# Overexpression of the *Candida albicans ALA1* Gene in *Saccharomyces cerevisiae* Results in Aggregation following Attachment of Yeast Cells to Extracellular Matrix Proteins, Adherence Properties Similar to Those of *Candida albicans*

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*Candida albicans* maintains a commensal relationship with human hosts, probably by adhering to mucosal tissue in a variety of physiological conditions. We show that adherence due to the *C. albicans* gene *ALA1* when transformed into *Saccharomyces cerevisiae*, is comprised of two sequential steps. Initially, *C. albicans* rapidly attaches to extracellular matrix (ECM) protein-coated magnetic beads in small numbers (the attachment phase). This is followed by a relatively slower step in which cell-to-cell interactions predominate (the aggregation phase). Neither of these phases is observed in *S. cerevisiae*. However, expression of the *C. albicans ALA1* gene from a low-copy vector causes *S. cerevisiae* transformants to attach to ECM-coated magnetic beads without appreciable aggregation. Expression of *ALA1* from a high-copy vector results in both attachment and aggregation. Moreover, transcriptional fusion of *ALA1* with the galactose-inducible promoters GALS, GALL, and GAL1, allowing for low, moderate, and high levels of inducible transcription, respectively, causes attachment and aggregation that correlates with the strength of the GAL promoter. The adherence of *C. albicans* and *S. cerevisiae* overexpressing *ALA1* to a number of protein ligands occurs over a broad pH range, is resistant to shear forces generated by vortexing, and is unaffected by the presence of sugars, high salt levels, free ligands, or detergents. Adherence is, however, inhibited by agents that disrupt hydrogen bonds. The similarities in the adherence and aggregation properties of *C. albicans* and *S. cerevisiae* overexpressing *ALA1* suggest a role in adherence and aggregation for *ALA1* and *ALA1*-like genes in *C. albicans*.

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*Candida albicans* is capable of maintaining commensal relationships with mammalian hosts, in part, by surviving in small numbers on mucous membranes (3, 26). Since, *C. albicans* has evolved in the normal host by maintaining a commensal state, it is likely to have developed a mechanism(s) to maintain long-term residence. Adherence of the fungus to host tissue is generally believed to be one such important mechanism, both for initiating and for maintaining the commensal state. Furthermore, the adherence of *C. albicans* to many substrates is often associated with aggregations of yeast cells. This is a particularly prominent feature of in vitro adherence assays when *C. albicans* is added in saturating numbers to such targets as buccal cells (25), basement membrane (21), endothelial cells (19), and fibroblasts (23).

Cell surface molecules that may serve as *C. albicans* adhesins for host tissue include glycoproteins (4), polysaccharides (7), and lipids (10). Genetic approaches have identified and isolated *C. albicans* genes that may encode potential adhesins. For example, the *C. albicans AAF1/CAD1* gene was isolated by expression cloning in *Saccharomyces cerevisiae*, and transformants exhibited enhanced adherence to polystyrene and to buccal epithelial cells. Transformants also autoaggregated with or without adherence (1). Further work by another group, however, disputed the direct involvement of the *AAF1/CAD1*

gene product in adherence to endothelial and epithelial cells and demonstrated that the autoaggregation was a form of flocculation inhibited by mannose (5). Another gene, α*INT1*, was isolated from *C. albicans* by screening a genomic library with a human α-integrin gene probe (8). Expression of α*INT1* in *S. cerevisiae* caused fungal adherence to epithelial cells, formation of germ tube-like structures, and autoaggregation. However, an observation has been made that a strong similarity exists between the α*INT1* gene sequence and the *S. cerevisiae* gene *BUD4*, which is involved in yeast cell bud site selection (28). Consequently, the function of this gene may be related more to morphology than to adherence (2). Another novel adhesive mechanism involving *C. albicans* is the recent description of the covalent interaction of a hypha-specific surface protein and mammalian cell proteins catalyzed by transglutaminases, thus forming a stable yeast cell-mammalian cell bond (29).

We have previously described the isolation of the *C. albicans* gene *ALA1* (agglutinin-like adhesin 1), by expression cloning in *S. cerevisiae* (9). Expression of *ALA1* in nonadherent *S. cerevisiae* caused transformants to adhere to extracellular matrix (ECM)-coated magnetic beads and to human buccal epithelial cells. The predicted Ala1p sequence shows features consistent with cell surface localization and similarities to the *S. cerevisiae* agglutinin protein (AGα1) which mediates cell-cell adhesion during the mating of haploid yeast cells. Recently, Fu et al. demonstrated that another *C. albicans* gene, *ALS1* (agglutinin-like sequence 1), causes *S. cerevisiae* trans-

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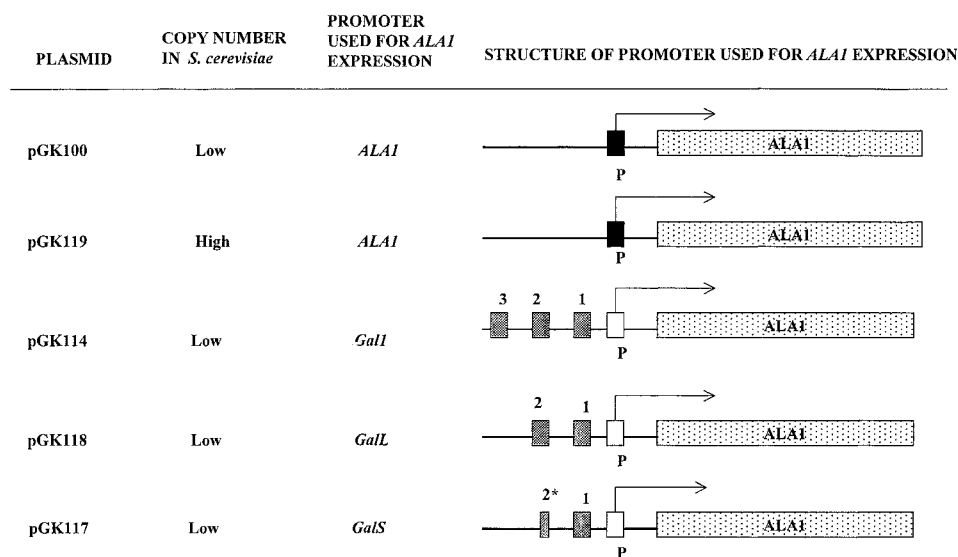
FIG. 1. Schematic representation of plasmids used in this study showing plasmid copy numbers and structures of the putative *ALA1* promoter and the galactose-inducible promoters. PGK100 is our original plasmid carrying *ALA1*. It is derived from pAUR112 (PanVera Corp., Madison, Wis.), which is incapable of conferring adherence properties on *S. cerevisiae*. The *ALA1* promoter is not mapped, but deletion of the putative promoter abolishes its expression. Three upstream activation sequences (UAS1, UAS2, and UAS3) required for full promoter induction by galactose are shown by the numbered shaded boxes. The promoter location is shown by a solid box for *ALA1* and an open box for the galactose promoter, and each is marked by a P. ∗, Partial deletion of UAS2.

formants to adhere to human endothelial and epithelial cells when expressed from a galactose-inducible promoter (6).

In the present investigation, we show that adherence of *C. albicans* and transformed *S. cerevisiae* to proteins and cells is characterized by two sequential steps: attachment followed by aggregation. This form of adherence of *C. albicans* and *S. cerevisiae* expressing *ALA1* from different plasmids occurs over a broad pH range to a number of protein ligands and is resistant to shear forces generated by vortexing and competition from free additives. The adherence of both yeasts is inhibited by hydrogen bond-disrupting agents.

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## MATERIALS AND METHODS

**Media, strains, and transformation.** A *C. albicans* CA1 strain (prototroph) isolated from a human source was used in adherence assays in these studies. *S. cerevisiae* YPH499 (*MATa ura3-52 lys2-801*[amber] *ade2-101*[ochre] *trp1-Δ63 his3-Δ200 leu2-Δ1*) was obtained from the American Type Culture Collection (ATCC). All fungal strains used in these studies were maintained as frozen stocks in 20% glycerol for the long-term storage. Media used for growing yeast strains were as follows: YPD (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose), and synthetic defined medium consisting of 0.67% yeast nitrogen base and 2% glucose with appropriate amino acid supplements (50 μg/ml). *S. cerevisiae* YPH499 was transformed with plasmid DNAs by the lithium acetate method (11). Competent *Escherichia coli* cells (XL10-Gold) were purchased from Stratagene (La Jolla, Calif.) and transformed with a plasmid according to the vendor's instructions. *E. coli* was grown at 37°C in Luria-Bertani broth (1% NaCl, 1% tryptone, 0.5% yeast extract).

**DNA manipulation.** Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.), and were used according to the vendor's instructions. DNA fragments were purified after they were resolved on a 1% low-melting-point agarose gel by using the Qiagen gel extraction kit according to the vendor's instructions (Qiagen, Inc., Chatsworth, Calif.). Plasmid DNA was purified from *E. coli* with the Qiagen Maxi Kit. Plasmid DNA from yeast was isolated by the method described by Hoffman and Winston (13).

**Construction of plasmids.** The various *ALA1* plasmids used in this study are shown in Fig. 1.

**Plasmid pGK100.** pGK100 (Fig. 1) is the original *ALA1* plasmid and is described in our previous publication (9).

**Plasmid pGK119.** Plasmid pGK119 (Fig. 1) was constructed by ligating the *Bam*HI-*Xho*I fragment from pGK102 into pRS426 (obtained from the ATCC) that had been restricted with *Bam*HI and *Xho*I and dephosphorylated. After ligation, DNA was directly transformed into *S. cerevisiae* YPH499, and the cell pellet was suspended in 5 ml of YPGal and grown at 28°C for 20 h. Cells were collected and washed three times with 5 ml of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA) and then suspended in 1 ml of TE buffer. Cells expressing *ALA1* were selected by using fibronectin (FN)-coated magnetic beads as described earlier (9). Two transformants of the six analyzed exhibited adherence with an aggregation phenotype when grown in YPD or YPGal, and one of these was used for the adherence studies.

**Plasmids pGK112 and pGK116.** Plasmids (p414GAL1, p414GALL, and p414GALS) containing galactose-inducible promoters were purchased from the ATCC. Several attempts to clone the *ALA1* gene in *E. coli* by using p414GAL1 were not successful. Therefore, we constructed plasmids pGK112 and pGK116 that contain GAL1 and GALS promoters, respectively. In these plasmids, *E. coli* and *S. cerevisiae* replicons of p414GAL1 and p414GALS were replaced with the replicons from pAUR112, in which *ALA1* is stable. For construction of pGK112, the *Sac*I-*Sna*BI fragment from p414GAL1 was gel purified after making blunt ends by using T4 DNA polymerase. This DNA fragment contains the TRP1 marker, the GAL1 promoter, and multiple cloning sites. Similarly, the *Xho*I-*Bgl*II fragment from pAUR112 was blunt ended with T4 DNA polymerase, dephosphorylated, and gel purified. This DNA fragment contains sequences for replication in *E. coli* and *S. cerevisiae* and the β-lactamase gene for selection in *E. coli*. After ligation of these two fragments and transformation into *E. coli*, a chimeric plasmid (pGK112) was isolated that could be transformed and selected for the TRP marker in *S. cerevisiae*. By a similar strategy, pGK116 was constructed by ligating *Sac*I-*Sna*BI and *Xho*I-*Bgl*II fragments from p414GALS and pAUR112, respectively.

**Plasmids pGK114, pGK118, and pGK117.** A DNA fragment containing the promoter-less *ALA1* gene was cloned into pGK112 and pGK116 to obtain plasmids pGK114 and pGK117, respectively. In contrast to the original plasmids, *ALA1* is stable in pGK114 and pGK117. For the construction of pGK114, the *Eco*RV-*Xba*I fragment from pGK102 was gel purified after filling in of the *Xba*I end by using DNA polymerase I (Klenow fragment). This fragment was ligated to pGK112 that had been restricted by *Sma*I and was then dephosphorylated. After transformation into *E. coli*, recombinant plasmids were analyzed, and a plasmid containing *ALA1* in the right orientation was called pGK114. The plasmid was transformed into *S. cerevisiae* YPH499, and transformants were analyzed for adherence to FN-coated magnetic beads. All six transformants analyzed exhibited adherence with an aggregation phenotype when grown in YPGal medium but not when grown in YPD medium. For the construction of pGK117, the promoter-less *ALA1*-containing *Eco*RV-*Xho*I fragment from pGK102 was isolated by gel purification. The pGK116 vector DNA was prepared by restricting with *Sma*I and *Xho*I and dephosphorylation. After ligation and transformation into *S. cerevisiae* YPH499, *ALA1*-expressing cells were isolated as described for the construction of pGK119. Three of six transformants selected for analysis

FIG. 2. Diagrammatic representation of Ala1p and approximate locations of the antigenic peptides used in this study. The N-terminal peptide begins with residue 258 (SPSDNNQYQLSYKND). The C-terminal peptide begins with residue 1309 (SKTKSIEESIMNPOS). The molecule includes an immunoglobulin G domain (IgG), a threonine-rich domain (T), a tandem repeat domain (TR), and a serine-threonine-rich domain in the carboxy terminus (S/T).

exhibited adherence with an aggregation phenotype when grown in YPGal but not in YPD. One of these transformants was used for further studies. Plasmid pGK118 was constructed by ligating the *Eco*RV-*Xho*I fragment containing *ALA1* into p414GALL that had been restricted with *Sma*I and *Xho*I and dephosphorylated. The ligated DNA was transformed into *S. cerevisiae* YPH499, and *ALA1*-expressing cells were isolated as described above. In this case, four of six transformants exhibited adherence with an aggregation phenotype when grown in YPGal but not in YPD, and one of these transformants was used in the adherence studies.

**Antibodies to Ala1p.** In order to demonstrate the presence of Ala1 on the surface of the *S. cerevisiae* transformants, rabbit polyclonal antibodies to 15-mer peptides predicted by Chou-Fasman analysis to be on the surface of the protein were obtained (Research Genetics, Huntsville, Ala.). The approximate locations of the 15-mer peptides in Ala1p are shown in Fig. 2.

Fungi were cultured as already described, and $\sim 5 \times 10^9$ cells of each strain were placed in 10 ml of Tris-HCl (pH 12) with 100 mM phenylmethylsulfonyl fluoride and shaken for 30 min at room temperature. After this treatment the cells are not capable of adhering or aggregating (see Results). The cells were centrifuged, and the supernatant was retained and concentrated (Centriprep; Amicon, Beverly, Mass.). All volumes were kept equal during the concentration, and samples derived from each yeast strain were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, incubated with the primary antibody (1:2,500 dilution) overnight, and treated with a secondary alkaline phosphatase-tagged antibody (1:250 dilution).

For fluorescent-antibody studies the cells were suspended in phosphate-buffered saline containing 3% bovine serum albumin (BSA; pH 7), incubated in a 1:10 dilution of primary antibody for 2 h, and then washed and treated with a secondary, fluorescein-tagged antibody at a dilution of 1:100.

**Preparation of cells for adherence assay.** An isolated *C. albicans* colony from a YPD agar plate was inoculated in 5 ml of YPD and grown for 16 to 20 h at 28°C. The overnight culture was transferred to 45 ml of YPD and grown for 6 to 7 h at 28°C. Cells were harvested by centrifugation, and the cell pellet was washed twice with 25 ml of TE buffer (pH 7.0; 10 mM Tris-HCl, 1 mM EDTA). Cells were suspended in 5 ml of TE buffer and stored at 4°C. *S. cerevisiae* YPH499 cells were prepared by inoculating an isolated colony into 50 ml of YPD and then grown at 28°C for 24 h. As described for *C. albicans*, cells were harvested, washed, and stored in 5 ml of TE buffer. Similarly, transformed *S. cerevisiae* YPH499 harboring pGK100 and pGK119 was grown in 50 ml of YPD at 28°C for 24 h. *S. cerevisiae* YPH499 harboring pGK114, pGK117, and pGK118 was grown in 50 ml of YPGal for 60 to 70 h at 28°C. Cells were harvested, washed, and stored in TE buffer as described for *C. albicans*.

**Adherence assay.** The adherence assay is based on a method that we had previously developed to isolate the *C. albicans ALA1* gene (9). We have now made further modifications in this method which enables us to perform a quantitative adherence assay measuring adherent cells with or without aggregation. The method utilizes ECM-coated, i.e., FN-laminin (LM)-, and type IV collagen (COL IV)-coated, magnetic beads to selectively isolate *C. albicans* and *S. cerevisiae* expressing *ALA1*. BSA is used to mask unoccupied sites according to the manufacturer's directions. Masking the unoccupied sites with ethanolamine produced the same results as did BSA; thus, the interactions were with the ECM proteins. The adherence assay was performed in a 15-ml glass tube to which 940 μl of TE buffer (pH 7.0), 10 μl of ECM-coated magnetic beads (final concentration, $\sim 10^6$ beads/ml), and 50 μl of cell suspension (final concentration, >$10^8$ cells/ml) were added in that order. *C. albicans* at a concentration of $\sim 10^7$ cells/ml was determined to be saturating under these conditions since adherence remained constant at higher cell concentrations (results not shown). After an immediate mixing by vortexing, the glass tube was placed on an end-to-end shaker and incubated at room temperature for 30 min with moderate shaking. The mixture was vortexed for 10 to 15 s; the tube was then immediately placed in a magnetic separator to attract magnetic beads on the side, and the supernatant was carefully removed. The separated magnetic beads were washed with 1 ml of TE by adding it along the wall while keeping the tube in the magnet. The supernatant was carefully removed, and magnetic beads were similarly washed with 1 ml of TE while keeping the tube in the magnetic separator. The separated magnetic beads and adherent cells were washed three times with TE as described above and, after the last wash, the cells and magnetic beads were suspended in
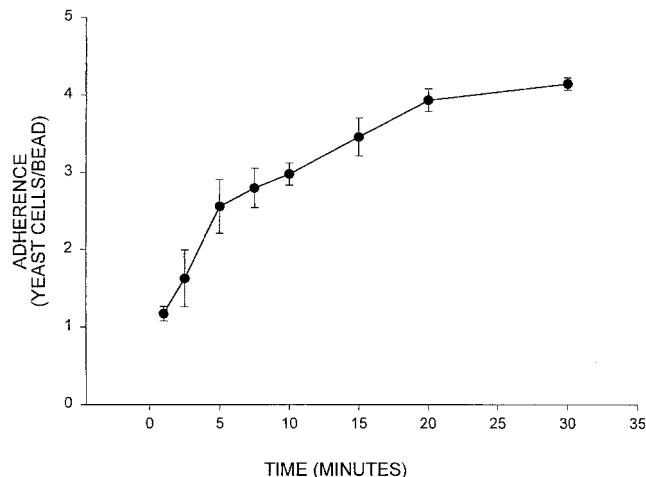


FIG. 3. Curve of *C. albicans* binding to FN-coated magnetic beads. Within minutes, aggregation of cells to the attached cells occurs. The error bar is equal to one standard deviation from the mean.

an appropriate volume of 0.1 N NaOH (0.1 to 2.0 ml). Suspension in NaOH dissociates adherent cells from the magnetic beads. Cells and magnetic beads in each sample were counted four times with a hemacytometer.

**Statistics.** Experiments involving the quantitation of cells were repeated a minimum of three times for each variable. Standard deviation was used as an expression of variation. Comparisons of the means were made by using the Student *t* test, and a *P* value of <0.05 was considered significant.

## RESULTS

**Attachment of yeast cells to ECM-coated magnetic beads induces aggregation of *C. albicans* and *S. cerevisiae* overexpressing *ALA1*. (i) *C. albicans*.** To gain insight into the relationship between attachment and aggregation, we investigated the kinetics of *C. albicans* adherence. Samples were taken at increasing times of incubation. The curve of *C. albicans* binding to FN-coated magnetic beads is shown in Fig. 3. A rapid increase in adherence was observed for the initial 5 min of incubation. The second and slower phase of adherence occurred from 5 to 20 min of incubation. No significant increase in adherence was observed after 20 min of incubation. Samples taken at different times were also observed microscopically. During the first several minutes, cells rapidly attached to magnetic beads but no aggregates formed (the attachment phase). In the second, slower phase, aggregates formed and became increasingly larger in size (the aggregation phase). No aggregation of cells occurred if FN-coated magnetic beads were not added during incubation. Similarly, no aggregation occurred if uncoated magnetic or polystyrene beads of different sizes were incubated with cells. The kinetics of adherence suggest (Fig. 3) and the microscopic observations confirm that the adherence of *C. albicans* cells to an FN-coated magnetic bead is the sum of two distinct steps: attachment (cell-bead interaction) and aggregation (cell-cell interaction). Incubation of *C. albicans* with FN-coated magnetic beads resulted in an average of four to six cells per bead, and aggregates of different sizes can be found (Fig. 4a).

**(ii) *S. cerevisiae* harboring a low- or high-copy *ALA1* plasmid.** *S. cerevisiae* harboring the original vector plasmid without *ALA1*, i.e., pAUR112, did not attach to or aggregate with ECM-coated magnetic beads in any way (Fig. 4b). As reported in our previous study (9), *S. cerevisiae* harboring pGK100, a low-copy *ALA1* plasmid, exhibited attachment but no appreciable aggregation (Fig. 4c). We interpreted these results to be
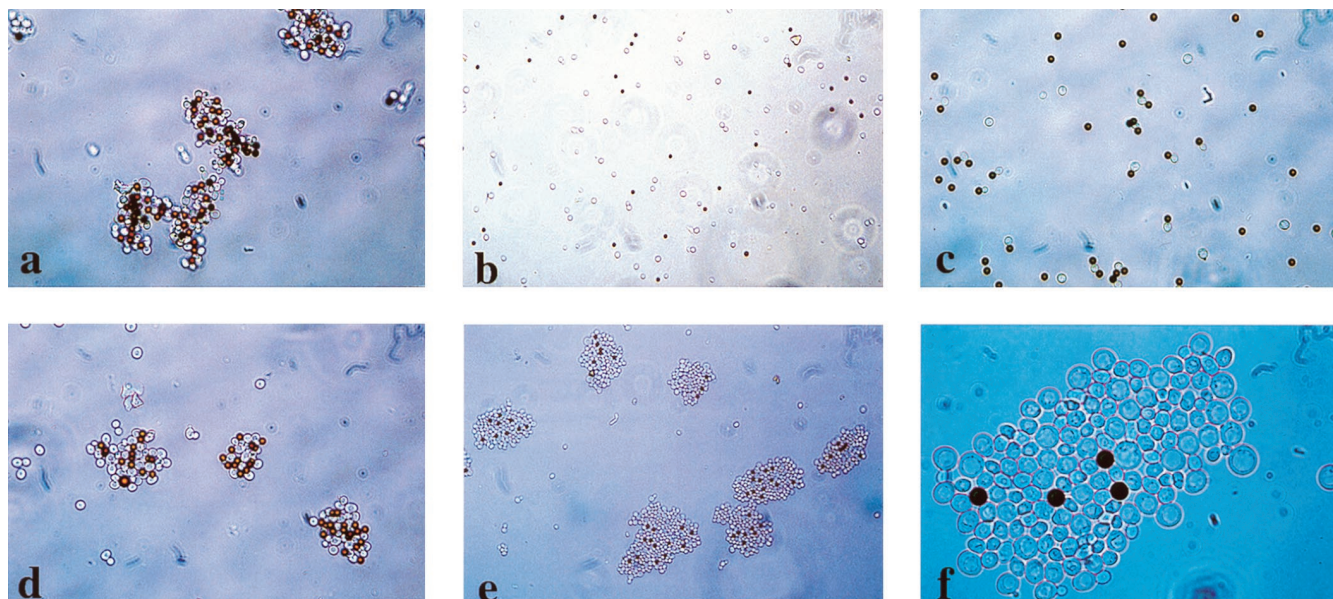
FIG. 4. Photomicrographs of the interaction of fungi with FN-coated magnetic beads. (a) *C. albicans* incubation with FN-coated magnetic beads (orange spheres) results in the formation of large aggregates of cells and beads. (b) *S. cerevisiae* harboring pAUR112 (the vector lacking *ALA1*) does not interact with FN-coated magnetic beads in any way. (c) Incubation of *S. cerevisiae* harboring pGK100 with FN-coated magnetic beads results predominantly in the attachment of one cell to one bead. Aggregates are not formed. This demonstrates the first step, the attachment phase, in adherence. (d) *S. cerevisiae* harboring pGK119, thus expressing greater amounts of *ALA1* than pGK100 (panel c), attaches and goes on to form aggregates with FN-coated magnetic beads, as did *C. albicans* (panel a), demonstrating the aggregation phase of adherence. (e) *S. cerevisiae* with pGK114 grown in the presence of galactose forms large aggregates of cells with FN-coated magnetic beads. Note the lack of aggregation of cells when the magnetic beads do not form a nidus for attachment. (f) Higher-power magnification of *S. cerevisiae* harboring pGK114 grown in galactose adhering to FN-coated magnetic beads. Note that few beads are present, and a remarkable number of cell-cell interactions are formed during the aggregation phase of adherence.

due to the low level of *ALA1* expression in *S. cerevisiae* compared to *C. albicans*. pGK100 is a low-copy plasmid in *S. cerevisiae* and may not have enough gene copies to produce sufficient amounts of Ala1p necessary to exhibit the aggregation phenotype. Therefore, we constructed a high-copy plasmid, pGK119, by cloning *ALA1* in pRS426 that uses a high-copy (2μm) replicon in *S. cerevisiae* (Fig. 1). In contrast to pGK100, *S. cerevisiae* harboring pGK119 exhibited attachment and aggregation phenotypes (Fig. 4d). Quantitative measurement of adherence indicated that expression of *ALA1* from a high-copy plasmid (pGK119) resulted in an approximately threefold increase in adherence compared to the adherence of *S. cerevisiae* carrying the low-copy plasmid, pGK100 (Fig. 5). However, the increase in adherence was not proportional to the expected increase in plasmid copy number, which is at least 20-fold higher for pGK119 than for pGK100 (12). This suggested that transcription from the *ALA1* promoter might be regulated in *S. cerevisiae*.

**(iii) *S. cerevisiae* expressing *ALA1* from a series of galactose-inducible promoters.** In order to solve the problem described above, we investigated the positive effect of increased Ala1p levels on the aggregation phenotype by replacing the putative *ALA1* promoter with a series of galactose-inducible promoters (GAL1, GALL, and GALS) (Fig. 1). GAL1 is a galactose-inducible promoter that contains three upstream activating sequences (UAS1, UAS2, and UAS3) (24). Deletion of UAS3 yields GALL, and deletion of UAS3 and one-half of UAS2 yields GALS. Therefore, GALS is the weakest promoter, GALL is a moderate promoter, and GAL1 is the strongest promoter.

Adherence to FN-coated magnetic beads of *S. cerevisiae* harboring different plasmids containing *ALA1* transcription fusions with GALS, GALL, and GAL1 promoters is shown in Fig. 5. As expected, there was no adherence in all three *ALA1*

fusions when yeast cells were grown in a medium containing glucose as the sole carbon source. *C. albicans* adherence was similar with either glucose or galactose as the carbon source (results not shown). However, when glucose was replaced with galactose, *S. cerevisiae* harboring the pGK117, pGK118, and
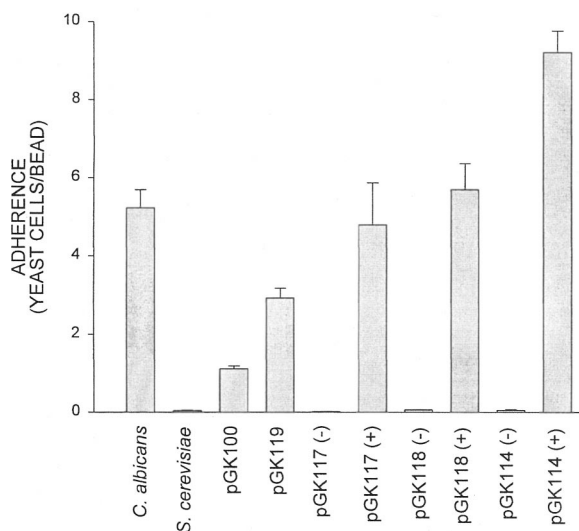


FIG. 5. Adherence of the different fungal strains to FN-coated magnetic beads. *C. albicans* and *S. cerevisiae* YPH499 harboring different plasmids were grown in YPD or YPGal. (See Fig. 1 for plasmid designations.) Adherence is the sum of the attached and aggregated cells. For *S. cerevisiae* strains harboring galactose-inducible *ALA1*, "−" indicates growth under a noninducible condition (glucose as the carbon source or YPD), and "+" indicates growth under an inducible condition (galactose as the carbon source or YPGal). The error bar equals one standard deviation from the mean.

TABLE 1. Relationship between the expression of *ALA1* as determined by the amount of adherence and aggregation of *S. cerevisiae* transformants to FN-coated magnetic beads and the detection of surface Ala1p by Western blot assay and cell surface fluorescence microscopy[a]

| Feature | Amt with *S. cerevisiae* plasmid: | | | |
|---|---|---|---|---|
| | pAUR112 | pGK117 | pGK118 | pGK114 |
| Attachment to beads | − | + | + | + |
| Aggregation of cells | − | ++ | +++ | ++++ |
| Western blot detection with antibody to IgG domain | − | + | ++ | +++ |
| Western blot detection with antibody to S/T domain | − | + | + | + |
| Surface fluorescence using antibody to IgG domain | − | + | + | + |
| Surface fluorescence using antibody to S/T domain | − | − | − | − |

[a] For the immunoglobulin G (IgG) and serine-threonine (S/T) domains of Ala1p, see Fig. 2. Qualitative estimates of the amount of aggregation range from none (−) to extensive, involving hundreds of yeast cells (++++), and qualitative estimates of the amount of protein range from none (−) to the maximum amount (+++) (see Fig. 6).

pGK114 plasmids exhibited adherence directly correlated with the strength of the GAL promoter (Fig. 5). This finding is corroborated by Fig. 6, demonstrating that the amount of surface extractable Ala1 from yeast cells is directly correlated with the strength of the GAL promoter. Aggregation after attachment of *S. cerevisiae* harboring pGK114 to FN-coated magnetic beads is shown in Fig. 4e and f. *S. cerevisiae* harboring pGK114 also attached and aggregated on human buccal epithelial cells (data not shown). As shown in Fig. 5, *S. cerevisiae* harboring pGK114 had more adherent cells (attached and aggregated) than did *C. albicans*. This may be due to the greater amount of Ala1p on the surface of the transformant than on *C. albicans*, thus resulting in greater adherence. The relationship of cell surface Ala1p detected by Western blot assay and cell surface fluorescence to adherence and aggregation is further shown in Table 1. Both antibodies detected Ala1p on a Western blot, with the intensity of the reaction proportional to the strength of the GAL promoter, as shown in Fig. 6. It is interesting that fluorescence did not occur on the cell surface using the anti-
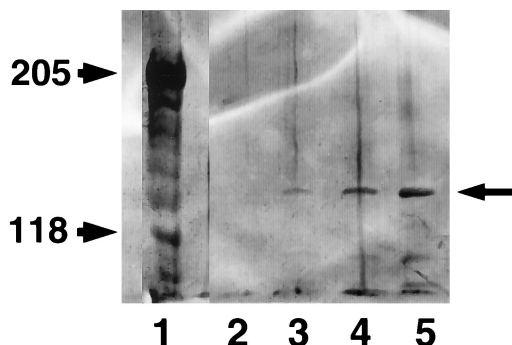


FIG. 6. Immunoblot of Ala1p antigen extracted from the surface of cells demonstrating a direct correlation of the amount of cell surface protein detected and the strength of the GAL promoter. Nitrocellulose was blotted with the antibody to the peptide from the IgG domain. Lane 1, molecular mass markers at 205 and 118 kDa; lane 2, *S. cerevisiae* with the original vector alone (pAUR112; i.e., there is no *ALA1*); lanes 3, 4, and 5, supernatants of strains of *S. cerevisiae* harboring pGK117, pGK118, and pGK114 representing low, medium, and strong GAL promoters, respectively. Ala1p is marked by an arrow and is ca. 135 kDa.
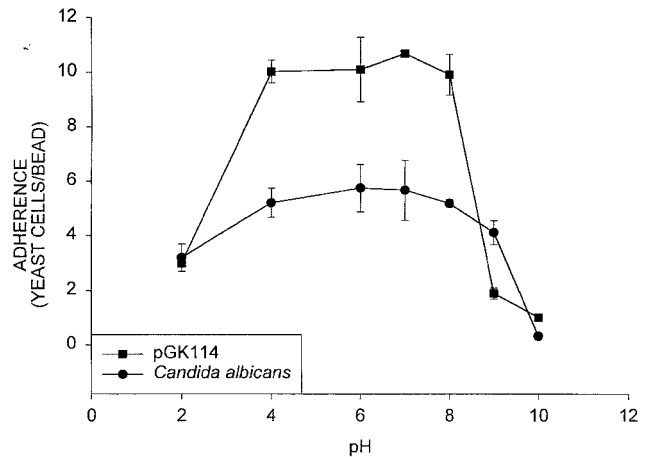


FIG. 7. Effect of pH on adherence and aggregation of *C. albicans* and *S. cerevisiae* harboring pGK114. A standard adherence assay was performed in 100 mM Tris-HCl buffer at the indicated pH value. The error bar equals one standard deviation from the mean. Values for pHs 2, 9, and 10 are significantly different from the nearest neighbor value.

body to the serine-threonine domain, a result likely due to the more interior location of the peptide, the heavy glycosylation which is predicted for this domain, and weaker interaction of this antibody.

The conclusion from these experiments is that the magnitude of adherence is directly correlated with the plasmid copy number and the strength of the galactose-inducible promoter driving the *ALA1* expression.

**Similarities of the adherence properties of *C. albicans* and *S. cerevisiae* overexpressing *ALA1*.** Since overexpression of *ALA1* in *S. cerevisiae* causes aggregation of cells after attachment to FN-coated magnetic beads and human buccal epithelial cells morphologically similar to *C. albicans*, we decided to investigate additional adherence properties.

**(i) Resistance to shear forces.** While developing this assay, we observed an unusual stability of *C. albicans* adherence to FN-coated magnetic beads. The adherent cells were resistant to shear forces generated by vortex mixing at full speed. We therefore compared the stability of adherence of *S. cerevisiae* harboring pGK114, pGK117, and pGK100 with that of *C. albicans*. In this experiment, tubes containing cells adherent to FN-coated magnetic beads were continuously vortexed at full speed for different times. *C. albicans* cells remained adherent to the beads after up to 5 min of vortexing. Similarly, *S. cerevisiae* cells expressing *ALA1* from pGK114, pGK117, or pGK100 remained adherent to the beads after up to 5 min of vortexing. Interestingly, resistance to shear forces was similar in *S. cerevisiae* harboring pGK114 and pGK100, which formed the most and the least aggregates, respectively. These results suggest that the stability of adherence is not affected by the level of Ala1p although, as shown, aggregation is proportional to the levels of Ala1p.

**(ii) Effect of pH.** We next compared the adherence properties of *C. albicans* and *S. cerevisiae* harboring pGK114 at different pH values. As shown in Fig. 7, both organisms exhibited adherence to FN-coated magnetic beads over a wide pH range. In both cases, significant adherence activity was detected at neutral and acidic pH values, and the activity rapidly declined at pH 9 or higher. Both fungi exhibited broad pH optima for adherence, i.e., from pH 4 to pH 8.

**(iii) Adherence to various ECM-coated magnetic beads.** We also compared the adherence of *C. albicans* and *S. cerevisiae*
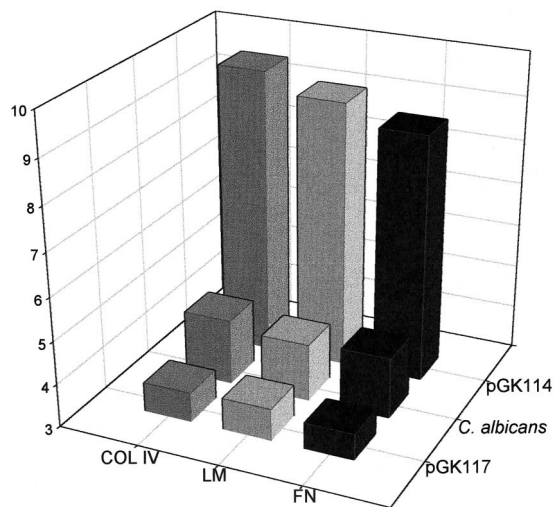
FIG. 8. Comparison of adherence of *C. albicans* and *S. cerevisiae* harboring pGK114 or pGK117 to magnetic beads coupled with the indicated proteins.

harboring pGK114 or pGK117 to FN-, LM-, and COL IV-coated magnetic beads. These are well-studied ligands that, when immobilized, allow *Candida* adherence (18). As shown in Fig. 8, the degrees of adherence of *C. albicans* and *S. cerevisiae* harboring pGK114 or pGK117 to ECM-coated magnetic beads were similar.

**(iv) Effects of additives on adherence and aggregation.** We compared the effects of different additives on adherence to FN-coated magnetic beads. Results obtained with *C. albicans* and *S. cerevisiae* overexpressing *ALA1* (pGK114) were qualitatively identical. The results with *S. cerevisiae* with pGK114 are shown in Fig. 9. The presence of 1% glucose, 1% galactose, 1% mannose, or 1 M NaCl during the adherence of *C. albicans* and *S. cerevisiae* overexpressing *ALA1* had no significant effect compared to the control, where no additive was present. We next determined the effect of the addition of the soluble ECM proteins FN, LM, and COL IV on adherence. As shown in Fig. 9, the addition of 100 μg of FN and COL IV per ml had no significant effect on adherence of *C. albicans* and *S. cerevisiae* expressing *ALA1*. Similarly, the addition of 1% L-fucose and 0.1% (vol/vol) Tween 20 had no effect on adherence (results not shown). However, there was a consistent negative effect of LM on the adherence of *C. albicans* and *S. cerevisiae* overexpressing *ALA1*. Reagents that disrupt hydrogen bonds, such as 50% formamide or 6 M urea, or a high pH caused the greatest reductions in the adherence of *C. albicans* and *S. cerevisiae* overexpressing *ALA1* (Fig. 9). Although FN-coated magnetic beads were used in these studies, similar results were observed with other ECM-coated magnetic beads.

To further understand the mechanism of the additive agents, *C. albicans* and *S. cerevisiae* harboring pGK114 were preincubated with the additives for 30 min and washed thoroughly to remove the unbound additives, and then the adherence assay was performed. An inhibitory effect on adherence by high pH buffer is maintained in preincubated cells. In contrast, preincubation of cells in 50% formamide, 6 M urea, and 100 μg of LM per ml did not retain their respective inhibitory effects on adherence. These results suggest that the effects of formamide, urea, and LM on adherence are not likely to be caused by permanent alteration of cell surface. In contrast, incubation at high pH causes permanent changes to the cell surface resulting in the inhibition of adherence.

## DISCUSSION

*C. albicans* maintains long-term commensal relationships with a number of hosts presumably, in part, by its ability to adhere to a variety of biological surfaces. It can be cultured from mucosal surfaces with great ranges in pH values. The residence of *C. albicans* on these mucosal surfaces is resistant to competition from other microorganisms, the presence of a variety of potentially competing molecules in biological fluids, and the shear forces generated by the flow of body fluids (26). In this report we characterize a form of *C. albicans* adherence consisting of two sequential steps: an attachment phase followed by an aggregation phase. This type of adherence is noteworthy for its occurrence in acidic to neutral pH ranges, its resistance to strong shear forces, and the presence of numerous competing molecules and applies to human cells and proteins, alike. It is possible that this type of adherence endows *C. albicans* with the ability to maintain a long-term commensal relationship with the host.

Our results imply that the expression of *ALA1* and *ALA1*-like genes is responsible for a type of adherence in *C. albicans* characterized by attachment and aggregation of yeast cells. The presence of the *ALS* gene family in *C. albicans* is inferred by the hybridization of multiple genomic DNA fragments to an *ALS* family-specific probe (15). The complete sequences of three members of the gene family, *ALA1*, *ALS1*, and *ALS3*, have been reported, but only *ALA1* and *ALS1* have been functionally characterized (6, 9). We isolated *ALA1* by its ability to confer adherence properties on *S. cerevisiae* transformants for ECM-coated magnetic beads (9), and Fu et al. isolated *ALS1* by its ability to cause *S. cerevisiae* transformants to adhere to endothelial and epithelial cells when the gene was expressed from a galactose-inducible promoter (6). We have found that *ALS1* behaves like *ALA1* in our assay (unpublished results). This adherence function appears to be reserved for *Candida* agglutinin-like proteins, since *S. cerevisiae* expressing α-agglutinin (encoded by *AGα1*, to which Ala1p shows similarity in the N terminus) does not adhere to ECM-coated magnetic beads (unpublished results).

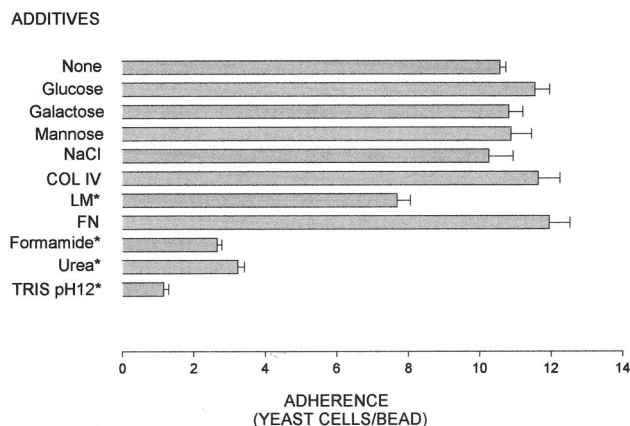Adherence of *C. albicans* to the ECM proteins has been well



FIG. 9. Effects of different additives on the attachment and aggregation of *S. cerevisiae* harboring pGK114. Additives were included in the adherence assay before the addition of yeast cells. The final concentrations of the additives were as follows: 1% each of D-glucose, D-galactose, or D-mannose; 1 M NaCl; 100-μg/ml concentrations of COL IV, LM, and FN; 50% formamide; 6 M urea; 100 mM Tris-HCl (pH 12.0). Data for L-fucose (1%) and Tween 20 at 0.1% (vol/vol) are not shown, but neither caused the inhibition of adherence or aggregation. The error bar equals one standard deviation from the mean. Significant differences ($P < 0.05$) from the control mean value (None) are marked with asterisks.

described by numerous groups and obviously encompasses the attachment step we describe. However, the aggregation of adherent *C. albicans* is also a well-recognized phenomenon, particularly in in vitro experiments (21, 22). The term aggregation has been used to describe the interaction of *C. albicans* germ tubes with one another (14, 27), and the term coaggregation has been used to describe yeast cells adhering to bacteria (16). Here, the term aggregation is reserved for yeast cell-cell interactions which occur when the microorganisms are already attached to a substrate. Cell-cell interactions that occur in suspension are more appropriately termed flocculation. Aggregation of *C. albicans* has been noted with such adherence targets as ex vivo porcine endothelium (19), human intestinal epithelial cells (22), and human buccal epithelial cells (for a full discussion, see reference 25). Aggregation as it occurs in our assay is the second of two steps in the adherence of cells to a target. First, cells attach to an appropriate target, such as an ECM-coated magnetic bead. The attachment of cells to these beads is a transitional step that, with or without the second step, culminates in what is measured as adherence. For example, *S. cerevisiae* harboring pGK100 apparently does not express sufficient amounts of Ala1p, and thus, cells harboring this low-copy plasmid attach to the beads but do not aggregate. However, when cells express sufficient amounts of Ala1p, both attachment and aggregation occur and the high levels of Ala1p increase the magnitude of adherence by increasing the extent of aggregation. Not all forms of cell adherence lead to aggregation. For example, adherence of *C. albicans* to uncoated plastic beads, cationic beads, or an oil-water interface does not result in aggregation (17, 20). The implication is that the form of adherence that we describe here, involving attachment and aggregation mediated by Ala1p, somehow involves specific recognition of the target surface by the cells.

Aggregation as described here is different from flocculation, a form of yeast cell-cell interaction governed by cell surface lectins and dependent upon the presence of divalent cations (30). The strain of *S. cerevisiae* used in our studies is readily induced to floc by the addition of $Ca^{2+}$, and this is reversed upon the addition of EDTA. However, even when flocculation is induced in *S. cerevisiae* by the addition of $Ca^{2+}$, the cells do not interact in any way with the ECM-coated magnetic beads. However, in flocculation, due to the sheer mass of the microorganisms forming a floc, cells may become physically entrapped and thus masquerade as adherent microorganisms, although this form of interaction did not occur in our assay.

The attachment step of adherence in our assay is due solely to the presence of Ala1p on the surface of the cell—without this protein *S. cerevisiae* is unable to adhere to the protein-coated beads. The domain of Ala1p involved in attachment is unknown. Similarly, how Ala1p effects aggregation is unknown. Perhaps aggregation is due to Ala1p-Ala1p interactions of separate cells or, alternatively, to the Ala1p–non-Ala1p cell wall interactions of adjacent cells. It is also possible that different domains of Ala1p are involved in adherence, one for attachment and another for aggregation.

The adherence properties of *C. albicans* and *S. cerevisiae* overexpressing *ALA1* are very similar. In both cases adherence can be observed over a wide pH range and is resistant to strong shear forces and to exogenously added competing molecules, such as sugars, free ligands, and detergents. It is likely that *C. albicans* on certain occasions utilizes *ALA1* or *ALA1*-like genes to effect adherence to a host surface. The properties of *ALA1*-mediated adherence suggest that this mechanism is well suited to maintaining long-term residence in the host. The aggregation step may assist in the colonization of the host by recruiting

cells to interact with small numbers of cells that have previously attached to tissue (26).

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