Published in final edited form as:

Microbiology (Reading). 2005 May ; 151(Pt 5): 1619–1630. doi:10.1099/mic.0.27763-0.

Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family

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Abstract

The ALS (agglutinin-like sequence) gene family encodes eight large cell-surface glycoproteins. The work presented here focuses on Als2p and Als4p, and is part of a larger effort to deduce the function of each Als protein. Both *ALS4* alleles were deleted from the *Candida albicans* genome and the phenotype of the mutant strain (*als4* /*als4* ; named 2034) studied. Loss of Als4p slowed germ tube formation of cells grown in RPMI 1640 medium and resulted in decreased adhesion of *C. albicans* to vascular endothelial cells. Loss of Als4p did not affect adhesion to buccal epithelial cells, biofilm formation in a catheter model, or adhesion to or destruction of oral reconstituted human epithelium (RHE). Although deletion of one *ALS2* allele was achieved readily, a strain lacking the second allele was not identified despite screening thousands of transformants. The remaining *ALS2* allele was placed under control of the *C. albicans* *MAL2* promoter to create an *als2* /*PMAL2-ALS2* strain (named 2342). Real-time RT-PCR analysis of strain 2342 grown in glucose-containing medium (non-inducing conditions) showed that although *ALS2* transcript levels were greatly reduced compared to wild-type cells, some *ALS2* transcript remained. The decreased *ALS2* expression levels were sufficient to slow germ tube formation in RPMI 1640 and Lee medium, reduce adhesion to vascular endothelial cells and to RHE, decrease RHE destruction, and impair biofilm formation. Growth of strain 2342 in maltose-containing medium (inducing conditions) restored the wild-type phenotype in all assays. Real-time RT-PCR analysis demonstrated that in maltose-containing medium, strain 2342 overexpressed *ALS2* compared to wild-type cells; however no overexpression phenotype was apparent. Microarray analysis revealed little transcriptional response to *ALS4* deletion, but showed twofold up-regulation of orf19.4765 in the glucose-medium-grown *als2* /*PMAL2-ALS2* strain. orf19.4765 encodes a protein with features of a glycosylated cell wall protein with similarity to *Saccharomyces cerevisiae* Ccw12p, although initial analysis suggested functional differences between the two proteins. Real-time RT-PCR measurement of *ALS2* and *ALS4* transcript copy number showed a 2.8-fold increase in *ALS2* expression in the *als4* /*als4* strain and a 3.2-fold increase in *ALS4* expression in the *als2* /*PMAL2-ALS2* strain, suggesting the potential for compensatory function between these related proteins.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that causes oral and vaginal mucosal infections as well as systemic disease (Odds, 1988). The ability of *C. albicans* to adhere to host surfaces is positively correlated with its pathogenicity (Calderone & Braun, 1991). The ALS family of *C. albicans* includes eight genes that encode large cell-surface glycoproteins (Hoyer, 2001; Zhao *et al.*, 2004). Adhesive function has been shown for Als1p and Als3p in *C. albicans* (Fu *et al.*, 2002; Zhao *et al.*, 2004). Overproduction of other Als proteins in *Saccharomyces cerevisiae* produced an adherent phenotype for this normally non-adherent organism, suggesting they may also play a role in *C. albicans* adhesion (Gaur & Klotz, 1997; Sheppard *et al.*, 2004).

Als proteins consist of a three-domain structure: the N-terminal domain, in which adhesive function is believed to reside, a central domain consisting of tandemly repeated copies of a conserved 36-amino acid sequence, and a C-terminal domain of varying length and sequence (Hoyer, 2001). The central and C-terminal domains are rich in serine and threonine residues and also contain many consensus sites for N-glycosylation, consistent with the observed heavy glycosylation of the mature proteins (Kapteyn *et al.*, 2000). The current working model for an Als protein is an adhesive N-terminal domain presented on the cell surface by extended, heavily glycosylated central and C-terminal domains. Functional analysis of Als1p in *S. cerevisiae* supported this model (Loza *et al.*, 2004). This model is also consistent with features of other fungal cell-surface glycoproteins that have a similar general structure and amino acid composition (Frieman *et al.*, 2002).

One major goal of our work on the ALS family is to disrupt each gene individually and study the phenotype of the resulting mutant *C. albicans* strain. Work presented here focuses on *ALS2* and *ALS4*, which were originally described in the same paper (Hoyer *et al.*, 1998). *ALS2* and *ALS4* are found on the 6C *Sfi*I fragment of the *C. albicans* genome (Chu *et al.*, 1993; Hoyer *et al.*, 1998). Examination of a set of 160 *C. albicans* isolates that represent the major *C. albicans* genetic clades (Soll & Pujol, 2003) showed that each strain encoded both *ALS2* and *ALS4* (J. A. Nuessen & L. L. Hoyer, unpublished data). Both *ALS2* and *ALS4* have a large, conserved tandem repeat domain and a 3' domain that is more than 95 % identical between the genes. Sequence conservation between the *ALS2* and *ALS4* loci extends at least 1 kb upstream of the start codon, where the loci are approximately 83 % identical (X. Zhao & L. L. Hoyer, unpublished observation). Similarly, for at least 500 bp downstream of the stop codon, the *ALS2* and *ALS4* loci are more than 95 % identical (Hoyer *et al.*, 1998). This high degree of sequence conservation within and outside of the *ALS2* and *ALS4* coding regions has complicated assembly of the *C. albicans* genome sequence in these regions (<http://candida.bri.nrc.ca/candida>). Previous work to study the expression patterns of *ALS2* and *ALS4* required oligonucleotide probes to distinguish between these closely related genes and found that *ALS4* expression increased during exponential phase in a culture grown at 30 °C in YPD (Hoyer *et al.*, 1998). *ALS2* message was never detected on a Northern blot, potentially due to large message size and transcript instability.

In this work, we created an *als4* /*als4* mutant strain using the Ura-blaster method in strain CAI4 (Fonzi & Irwin, 1993), and analysed its phenotype in assays for germ tube formation and adhesion, an *in vitro* disease model and a catheter model of biofilm formation. The same assays were used to study a strain in which *ALS2* expression was diminished by deletion of one allele and regulation of the remaining allele by the *C. albicans* *MAL2* promoter (Brown *et al.*, 1996). The mutant strains were subjected to transcriptional profiling and results validated by real-time RT-PCR. The real-time RT-PCR assay was also used to study transcription of *ALS2* and *ALS4* under different growth conditions and in the mutant strains. Data presented here demonstrate contributions of Als2p and Als4p to *C. albicans* adhesion and suggest the potential for compensatory function within the ALS family.

METHODS

Construction of an *ALS4* gene deletion and replacement strain

All *C. albicans* strains used or constructed in this study are shown in Table 1. All PCR amplifications used *Pfu* polymerase (Stratagene). *C. albicans* *ALS4* was disrupted using a *hisG-URA3-hisG* cassette in plasmid pHUL, which is a derivative of pMB7 (Fonzi & Irwin, 1993; Fig. 1). Construction of pHUL was described previously (Zhao *et al.*, 2004). Briefly, pHUL contains restriction sites 5' and 3' of the Ura-blaster cassette into which the flanking regions from any ALS gene can be cloned. To disrupt *ALS4*, primers ALS4upF and ALS4upR (Table 2) were used to amplify a 417 bp fragment from 5' of the *ALS4* coding region. This fragment was digested with *AvrII/XhoI* and cloned into pHUL that was cut with the same enzymes. Primers ALS4dnF and ALS4dnR were used to amplify 435 bp of DNA from downstream of *ALS4*. The fragment was cut with *SstII/NgoMIV* and cloned into the growing pHUL construct. Digestion of the final plasmid with *AvrII/NgoMIV* released the cassette that was used for transformation of CAI4. The method for transformation and screening of clones was described before (Zhao *et al.*, 2004). The heterozygous strain 1517 was grown on medium containing 5-fluoroorotic acid (5-FOA; Boeke *et al.*, 1984) to select a Uri⁻ isolate. The resulting strain, 2035, was transformed with the *ALS4* disruption cassette and clones screened as before. Strain 2034 was verified as an *als4* /*als4* mutant by Southern blotting (Fig. 1). The *ALS4* upstream probe used in this blot was synthesized by PCR with primers ALS4gfpF and ALS4upR (Table 2; Fig. 1). Deletion of *ALS4* from nt -184 to 86 nt 3' of the *ALS4* stop codon was verified by PCR and DNA sequencing using primers ALS4gfpF and ALS4dn2.

To create a cassette for integration of a wild-type allele into the *als4* /*als4* strain, a wild-type copy of the small *ALS4* allele from strain SC5314 (*ALS4SA*) with 602 bp of upstream sequence was amplified by PCR using primers ALS4upF and ALS4R (Table 2). The PCR product was digested with *AvrII/XhoI* and cloned into pUL that had been digested with the same enzymes. Construction of pUL was described previously (Zhao *et al.*, 2004). Briefly, the pUL plasmid was made by cutting the *hisG-URA3-hisG* cassette from pHUL and replacing it with a functional copy of *URA3*. Sequence from downstream of *ALS4* was amplified using primers ALS4dnF2 and ALS4dnR (Table 2). The resulting 710 bp fragment was digested with *SstII/NgoMIV* and cloned into the growing pUL construct that had been cut with the same enzymes. Digestion of the final plasmid with *AvrII/NgoMIV* released a

fragment encoding the *ALS4* upstream sequence, coding region and downstream sequence as well as *URA3*. This fragment was transformed into strain 2066, which was derived by plating strain 2034 on 5-FOA. In strain SC5314, *ALS4* alleles are approximately 6300 bp and 4569 bp, due mainly to differences in the number of copies of the 108 bp tandem repeat sequence in the central domain of the coding region. The replacement strain 2093 encodes the small *ALS4* allele from strain SC5314; its construction was verified by Southern blotting (Fig. 1).

Construction of a PMAL-ALS2 strain

To construct the *ALS2* disruption cassette, a 399 bp upstream fragment of *ALS2* was amplified by PCR using primers ALS2upF and ALS2upR (Table 2; Fig. 2). The PCR products were digested with *AvrII/XhoI* and ligated into pHUL that was digested with the same enzymes. For the downstream flanking sequence, a 378 bp fragment was amplified using primers ALS2dnR and ALS2dn2 (Table 2), digested with *SstII/NgoMIV* and cloned into the growing pHUL construct. Digestion of the final plasmid with *AvrII/NgoMIV* released a disruption cassette that was used to transform CAI4. Selection and screening of transformants followed previous methods (Zhao *et al.*, 2004). Correct transformants were identified by Southern blotting using a probe synthesized by PCR with primers ALS2gfpF and ALS2upR (Table 2). The correct transformant, strain 1442, was grown on medium containing 5-FOA to select the Uri⁻ strain 1443 (Table 1). Strain 1443 was transformed with the Ura-blaster cassette to delete the second *ALS2* allele. However, after more than 20 transformations and screening over 2000 clones, a strain in which the second allele of *ALS2* was disrupted could not be identified. Southern blot analysis of nearly all transformants derived with this method showed an additional *ALS2*-hybridizing band at the *ALS2* locus following the secondary transformation step. Attempting *ALS2* deletion using the PCR-mediated method of Wilson *et al.* (2000) yielded only three colonies from many independent transformations; none of these transformants had the correct restriction digestion pattern on Southern blots. Therefore, a strategy was developed to place the remaining *ALS2* allele under control of a regulated promoter.

Plasmid pUL was used to make the promoter construct. A fragment from upstream of *ALS2* was amplified using primers ALS2upF and ALS2upR (Table 2), digested with *AvrII/XhoI* and cloned upstream of *URA3* in pUL (Fig. 2). The *MAL2* promoter (Brown *et al.*, 1996) was amplified by PCR with the MAL2F and MAL2R primer pair using SC5314 genomic DNA as the template. The product was digested with *KpnI/NgoMIV* and cloned into similarly digested pUL, immediately downstream of the *URA3* coding region. The 5' end of the *ALS2* coding region (369 bp) was amplified with primers ALS2NgoF and ALS2SstIR (Table 2), digested with *NgoMIV/SstII* and cloned immediately downstream of the *MAL2* promoter. Digestion of the final plasmid with *AvrII/SstII* released the transformation cassette. Strain 1443 was transformed with this fragment; selection and screening of the transformants was as described above. The resulting strain, 2342, had one deleted *ALS2* allele and the other under control of the *MAL2* promoter (Table 2). Constructs were verified by Southern blots as described above (Fig. 2). Growth rates of strains created in this work were matched to CAI12 (Table 1). Zhao *et al.* (2004) described methods for growth rate measurements and statistical analysis of the data.

Phenotypic assays

Methods to evaluate germ tube formation and cellular aggregation of the mutant strains were described previously (Zhao *et al.*, 2004). Starter cultures for this work were grown overnight in YPD medium (per litre: 10 g yeast extract, 20 g peptone, 20 g glucose). Assays were conducted for 1 h in either RPMI 1640 without L-glutamine (RPMI; Invitrogen catalogue number 11875-085) or the medium described by Lee *et al.* (1975). Since the *MAL2* promoter is repressed by glucose and induced by maltose, maltose-containing media were also used to assay strain 2342 and the CAI12 control. Maltose was added to RPMI without glucose (Invitrogen catalogue no. 11879-020) at a concentration equal to that of glucose in the original RPMI medium to make RPMI-Mal. Similarly, maltose was substituted for glucose in the Lee medium recipe to make Lee-Mal. Starter cultures for maltose experiments were grown overnight in YPMal medium (per litre: 10 g yeast extract, 20 g peptone, 40 g maltose). Washed and counted cells were inoculated into either of the maltose-containing assay media above. Following 1 h of incubation, cells in all assays were fixed in 1 % glutaraldehyde. A positive reading was assigned to cells that had a germ tube equal to or longer than one diameter of the mother yeast cell; all other cells were scored as negative. A mixed model analysis of variance (PROC MIXED in SAS, version 8; SAS Institute) was used to assess differences in germ tube formation among the *C. albicans* strains.

Adhesion assays to human vascular endothelial cells were done in a 6-well plate format as described before (Zhao *et al.*, 2004). Adhesion to buccal epithelial cells (BEC) was studied with fresh human cells collected in accordance with the policies of the Institutional Review Board of the University of Illinois. The method, described previously, involved incubation of fungal and human buccal cells in a flask, followed by removal of non-adherent fungal cells by filtration and washing (Zhao *et al.*, 2004). For both the endothelial and epithelial adhesion assays, fungal strains were incubated in RPMI or RPMI-Mal for 1 h to form germ tubes prior to the assay.

Methods for the reconstituted human epithelium (RHE) model and specimen processing were described previously (Zhao *et al.*, 2004). Yeast forms were used as the inoculum. Replicate RHE samples were incubated at 37 °C and saturated humidity for 1 or 8 h.

Model catheter biofilms were grown for dry weight analysis according to the protocol of Kuhn *et al.* (2002) with the following minor modifications. The growth medium for this model is YNB+50 mM glucose, which was altered to YNB+50 mM maltose when induction of *ALS2* expression was required. Prior to incubation in fetal bovine serum, the autoclaved silicone elastomer disks were baked overnight in an 80 °C oven to remove residual moisture. Each disk was preweighed using sterile technique. After 48 h growth of the model biofilm, the medium in each well was removed carefully. The catheter disks on which the biofilm was grown were transferred onto Whatman chromatography paper and dried overnight in an 80 °C oven. Each disk was weighed immediately after removal from the oven. Biofilm dry weight was calculated by subtracting the preweight of each disk from the disk weight following biofilm growth and baking. Biofilms were grown in triplicate in three separate experiments. Means and standard errors of the mean were calculated using the LSMEANS procedure in SAS (version 8; SAS Institute).

Microarray analysis

The microarray used in this work was produced by a working group funded by the National Institute of Dental and Craniofacial Research. *C. albicans* ORFs were obtained from assembly 6 of the *C. albicans* genome sequence (<http://www-sequence.stanford.edu/group/candida>). ORFs encoding proteins greater than 150 aa and that started with an ATG codon were included in the array. ORFs were sorted into two categories: those that were unique by BLAST comparisons, and those that were likely to represent a group of closely related genes or a gene family. Primers to amplify each of the unique ORFs were designed using PRIMER3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html). Primers for the gene family ORFs were designed by hand and checked using the PRIMER3 program. Primers were designed to have a T_m of approximately 60 °C and to amplify a region of approximately 300 bp. When possible, amplification products were targeted to the 3' end of the coding region. The database of ORFs represented on the array is available at <http://www.cbs.umn.edu/labs/berman>.

Two separate rounds of PCR were conducted with each primer pair and *C. albicans* SC5314 genomic DNA as the template. PCR products from the reactions were combined and checked for quality by agarose gel electrophoresis. PCR products were purified using the MultiScreen-PCR 96-well filtration system (Millipore), eluted in water, dried in a SpeedVac (ThermoLabs) and resuspended at approximately 0.1 µg µl⁻¹ in 3× SSC (0.45 M NaCl, 0.045 M sodium citrate). A total of 6392 DNA samples were spotted onto SuperAmine Substrate slides (Telechem) at Microarrays Inc. Controls included *Arabidopsis thaliana* spot report genes and mammalian DNA fragments (Stratagene), *C. albicans* genes of known expression profiles, and buffer blanks. Detailed information on the array and its construction is available at <http://candida.cvm.uiuc.edu>

C. albicans strains for microarray analysis were grown from YPD plates freshly streaked from –80 °C stocks. One well-isolated colony was resuspended in 1 ml YPD and 20 µl of this suspension was used to inoculate 20 ml YPD in a 50 ml flask. All glassware for this experiment was acid-washed and sterile. Flasks were incubated for 16 h at 37 °C and 200 r.p.m. shaking. Cells were counted after incubation to ensure that all strains grew to the same density, which was expected since the growth rates of the strains were matched to each other (Table 1). Cultures were also checked microscopically to ensure lack of germ tubes or hyphae for any of the strains. Cells were washed and resuspended in Dulbecco's phosphate-buffered saline without Ca²⁺ or Mg²⁺ (DPBS; Cambrex catalogue number 17-512Q) and counted in triplicate. Flasks containing 225 ml prewarmed RPMI were inoculated at a density of 5×10⁶ cells ml⁻¹ and incubated at 37 °C and 200 r.p.m. shaking for 1 h. Inoculation of cultures was staggered so time points could be collected precisely. Cells were collected by filtration, immediately flash frozen and stored at –80 °C until RNA was extracted. All cultures were grown in duplicate from independent starter cultures of each strain.

Total RNA was extracted from cells using a hot phenol method (Collart & Oliviero, 1993). Total RNA was further purified using the RNeasy kit (Qiagen) according to the manufacturer's suggested protocol (MacKenzie *et al.*, 1997). The methods for labelling probes and hybridization to microarrays were adapted from protocols developed by The

Institute for Genomic Research (TIGR, <http://pga.tigr.org/protocols.html>). cDNA was synthesized using Superscript II RT (Invitrogen; 100 U reverse transcriptase) in reaction mixtures containing 0.5 mM (total) deoxynucleoside triphosphates [aminoallyl-dUTP and deoxynucleoside triphosphates (4: 1)]. After cDNA synthesis overnight at 42 °C, the RNA was hydrolysed with 1 M NaOH and 0.5 M EDTA. The reaction was neutralized with 1 M HCl. The cDNA was purified using the Qiagen QIAquick PCR purification kit and modified protocol (TIGR). The cDNA was recovered and dried in a SpeedVac (ThermoLabs). The cDNA was coupled with fluorescent dyes by resuspension in 0.05 M sodium bicarbonate buffer, pH 9.0, and incubation with either Cy3 or Cy5 dye (GE Healthcare) for 1 h at room temperature in the dark. Unincorporated dye was removed using a Qiagen QIAquick PCR purification kit, and the samples were dried in a SpeedVac. The samples of cDNA coupled with fluorescent dyes were resuspended in a solution containing 50 % formamide, 10× SSC, and 0.2 % SDS. The appropriate Cy3-cDNA and Cy5-cDNA samples were then mixed and heated for 2 min at 100 °C before the samples were applied to the microarray slide. Samples were hybridized for 20 h at 42 °C, and the slides washed, dried, and analysed as described below. For the direct comparison of two samples (CAI12 vs *als2* /*PMAL2-ALS2* and CAI12 vs *als4* /*als4*), a replicated dye swap of the two individual comparisons was performed.

Arrays were scanned on a GenePix 4000B scanner (Axon Instruments), and data were quantified by using GENEPIX PRO (version 4.0). To determine an appropriate normalization method, the fluorescence (R [cy5], G [cy3]) data were analysed using *MA* plots (Dudoit *et al.*, 2002) that show the intensity log-ratio $M = \log_2(R/G)$ vs the mean log intensity $A = \log_2(R/G)$. A global normalization model of $\log_2$ transformed fluorescence data was used (Wolfinger *et al.*, 2001). The residuals from this model, which are regarded as a crude indicator of relative expression level and are referred to as ‘normalized expression levels’, were then subjected to individual gene-specific models. The normalization and gene models were fit using the MIXED procedure of SAS (version 8; SAS Institute). SAS code for this analysis was based on code available in the introductory Manual for Mixed Model Analysis of Microarray Data (MANMADA 2002; <http://statgen.ncsu.edu/ggibson/Manual.htm>). Data files and array images are available at <http://candida.cvm.uiuc.edu>

To assess the magnitude and significance of the effects of the strains on the normalized expression level of each gene, least-square means using the LSMEANS option of SAS (version 8; SAS Institute) were calculated as described in the MANMADA (2002; <http://statgen.ncsu.edu/ggibson/Manual.htm>). The LSMEANS statement calculates the mean value for each target category, adjusted for the other terms in the model. For each pairwise comparison, the difference between the two normalized expression levels and their standard errors is calculated along with the results of *t*-tests for the statistical significance of the difference between the means in each pair (Littel *et al.*, 1996). Genome annotation and sequence data available at <http://www-sequence.stanford.edu/group/candida>, <http://agabian.ucsf.edu/canoDB/anno.php>, <http://genolist.pasteur.fr/CandidaDB>, <http://candida.bri.nrc.ca/candida> and <http://www.cbs.umn.edu/labs/berman> were used to identify genes and assign putative function to gene products.

Real-time RT-PCR analysis

Primers for real-time RT-PCR analysis were designed using PRIMEREXPRESS (Applied Biosystems). Each primer had a T_m between 59 and 60 °C and an amplicon between 50 and 100 bp. Primers for analysis of *ALS2*, *ALS4*, *TEF1*, *CCW12* and ORF 19.529 expression are shown in Table 2. Two different general cell culture methods were used for the real-time RT-PCR experiments described in this work. The first involved growing yeast forms in either YPD or YPMal medium. For each condition, a single colony from a 24 h YPD plate of CAI12 was inoculated into 1 ml YPD medium and vortexed to resuspend the cells. Twenty microlitres of this suspension was used to inoculate 10 ml fresh YPD. Cultures were incubated for 16 h at 30 or 37 °C and 200 r.p.m. shaking. An aliquot of each starter culture was observed microscopically to ensure that only yeast forms were present. Cells were collected by centrifugation, washed twice in sterile DPBS and counted in duplicate. Fresh YPD growth medium was inoculated at a density of 1×10^6 cells ml⁻¹ and incubated at 30 or 37 °C and 200 r.p.m. Aliquots of the culture were collected by filtration at 1, 8 and 16 h, flash frozen and stored at -80 °C until RNA was extracted. The second cell culture method involved growing germ tubes in RPMI or RPMI-Mal medium. Preparation of the starter culture, observing, washing and counting cells followed the method above. Cells were inoculated into prewarmed medium at a density of 5×10^6 cells ml⁻¹ and incubated at 37 °C with 200 r.p.m. shaking for 1 h. Collection and flash-freezing methods were the same as described above. Real-time RT-PCR was also used to confirm gene expression results from microarray analysis. For this work, the same total RNA samples from the microarray analysis (see above) were used.

Total RNA was DNase treated (Ambion) and purified with the RNeasy kit (Qiagen). cDNA was made using the SuperScript First-Strand Synthesis System (Invitrogen) and 1 µg total RNA template. cDNA was diluted 1: 5 with RNase-free water (Ambion). PCR reactions contained 100 nM each primer, 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 1× ROX Reference dye (Invitrogen) and 5 µl diluted cDNA in a final volume of 25 µl. PCR reactions were run on the ABI PRISM 7000 Sequence Detection System (SDS) with a 50 °C UDG incubation step for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer specificity was verified with a dissociation profile of each amplicon. A standard curve for each primer set was performed with 1:10, 1:25, 1:50, 1:100, 1:250 and 1:500 dilutions of the cDNA. The slopes of the standard curves were within 10% of 100% efficiency. C_T values were determined using the AUTOANALYSE feature of the SDS software.

Relative gene expression was quantified using the C_T method (Livak & Schmittgen, 2001) where $C_T = (C_{T, \text{Mutant}} - C_{T, \text{TEF}}) - (C_{T, \text{Control}} - C_{T, \text{TEF}})$. The fold change in the target gene, normalized to TEF1, was calculated for each sample using the equation 2^{-C_T} . Two separate RNA preparations (independent samples) were made for each target and carried through the analysis. Each RNA replication was treated separately and the results were averaged after the 2^{-C_T} calculation of each PCR run. The mean and standard error were then determined from two independent replications with two individual runs of triplicate samples per RNA preparation.

Absolute quantification of transcript copy number was also performed. Each primer pair was run with the corresponding cloned DNA sequence. Plasmids were isolated, treated with RNase and purified using the Wizard DNA Clean-Up System (Promega). Plasmid concentrations were determined spectrophotometrically and copy numbers calculated based on plasmid mass. A dilution series from 10^2 to 10^6 plasmid copies was used to generate a standard curve. Absolute quantification determined the input transcript copy number per 0.05 µg RNA by relating the C_T value to the standard curve as demonstrated by Heid *et al.* (1996). Reactions to calculate transcript copy number were run in duplicate from two separate RNA preparations. Means and standard errors were calculated in SAS (version 8; SAS Institute). Data were normalized to *TEF1* transcript copy number to allow comparisons between copy numbers at each growth condition and time point. For normalization, the mean for *ALS2* or *ALS4* transcript copy number was divided by the corresponding *TEF1* mean and the result multiplied by 10^6 , which was the general magnitude of *TEF1* transcript copy number values. Normalized values were graphed using GRAPHPAD PRISM software (GraphPad Software).

RESULTS

Use of real-time RT-PCR to compare transcription of *ALS2* and *ALS4* in wild-type *C. albicans* cells

Previous analysis of *ALS2* and *ALS4* expression patterns used Northern blots of RNA isolated from cultured cells. These studies showed increased *ALS4* expression as *C. albicans* cells exited exponential growth in 30 °C YPD cultures; *ALS2* transcript was not observed by Northern blotting (Hoyer *et al.*, 1998). Subsequent analyses using RT-PCR and real-time RT-PCR showed an abundance of *ALS2* transcript under various culture conditions (Green *et al.*, 2004, Green *et al.*, 2005), suggesting that previous failures to visualize *ALS2* transcript by Northern blotting were potentially due to the large size of *ALS2* transcripts or transcript instability. To better understand the relative levels of *ALS2* and *ALS4* transcript present in *C. albicans*, transcript copy number was quantified in YPD-grown CAI12 cells at either 30 or 37 °C. Timepoints of 1, 8 and 16 h were examined. Real-time RT-PCR analysis of total RNA extracted from each of these samples, absolute quantification of the results and normalization to *TEF1* transcript levels showed that *ALS4* expression increased toward stationary phase of a 30 °C culture as previously demonstrated by Northern blot (Hoyer *et al.*, 1998), but was highest at 8 h when cells were grown at 37 °C. *ALS4* transcription was considerably lower at 37 °C than at 30 °C in a YPD culture (Fig. 3). In general, the transcription of *ALS2* was stronger than that of *ALS4* under the conditions tested. *ALS2* transcript copy number increased over time in cells grown at 30 °C, and was highest at 8 h in cells grown at 37 °C (Fig. 3). These results showed the relative transcript levels for *ALS2* and *ALS4* and highlighted a temperature-dependence of *ALS4* transcription that was previously not characterized.

Quantification of altered *ALS2* and *ALS4* expression in mutant *C. albicans* strains

The Ura-blaster method was used to construct a *C. albicans* strain with both *ALS4* alleles deleted (strain 2034; *als4* Δ *als4* Δ) and a strain with one copy of the *ALS4* small allele reintegrated at the *ALS4* locus (strain 2093; *als4* Δ *ALS4SA*; Fig. 1). Despite numerous

attempts, a strain with both *ALS2* alleles deleted was not isolated (see Methods) so the remaining *ALS2* allele was placed under control of the *C. albicans* *MAL2* promoter to create strain 2342 (*als2* /*PMAL2-ALS2*; Fig. 2; Brown *et al.*, 1996). Restriction digests and Southern blotting, followed by PCR amplification and DNA sequence analysis, showed that construction of strains 2034 (*als4* /*als4*), 2093 (*als4* /*ALS4SA*) and 2342 (*als2* /*PMAL2-ALS2*) was correct (Figs 1 and 2).

These strains were further validated using real-time RT-PCR to determine the level of gene expression from each target locus. These quantitative assessments of gene expression were made under growth conditions that were used for phenotypic assays of the mutant strains (see below). RPMI-induced germ tubes were used for phenotypic assays because previous work showed that both *ALS2* and *ALS4* are transcribed within the first hour of growth in this medium (Green *et al.*, 2005). Strains CAI12, 2034 and 2342 were grown in YPD overnight, washed in DPBS, and cultured in RPMI medium for 1 h as described in Methods. Real-time RT-PCR analysis of total RNA from strains 2034 and CAI12 showed that the fold change in *ALS4* transcription was 0.0001 ± 0.0001 . A fold change value of 1.0 would indicate equivalent *ALS4* transcriptional levels in the two strains, while a fold change of 10 would indicate far greater *ALS4* transcription in strain 2034 than CAI12. Consequently, the value of 0.0001 ± 0.0001 , which is essentially zero, showed that there is an unmeasurably low level of *ALS4* transcription in strain 2034 (*als4* /*als4*) compared to the wild-type strain CAI12. This result was expected for a strain in which the *ALS4* coding region was completely deleted. Analysis of strain 2093, in which a wild-type copy of the *ALS4* small allele was reintegrated at the *ALS4* locus, showed a fold change of 0.24 ± 0.14 compared to CAI12, consistent with replacement of only one gene copy.

Strain 2342, in which one *ALS2* allele was deleted and the other controlled by the *MAL2* promoter, showed a fold change in *ALS2* expression of 0.36 ± 0.21 compared to strain CAI12 when both were grown in RPMI with glucose (non-inducing growth conditions for the *MAL2* promoter). This result indicated that *ALS2* transcription is not completely shut down under non-inducing growth conditions, but that far less *ALS2* transcriptional activity is present in strain 2342 than in the wild-type CAI12. When strains CAI12 and 2342 were grown in RPMI-Mal medium (inducing conditions), the fold change in *ALS2* expression was 21 ± 4.5 , suggesting that, under inducing conditions, strain 2342 is essentially an *ALS2* overexpression strain compared to strain CAI12.

Similar analyses were conducted for *C. albicans* strains grown under other conditions in order to provide a context for the phenotypic analysis of the strains with reduced *ALS* transcriptional activity. Real-time RT-PCR analysis of strains 2342 and CAI12 in YPMal medium showed an approximately eightfold increase in *ALS2* transcript copy number for strain 2342 compared to that in CAI12. The large increase in *ALS2* expression when strain 2342 was grown in RPMI-Mal or YPMal emphasized that the strength of the *MAL2* promoter was much greater than that of the native *ALS2* promoter under these growth conditions. Additional real-time RT-PCR reactions demonstrated that, in strain CAI12 grown for 1 h in RPMI, there is approximately 44-fold more *ALS2* transcript than *ALS4* transcript and that growth of CAI12 in YPMal medium results in only approximately a 1.4-fold change in *ALS2* transcript copy number compared to that observed for growth in YPD.

Germ tube formation and aggregation assays

Phenotypic analysis of strains 2342 (*als2* /*PMAL2-ALS2*), 2034 (*als4* /*als4*) and 2093 (*als4* /*ALS4SA*) started with assessments of germ tube formation and cellular aggregation of each strain compared to the CAI12 wild-type control because both of these parameters may affect adhesion assay results. Strains were incubated in RPMI or Lee medium for 1 h. Cells with a germ tube of length equal to or greater than the diameter of the mother yeast were counted as positive. RPMI-Mal and Lee-Mal were also used to induce expression from the *MAL2* promoter and increase *ALS2* expression in strain 2342. In RPMI, loss of wild-type *ALS2* and *ALS4* expression resulted in a significant decrease in germ tube formation (Table 3). A similar result was observed for strain 2342 in Lee medium, but strain 2034 did not differ from the wild-type control under this growth condition. In cases where reduced germ tube formation was observed, restoring function of the *ALS* gene also restored wild-type levels of germ tube formation. No differences from wild-type cellular aggregation were observed for any of the strains assayed (Table 3).

Adhesion to vascular endothelial or oral epithelial cells

Loss of wild-type levels of *ALS2* or *ALS4* expression reduced the ability of *C. albicans* to adhere to vascular endothelial cells (Fig. 4), although the reduction for strain 2034 was barely significant ($P=0.05$). Restoration of *ALS2* and *ALS4* returned adhesion to wild-type levels. There was no significant difference in adhesion of either strain 2034 or strain 2342 to BEC (Fig. 4).

RHE model of oral candidiasis

Although there was no significant difference in adhesion to BEC in the *in vitro* adhesion assays (Fig. 4), loss of wild-type *ALS2* activity resulted in decreased adhesion of strain 2342 to the buccal RHE layer ($P<0.0001$). Assessment of the number of fungal cells that adhered to the RHE layer at the 1 h time point showed a mean of 9.1 ± 0.5 for strain CAI12, 9.3 ± 0.5 for 2034 and 0.9 ± 0.2 for 2342 (from RPMI with glucose). Loss of wild-type *ALS4* activity did not affect epithelial destruction in the RHE model, while reduction of *ALS2* expression reduced epithelial damage (Fig. 5). Assessment of the percentage of cells that formed germ tubes in the RHE model at the 1 h time point showed an approximately 50 % reduction in the number of germ tubes present for strain 2342 compared to strains CAI12 and 2034. The decrease in germ tube formation for strain 2342 in RHE maintenance medium may affect the overall ability of this strain to adhere to and destroy the epithelial layer.

Model catheter biofilm formation

The *als4* /*als4* (2034) and *als2* /*PMAL2-ALS2* (2342) strains were tested in a model of catheter biofilm formation (Kuhn *et al.*, 2002) to see if the biofilm mass produced by each strain was similar to that made by a wild-type control. The dry weight of the biofilm made by the control strain CAI12 was 1.40 ± 0.16 mg compared to 1.28 ± 0.16 mg ($P=0.25$) for 2034 and 1.09 ± 0.16 mg ($P=0.004$) for 2342. The significant decrease in biofilm dry weight for strain 2342 was reversed when the strain was grown in maltose-containing medium (1.42 ± 0.09 mg, $P=0.24$, compared to the CAI12 control at 1.27 ± 0.09 mg). These data

demonstrate that loss of wild-type *ALS2* transcription affects biofilm formation, but that loss of wild-type *ALS4* levels does not affect this process.

Microarray analysis

Transcriptional profiling of strains 2034 (*als4* /*als4*) and 2342 (*als2* *PMAL2-ALS2*) was conducted to identify transcriptional changes associated with reduced *ALS2* or *ALS4* expression. All strains for microarray analysis were grown in RPMI medium for 1 h. This growth condition was selected because it was used for the adhesion assays and also was shown by real-time RT-PCR (see above) to be a growth condition where both *ALS2* and *ALS4* were transcribed. Arbitrary cut-off levels for microarray analysis are commonly set at a twofold change in gene expression between strains and a significance level (*P* value) < 0.05. For analysis of the *als4* /*als4* strain (2034) compared to CAI12, no genes met these criteria.

Analysis of strain 2342 showed up-regulation of a single gene that met the cut-off criteria of twofold change in gene expression and a *P* value < 0.05. This gene was orf19.4765 (orf6.4590, twofold change, *P* < 0.0001). Up-regulation of orf19.4765 in strain 2342 was confirmed by real-time RT-PCR analysis that showed a 3.7-fold increase in expression compared to strain CAI12. The best *S. cerevisiae* match to this gene is *CCW12* (*ScCCW12*), although the *ScCCW12* gene encodes a protein of 133 aa (Mrsa *et al.*, 1999) while the *C. albicans* ORF encodes a 219 aa product. Alignment of these two amino acid sequences showed 38 % identity across 133 aa. In *S. cerevisiae*, Ccw12p is GPI-anchored and highly glycosylated (Mrsa *et al.*, 1999). There is potential interest in up-regulation of orf19.4765 since ScCcw12p was initially characterized as a laminarinase-released cell wall mannoprotein that when disrupted resulted in sensitivity of cells to calcofluor white and Congo red (Mrsa *et al.*, 1999). *S. cerevisiae* mutant cells also showed a decreased growth rate and mating efficiency, defective agglutination, and 2.5 times more chitin than wild-type cells (Hagen *et al.*, 2004). Treatment of intact ccw12 cells with DTT released many more proteins than observed from the same treatment of wild-type cells (Hagen *et al.*, 2004). Like ScCcw12p, the predicted *C. albicans* protein encoded three consensus *N*-glycosylation sites and showed an amino acid composition that was rich in alanine, valine, serine and threonine. The presence of a hydrophobic N- and C-terminus in the *C. albicans* protein suggested that this protein is localized in the cell wall. However, construction and initial phenotypic analysis of a *C. albicans* orf19.4765 /orf19.4765 strain showed that loss of the putative CCW12 gene did not result in sensitivity to calcofluor white or Congo red (X. Zhao, S.-H. Oh & L. L. Hoyer, unpublished data). These observations suggest that the role of orf19.4765 in *C. albicans* may differ from that of *CCW12* in *S. cerevisiae*.

The microarray used for this work included probes that can report transcriptional changes for all ALS genes with the exception of *ALS2* and *ALS4* since only an oligonucleotide can distinguish these genes from each other and from the rest of the ALS family (Hoyer *et al.*, 1998). The array probe that corresponds to *ALS2* and *ALS4* was derived from the 3' end of the gene and hybridized with both sequences. Although this probe should be detected as down-regulated in either mutant strain, it only appeared in the list of genes down-regulated in strain 2034 (1.2-fold, *P* = 0.007), however, with an expression level similar to that observed

in the wild-type strain. One possible explanation for this result was compensatory regulation of each gene in response to altered expression of the other. This relationship was demonstrated by real-time RT-PCR that showed *ALS2* expression increased in strain 2034 (*als4* Δ *als4*) by 2.8-fold and *ALS4* expression increased in strain 2342 (*als2* Δ *PMAL2-ALS2*) by 3.2-fold. Such compensatory responses suggest the potential for functional redundancy within the ALS family. Microarray results did not show significantly up-regulated expression of any other ALS genes in response to altered expression of *ALS2* or *ALS4*.

DISCUSSION

This study focused on analysis of the *C. albicans* Als2p and Als4p proteins. Although a strain lacking the *ALS4* coding region was constructed readily, a strain in which the second *ALS2* allele was deleted was not identified despite screening thousands of transformants. Other experimental efforts also demonstrated that integrating constructs into the *ALS2* locus is more difficult than for the other ALS loci. For example, efforts to construct a *PALS2*-green fluorescent protein (GFP) reporter strain by integrating a GFP cassette into the *ALS2* locus yielded only one clone with a correct Southern blot pattern after screening over 800 transformants (Green *et al.*, 2005). Since an *als2* Δ *als2* strain was not identified, an alternative strategy that involved integration of the *C. albicans* *MAL2* promoter (Brown *et al.*, 1996) to drive *ALS2* expression was used. This effort led to the creation of strain 2342 (*als2* Δ *PMAL2-ALS2*). Placing strain 2342 into maltose-containing medium resulted in an 8- or 21-fold induction of *ALS2* expression compared to wild-type CAI12 cells. Thus, in a maltose-containing medium, strain 2342 is really an *ALS2*-overexpression strain. Overexpression of *ALS2* restored wild-type function but did not produce a phenotype different from wild-type *C. albicans* cells in the assays reported here. Previous work suggested that *ALS2* transcript is unstable compared to other ALS transcripts since it was not detected on Northern blots (Hoyer *et al.*, 1998) despite its relative abundance (Green *et al.*, 2004, Green *et al.*, 2005). The relationship between the increased *ALS2* expression in strain 2342 and quantity of cell-surface Als2p requires additional investigation. Also, since *ALS2* is still transcribed in glucose-grown strain 2342, we cannot conclude whether loss of Als2p is lethal for *C. albicans*.

Data from this work suggest that Als2p contributes to *C. albicans* adhesion. Reduction of *ALS2* expression significantly decreased *C. albicans* adhesion to vascular endothelial cells (Fig. 4). Conflicting results were noted for epithelial cells since reduction of *ALS2* expression did not affect adhesion to BEC in a flask-based assay (Fig. 4), but reduced adhesion in the oral RHE model (Fig. 5). These data indicate differences between the two assay systems and suggest that use of several assay formats is beneficial in reaching conclusions about protein function.

Data from this work also suggested that Als4p has adhesive function; however the effects were more subtle than for Als2p. Deletion of the *ALS4* locus decreased adhesion of *C. albicans* to vascular endothelial cells (Fig. 4), although this result was barely significant statistically. There was no difference in adhesion between the *als4* Δ *als4* strain and the wild-type control in the buccal epithelial cell assay (Fig. 4) or in the RHE model (Fig. 5).

Several factors may contribute to this more subtle phenotypic effect. First, transcript copy number is far lower for *ALS4* than for *ALS2* (Fig. 3). This trend is true particularly at 37 °C, the temperature at which the phenotypic assays are conducted. Although the relationship between transcript copy number and protein production still must be determined, it is likely that there is less Als4p than Als2p on the *C. albicans* surface. It may be more difficult to detect the adhesive capacity of a protein that is present in lesser abundance. Another possible reason for the subtle phenotypic change in the *als4 / als4* strain is the resulting increase in *ALS2* expression. Since *ALS2* expression levels are very high compared to *ALS4* levels at 37 °C (Fig. 3), *ALS2* could be masking the effect of the *ALS4* deletion. This argument suggests the possibility of functional redundancy within the Als family.

Although redundancy of function is often postulated as one reason *C. albicans* has gene families, it has yet to be demonstrated among the Als proteins. Analysis of Als1p and Als3p showed that they both function as adhesins (Fu *et al.*, 2002; Zhao *et al.*, 2004), yet neither one is up-regulated in response to mutation in the other locus (K. M. Yeater and others, unpublished). For functional redundancy to exist between two Als proteins, the protein features must be similar enough to compensate for lost function and also be produced in response to appropriate regulatory signals. Although Als adhesive function is postulated to reside in the N-terminal domain (Hoyer, 2001; Loza *et al.*, 2004), this portion of the protein is the least conserved between Als2p and Als4p. Much more notable are the large tandem repeat regions that have a mean number of repeat copies of approximately 34 for Als2p and are of a similar size for Als4p (J. A. Nuessen & L. L. Hoyer, unpublished observation), and the nearly identical Als2p and Als4p C-terminal domains. The tandem repeat and C-terminal regions of the Als proteins are heavily glycosylated (Kapteyn *et al.*, 2000) and would add considerably to the carbohydrate present on the *C. albicans* cell surface. This heavy glycosylation could affect the adhesive phenotype of the fungal cell. Additional experimentation is required to test this hypothesis. With respect to the regulation of *ALS2* and *ALS4* expression, sequence comparisons showed that the promoter regions are 83 % identical. Unique responses to temperature and culture conditions were demonstrated quantitatively in this work (Fig. 3) but may be overridden when one locus is compromised. Additional experimentation is required to deduce sequence elements responsible for this altered regulation. However, increased *ALS4* expression in response to changes in *ALS2* transcript levels cannot mask the resulting mutant phenotype. Similarly, up-regulation of other *C. albicans* genes such as *CCW12* is not sufficient to compensate for the phenotypic effects of altered *ALS2* expression. The analyses presented in this paper considered a limited number of growth conditions. Greater insight may be gained from examination of additional culture conditions.

Although the phenotype of the *als4 / als4* strain was subtle, integration of a wild-type copy of *ALS4* restored wild-type adhesion to vascular endothelial cells (Fig. 4). This restoration of wild-type function was achieved with the smaller *ALS4* allele from strain SC5314. An effect of allele size was noted for *ALS3* in strain SC5314, where the smaller allele (9 tandem repeat copies) showed a weak adhesive effect while the larger allele (12 tandem repeat copies) had a strong adhesive effect (Oh *et al.*, 2005). The *ALS4* allele used in this work differs by 16 tandem repeat copies from the larger *ALS4* allele in strain SC5314, suggesting that the effect of allele length may not be as important for this gene.

The results presented here and in our previous work (Zhao *et al.*, 2004) complete the functional analysis of proteins in the first half of the Als family (Als1p to Als4p) in *C. albicans*. The genes encoding these proteins share a cross-hybridizing tandem repeat domain and constitute one of the ALS subfamilies (Hoyer, 2001). Data have shown that each protein has adhesive function. Together, these data demonstrate unique features of each protein in *C. albicans* and suggest the potential for compensatory function within the Als family. Ongoing analysis will determine which of these themes apply to the remainder of the Als family.

ACKNOWLEDGEMENTS

We thank Bill Schnitzlein for his expert advice regarding real-time RT-PCR. We thank Mira Edgerton and the other members of the Consortium for *Candida* DNA Microarray Facilities (J. Berman, M. A. Ghannoum, P. Orlean, P. T. Magee and P. Sundstrom) for their efforts in constructing the *C. albicans* microarray. Judith Berman, Suzanne Grindle and John Crow contributed bioinformatics analysis that was essential for microarray construction. The skilled and highly accurate efforts of Lynne Herron and Georgina Cheng were also instrumental in microarray construction. This project was funded by United States Public Health Service grant R01 DE14158 from the National Institute of Dental and Craniofacial Research (NIDCR). Microarray construction was funded by grant R01 DE10641-S1 (to M. Edgerton) from NIDCR. Sequence data for *C. albicans* were generated at the Stanford DNA Sequencing and Technology Center with the support of NIDCR and the Burroughs Wellcome Fund.

Abbreviations

BEC	buccal epithelial cells
5-FOA	5-fluoroorotic acid
RHE	reconstituted human epithelium

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Fig. 1.

Construction and Southern blot verification of *C. albicans als4 /als4* and *als4 /ALS4SA* strains. The locations of the 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) *ALS4* alleles from strain SC5314. The length of the alleles differs due to variation in the number of tandem copies of the 108 bp sequence in the central domain. *ALS4* alleles in strain SC5314 are approximately 6300 bp (large allele; LA) and 4569 bp (small allele; SA). (b) The *hisG*-*URA3*-*hisG* cassette constructed by cloning PCR-amplified *ALS4* upstream and downstream sequences on either side of the parent cassette. (c) The *ALS4* locus with the disruption cassette integrated to replace the *ALS4LA* coding region as in strain 1517. Growth of this strain on 5-FOA, followed by transformation of the resulting strain with the disruption cassette shown in (b), eliminated the second *ALS4* allele to create strain 2034. (d) Construct built to replace a wild-type copy *ALS4SA* in the mutant strain. (e) *ALS4* locus with the replacement wild-type allele integrated. (f) Southern blot of *Bgl*II-digested genomic DNA from the indicated strains hybridized with the *ALS4* upstream probe to demonstrate correct strain construction. Molecular sizes (in kb) are shown on the left. Cross-hybridization between sequences upstream of *ALS2* and *ALS4* resulted in the appearance of the band above 12 kb, which corresponds to *ALS2*.

Fig. 2.

Construction and Southern blot verification of a *C. albicans als2* /*PMAL2-ALS2* strain. The locations of the 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) Both *ALS2* alleles from strain SC5314 are approximately 6.2 kb and are represented in a single diagram. (b) The *hisG-URA3-hisG* cassette constructed by cloning PCR-amplified *ALS2* upstream and downstream sequences on either side of the parent cassette. (c) The *ALS2* locus with the disruption cassette integrated to replace one *ALS2* allele to create strain 1442. Growth of this strain on 5-FOA created strain 1443, which was transformed with the *PMAL2* construct in (d) to create strain 2342. The *PMAL2*-regulated *ALS2* locus is shown in (e). (f) Southern blot of *Bgl*II-digested genomic DNA from the indicated strains hybridized with an *ALS2* upstream probe to demonstrate correct strain construction. Molecular sizes (in kb) are shown on the left. Cross-hybridization between sequences upstream of *ALS2* and *ALS4* resulted in the appearance of the bands at approximately 9 and 11 kb, which correspond to the alleles of *ALS4*.

Fig. 3.

Analysis of *ALS2* and *ALS4* transcription using real-time RT-PCR. *C. albicans* strain CAI12 was grown in YPD medium at 30 (a) or 37 °C (b) and cells harvested at 1, 8 and 16 h. Total RNA was analysed using real-time RT-PCR assays for *ALS2*, *ALS4* and *TEF1* transcription. Absolute quantification of transcript copy number was accomplished as described in Methods and the results normalized to the copy number of the *TEF1* housekeeping gene. Normalized transcript copy numbers from triplicate assays of independently replicated RNA samples are shown.

Fig. 4.

Adhesion assay data. Histograms showing the adherence of wild-type (CAI12), *als2* / *PMAL2-ALS2* (2342), *als4* / *als4* (2034) and *als4* / *ALS4SA* (2093) strains to vascular endothelial cells (a) or to BEC (b). Prior to each adhesion assay, strains were incubated in RPMI medium for 1 h to form germ tubes. For endothelial cell assays (a), a significant decrease in adherence of the *als2* / *PMAL2-ALS2* strain was observed ($P=0.01$). This strain and the CAI12 control were grown in RPMI-Mal medium to induce *ALS2* expression and restore a wild-type phenotype to the strain. Strain 2034 also showed decreased adherence to vascular endothelial cells, but the difference was less pronounced ($P=0.05$). Loss of wild-type *ALS2* or *ALS4* expression did not affect adherence to BEC in this assay. *, Significant differences from wild-type adhesion ($P < 0.05$).

Fig. 5.

Light micrographs of buccal RHE incubated with various *C. albicans* strains for 8 h. Destruction of the epithelial layer is most evident for the wild-type strain CAI12 (a) and the *als4 /als4* strain 2034 (c). Deletion of one *ALS2* allele and control of the other with the inducible *MAL2* promoter to create strain 2342 decreased adhesion and epithelial damage in this model system (b).

Table 1

C. albicans strains used in this study

Strain	Parent	Genotype*	Doubling time (h) [†]	Source/reference
CAI4	SC5314	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴		Fonzi & Irwin (1993)
CAI12	CAI4	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>IRO1 URA3</i>	1.79±0.05 ^a / 1.79±0.07 ^b	Porta <i>et al.</i> (1999)
1517	CAI4	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>ALS4SA/als4la</i> - <i>URA3</i>		This study
2035	1517	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>ALS4SA/als4la</i> - <i>ura3</i>		This study
2034	2035	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>als4sa</i> / <i>als4la</i> - <i>URA3</i>	1.75±0.04 ^a	This study
2093	2066	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>als4</i> / <i>als4</i> - <i>ALS4SA-URA3</i>	1.75±0.04 ^a	This study
1442	CAI4	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>ALS2/als2</i> - <i>URA3</i>		This study
1443	1442	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>ALS2/als2</i> - <i>ura3</i>		This study
2342	1443	<i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ / <i>iro1-ura3</i> :: λ <i>imm</i> ⁴³⁴ <i>als2</i> / <i>PMAL2-ALS2-URA3</i>	1.74±0.06 ^b	This study

* *ALS4* alleles are marked with LA to designate the large allele (approx. 6300 bp) or SA for the small allele (4569 bp; GenBank accession number AF272027) in strain SC5314. Allele sizes vary due to the number of copies of the 108 bp tandem repeat sequence present in the central domain of the coding region. *ALS2* allele sizes are very similar in strain SC5314 and were not distinguished from each other in this work.

[†] Doubling times (±SE) for *ALS2* and *ALS4* constructs were compared to the wild-type CAI12 control in two separate experiments. Experiments are discriminated with either ^a or ^b. There was no significant difference between doubling times for any of the strains (*P*>0.05).

Table 2

Oligonucleotide primers

Primer name	Label*	Primer sequence (5'→3')
ALS4upF	(1)	CCC CCT AGG CAA ATT GAT AGT TCT CTC CAG
ALS4upR	(2)	CCC CTC GAG CCA TAA AGT AGC CAA TAG AGC
ALS4dnF	(3)	CCC CCG CGG TGG CAA ATT GCA TTG CTG AG
ALS4dnR	(4)	CCC GCC GGC GGG ACA TAG ATA ACA ATA ATT GG
ALS4gfpF	(5)	CCC GGT ACC TGC TCT CTT TGC TTC GTT T
ALS4R	(6)	CCC CTC GAG CTC AGC AAT GCA ATT TGC CA
ALS4dn2	(7)	GTG TGA TGT AAT GCT ACC AAA TTG C
ALS4dnF2	(8)	CCC CCG CGG AAT CAG TAC GCC TTT GGC TTC TAC
ALS2upF	(9)	CCC CCT AGG TAT GTT TGC ATT ACG CTG TTG
ALS2upR	(10)	CCC CTC GAG CCA TAA GGT TGT TAG AAA CAT C
ALS2dnF	(11)	CCC CCG CGG TAG TAC GTT GTT TGT AAC TAA GTA
ALS2dnR	(12)	CCC GCC GGC TTA TGT GAT ACT TGA GAT GGT
ALS2dn2	(13)	GTG ATC AGC GTT ACC ATC GTA GC
ALS2gfpF	(14)	CCC GGT ACC CAA ACC AAA CCA GAC AAG AC
MAL2F	(15)	CCC GGT ACC TTG ATA TTT TTG TCT AGT ACC ATC
MAL2R	(16)	CCC GCC GGC TGT AGT TGA TTA TTA GTT AAA CCA
ALS2NgoF	(17)	CCC GCC GGC ATG CTT TTA CAA TTT TTG TTG CTA
ALS2SstIR	(18)	CCC GAG CTC CTT AAT AGA TGA TGT TAA AGT ATT GC
qRT-ALS2F	(19)	TTC CAA GTA TTA ACA AAG TTT CAA TCA
qRT-ALS2R	(20)	ACC AGA TGT GTA GCC ATT TGC AC
qRT-ALS4F	(21)	TCT GCA ACA CGA GTC AGC TCA
qRT-ALS4R	(22)	CCG CAC CAA CAC AAG CAT ATA
qRT-TEF1F	(23)	CCA CTG AAG TCA AGT CCG TTG A
qRT-TEF1R	(24)	CAC CTT CAG CCA ATT GTT CGT
qRT-CCW12F	(25)	CCG ACA ACA AAT GTG CTA CTA GTG T
qRT-CCW12R	(26)	TGT TGA CTG ATT GAA CAG CTG GA
qRT-orf19.529F	(27)	CAA CGT CAT AAC GAG GGA TCA ATA G
qRT-orf19.529R	(28)	GGA TTG TTG GCA GTA TTG GTT TG

* Numbers indicate the primer locations as shown in the figures.

Table 3

Germ tube formation and cellular aggregation of *C. albicans* strains

Strain	Growth medium	Time (min)	Germination* (%)	P value	Cell aggregation*	P value
CA112	RPMI	60	86.2±2.3		1.33±0.03	
2342			78.0±2.3	0.02	1.38±0.03	0.19
2034			77.8±2.3	0.02	1.40±0.03	0.11
2093			85.8±2.3	0.92	1.34±0.03	0.84
CA112	RPMI-Mal		67.8±2.3		1.09±0.03	
2342			69.0±2.3	0.72	1.09±0.03	1.00
CA112	Lee	60	72.5±3.6		1.28±0.04	
2342			62.2±3.6	0.02	1.21±0.04	0.21
2034			67.0±3.6	0.19	1.28±0.04	0.92
2093			73.8±3.6	0.74	1.31±0.04	0.54
CA112	Lee-Mal		52.1±3.6		1.08±0.04	
2342			54.3±3.6	0.59	1.07±0.04	0.76

* Values are reported as the mean±SEM as calculated by PROC MIXED in SAS (version 8; SAS Institute).