The second publication describing Als1p adhesion to epithelial surfaces used a murine model of oropharyngeal candidiasis and demonstrated that the als1/als1 strain was less adherent to oral tissues and to the tongue ex vivo (Kamai et al., 2002). In this model, the complex environment of the oral cavity may affect interaction of Als1p with host surfaces and adhesion may not be directly to epithelial ligands. The epithelial cell adhesion comparisons for als1/als1 and als3/als3 strains presented here predict that als3/als3 strains should show an even greater reduction in oral pathology than als1/als1 strains. This relationship remains to be tested.

Construction of the als1/als1 mutant described here and the one tested in previous studies (Fu et al., 2002; Kamai et al., 2002) differs in a way that may be significant to conclusions about adhesion and pathogenesis. In the previously published strain, disruption of the ALS1 coding region left the 5’ end of one allele intact. Transcription from the ALS1 promoter in this strain can result in export of a protein that includes the N-terminal 285 amino acids of Als1p. This portion of the N-terminal domain of Als proteins has been proposed to be involved in adhesive interactions by comparisons to the structure of the S. cerevisiae cell-surface adhesion glycoprotein α-agglutinin (Chen et al., 1995; Hoyer, 2001). Under conditions where ALS1 is transcribed, the exported N-terminal fragment would be delivered to the local site occupied by C. albicans, with the potential to interfere with adhesive interactions between C. albicans and other cells or surfaces. Therefore, pathogenesis effects observed with this strain may reflect more factors than simple loss of cell-surface-bound Als1p. To avoid the potential problems of creating soluble N-terminal Als fragments, the ALS coding regions in the mutant strains constructed in this work were completely removed. The
relative pathogenesis of *C. albicans* strains that produce N-terminal Als fragments is being investigated (C. B. Green & L. L. Hoyer, unpublished results).

The studies described here outline similarities and differences between Als3p and Als1p, two of the most closely related proteins in the Als family. The direct comparison of the *als1/als1* and *als3/als3* mutant strains in the adhesion assays presented here could lead to the conclusion that Als3p is the stronger adhesin. However, in the context of the Als family, it is possible that some of the proteins have redundant function. Another protein may partially compensate for the loss of Als1p, resulting in a less severe phenotype for the *als1/als1* strain. Phenotypic analysis of our collection of strains mutant in the other ALS genes and construction of selected double mutant strains will reveal the interrelationships between the Als proteins and their roles in *C. albicans* biology and pathogenesis.

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