EUKARYOTIC CELL, Jan. 2006, p. 140–147 Vol. 5, No. 1
1535-9778/06/$08.00+0 doi:10.1128/EC.5.1.140–147.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

# *Candida albicans* Ecm33p Is Important for Normal Cell Wall Architecture and Interactions with Host Cells

Raquel Martinez-Lopez,[1] Hyunsook Park,[2] Carter L. Myers,[2] Concha Gil,[1] and Scott G. Filler[2,3]*

*Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, 28040-Madrid, Spain[1]; St. Johns Cardiovascular Research Center, Division of Infectious Diseases, Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California 90502[2]; and The David Geffen School of Medicine at UCLA, Los Angeles, California 90024[3]*

Received 2 September 2005/Accepted 8 November 2005

***Candida albicans ECM33*** **encodes a glycosylphosphatidylinositol-linked cell wall protein that is important for cell wall integrity. It is also critical for normal virulence in the mouse model of hematogenously disseminated candidiasis. To identify potential mechanisms through which Ecm33p contributes to virulence, we investigated the interactions of *C. albicans ecm33*Δ mutants with endothelial cells and the FaDu oral epithelial cell line in vitro. The growth rate of blastospores of strains containing either one or no intact copies of *ECM33* was 50% slower than that of strains containing two intact copies of *ECM33*. However, all strains germinated at the same rate, forming similar-length hyphae on endothelial cells and oral epithelial cells. Strains containing either one or no intact copies of *ECM33* had modestly reduced adherence to both types of host cells, and a markedly reduced capacity to invade and damage these cells. *Saccharomyces cerevisiae* expressing *C. albicans ECM33* did not adhere to or invade epithelial cells, suggesting that Ecm33p by itself does not act as an adhesin or invasin. Examination of *ecm33*Δ mutants by transmission electron microscopy revealed that the cell wall of these strains had an abnormally electron-dense outer mannoprotein layer, which may represent a compensatory response to reduced cell wall integrity. The hyphae of these mutants also had aberrant surface localization of the adhesin Als1p. Collectively, these results suggest that Ecm33p is required for normal cell wall architecture as well as normal function and expression of cell surface proteins in *C. albicans*.**

*Candida albicans* causes both hematogenously disseminated and oropharyngeal disease. During hematogenously disseminated candidiasis, the blood-borne organisms must adhere to and penetrate the endothelial cell lining of the blood vessels to invade the deep tissues of the target organs. These target organs include the kidney, liver, spleen, heart, brain, and eye (6). During oropharyngeal candidiasis, *C. albicans* not only adheres to the epithelial cells of the oral mucosa, but also invades these cells. Invasion into the epithelial cell lining of the oral mucosa is characteristic of both human and experimental animal models of oropharyngeal candidiasis (3, 7, 15, 18, 23).

The mechanism by which *C. albicans* invades endothelial cells and oral epithelial cells has been investigated in vitro. These in vitro studies demonstrate that one mechanism by which *C. albicans* invades both endothelial cells and oral epithelial cells is by inducing its own endocytosis (8, 20, 21). Once *C. albicans* is endocytosed by either endothelial cells or oral epithelial cells, it damages these cells. In vitro, host cell damage requires the endocytosis of live organisms (8, 20). Importantly, we have found that mutants of *C. albicans* that are endocytosed poorly and cause little damage to endothelial cells and oral epithelial cells have significantly reduced virulence in mouse models of hematogenously disseminated or oropharyngeal candidiasis (20, 21, 25). The association between the inability of these strains to invade and damage host cells in vitro and their attenuated virulence suggests that fungal invasion and damage of endothelial cells and oral epithelial cells contribute to the pathogenesis of hematogenously disseminated and oropharyngeal candidiasis, respectively.

The cell wall represents the initial point of interaction between the host and pathogen. The *C. albicans ECM33* gene product is predicted to be a glycosylphosphatidylinositol (GPI)-linked cell wall protein (5). Recently, it was found that Ecm33p is required for normal cell wall integrity and the yeast-to-hypha transition in vitro (16). Blastospores of a *C. albicans ecm33*Δ/*ecm33*Δ mutant were larger and flocculated more extensively than blastospores of the wild-type strain. The mutant also exhibited enhanced susceptibility to compounds, such as calcofluor white and Congo red, that interfere with cell wall integrity. In addition, the *ecm33*Δ/*ecm33*Δ mutant had delayed hypha formation in liquid and solid media. Consistent with these in vitro defects, this mutant had severely attenuated virulence in the mouse model of hematogenously disseminated candidiasis. Finally, these investigations showed that both copies of *ECM33* were required for a normal phenotype. The phenotype of strains that contained only one copy of *ECM33* was more similar to that of the homozygous *ecm33*Δ/*ecm33*Δ mutant than to that of strains that contained two copies of *ECM33*.

We hypothesized that an additional reason Ecm33p is required for normal virulence is that this protein is necessary for specific interactions of *C. albicans* with endothelial and oral epithelial cells. Therefore, we investigated the role of Ecm33p in the ability of *C. albicans* to adhere to, invade, and damage endothelial cells and an oral epithelial cell line in vitro.

* Corresponding author. Mailing address: Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, 1124 W. Carson St., Torrance, CA 90502. Phone: (310) 222-3813. Fax: (310) 782-2016. E-mail: sfiller@ucla.edu.

TABLE 1. Strains of *C. albicans* and *S. cerevisiae* used in this study

| Species | Strain | Relevant genotype | Doubling time (h) | Reference |
|---|---|---|---|---|
| *C. albicans* | CAI4 | *ECM33/ECM33 ura3Δ::imm434/ura3Δ::imm434* | | 9 |
| | CAI4-URA | *ECM33/ECM33 ura3Δ::imm434/ura3Δ::imm434::URA3* | 1.3 | 20 |
| | RML1a | *ECM33/ecm33Δ::hisG ura3Δ::imm434/ura3Δ::imm434* | | 16 |
| | RML1U | *ECM33/ecm33Δ::hisG ura3Δ::imm434/ura3Δ::imm434::URA3* | 2.0 | This study |
| | RML2a | *ecm33Δ::hisG/ecm33Δ::hisG ura3Δ::imm434/ura3Δ::imm434* | | 16 |
| | RML2U | *ecm33Δ::hisG/ecm33Δ::hisG ura3Δ::imm434/ura3Δ::imm434::URA3* | 2.0 | This study |
| | RML3a | *ecm33Δ::hisG/ecm33Δ::hisG::ECM33-cat ura3Δ::imm434/ura3Δ::imm434* | | 16 |
| | RML3U | *ecm33Δ::hisG/ecm33Δ::hisG::ECM33-cat ura3Δ::imm434/ura3Δ::imm434::URA3* | 2.0 | This study |
| | RML4 | *ecm33Δ::hisG::ECM33-cat/ecm33Δ::hisG::ECM33-cat-URA3-cat ura3Δ::imm434/ura3Δ::imm434* | | 16 |
| | RML4a | *ecm33Δ::hisG::ECM33-cat/ecm33Δ::hisG::ECM33-cat ura3Δ::imm434/ura3Δ::imm434* | | This study |
| | RML4U | *ecm33Δ::hisG::ECM33-cat/ecm33Δ::hisG::ECM33-cat ura3Δ::imm434/ura3Δ::imm434::URA3* | 1.3 | This study |
| | CAYF4 | *als1Δ::hisG/als1Δ::hisG ura3Δ::imm434/ura3Δ::imm434::URA3* | ND[a] | Unpublished |
| *S. cerevisiae* | BY4741 | *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | ND | EUROSCARF |
| | Y03215 | *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ecm33::kanMX4* | ND | EUROSCARF |

[a] ND, not determined.

## MATERIALS AND METHODS

**Organisms, media, and plasmids.** The *C. albicans* and *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. All *C. albicans* strains were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) agar plates at 37°C. This medium was supplemented with 80 μg of uridine per ml for growing $Ura^-$ strains. $Ura^+$ transformants were selected on synthetic complete medium without uridine (2% glucose, 0.67% yeast nitrogen base without amino acids, 0.065% synthetic complete supplement mixture without uridine) (Qbiogene, Carlsbad, CA). To select $Ura^-$ strains, the organisms were grown on synthetic complete medium containing uridine and 0.1% 5-fluoroorotic acid (Zymo Research, Orange, CA). For use in the experiments, all organisms were grown in liquid YPD medium on a rotary shaker overnight at 30°C, harvested by centrifugation, and washed twice in Dulbecco's phosphate-buffered saline (PBS). The organisms were resuspended to the appropriate concentration in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA).

**Strain construction.** The *C. albicans ECM33* mutants used in the previous study all had *URA3* integrated at the *ECM33* locus (16). The chromosomal locus at which *URA3* is integrated can influence the proteome of *C. albicans*, its adherence to host cells in vitro, and its virulence (2, 4, 28, 29). Therefore, to avoid the potential confounding effects of the chromosomal location of *URA3* on the phenotypes of the *ECM33* mutants, we modified them so that *URA3* was integrated at its native locus in all strains. The *ura3Δ/ura3Δ* strains RML1a (*ECM33/ecm33Δ*), RML2a (*ecm33Δ/ecm33Δ*), and RML3a (*ecm33Δ/ecm33Δ::ECM33*) were transformed with a 3.9-kb NheI/PstI fragment encompassing the *C. albicans URA3* and adjacent *IRO1* genes, as described previously (20), to produce RML1U, RML2U, and RML3U, respectively. Integration of the *URA3-IRO1* fragment into the correct chromosomal locus was confirmed by PCR using the primers 5′-TGCTGGTTGGAATGCTTATTTG-3′ and 5′-TGCAAATTCTGCTACTG GAGTT-3′.

A $Ura^-$ clone of strain RML4 (*ecm33Δ::ECM33/ecm33Δ::ECM33*) was selected by plating it on 5-fluoroorotic acid. This $Ura^-$ clone was named RML4a. The absence of *URA3* in RML4a was confirmed by PCR using the primers 5′-TAGGACGTGACAAGATACAGGATCGCA-3′ and 5′-GTTTTCCGCCA TCGCAATCAGGC-3′. Next, RML4a was transformed with the *URA3-IRO1* fragment as outlined above to make RML4U. The integration of *URA3* into the correct locus was confirmed by Southern blotting (Fig. 1).

**Growth rate determination.** The doubling times of the *C. albicans* strains were determined by growing them in liquid YPD medium on a rotary shaker at 30°C. At 1-hour intervals, an aliquot was removed and sonicated briefly, and then the optical density at 600 nm was measured.

**Expression of *C. albicans ECM33* in *S. cerevisiae*.** To analyze the effects of expression of *C. albicans ECM33* (Ca*ECM33*) in *S. cerevisiae*, the plasmid pYEPCaECM33, which contained the Ca*ECM33* protein coding sequence, 330 bp of promoter sequence, and 495 bp of 3′ untranslated region, was used (16). As a control, plasmid pYEPScerevisiaeECM33, containing the protein coding sequence of *S. cerevisiae ECM33* (Sc*ECM33*), 1,102 bp of promoter, and 565 bp of 3′ untranslated region, was used (16). An additional control was the empty plasmid pYEP352. The plasmids containing Ca*ECM33* and Sc*ECM33* were transformed into strains of *S. cerevisiae* with either an intact copy of Sc*ECM33* (strain BY4741) or a disrupted copy of this gene (strain Y03215) (Table 1).

**Endothelial cells and oral epithelial cells.** Endothelial cells were isolated from the veins of human umbilical cords by the method of Jaffe et al. (14). They were grown in M-199 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 10% defined bovine calf serum (both from Gemini BioProducts, Inc., Calabasas, CA), and supplemented with 2 mM L-glutamine with penicillin and streptomycin (Irvine Scientific) as described previously (8).

The FaDu oropharyngeal epithelial cell line was purchased from the American Type Culture Collection (Manassas, VA). It was maintained in Eagle's minimal essential medium with Earle's balanced salt solution (Irvine Scientific) adjusted to contain 1.5 g/liter sodium bicarbonate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate. This medium was supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Both cell types were grown at 37°C in a humidified environment containing 5% $CO_2$ and used at 95% confluence in all experiments.

**Endocytosis assay.** The number of organisms that were endocytosed by the endothelial cells and FaDu oral epithelial cells was determined using a differential fluorescence assay exactly as described previously (20, 22). Briefly, endothelial cells or FaDu cells were grown on fibronectin-coated glass coverslips and infected with $10^5$ cells of either *C. albicans* or *S. cerevisiae* in RPMI 1640 medium. After incubating for 90 min, the cells were rinsed once with Hanks' balanced salt solution (Irvine Scientific) and then fixed in 3% paraformaldehyde. The nonendocytosed organisms were stained with an anti-*C. albicans* rabbit serum (Biode-

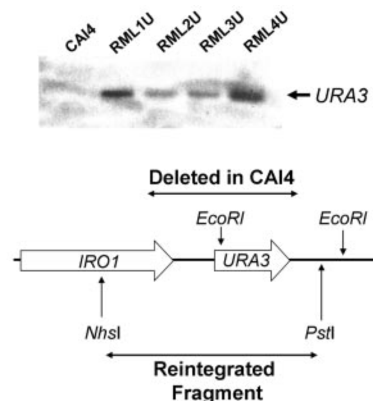


FIG. 1. Southern blot analysis of restoration of the *URA3* locus in the various *C. albicans* strains. Genomic DNA from the indicated strains was digested with EcoRI. and the blot was probed with an EcoRI fragment encompassing *URA3* and its 3′-flanking region.

sign International, Kennebunkport, ME) conjugated with Alexa 568 (Molecular Probes, Eugene, OR). This antiserum recognizes both *C. albicans* and *S. cerevisiae* (27). Afterwards, the FaDu cells were rinsed extensively with PBS and then permeabilized with 0.2% (vol/vol) Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS. Next, the cell-associated organisms (the endocytosed plus nonendocytosed organisms) were stained with the anti-*C. albicans* rabbit serum conjugated with Alexa 488 (Molecular Probes). The coverslips were observed with an epifluorescence microscope. The number of organisms endocytosed by the host cells was determined by subtracting the number of cell-associated organisms (labeled with Alexa 568, which fluoresces red) from the total number of organisms (labeled with Alexa 488, which fluoresces green). At least 100 organisms were counted on each coverslip, and all experiments were performed in triplicate.

**Damage assay.** The extent of damage caused by the various *C. albicans* strains to the endothelial cells and the FaDu cell line was measured using a $^{51}$Cr release assay as described previously (1, 20, 21). The host cells were grown in a 96-well tissue culture plate with detachable wells and incubated overnight with $Na_2{}^{51}CrO_4$ (MP Biomedicals, Inc., Irvine, CA) per well. The following day, the unincorporated tracer was removed by rinsing. When endothelial cells were used, they were infected with $4 \times 10^4$ organisms in RPMI 1640. Because FaDu cells are less susceptible to damage by *C. albicans*, they were infected with $10^5$ organisms in the same medium. To measure the spontaneous release of $^{51}$Cr, uninfected host cells were exposed to medium alone. After a 3-h incubation, the upper 50% of medium was removed from each well and then the wells were manually detached from one another. The amount of $^{51}$Cr in the aspirates and the well was determined by gamma counting. After correcting for the amount of $^{51}$Cr incorporated in each well, the specific release of $^{51}$Cr was calculated by the formula (experimental release − spontaneous release)/(total incorporation − spontaneous release). Experimental release was the amount of $^{51}$Cr released into the medium by cells infected with *C. albicans*. Spontaneous release was the amount of $^{51}$Cr released into the medium by uninfected host cells. Total incorporation was the sum of the amount of $^{51}$Cr released into the medium and remaining in the host cells.

**Transmission electron microscopy.** Samples for transmission electron microscopy were prepared according to the protocol described by Miret et al. (17). Briefly, $2 \times 10^7$ blastospores of each strain were collected after 24 h of growth in liquid YPD medium and fixed at 4°C for 24 h in 500 μl fixative solution (sodium cacodylate buffer, pH 7.2, containing 1.2% glutaraldehyde and 2% paraformaldehyde). The samples were then washed with saline and postfixed for 90 min with 1% potassium permanganate. The fixed cells were dehydrated through a graded series of ethanol and embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA). Thin sections were stained with uranyl acetate and lead citrate and then imaged with a Zeiss EM902 electron microscope. Multiple cells of each strain were imaged.

**Detection of Als1p on the surface of *C. albicans*.** The localization of the adhesin, Als1p on the surface of the various strains of *C. albicans* was determined by direct immunofluorescence using a modification of our previously described method (10). Briefly, $2 \times 10^5$ blastospores of the different *C. albicans* strains were incubated for 90 min with endothelial cells on glass coverslips as in the endocytosis assay. Next, the cells were rinsed once with Hanks' balanced salt solution, fixed with 3% paraformaldehyde, and blocked with 1% goat serum containing 1% Triton X-100. The cells were incubated with an Alexa 488-conjugated murine monoclonal antibody directed against the N terminus of Als1p (10). After extensive rinsing with PBS, the cells were stained with the Alexa 568-conjugated rabbit anti-*C. albicans* antiserum and viewed by confocal microscopy. To enable comparison of the intensity of staining among the different strains, the image acquisition parameters were set using the wild-type strain and these parameters were used for imaging all of the other strains. The final confocal images were produced by combining optical sections taken through the *z* axis.

To determine if some fraction of Als1p was not detected by the anti-Als1p antibody because it was buried in the cell wall, $3 \times 10^6$ blastospores of the different strains were added to endothelial cells in a six-well tissue culture plate and allowed to germinate for 90 min. Next, the medium was aspirated and replaced with distilled water to lyse the endothelial cells. The germ tubes were scraped from the wells into 1.5-ml centrifuge tubes, fixed in 3% paraformaldehyde for 15 min, and washed twice with PBS. They were resuspended in KS buffer (0.1 M potassium phosphate buffer, 1 M sorbitol, pH 7.0,) containing 0.5 units of zymolyase (Zymo Research Corporation, Orange, Calif.) per ml. The germ tubes were allowed to settle onto poly-L-lysine coated coverslips where they were incubated for 5 to 30 min. At each time point, the coverslips were washed three times with KS buffer, blocked with 1% bovine serum albumin in PBS, and then stained with the anti-Als1p monoclonal antibody, followed by the rabbit anti-*C. albicans* antiserum as above.

**Statistical analysis.** Differences among the various strains of *C. albicans* in their interactions with endothelial cells and the FaDu oral epithelial cell line were compared by analysis of variance. *P* values of ≤0.05 were considered significant.

## RESULTS

**Strains lacking one or both copies of *ECM33* had slower growth rates in vitro than strains containing two copies of *ECM33*.** Strains of *C. albicans* that grow slowly in vitro frequently have attenuated virulence when tested in the mouse model of hematogenously disseminated candidiasis (24). Growth rate is also likely to be an important factor for virulence during oropharyngeal candidiasis. Therefore, we determined the growth rates of blastospores of the various *ECM33* strains in vitro. The doubling time of all *C. albicans* strains containing either one or no copies of *ECM33* was approximately 50% longer than that of either the wild-type strain or the *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* double-complemented strain (Table 1). Therefore, both copies of *ECM33* are critical for the normal growth of *C. albicans* blastospores in vitro and probably in vivo.

**Hyphal formation on endothelial cells and the FaDu oral epithelial cell line is independent of *ECM33*.** The slow growth rates of the *ecm33*Δ strains precluded a meaningful analysis of the contribution of Ecm33p to virulence in relevant animal models of candidiasis. Therefore, for a surrogate for virulence studies, we examined the interactions of the *ECM33* mutants with endothelial cells and an oral epithelial cell line in vitro. Although the organisms were added to these host cells as blastospores, they began germinating within 45 min. Previously, it was reported that *ecm33*Δ mutants had filamentation defects on some liquid and solid media (16). Interestingly, we observed that all of the mutant strains produced normal length hyphae on the endothelial cells and oral epithelial cells after 90 min of incubation (Fig. 2). Therefore, the slow doubling time of the blastospores did not translate into delayed hyphal formation or elongation under the conditions used in the assays.

***ECM33* is required for maximal *C. albicans* adherence to and invasion of endothelial cells and the FaDu oral epithelial cell line.** The capacity of each *ECM33* mutant strain to adhere to endothelial cells and the FaDu oral epithelial cell line after 90 min of infection was investigated. Fewer organisms of the heterozygous *ecm33*Δ/*ECM33* mutant were cell-associated (defined as the sum of the adherent and endocytosed organisms) with either type of host cell compared to the wild-type strain (Fig. 3). The number of cell-associated organisms was even lower when the host cells were infected with the homozygous *ecm33*Δ/*ecm33*Δ strain, suggesting that there was a gene dosage effect. When the homozygous *ecm33*Δ/*ecm33*Δ strain was complemented with a single copy of wild-type *ECM33*, the number of organisms that were cell associated with either type of host cell was similar to that of the heterozygous *ecm33*Δ/*ECM33* mutant. Complementing the homozygous *ecm33*Δ/*ecm33*Δ strain with two copies of *ECM33* resulted in a phenotype that was indistinguishable from that of the wild-type strain. These results indicate that both copies of *ECM33* are required for maximal adherence to both endothelial cells and oral epithelial cells.

Strains containing either one or no copies of *ECM33* were also endocytosed poorly by both endothelial cells and FaDu

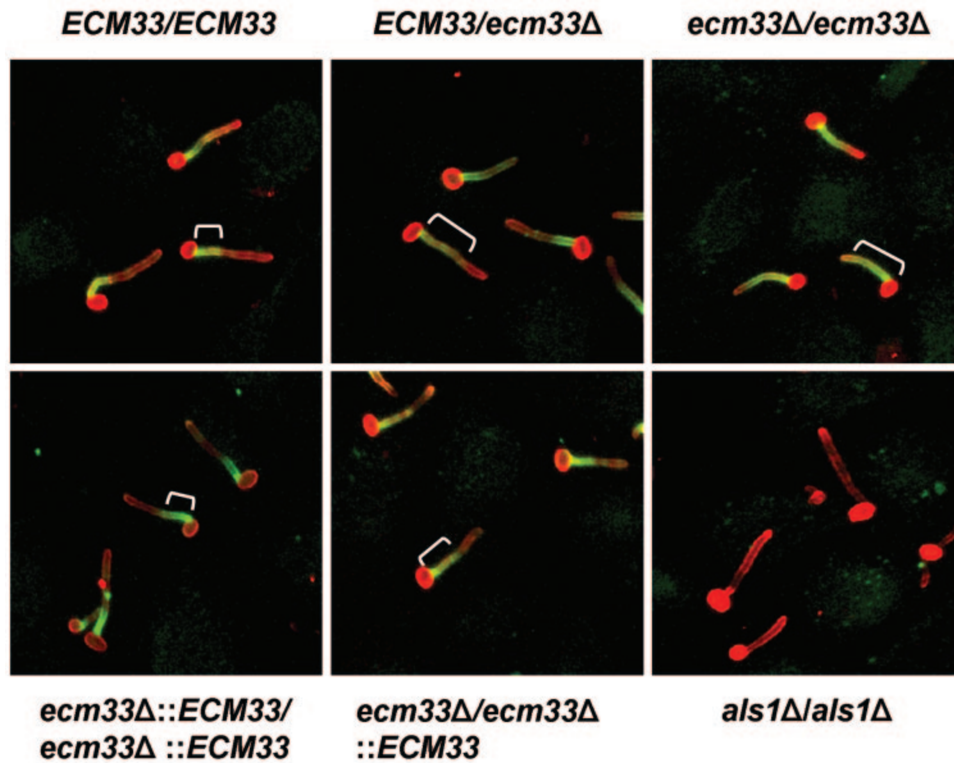


FIG. 2. *ECM33* is not required for hyphal formation on endothelial cells, but it does influence the localization of Als1p on the hyphal surface. Endothelial cells were infected with the indicated strains of *C. albicans*. After 90 min, the cells were fixed and stained with an anti-Als1p monoclonal antibody (green) and an anti-*C. albicans* antiserum (red). The cells were viewed by confocal microcopy and the images were produced by merging the red and green channels. The white brackets indicate the extent of Als1p expression on the surface of the hyphae.

oral epithelial cells (Fig. 3). The homozygous *ecm33*Δ/*ecm33*Δ strain had a greater endocytosis defect than did the strains containing a single copy of wild-type *ECM33*. Paralleling the adherence results, the endocytosis defect of the *ecm33*Δ/*ecm33*Δ:: *ECM33* singly complemented strain was similar to that of the *ECM33*/*ecm33*Δ heterozygous strain, whereas the *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* double-complemented strain had no endocytosis defect.

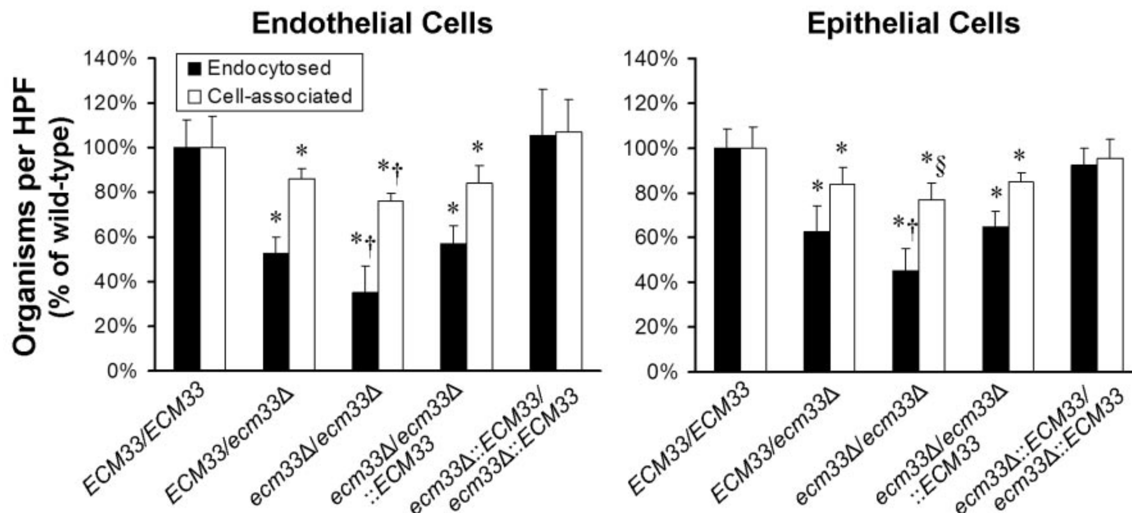


FIG. 3. Two copies of *ECM33* are required for maximal *C. albicans* adherence to and endocytosis by endothelial cells and FaDu oral epithelial cells. The indicated strains of *C. albicans* were incubated with either endothelial cells or the FaDu oral epithelial cell line for 90 min, after which the numbers of endocytosed and cell-associated organisms were determined by a differential fluorescence assay. Results are the mean ± standard deviation of three experiments, each performed in triplicate. *, $P < 0.01$ compared to either the wild-type *ECM33*/*ECM33* or the *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* strain. †, $P < 0.02$ compared to all other strains. §, $P = 0.06$ compared to the *ECM33*/*ecm33*Δ strain and $P < 0.02$ compared to all other strains. HPF, high-power field.

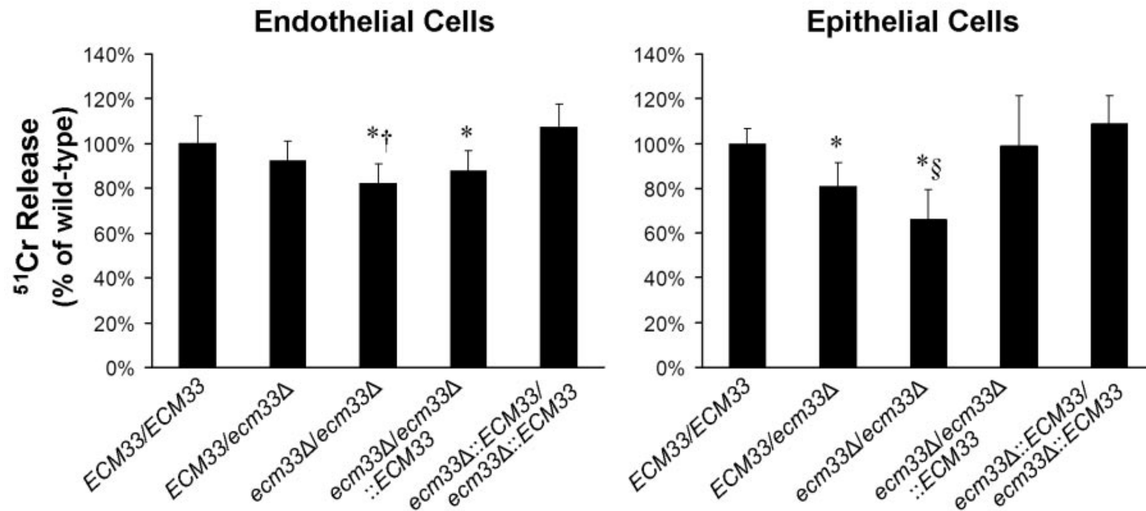


FIG. 4. Role of *ECM33* in *C. albicans*-induced damage to endothelial cells and FaDu oral epithelial cells. The indicated host cells were loaded with $^{51}$Cr and then incubated with the indicated strains of *C. albicans* for 3 h. The extent of host cell damage was measured by the release of $^{51}$Cr into the medium. Results are the mean ± standard deviation of at least independent experiments, each performed in triplicate. *, $P < 0.04$ compared to both the wild-type *ECM33*/*ECM33* and the *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* strain. †, $P = 0.03$ compared to the *ecm33*Δ/*ECM33* strain and $P = 0.19$ compared to the *ecm33*Δ/*ecm33*Δ::*ECM33* strain. §, $P < 0.002$ compared to both the *ECM33*/*ecm33*Δ and the *ecm33*Δ/*ecm33*Δ::*ECM33* strains.

For strains containing either one or no intact copies of *ECM33*, the reduction in endocytosis was greater than the reduction in adherence (Fig. 3). For example, 24% fewer cells of the homozygous *ecm33*Δ/*ecm33*Δ mutant were cell associated with endothelial cells, compared to the wild-type strain. In contrast, 65% fewer cells of the *ecm33*Δ/*ecm33*Δ mutant were endocytosed by endothelial cells compared to the wild-type strain. Therefore, the endocytosis defect of the *ecm33*Δ strains was not merely the result of decreased adherence.

***ECM33* is essential for *C. albicans* to cause maximal damage to oral epithelial cells in vitro.** We compared the extent of damage caused by the different *C. albicans* strains to endothelial cells and FaDu oral epithelial cells. Strains containing a single intact copy of *ECM33* caused slightly less damage to both types of host cells compared to the wild-type strain. The homozygous *ecm33*Δ/*ecm33*Δ mutant was the only strain that consistently caused significantly less damage to both endothelial and epithelial cells than did the wild-type strain ($P = 0.003$ and $P < 0.0001$, respectively) (Fig. 4).

***ecm33*Δ mutants have aberrant cell wall architecture.** There are at least two nonexclusive explanations for the defects in the interactions of the *ecm33*Δ mutants with endothelial and oral epithelial cells. Biochemical studies have indicated that Ecm33p is present in the cell wall of *C. albicans*, and it may be expressed on the cell surface (5). Therefore, it is possible that Ecm33p functions as an adhesin or invasin. However, it is also known that Ecm33p is required for normal cell wall integrity in *C. albicans* (16). Similarly, in *Saccharomyces cerevisiae*, Ecm33p is necessary for normal cell wall architecture (19). Thus, it is also possible that Ecm33 is required for the normal functioning of other *C. albicans* cell wall proteins that are adhesins or invasins.

To investigate whether Ecm33p itself mediates adherence to or invasion of host cells, we expressed either Ca*ECM33* or Sc*ECM33* in both a wild-type and an Sc*ecm33*Δ mutant strain of *S. cerevisiae*. We then measured the adherence to and endocytosis of these strains by the FaDu oral epithelial cell line. For a negative control, we also tested the same strains of *S. cerevisiae* transformed with empty plasmid. We found that all strains had similarly low levels of adherence and endocytosis (data not shown). Ca*ECM33* was functional in *S. cerevisiae* because it reversed the cell wall integrity defects of the Sc*ecm33*Δ strain (16). These results suggest that Ecm33p by itself is not sufficient to mediate adherence or endocytosis, at least in *S. cerevisiae*.

Next, we used transmission electron microscopy to investigate the structure of the cell walls of the *ecm33*Δ mutants. Multiple cells of each strain were imaged and several different sections were viewed. The wild-type and *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* double-complemented strains both had normal cell wall architecture, consisting of an electron dense outer layer of mannoproteins, and an electron lucent inner layer composed mainly of 1,3 β-glucan and chitin (Fig. 5A and B). In contrast, the cell walls of all strains containing either one or no functional copies of *ECM33* were consistently abnormal. In the strains with only one copy of *ECM33*, the outer mannoprotein layer of the cell wall was abnormally wide and electron dense (Fig. 5C and D). In the homozygous *ecm33*Δ/*ecm33*Δ mutant, not only was the outer layer of the cell wall abnormally electron dense, but the electron lucent inner layer was also much wider and appeared to be less electron dense than that of the other strains (Fig. 5E and F). These results indicate that *ECM33* is required for the normal cell wall architecture of *C. albicans*.

***ECM33* is necessary for normal Als1p localization on hyphae.** The aberrant cell wall architecture of the *ecm33*Δ mutants suggested that there may be alteration in the surface expression or localization of other proteins in these strains.

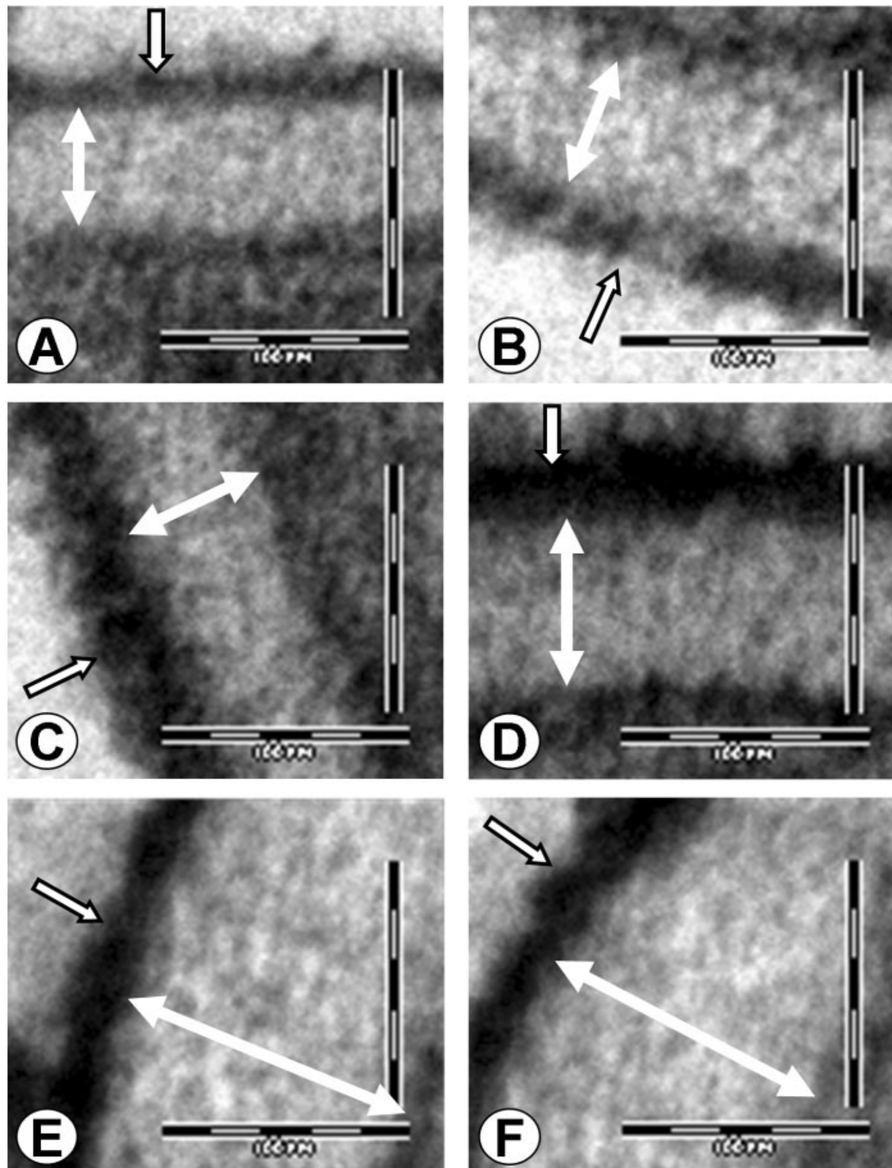


FIG. 5. *ECM33* is necessary for the normal cell wall architecture of *C. albicans*. Representative transmission electron micrographs of the cell walls of blastospores of the wild-type (A), *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* (B), *ecm33*Δ/*ECM33* (C), *ecm33*Δ/*ecm33*Δ::*ECM33* (D), and *ecm33*Δ/*ecm33*Δ (E and F) strains. White, double-headed arrows indicate the thickness of the internal layer of 1,3 β-glucans and chitin. Open, single-headed arrows indicate the outer mannoprotein layer. Scale bars indicate 100 nm.

Therefore, we analyzed the expression of Als1p on the surface of the various strains by using direct immunofluorescence with a monoclonal antibody directed against the N terminus of Als1p. Als1p is a GPI-linked protein that is expressed on the surface of *C. albicans* hyphae, where it functions as an adhesin (10, 11, 27). In the wild-type and the *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* double-complemented strains, Als1p was observed to be present in a relatively narrow band at the base of the hyphae, adjacent to the blastospore-hyphal junction (Fig. 2). In the strains containing a single functional copy of *ECM33*, this band of Als1p extended slightly further along the length of the hyphae. Importantly, in the homozygous *ecm33*Δ/*ecm33*Δ mutant, Als1p was expressed diffusely over the majority of the hyphal surface, except at the very tip. Therefore, *ECM33* is necessary for the normal localization of Als1p.

Although Als1p is known to be expressed on the cell surface of *C. albicans* (10), it is possible that some fraction of the total pool of Als1p is buried within the cell wall and is therefore not accessible to the monoclonal antibody that was used to detect this protein. If this possibility were true, then the aberrant localization of Als1p in the *ecm33*Δ mutants could have been due to the exposure of some Als1p that is normally inaccessible to the anti-Als1p antibody. To test this possibility, we incubated germ tubes of the wild-type, homozygous *ecm33*Δ/*ecm33*Δ mutant, and *ecm33*Δ::*ECM33*/*ecm33*Δ::*ECM33* double-complemented strain for various times in zymolyase to

remove the cell wall. After a 5-min exposure to this enzyme, there was a slight reduction in the intensity of Als1p staining in all strains. However the distribution of Als1p along the hyphae of the different strains remained similar to that of control hyphae that were not exposed to the enzyme (data not shown). Longer exposure to the enzyme resulted in the progressive loss of Als1p staining on all hyphae, but it did not change the distribution of the remaining Als1p. Therefore, the aberrant localization of Als1p in the *ecm33*Δ strains was not due to the cell surface expression of protein that was normally buried within the cell wall of the wild-type strain.

## DISCUSSION

Ecm33p is important for several factors that contribute to the virulence of *C. albicans*. One of these factors is growth rate. The growth rate of *C. albicans* strains is known to have a significant effect on their virulence in the mouse model of hematogenously disseminated infection (24). Although the relationship between *C. albicans* growth rate and virulence during oropharyngeal infection has not been reported, it is highly probable that strains that grow slowly have attenuated virulence during this infection, as well.

It is unlikely that the slow growth rate of blastospores of the *ecm33*Δ mutants played a significant role in their interactions with endothelial cells and oral epithelial cells in vitro. These organisms germinated normally on both types of host cells and the resultant hyphae were of normal length. Previously, it was reported that *ecm33*Δ mutants had filamentation defects in both liquid and solid media (16). However, these prior experiments were performed under different conditions from the ones used in the current studies. Also, the previous *ecm33*Δ mutants had *URA3* integrated at a different chromosomal locus. Thus, our current results are not directly comparable to the previous data.

We observed that hyphae of the *ecm33*Δ mutants adhered to and were endocytosed poorly by both endothelial cells and oral epithelial cells. The adherence defect of these mutants was not as great as their endocytosis defect, indicating that their reduced endocytosis was not solely the result of decreased adherence.

Ecm33p is located in the *C. albicans* cell wall, and it is known to be important for cell wall integrity (5, 16). We found that the *ecm33*Δ strains had markedly aberrant cell wall architecture with an abnormally electron dense outer mannoprotein layer. We speculate that the enhanced mannoprotein synthesis in these mutants may represent a compensatory response to a weakened cell wall. This abnormal mannoprotein layer likely contributed to the reduced adherence and endocytosis of the *ecm33*Δ strains by interfering with the normal expression and/or function of *C. albicans* adhesin and invasin proteins. Consistent with this theory, we found that the Als1p adhesin was abnormally distributed on the surface of the *ecm33*Δ strains, probably as a result of either abnormal trafficking of Als1p or prolonged *ALS1* gene expression. Furthermore, we found no evidence that Ecm33p by itself was able to mediate adherence or invasion of oral epithelial cells.

The *ecm33*Δ mutants also had diminished capacity to damage oral epithelial cells. Because endocytosis is required for *C. albicans* to damage endothelial cells and FaDu oral epithelial cells (8, 20), the decreased host cell damage caused by the *ecm33*Δ mutants is likely due in part to the reduced endocytosis of these strains. It is also possible that the *ecm33*Δ mutants have reduced secretion of lytic enzymes, such as secreted aspartyl proteases and phospholipases (12, 13, 26), which contributed to the host cell damage defect of these mutants.

A notable finding was that the phenotype of strains that still possessed one functional copy of *ECM33* was much closer to the phenotype of the strain that had no functional *ECM33* than to phenotype of the strains containing two copies of *ECM33*. Previous Northern blot analysis of *ECM33* transcript levels in the various strains confirmed that strains containing one intact copy of *ECM33* expressed approximately 50% less *ECM33* mRNA than did strains containing two intact copies of *ECM33* (16). Although the exact amount of Ecm33p expressed by these stains is currently unknown, it is likely that at least some of this protein is expressed by strains containing a single functional copy of *ECM33*. Therefore, there appears to be a critical threshold of cellular Ecm33p content. When the amount of Ecm33p drops below this threshold, there is a significant alteration in cell wall architecture and growth rate. These changes result in a reduction in virulence-related traits, including the capacity of *C. albicans* to adhere to, invade, and damage host cells. Why Ecm33p is so vital for *C. albicans* pathogenicity is currently under investigation.

## ACKNOWLEDGMENTS

We thank Norma Solis and Quynh Trang Phan for help with tissue culture studies, Donald Sheppard for invaluable discussion, and the perinatal nurses at the Harbor-UCLA Medical Center Pediatric Clinical Research Center for collection of umbilical cords.

This work was supported in part by grants R01DE013974, DE017088, R01AI054928, R01AI19990, and MO1RR00425 from the U.S. National Institutes of Health and by BIO 2003-00030 from the Comision Interministerial de Ciencia y Tecnologýa (CYCIT, Spain), and CPGE 1010/2000 for Strategic Groups from Comunidad Autonoma de Madrid. Raquel Martinez-Lopez is the recipient of a fellowship from the Ministerio de Educacion y Ciencia de España.

## REFERENCES

1. **Bensen, E. S., S. G. Filler, and J. Berman.** 2002. A forkhead transcription factor is important for true hyphal as well as yeast morphogenesis in *Candida albicans*. Eukaryot. Cell **1:**787–798.
2. **Brand, A., D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds.** 2004. Ectopic Expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of URA3 at the RPS10 locus. Eukaryot. Cell **3:**900–909.
3. **Cawson, R. A., and K. C. Rajasingham.** 1972. Ultrastructural features of the invasive phase of *Candida albicans*. Br. J. Dermatol. **87:**435–443.
4. **Cheng, S., M. H. Nguyen, Z. Zhang, H. Jia, M. Handfield, and C. J. Clancy.** 2003. Evaluation of the roles of four *Candida albicans* genes in virulence by using gene disruption strains that express *URA3* from the native locus. Infect. Immun. **71:**6101–6103.
5. **de Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. Eukaryot. Cell **3:**955–965.
6. **Edwards, J. E., Jr.** 2000. Candida species, p. 2656–2674. In G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 5th ed. Churchill Livingston, Philadelphia, Pa.
7. **Eversole, L. R., P. A. Reichart, G. Ficarra, A. Schmidt-Westhausen, P. Romagnoli, and N. Pimpinelli.** 1997. Oral keratinocyte immune responses in HIV-associated candidiasis. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endodont. **84:**372–380.
8. **Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Luckett.** 1995. Penetration and damage of endothelial cells by *Candida albicans*. Infect. Immun. **63:**976–983.
9. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.

10. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. Mol. Microbiol. **44:**61–72.
11. **Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi.** 1995. *Candida albicans ALS1*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. **15:**39–54.
12. **Ibrahim, A. S., S. G. Filler, D. Sanglard, J. E. Edwards, Jr., and B. Hube.** 1998. Secreted aspartyl proteinases and interactions of *Candida albicans* with human endothelial cells. Infect. Immun. **66:**3003–3005.
13. **Ibrahim, A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards, Jr., Y. Nozawa, and M. A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. Infect. Immun. **63:**1993–1998.
14. **Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick.** 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Investig. **52:**2745–2756.
15. **Kamai, Y., M. Kubota, T. Hosokawa, T. Fukuoka, and S. G. Filler.** 2001. New model of oropharyngeal candidiasis in mice. Antimicrob. Agents Chemother. **45:**3195–3197.
16. **Martinez-Lopez, R., L. Monteoliva, R. Diez-Orejas, C. Nombela, and C. Gil.** 2004. The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. Microbiology **150:** 3341–3354.
17. **Miret, J. J., A. J. Solari, P. A. Barderi, and S. H. Goldemberg.** 1992. Polyamines and cell wall organization in *Saccharomyces cerevisiae*. Yeast **8:**1033–1041.
18. **Montes, L. F., and W. H. Wilborn.** 1968. Ultrastructural features of host-parasite relationship in oral candidiasis. J. Bacteriol. **96:**1349–1356.
19. **Pardo, M., L. Monteoliva, P. Vazquez, R. Martinez, G. Molero, C. Nombela, and C. Gil.** 2004. *PST1* and *ECM33* encode two yeast cell surface GPI proteins important for cell wall integrity. Microbiology **150:**4157–4170.
20. **Park, H., C. L. Myers, D. C. Sheppard, Q. T. Phan, A. A. Sanchez, J. E. Edwards, Jr., and S. G. Filler.** 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell. Microbiol. **7:**499–510.
21. **Phan, Q. T., P. H. Belanger, and S. G. Filler.** 2000. Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. Infect. Immun. **68:**3485–3490.
22. **Phan, Q. T., R. A. Fratti, N. V. Prasadarao, J. E. Edwards, Jr., and S. G. Filler.** 2005. N-cadherin mediates endocytosis of *Candida albicans* by endothelial cells. J. Biol. Chem. **280:**10455–10461.
23. **Reichart, P. A., H. P. Philipsen, A. Schmidt-Westhausen, and L. P. Samaranayake.** 1995. Pseudomembranous oral candidiasis in HIV infection: ultrastructural findings. J. Oral Pathol. Med. **24:**276–281.
24. **Rieg, G., Y. Fu, A. S. Ibrahim, X. Zhou, S. G. Filler, and J. E. Edwards, Jr.** 1999. Unanticipated heterogeneity in growth rate and virulence among *Candida albicans AAF1* null mutants. Infect. Immun. **67:**3193–3198.
25. **Sanchez, A. A., D. A. Johnston, C. Myers, J. E. Edwards, Jr., A. P. Mitchell, and S. G. Filler.** 2004. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. Infect. Immun. **72:**598–601.
26. **Schaller, M., H. C. Korting, W. Schafer, J. Bastert, W. Chen, and B. Hube.** 1999. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol. Microbiol. **34:**169–180.
27. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. J. Biol. Chem. **279:**30840–30849.
28. **Staab, J. F., and P. Sundstrom.** 2003. *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. Trends Microbiol. **11:**69–73.
29. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. Infect. Immun. **70:** 3281–3283.