Cloning and Characterization of *CSP37*, a Novel Gene Encoding a Putative Membrane Protein of *Candida albicans*

MARIA SENTANDREU,¹ ALMUDENA NIETO,¹ ASUNCION IBORRA,¹ M. VICTORIA ELORZA,¹
JOSE PONTON,² WILLIAM A. FONZI,³ AND RAFAEL SENTANDREU^{1*}

*Secció Departamental de Microbiologia, Facultat de Farmàcia, Universitat de València, València,¹ and
Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Odontología,
Universidad del País Vasco, Bilbao,² Spain, and Department of Microbiology and Immunology,
School of Medicine, Georgetown University Medical Center, Washington, D.C.³*

Received 5 March 1997/Accepted 29 May 1997

In the course of an analysis of the functions and assembly of the cell wall of *Candida albicans*, we have cloned and characterized a gene, which we designated *CSP37* (cell surface protein), encoding a 37-kDa polypeptide which is a membrane-associated protein. The gene was isolated by immunological screening of a DNA library constructed from mycelial cells with a polyclonal serum raised against cell walls of this morphology. Analysis of the nucleotide sequence of a corresponding genomic DNA fragment revealed a single open reading frame which encodes a predicted protein of 321 amino acids with no significant homology to others in the databases. Disruption of the *CSP37* gene by the method described by Fonzi and Irwin (Genetics 134:717–728, 1993) eliminated expression of the Csp37 protein. The mutant strains showed no apparent defect in cell viability, growth, or cell wall assembly but displayed attenuated virulence in systemic infections induced in mice and reduced the ability to adhere to polystyrene.

Candida albicans is an imperfect fungus capable of causing life-threatening infections in immunocompromised patients as well as a variety of mucosal infections in generally healthy individuals (50). Due to its importance as a human pathogen and the limited number of safe drugs to control deep-rooted infections, many laboratories have undertaken cellular, molecular, and genetic studies to understand the mechanisms governing *C. albicans* biology. However, the diploid nature of this fungus and the failure to identify a sexual cycle have hampered many classical genetic approaches (59).

A number of factors are thought to contribute to the virulence of *C. albicans*, but their relative importance during pathogenesis remains unclear (14). Potential virulence determinants include the ability to switch from a yeast to a mycelial form (31, 62) and also between different colonial morphologies (64), the production of extracellular hydrolytic enzymes (28, 36), synthesis of receptor-ligand molecules required for recognition, and adhesion to the host tissues (7, 8), etc. Since the cell wall is involved in these processes, some groups have attempted to elucidate the mechanism of synthesis of its different constituents, their assembly, and the modifications that occur during the morphological transition. Thus, a number of cell surface antigens have been reported to be preferentially expressed in hyphal or blastoconidial cell morphologies (10, 52, 66, 67), and several genes related to cell wall architecture (assembly and functions) have been cloned. These genes have been identified by various methods, including differential hybridization screening (27), complementation in *Saccharomyces cerevisiae* (3), cross-hybridization with genes cloned from other organisms (heterologous genes) (20, 65), hybridization with sequence-specific oligonucleotides (9), and PCR amplification (11, 12). We have used another approach to clone morphology-specific *C. albicans* genes related to the cell wall. We have

isolated cDNA clones by screening with polyclonal antibodies raised against isolated cell walls of blastoconidia and mycelial cells (17, 61).

In this paper, a cDNA clone that reacted with polyclonal antibodies specific for mycelial cell walls was studied. It encodes a novel protein with no significant homology to known sequences and absent from the *S. cerevisiae* genome. $\Delta csp37$ null mutants were constructed and subsequently phenotypic analysis and virulence testing were conducted. Location of the protein codified and potential functions are discussed.

MATERIALS AND METHODS

Microorganisms and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. Cells were routinely grown in YPD (2% glucose, 1% yeast extract, 2% Bacto Peptone [Difco, Detroit, Mich.]) with shaking at the selected temperature. For specific experiments, cells were also cultured in medium 199 containing Earle's salts and glutamine but lacking sodium bicarbonate (GIBCO-BRL), modified Lee's medium (41) without amino acids and 0.5-g/liter proline, or SD minimal medium (2% glucose, 0.67% yeast nitrogen base without amino acids [Difco]). The 199 medium was buffered to different pHs with 150 mM HEPES. Media were supplemented with uridine (25 μ g/ml) when needed, and Urd[−] auxotrophs were selected on medium containing 5-fluoroorotic acid (5-FOA) as described elsewhere (6).

For germ tube induction, cells were cultured in the modified Lee's medium as described elsewhere (16). Alternatively, blastoconidia were inoculated in YPD and shaken at 180 rpm at 25°C until the culture had reached the late-logarithmic-early-stationary growth phase. Hyphae were formed after transfer of the cells to modified Lee's medium (pH 6.8), SD medium containing *N*-acetylglucosamine (25 mM), or medium 199 (pH 6.8), and the medium was incubated at 37°C.

Protoplasts were obtained and regenerated as previously described (16).

S. cerevisiae and *Yarrowia lipolytica* strains used to obtain genomic DNA were grown in YPD at 28°C. *Escherichia coli* DH5 α was used for most transformations and Novablu DE3 was used for recombinant protein expression and purification.

Screening of libraries and sequencing. A cDNA clone (24M) was isolated by immunoscreening of a mycelial library with rabbit polyclonal antiserum raised against walls of mycelial cells as described elsewhere (61). 24M contained a 0.5-kb insert released by *Eco*RI digestion that was employed for hybridization screening of a genomic library of *C. albicans* ATCC 26555 constructed in the multicopy plasmid YRp7 (49). Colony hybridizations were carried out with a nonradioactive labeling and detection kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. A hybridizing plasmid containing a 9-kb insert (pGEV2.1) was characterized by restriction endonuclease mapping and Southern blot hybridization (Fig. 1). A number of overlapping, restriction endonuclease-generated fragments from the pGEV2.1 plasmid were subcloned

* Corresponding author. Mailing address: Secció de Microbiologia, Facultat de Farmàcia, Universitat de València, Avda. Vicent Andrés Estellés s/n., 46100 Burjassot, València, Spain. Phone: 34 6 3864299. Fax: 34 6 3864682. E-mail: rafael.sentandreu@uv.es.

TABLE 1. *C. albicans* strains used in this study

Strain	Genotype	Reference ^a
26555	Wild type	ATCC
3153	Wild type	NCPF
SC5314	Wild type	23
CAI4	$\Delta ura3::imm434/\Delta ura3::imm434$	19
CAIS2	$\Delta csp37::hisG-URA3-hisG/CSP37$	This work
	$\Delta ura3::imm434/\Delta ura3::imm434$	
CAIS2.1	$\Delta csp37::hisG/CSP37$	This work
	$\Delta ura3::imm434/\Delta ura3::imm434$	
CAS1	$\Delta csp37::hisG/\Delta csp37::hisG-URA3-hisG$	This work
	$\Delta ura3::imm434/\Delta ura3::imm434$	
CAS1.1	$\Delta csp37::hisG/\Delta csp37::hisG \Delta ura3::imm434/\Delta ura3::imm434$	This work

^a ATCC, American Type Culture Collection; NCPF, National Collection of Fungal Pathogens, London, United Kingdom.

into pUC and M13 cloning vectors (55), and single-stranded and double-stranded DNAs were sequenced by the dideoxy chain termination method (56) with T7 DNA polymerase and with the automated DNA Sequencer (model 370A; Applied Biosystems).

Sequence analyses were performed with the software system PC/Gene (release 6.85; IntelliGenetics, Inc., Geneva, Switzerland) and PSORT program (47) from the National Institute for Basic Biology (Okazaki, Osaka, Japan). Homology searches of databases were conducted with the BLAST program (2) and BLOCKS (26).

Southern blot analysis. Genomic DNA was extracted from *C. albicans*, *Y. lipolytica*, and *S. cerevisiae* by the method described by Scherer and Stevens (60). Southern blot hybridizations were carried out with a nonradioactive labeling and detection kit (Boehringer) according to the manufacturer's recommendation on positively charged nylon membranes.

Northern blot analysis. Total RNA was prepared by the method described by Langford and Gallwitz (39). RNA was separated by electrophoresis in formaldehyde gels, and transcript size was determined by comparison with rRNA species. Blotting was carried out with Hybond-N nylon membranes. The 24M 0.5-kb cDNA insert was radioactively labeled by random priming and was used as a hybridization probe. The *C. albicans* actin gene, *ACT*, was used in control hybridizations.

Production and purification of the fusion protein in *E. coli*. *E. coli* Novablue DE3 was used as the host, and the pET 15b plasmid carrying the His Tag sequence, a stretch of six consecutive histidine residues, was used as a vector (Novagen, Inc., Madison, Wis.). A fragment of 997 bp, containing the whole 24M open reading frame (ORF) and two additional in-phase stop codons, was amplified by PCR. The amplification primers 5' CAG GGA TCC CAT GTC TGC TGG AAA AA 3' and 5' CAG GGA TCC CTG TTT ATG CTA ACA TC 3' contained a *Bam*HI restriction site at the 5' end. After an initial denaturation step of 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 40°C for 2 min, and 72°C for 2 min, the PCR product was *Bam*HI digested and ligated at the same restriction site of dephosphorylated vector pET 15b. Transformation of *E. coli* Novablue DE3 was carried out following standard procedures, and screening of transformants was done by colony hybridization with the PCR product as a probe. Plasmids from four positive clones were purified and sequenced. One of them, named pET 49, was chosen for protein production.

E. coli transformed with pET 49 was grown at 37°C to an optical density at 600 nm of 1.0, and then expression of the recombinant protein was induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG; Boehringer) at a final concentration of 100 mM. At several intervals following induction, cells from 1 ml of culture were harvested by centrifugation to monitor protein production. Total cell protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose paper, and either stained with Ponceau or reacted with polyclonal antibodies against mycelial cell walls adsorbed against an *E. coli* extract.

For protein purification, cells from 500 ml of medium induced with IPTG for 2 h at 37°C were harvested by centrifugation, resuspended in binding buffer (40 mM imidazole, 4 M NaCl, 160 mM Tris-HCl [pH 7.9]), and lysed by sonication. The lysate was centrifuged at 39,000 $\times$ g for 20 min, and the pellet was suspended in 20 ml of binding buffer containing 8 M urea and was sonicated to solubilize the recombinant protein. Affinity column chromatography was carried out by using His $\cdot$ Bind metal chelation resin following the instructions of the manufacturer (Novagen, Inc.). After unbound proteins were washed away, the target protein was recovered by elution with 1 M imidazole. Proteins in each fraction were subjected to SDS-PAGE to verify the efficiency of the process.

The fusion protein encodes a protease cleavage site that allows removal of the oligohistidine domain by treatment with thrombin. The eluate from His $\cdot$ Bind resin containing the fusion protein was dialyzed against several concentrations of urea (2 to 0.5 M). The amount of protein was determined with the Bio-Rad

dye-binding assay kit. Digestion of the recombinant protein with thrombin was carried out with 0.5 U per mg of recombinant protein and incubation at 20°C for 16 h. Samples were analyzed by SDS-PAGE fractionation and immunodetection.

Production of polyclonal antibodies against the recombinant protein. Two New Zealand White rabbits were immunized with the purified recombinant protein. Immunization was carried out with four doses of 100 μ g of protein injected subcutaneously at 2-week intervals. The protein was suspended in complete (the first dose) and incomplete (the last three doses) Freund adjuvant in a total volume of 0.5 ml. The animals were bled 3 days after the fourth inoculation, the sera were centrifuged for 10 min at 27,000 $\times$ g, and the supernatant was treated with ammonium sulphate at a concentration of 40%. After 18 h at 4°C, the precipitate was collected by centrifugation for 30 min at 27,000 $\times$ g. The pellet was dissolved in phosphate-buffered saline (PBS) and dialyzed against the same buffer.

Preparation of cell walls and isolation of membrane fractions from *C. albicans*. Subcellular fractions were obtained as previously described (10, 43). Briefly, blastoconidia and mycelial cells were collected by centrifugation at 3,000 $\times$ g for 10 min, washed twice with chilled distilled water, suspended in a small volume of 0.001 M phenylmethylsulfonyl fluoride (PMSF) in 0.01 M Tris-HCl buffer (pH 7.2), and broken by shaking with glass beads. The procedure resulted in nearly complete cell breakage. The cell walls were sedimented (1,200 $\times$ g for 10 min) to eliminate membranous and cytoplasmic proteins, washed in chilled 0.001 M PMSF, and treated with boiling 2% (wt/vol) SDS to remove noncovalently bound proteins. The supernatant fluid following breakage was centrifuged at 40,000 $\times$ g for 40 min to obtain the mixed membrane and cytosol fractions. Purification of plasma membranes and internal membranes was achieved by the concanavalin A method (57). Yeast protoplasts were suspended in 50 mM Tris-HCl (pH 7.4) containing 0.9 M sorbitol and 10 mM MgSO₄ and supplemented with 0.3-mg/ml concanavalin A. The mixture was maintained at 30°C for 30 min until the protoplasts flocculated. The protoplasts were collected by centrifugation at 200 $\times$ g for 1 min, and the pellet was resuspended in 10 mM Tris-HCl (pH 7.4) containing 5 mM MgSO₄. The protoplast suspension was homogenized in a Sorvall Omnimixer at 0°C, and the lysate obtained was incubated at 30°C for 15 min to degrade the nuclear structure. Aliquots of the lysate were layered over 3 volumes of 0.1 M Tris-HCl buffer (pH 7.4)–0.5 M mannitol and centrifuged at 150 $\times$ g for 30 min. The supernatant containing the intracellular membranes (internal membranes) was removed and centrifuged at 75,000 $\times$ g for 80 min in the SW-25-2 rotor of an L2 65B Beckman ultracentrifuge. The pellet containing the plasma membranes was resuspended in 0.01 M Tris-HCl (pH 7.4)–1 M α -methylmannoside in order to eliminate concanavalin A. The suspension was incubated for 30 min and centrifuged, and the pellet was resuspended in the appropriate buffer.

Protein gel electrophoresis and Western blot techniques. Proteins were separated by SDS-PAGE performed basically as described elsewhere (38) in 10% (wt/vol) acrylamide gels loaded with 20 μ g of protein. After transfer to nitrocellulose, the proteins were immunodetected with monospecific rabbit antiserum obtained against the recombinant protein. Detection was carried out with the enhanced chemiluminescence (ECL) method from Amersham Life Sciences (Little Chalfon, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. In some cases, the membranes were stripped of bound antibodies and reprobed following the method described in ECL Western blotting protocols (Amersham Life Sciences). The membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH

FIG. 1. Restriction map of the 9.0-kb *Bam*HI genomic DNA fragment of pGEV2.1 containing the *CSP37* gene and strategy for the disruption of *CSP37*. The two *CSP37* alleles of *C. albicans* CAI4 were disrupted by replacing a 1.0-kb *Ava*I-*Eco*RI fragment with the *Ura*-blaster cassette (see Materials and Methods). The 5.5-kb *Pvu*II-*Bgl*II fragment containing the deletion-disruption cassette was used to transform the CAI4 Urd⁺ strain.

6.7)], incubated at 50°C for 30 min, and washed twice for 10 min in 0.01 M Tris-HCl (pH 7.4).

Immunodetection of the recombinant protein in *E. coli* extracts was conducted with polyclonal antibodies raised against mycelial cell walls (44) adsorbed for 30 min at room temperature with an *E. coli* protein extract to avoid cross-reactivity.

Indirect IFA. Immunofluorescence assays (IFAs) were carried out as described elsewhere (10). Cells were washed twice in PBS and fixed with 0.5% formalin in PBS. Drops (10 µl) of antiserum in PBS were placed on the cells. Fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Ortho-Diagnostic Systems) was added to the slide, which was maintained at 37°C for 30 min, washed with PBS, ringed with glass-distilled water, and examined in a Zeiss Photomicroscope III equipped with epifluorescence (UV filter no. 487702; excitation line, 365/366 nm). Protoplasts were fixed for 30 min with 2% glutaraldehyde in 0.6 M KCl and then processed for IFA as described above.

Gene disruption procedures. Disruption of *CSP37* was achieved by the procedure described by Fonzi and Irwin (19). To construct a *CSP37* null mutant, plasmid pGEV2.1 was digested with *EcoRI* to release the 1.5-kb fragment containing the 3' region of the gene. This fragment was blunt end ligated into the *BglII* site of pMB7 (19) to generate plasmid pAL8. A 2.3-kb *Aval-HindIII* fragment from pGEV2.1 located upstream of *CSP37* was then ligated into the *SalI-HindIII* sites of pAL8 to generate pAL9. This plasmid was digested with *BglII* and *PvuII*, releasing a DNA fragment containing the *CSP37* deletion-disruption cassette (Fig. 1). Approximately 20 µg of this linear DNA was used to transform the Urd⁻ strain CA14 (19, 22). Transformed cells were selected as Urd⁺ in SD minimal medium lacking uridine, and integration of the transforming DNA at the *CSP37* locus was verified by Southern blot analysis. Spontaneous Urd⁻ derivatives of this strain were selected on medium containing 5-FOA (6). These clones were analyzed by Southern blot hybridization to identify those which had undergone intrachromosomal recombination. One of these Urd⁻ derivatives was used for mutagenesis of the second allele of *CSP37*. A null mutation was introduced into the remaining functional allele of *CSP37* with the same disruption cassette. Transformed cells were selected as Urd⁺, and integration into the undisrupted *CSP37* allele was verified by Southern blot analysis. Spontaneous Urd⁻ segregants were selected by resistance to 5-FOA. Urd⁻ segregants that lost the wild-type copy of *CSP37* and retained the deletion mutation were identified by Southern blot analysis.

Virulence studies. Survival of BALB/c mice following intravascular injection with the CAIS2, CAS1, and the clinical isolate SC5314 Urd⁺ strains was examined. The strains were grown to stationary phase at 30°C in Sabouraud dextrose broth (Difco) adjusted to pH 4.5. Following a second passage in this medium, the cells were washed twice in PBS, collected by centrifugation, and suspended to a density of 3×10^6 per ml as determined by hemocytometer counting. In one experiment, 10 BALB/c mice for each strain were injected via the lateral tail vein with 1.5×10^6 cells suspended in 0.5 ml of saline. The effect of lower doses (1×10^6 and 5×10^5 cells) on virulence on groups of five mice was studied. The cages were checked twice daily for dead mice.

Adherence assay. The adhesion of *C. albicans* cells to polystyrene was quantified spectrofluorometrically. Yeast cells in the stationary phase were inoculated in 350 µl of medium 199 (pH 6.7) at a final concentration of 10^6 ml⁻¹, and the medium was incubated for 2 h at 37°C in 24-well tissue culture polystyrene plates. After washing, the plates were stained with 1% calcofluor white in saline for 20 min at room temperature. The plates were washed again, and the fluorescence was read in a spectrofluorometer (Fluoroskan Ascent; Labsystem, Helsinki, Finland), with an excitation filter of 358 nm and an emission filter of 460 nm. Controls were used to examine possible interference from nonspecific binding and consisted of wells with medium 199. The results were expressed as relative fluorescence, which was obtained by subtracting the reactivity obtained in the control wells from that shown by the test wells. All values are mean values derived from at least four independent assays.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank under accession no. U89676.

RESULTS

Isolation of the gene and sequence analysis. We had previously used polyclonal antibody preparations against mycelial cell walls to immunoscreen a *C. albicans* cDNA library from germ tube cells and isolated 18 positive clones (61). One-third of them encoded part of a 70-kDa heat shock protein (17). In the present work, a novel gene encoded by a unique positive clone (24M) was characterized. The 24M phage DNA was digested with *EcoRI*, and the released insert of 491 bp was used as a probe to screen a *C. albicans* genomic library constructed in the vector YRp7 (49). Several positive colonies were identified, and a plasmid from one of them, pGEV2.1, which contained a 9-kb insert, was chosen for further analysis (Fig. 1).

FIG. 2. DNA sequence of the *DraI*-*BglII* fragment of pGEV2.1 containing the *CSP37* gene and flanking regions. A highly AT-rich region in the promoter is underlined. The potential Cap signal (thick line) is indicated. Predicted amino acid sequence is given below the nucleotide sequence. Boxed dashed lines, potential transmembrane domain; circles, N-glycosylation sites; boxes, potential ATP-GTP binding sites.

The nucleotide sequence of a *DraI*-*BglII* fragment (1,862 nucleotides) identified an ORF that extended from an ATG initiation codon at nucleotide 418 and ended at nucleotide 1381 (Fig. 2). The nucleotide sequence at the 3' end was identical to that of the 24M cDNA (61). In common with other *C. albicans* genes, the promoter of this ORF, named *CSP37* (cell surface protein of 37 kDa; see below), was highly AT rich (nucleotides 328 to 342 [90 to 70 upstream of the ORF]; ATTTATATATATTTCA), and a Cap signal (nucleotides 364 to 371 [54 to 47 upstream of the ORF]; TCATTAGA) was also detected. TATA boxes with the consensus sequences TATA (A/T)A (18, 30) were found in positions 170 to 164 and 87 to 81 upstream of the translational start codon. No consensus splice signals characteristic of introns were found within the coding region. Polyadenylation of the transcript is predicted to occur 67 nucleotides downstream of the first stop codon, as determined by sequence analysis of the cDNA (61).

The gene encodes a putative polypeptide of 321 amino acids with a calculated molecular weight of 37,072 (Csp37p) and one potential transmembrane domain (positions 2 to 17; SAGKY-LLGTAALVGGV) (54). Analysis of the hydrophathy of Csp37p (37) also revealed a hydrophobic region at the amino terminus (Fig. 3). This amino-terminal sequence could have been considered a signal peptide, but no cleavage site that conformed with Von Heijne's rule (69) was identified. Membrane proteins with uncleavable signal peptide are often anchored to the endoplasmic reticulum (47); however, no consensus signals for endoplasmic reticulum retention (HDEL [15] or dilysine motif [29]) were identified at the carboxy terminus. Further analysis by the method described by Klein et al. (32) classified Csp37p as a membrane peripheral protein and not as an integral membrane protein.

The net charge balance of the signal anchor flanking se-

FIG. 3. (A) Kyte and Doolittle (37) hydropathy plot of CSP37-encoded protein computed with a window size of six amino acids. Bars denote predicted membrane-spanning region. (B) Secondary-structure prediction for the CSP37 sequence with the GGBSM program (21). coil, random coil; extended, β -strands. (C) Coiled-coil probability based on a seven-residue heptad repeat (42). The y axis represents probability. Numbering starts with the first amino acid translated from the ORF. Extents of regions predicted in a conformation are indicated at the top of the corresponding panel.

quence [$\epsilon(C_{\text{cyt}}-N_{\text{exo}})$] determines the orientation of the membrane-spanning segment (25, 70) and in our case gave a $\epsilon(C-N)$ value of less than zero, which is proposed to be necessary for an $N_{\text{cyt}} C_{\text{exo}}$ orientation of the signal anchor sequence (25).

Other features identified in the protein sequence included two potential N-glycosylation sites ($N_{204}-W-T$ and $N_{240}-D-S$)

and an ATP-GTP binding site motif A (P loop [288 to 295]), and three potential MIDAS motifs (DxSx) for coordination of divalent cations were also detected (153 to 156 [DKSD], 234 to 237 [DLSK], and 278 to 281 [DASK]) (20, 40).

Database comparisons with the Csp37p sequence did not yield any obvious homolog. However, we noticed that align-

FIG. 4. Southern blot analysis of *C. albicans* (lanes 1 to 8), *S. cerevisiae* (lane 9), and *Y. lipolytica* (lane 10). DNAs were prepared (60) and digested with *Eco*RI (lanes 1, 9, and 10), *Sac*I (lane 2), *Cla*I (lane 3), *Hind*II (lane 4), *Nde*I (lane 5), *Hinc*II (lane 6), *Bst*EII (lane 7), and *Bam*HI (lane 8). The 2.0-kb *Ava*I-*Bgl*II fragment of PGEV2.1 containing the *CSP37* gene was used as a hybridization probe.

ments yielded mainly coiled-coil proteins such as myosin II heavy chains from different organisms (MYS2_DICDI; SwissProt), a yeast integrin homolog (L03188) or an outer membrane protein from *Thermotoga maritima* (OMPA_THEME; SwissProt). Best alignments showed an amino acid identity of approximately 25%, distributed randomly over the sequences and probably indicative of structural analogy rather than true homology. As expected from the sequence alignments, a secondary-structure analysis of the deduced sequence suggested a relatively high content of α -helix (56% predicted by the GGBSM [Gascuel and Golmard Basic Statistical Method] program [21]) and, in particular, a probability of 0.9 (90%) to form coiled-coil structures in the regions from amino acids 157 to 193 and 219 to 290 (Fig. 3). A gap of possible discontinuity between the two coiled-coil regions is found between amino acids 193 and 219.

Interestingly, no ORF within the entire *S. cerevisiae* genome was found to encode a protein with significant similarity to Csp37p, suggesting that this protein is not common to all fungi. Southern blot hybridization of *S. cerevisiae* genomic DNA with the *CSP37* probe under low-stringency conditions confirmed the comparison analysis (Fig. 4). No cross-hybridization with *Y. lipolytica* genomic DNA either (Fig. 4) was detected, but this could be due to the high differences in GC content between *C. albicans* and this dimorphic yeast (1, 35).

Northern blot analysis. The 24M cDNA clone was selected by its reactivity with hypha-specific antiserum, suggesting that it was differentially expressed in relation to cell morphology or the culture conditions used to induce the hyphal morphology. This hypothesis was tested by Northern blot hybridization. A 1.1-kb transcript that hybridized with the cDNA was expressed when strain SC5314 was induced to form hyphae at pH 6.8 and at 37°C in either modified Lee's medium (41) or in 199 medium (Fig. 5). The transcript was also present when RNA was obtained from cells grown as blastoconidia at 25°C in the same medium. Even though there were basal levels of *CSP37* mRNA under all conditions tested, expression of the gene was reduced at 25°C and at pH 4.5 in Lee's medium and at 25°C and at pH 7.0 in medium 199.

In addition, RNA was prepared from protoplasts incubated for 3 h at 28 or 37°C in Lee's medium and stabilized with 0.6 M KCl, and, as can be seen in Fig. 5, no variation in *CSP37* mRNA expression was detected in relation to the de novo synthesis of the cell wall.

FIG. 5. Northern blot analysis of *CSP37* transcript. Stationary-phase cells of *C. albicans* SC5314 were inoculated into the indicated media and incubated for 5 h. Protoplasts (Prot) were regenerated for 3 h as described elsewhere (16). RNA isolation and Northern blot hybridizations were carried out as described in Materials and Methods. Upper panel, results of hybridization with *CSP37*; lower panel, results of the hybridization with actin DNA. The electrophoretic positions of rRNA are shown on the right.

Expression of the recombinant protein in *E. coli*. The *CSP37* gene was cloned into the expression vector pET 15 to generate a fusion protein containing a carboxy-terminal His Tag. After 30 min of induction, two proteins of 37 and 32 kDa that reacted specifically with the germ tube antiserum (used to screen the cDNA library) were detected (Fig. 6). However, these proteins did not react with polyclonal antiserum raised against yeast cell walls (data not shown). These proteins were absent when the inducer was not present (0 min), meaning that they were encoded by the pET 49 recombinant plasmid (Fig. 6B).

The 37-kDa protein (previous solubilized with 8 M urea) was purified by affinity chromatography in a His·Bind metal chelation resin. The 32-kDa recombinant protein was not purified by this procedure, suggesting that it could represent a proteolytic cleavage product of the 37-kDa protein which had lost the His Tag. Treatment of the 37-kDa purified fusion protein with thrombin cleaved the histidine tail, releasing a protein with an apparent molecular mass of 35 kDa (Fig. 6C).

Cellular localization of Csp37p in *C. albicans*. To elucidate the location of Csp37p in the cell, cells were fractionated and the presence of native Csp37 was detected with polyclonal antiserum raised against the recombinant protein. Since the *CSP37* transcript was present in both morphologies, we analyzed subcellular fractions from both yeast and germ tubes to investigate whether there was a differential expression or localization of the protein related to the specificity observed in

FIG. 6. Time course of fusion protein expression and effect of thrombin digestion. *E. coli* with the pET construction was grown at 37°C in Luria-Bertani medium containing ampicillin. After induction with 100 mM IPTG, 1 ml of the culture was taken at 0 and 30 min. Cells were collected by centrifugation, and supernatant was removed. Proteins were detected with Ponceau (A) or were immunodetected with polyclonal antibodies (pAb) (B) against cell walls of *C. albicans*. Eluates obtained from the His binding resin (C) were digested with thrombin, analyzed by SDS-PAGE, and immunodetected with pAb against mycelial cell walls of *C. albicans* (lane 2). Lane 1, control without thrombin.

FIG. 7. Western blot analysis of Csp37p. *C. albicans* SC5314 was incubated in the Lee's medium (41) at 28°C (blastconidia [y]) and 37°C (germ tubes [m]). After 3 h, the cells were collected and broken, and the cell wall, mixed membrane (M), and cytosol (C) preparations were collected. The cell walls were extracted with SDS. The materials obtained were analyzed by SDS-PAGE and blotting to nitrocellulose paper and were stained with polyclonal antibodies against the recombinant fusion Csp37p (A) and polyclonal antibodies against mycelial cell walls (B).

the initial screen of the cDNA library. Differential localization of several antigens in yeast and germ tube cells has been reported elsewhere (45, 51, 52). However, in the case of Csp37p, polyclonal serum against the recombinant protein recognized a molecular species of 37 kDa in SDS-extracted cell walls and mixed membrane preparations prepared from yeast or hyphae. In this analysis, cytosol fractions were included as a control. No reactive protein was detected in these fractions (Fig. 7), indicating that the presence of the protein in the particulate fraction was not due to contamination with a cytosolic protein.

To further elucidate the membrane localization of the antigen, plasma membranes and internal membranes were isolated and examined by immunoblotting. In this paper, the term internal membranes is applied to all yeast membrane systems, with the exception of plasma membranes. Polyclonal antiserum raised against the recombinant protein recognized a band of 37 kDa from the plasma membrane, while almost no positive signal in internal membranes was obtained (Fig. 8, lanes 1 and 2). After stripping, the nitrocellulose membranes were re-probed with polyclonal serum against mycelial cell walls. In the plasma membrane preparation, a prominent band of 37 kDa was found; in internal membranes, a faint band of the same size was present. Both preparations contained several antigens that cross-reacted with the antibody (Fig. 8, lanes 3 and 4).

FIG. 8. Western blot analysis of plasma membranes (lanes 1 and 3) and internal membranes (lanes 2 and 4) of yeast cells of *C. albicans* SC5314 detected with polyclonal antibodies against the recombinant fusion Csp37p (A) and polyclonal antibodies against mycelial cell walls (B).

FIG. 9. Bright-field (A) and immunofluorescence (B) optics of yeast cell protoplasts formed from exponentially growing cells of *C. albicans* SC5314. Cells were incubated with polyclonal antibodies against the recombinant fusion Csp37p (1:100 dilution) and stained with a fluorescein isothiocyanate-conjugated secondary antibody. Note that incomplete protoplasts with most of the cell wall still present (arrows) did not stain with the antibody.

Indirect IFAs on mycelial and yeast cells were also conducted to verify the cell surface localization of the protein. No immunofluorescence was detected in the surface of intact cells of either morphology (data not shown).

Although the antigen was not expressed on the surfaces of yeast cells, its presence on the plasma membrane was examined. For this purpose, yeast cells were sequentially treated with β -mercaptoethanol and Zymolyase, as described by Elorza et al. (16), until protoplasts were formed. After protoplast formation with Zymolyase for 30 min, 40 to 60% of the cells showed a positive signal (Fig. 9), and under such conditions, 20% of the yeast cells did not convert to protoplasts. These cells did not react with the polyclonal antiserum raised against the recombinant protein.

Construction of CSP37 null mutants. As an additional approach to characterization of the function of the Csp37p, construction of null mutants and analysis of the resulting phenotype were carried out. To disrupt *CSP37*, a 1.0-kb *AvaI*-*EcoRI* fragment including the promoter and the 5' end of *CSP37* was replaced by the Ura blaster cassette consisting of the *C. albicans* *URA3* gene flanked by direct repeats of the *Salmonella typhimurium* *hisG* gene (Fig. 1). A linear DNA fragment including the cassette flanked by the remaining *CSP37* sequences was used to transform strain CAI4 to Urd⁺.

Ten of the resulting Urd⁺ isolates were examined, and each contained the desired insert at the *CSP37* locus. Southern blot analysis of a representative isolate, CAIS2, is shown in Fig. 10. DNA from strain CAIS2 digested with *EcoRI* exhibited the anticipated hybridization bands when hybridized with an *AvaI*-*BglII* fragment containing the 24M ORF and the *hisG*-*URA3*-*hisG* cassette. Genomic DNA from this strain contained two 24M-hybridizing bands, a 4.2-kb band and a 1.7-kb band characteristic of the parental strain CAI4, and an additional fragment of 3.6 kb. The size of the last fragment is consistent with replacement of one allele of the *CSP37* gene with the transforming DNA. This band also hybridized with the cassette. Ura⁻ segregants of strain CAIS2 were selected on 5-FOA-containing medium (6) and examined by Southern blot analysis. Of 13 independent segregants examined, 10 had undergone intrachromosomal recombination between the *hisG* repeats,

FIG. 10. Disruption of the *C. albicans* *CSP37* locus. Southern blot analysis of genomic DNAs from CAI4 (lane 1), CAIS2 (lane 2), CAIS2.1 (lane 3), CAS1 (lane 4), and CAS1.1 (lane 5) digested with *Eco*RI and hybridized with an *Ava*I-*Bgl*II fragment containing the *CSP37* gene (A) and the *hisG-URA3-hisG* cassette (B). On the left, the wild-type and disrupted alleles are represented opposite the corresponding *Eco*RI fragments that hybridized with each probe (arrows). Thin black lines, *CSP37* 5' and 3' flanking regions; E, *Eco*RI sites; A, *Ava*I site; black rectangles, *CSP37*-deleted region; shaded rectangles, *hisG* sequences; white rectangles, *URA3* sequences; thick black lines below, hybridizing fragments. The lengths of the *Eco*RI fragments detected are shown on the right.

resulting in excision of the *URA3* marker and one copy of *hisG*. Three had experienced an interchromosomal recombination event, reverting to the parental genotype. Southern blot analysis of a representative intrachromosomal recombinant, strain CAIS2.1, is shown in Fig. 10. The 3.6-kb *Eco*RI fragment seen in strain CAIS2 was absent, and a new hybridizing fragment, 5.8 kb in length, was present. The size of this latter fragment is consistent with the desired intrachromosomal event.

The homozygous *CSP37* null mutants were generated after transformation of CAIS2.1 with the same deletion-disruption cassette. Genomic DNA was isolated from transformants and screened by Southern blot hybridization to determine whether the transforming DNA had replaced the previously disrupted allele or had integrated into the remaining parental allele. Southern blot analysis of a representative *Urd*⁺ isolate that exhibited a hybridization pattern consistent with targeting of the previously undisrupted allele, strain CAS1, is shown in Fig. 10. The 4.2- and 1.7-kb *Eco*RI fragments characteristic of the undisrupted gene were missing when hybridizing with the *Ava*I-*Bgl*II probe. The 5.8-kb band indicative that the *hisG* disrupted allele was present as well as the 3.6-kb band expected for insertion of the *hisG-URA3-hisG* sequences was detected with both probes. CAS1 was plated on 5-FOA-containing medium to select *Urd*⁻ segregants. Three *Urd*⁻ isolates were screened by Southern blot hybridization, and each exhibited a single 5.8-kb *Eco*RI fragment that hybridized with both the *Ava*I-*Bgl*II probe and the cassette (CAIS1.1).

Northern blot analysis demonstrated that no mRNA was present when hybridized with the 24M cDNA probe in RNA samples from the null mutant CAIS1.1 incubated at 28 and 37°C in modified Lee's medium (data not shown).

Phenotypic analysis of *CSP37* heterozygous and homozygous mutants. The specific growth rates of the yeast form of the parental strain SC5314 and the *Urd*⁺ strains CAIS2 and CAS1 were similar in YPD and Lee's modified medium at 30°C. The yield of cells after overnight growth of the yeast form in these media was again similar. No differences in the kinetics of germ tube formation were observed between the mutants and the wild type in modified Lee's medium or in *N*-acetylglucosamine-containing medium at 37 and 42°C.

We also examined the sensitivities to heat shock, caffeine (8

to 20 mM), and substances that interfered with cell wall assembly (Calcofluor white and Congo red), and no significant differences were found between mutants and parental strains either at 28 or at 37°C. Growth was inhibited on plates containing more than 500 µg of Calcofluor and Congo red per ml.

The behavior of *CSP37*-deficient strains on high-osmolarity medium was also analyzed. When incubated either at 28 or at 37°C, no differences between the parent and the deletion mutants on plates containing 0.25 to 0.5 M NaCl or CaCl₂ were found.

Growth in nonfermentable carbon sources (glycerol and potassium acetate) was also analyzed following incubation at 28, 37, or 42°C. No differences were found.

Effect of *CSP37* deletion on virulence. The virulence of the *Urd*⁺ parental and disrupted strains was studied in a systemic infection model as described in Materials and Methods. The survival of mice infected with 1.5×10^6 SC5314 cells (control) was initially reduced compared to that with CAIS2- and CAS1-infected mice (Fig. 11A), with 60% of the mice succumbing by day 3, whereas 100 and 90% of the mice injected with CAIS2 and CAS1 *Urd*⁺ strains survived. However, after 4 days, mortality reached about 80% in the control strain (SC5314) and 70% in the case of both CAIS2 and CAS1. The results suggested that the disruptants (homo- and heterozygous strains) may be compromised in establishing the initial stages of infection. When lower doses were inoculated, the survival time of the mice was increased, but the lag in mortality of the *csp37* double-deleted strain was still evident (Fig. 11B and C).

Effect of *CSP37* deletion on the adhesion of the fungus to polystyrene. Adherence to polystyrene of *C. albicans* wild-type SC5314, CAIS2, and CAS1 was studied as described in Materials and Methods. Values for CAIS2 and CAS1 were significantly lower ($P < 0.05$ and $P < 0.01$, respectively) compared to those for parental SC5314 and a control strain, 3153 (Fig. 12).

DISCUSSION

We have isolated a novel gene of *C. albicans* by screening a mycelial cDNA library with polyclonal antisera raised against mycelial and yeast cell walls. The 24M clone reacted only with antiserum specific for the hyphal morphology, suggesting that it was differentially expressed in relation to cell morphology or

FIG. 11. Survival of mice following intravascular injection with *C. albicans* CAIS2 (●) and CAS1 (■) and the clinical isolate SC5314 (□). Totals of 1.5×10^6 (A), 1×10^6 (B), and 0.5×10^6 (C) cells per mouse were administered.

the culture conditions used to induce the morphological transition. Analysis of expression indicated that a CSP37 transcript was present not only in hyphae but also in cells grown in the yeast form.

Two observations suggest that CSP37 encodes a bitopic integral membrane protein and not a cell wall protein. First, its sequence showed an uncleavable signal peptide as a unique potential membrane-spanning segment. Second, use of specific antibodies allowed identification in Western blot analysis of a single polypeptide in membrane fractions of blastoconidia and mycelial cells.

The Hartmann's prediction (25) for eukaryotic membrane-spanning proteins indicates an $N_{\text{cyt}}-C_{\text{exo}}$ orientation of the signal anchor sequence of a type II topology for Csp37p according to Singer's classification (63), with the NH_2 terminus anchored to the membrane, whereas the bulk of the molecule is on the exterior side. This type II topology precludes having

enzymatically active domains on the cytoplasmic side of the membrane and suggests an extracellular function.

Comparison of the CSP37 nucleotide and amino acid sequences with those from different databases did not reveal any significant homologies between Csp37p and other known proteins. Furthermore, no homology to any of the ORFs of the *S. cerevisiae* genome was found. This is of particular interest, since *C. albicans* and *S. cerevisiae* differ in their natural environment and their morphogenetic properties. *C. albicans* can form true mycelial morphology, whereas *S. cerevisiae* produces pseudohyphae only (34). Thus, Csp37p may contribute to the unique biological properties of *C. albicans*.

To date, only a limited number of *C. albicans* genes involved in cell morphogenesis have been described. Some genes whose expression correlates with germ tube formation do not seem to be essential in the yeast-to-mycelium transition (4, 5, 24), whereas the lack of genes expressed in relation to external environmental changes leads to morphological aberrances under these defined conditions (58). In our case, CSP37 expression seemed to be influenced by the culture conditions; therefore, we decided to explore its potential role in morphogenesis and pathogenesis. With this aim, we constructed CSP37 null mutants by sequential gene disruption. Mutant cells lacking CSP37 grew in the same manner as wild-type cells on a number of different carbon sources, on both rich and minimal media, and at various temperatures. Our conclusion is that CSP37 is not required for normal cellular growth or morphogenesis.

To gain information about the possible physiological function of Csp37p, we also tested the heterozygous and homozygous mutants under different stress conditions, such as heat shock, hyperosmolarity, or sensitivity to drugs reported to interfere in different processes related to cell wall assembly (Calcofluor white and Congo red) (33, 46, 68) and cellular integrity and growth control mediated by the PKC-MPK1 signal transduction pathway (13, 48, 53). One explanation for the apparent lack of phenotype of the deleted strains in vitro is that a second protein in the cell performs essentially the same function as Csp37p. Such a situation exists for chitin synthase (24). Northern blot analysis of CSP37 mutants and Southern blot hybridization with the CSP37 gene failed to detect homologous sequences in the *C. albicans* genome, but this is not proof for the absence of homologs. A third possibility is that Csp37p is necessary for growth or survival under conditions which we have not duplicated in the laboratory or is involved in a func-

FIG. 12. Adhesion to polystyrene of *C. albicans* 3153 (lane 1), SC5314 (lane 2), CAIS2 (lane 3), and CAS1 (lane 4). The number of input cells in each assay was controlled spectrophotometrically, and the inoculum was made with yeast cells (10^6 ml^{-1}) incubated at 37°C for 140 min, inducing their germination and promoting adhesion to polystyrene. Results are mean values derived from at least four independent assays. Bars, standard deviations.

tion for which we have no assay. In this respect, virulence studies have suggested a potential role for Csp37p in systemic infections. *csp37* null mutants are less virulent in the first stages of infection, independently of the dose. However, the differences in virulence between the parent and the heterozygous and homozygous null mutants are restricted to the first stages of infection. This delay in mortality might be related to delayed or low-level expression of functional homologs of Csp37p or to a contributory but nonessential role of Csp37p in the initiation of infection. In relation to the virulence results, it is interesting to note that adhesion of the mutant strains to polystyrene was significantly reduced compared with that for the parental strain. Attachment of *C. albicans* to medical implants and host cells has been suggested to be a critical step in the initiation of colonization and infection. Csp37p might play a key role in these processes.

Despite the lack of a discernible function for Csp37p, the data reported here are significant in that they provide the first evidence indicating a potential role in virulence for a novel *C. albicans* gene that could help to understand the mechanisms of host-parasite interaction during *Candida* infections.

ACKNOWLEDGMENTS

This work was partially supported by grants from Dirección General de Investigación Científica y Técnica (PB93-0051), Fondo de Investigaciones Sanitarias de la Seguridad Social (95/1602), BIOMED BMH4-CT96-0310 (Brussels), and NIH grant AI3794. M. V. Elorza is Research Investigator of the Consejo Superior de Investigaciones Científicas, and M. Sentandreu was supported by a doctoral grant from Conselleria d'Educació i Ciència de la Generalitat Valenciana.

REFERENCES

- Ahearn, D. G. 1978. Medically important yeasts. *Annu. Rev. Microbiol.* **32**:59–68.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Au Young, J., and P. W. Robbins. 1990. Isolation of a chitin synthase gene (*CHS1*) from *Candida albicans* by expression in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **4**:197–207.
- Bailey, D. A., P. J. F. Feldmann, M. Bovey, N. A. R. Gow, and A. J. P. Brown. 1996. The *Candida albicans* *HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178**:5353–5360.
- Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd. 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. *Infect. Immun.* **61**:3648–3655.
- Boeke, J. D., F. LaCrute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**:345–346.
- Calderone, R. A., and P. C. Braun. 1991. Adherence and receptor relationships of *Candida albicans*. *Microbiol. Rev.* **55**:1–20.
- Calderone, R. A., R. Diamond, J. M. Senet, J. Warmington, S. Filler, and J. E. Edwards. 1994. Host cell-fungal cell interactions. *J. Med. Vet. Mycol.* **32**(Suppl. 1):151–168.
- Cannon, R. D., K. Niimi, H. F. Jenkinson, and M. G. Shepherd. 1994. Molecular cloning and expression of the *Candida albicans* beta-N-acetylglucosaminidase (*HEX1*) gene. *J. Bacteriol.* **176**:2640–2647.
- Casanova, M., M. L. Gil, L. Cardenoso, J. P. Martinez, and R. Sentandreu. 1989. Identification of wall-specific antigens synthesized during germ tube formation by *Candida albicans*. *Infect. Immun.* **57**:262–271.
- Chambers, R. S., M. J. Broughton, R. D. Cannon, A. Carne, G. W. Emerson, and P. A. Sullivan. 1993. An exo-beta-(1,3)-glucanase of *Candida albicans*: purification of the enzyme and molecular cloning of the gene. *J. Gen. Microbiol.* **139**:325–334.
- Chen Wu, J. L., J. Zwicker, A. R. Bowen, and P. W. Robbins. 1992. Expression of chitin synthase genes during yeast and hyphal growth phases of *Candida albicans*. *Mol. Microbiol.* **6**:497–502.
- Costigan, C., S. Gehrung, and M. Snyder. 1992. A synthetic lethal screen identifies *SLK1*, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. *Mol. Cell. Biol.* **12**:1162–1178.
- Cutler, J. E. 1991. Putative virulence factors of *Candida albicans*. *Annu. Rev. Microbiol.* **45**:187–218.
- Dean, N., and H. R. Pelham. 1990. Recycling of proteins from the Golgi compartment to the ER in yeast. *J. Cell Biol.* **111**:369–377.
- Elorza, M. V., A. Marcilla, and R. Sentandreu. 1988. Wall mannoproteins of the yeast and mycelial cells of *Candida albicans*: nature of the glycosidic bonds and polydispersity of their mannan moieties. *J. Gen. Microbiol.* **134**:2393–2403.
- Eroles, P., M. Sentandreu, M. V. Elorza, and R. Sentandreu. 1995. Cloning of a DNA fragment encoding part of a 70-kDa heat shock protein of *Candida albicans*. *FEMS Microbiol. Lett.* **128**:95–100.
- Ferguson, J., J. Y. Ho, T. A. Peterson, and S. I. Reed. 1986. Nucleotide sequence of the yeast cell division cycle start genes *CDC28*, *CDC36*, *CDC37*, and *CDC39*, and a structural analysis of the predicted products. *Nucleic Acids Res.* **14**:6681–6697.
- Fonzi, W. A., and M. Y. Irwin. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**:717–728.
- Gale, C., D. Finkel, N. Tao, M. Meinke, M. McClellan, J. Olson, K. Kendrick, and M. Hostetter. 1996. Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **93**:357–361.
- Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**:97–120.
- Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**:1425.
- Gillum, A. M., E. Y. Tsay, and D. R. Kirsch. 1984. Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *Saccharomyces cerevisiae* *ura3* and *Escherichia coli* *pyrF* mutations. *Mol. Gen. Genet.* **198**:179–182.
- Gow, N. A., P. W. Robbins, J. W. Lester, A. J. Brown, W. A. Fonzi, T. Chapman, and O. S. Kinsman. 1994. A hyphal-specific chitin synthase gene (*CHS2*) is not essential for growth, dimorphism, or virulence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **91**:6216–6220.
- Hartmann, E., T. A. Rapoport, and H. F. Lodish. 1989. Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl. Acad. Sci. USA* **86**:5786–5790.
- Henikoff, S., and J. G. Henikoff. 1991. Automated assembly of protein blocks for database searching. *Nucleic Acids Res.* **19**:6565–6572.
- Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi. 1995. *Candida albicans* *ALSI*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. *Mol. Microbiol.* **15**:39–54.
- Hube, B., M. Monod, D. A. Schofield, A. J. Brown, and N. A. Gow. 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol. Microbiol.* **14**:87–99.
- Jackson, M. R., T. Nilsson, and P. A. Peterson. 1990. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. *EMBO J.* **9**:3153–3162.
- Kim, S., J. Mellor, A. J. Kingsman, and S. M. Kingsman. 1986. Multiple control elements in the *TRP1* promoter of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **6**:4251–4258.
- Kimura, L. H., and N. N. Pearsall. 1980. Relationship between germination of *Candida albicans* and increased adherence to human buccal epithelial cells. *Infect. Immun.* **28**:464–468.
- Klein, P., M. Kanehisa, and C. DeLisi. 1985. The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta* **815**:468–476.
- Kopecka, M., and M. Gabriel. 1992. The influence of Congo red on the cell wall and (1→3)-beta-D-glucan microfibril biogenesis in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **158**:115–126.
- Kron, S. J., C. A. Styles, and G. R. Fink. 1994. Symmetric cell division in pseudohyphae of the yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **5**:1003–1022.
- Kurtzman, C. P., H. J. Phaff, and S. A. Mayer. 1983. Nucleic acid relatedness among yeast, p. 139–166. In J. F. T. Spencer, D. M. Spencer, and A. R. W. Smith (ed.), *Yeast genetics*. Springer-Verlag, New York, N.Y.
- Kwon Chung, K. J., D. Lehman, C. Good, and P. T. Magee. 1985. Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. *Infect. Immun.* **49**:571–575.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic of a protein. *J. Mol. Biol.* **157**:105–132.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Langford, C. J., and D. Gallwitz. 1983. Evidence for an intron-containing sequence required for the splicing of yeast RNA polymerase II transcripts. *Cell* **33**:519–527.
- Lee, J. O., P. Rien, M. A. Arnaut, and R. Liddington. 1995. Crystal structure of the A domain from the alpha subunit of integrin CR3 (CD 11b/CD 18). *Cell* **80**:631–638.
- Lee, K. L., H. R. Buckley, and C. C. Campbell. 1975. An amino acid liquid synthetic for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**:148–153.
- Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science* **252**:1162–1164.
- Marcilla, A., M. V. Elorza, S. Mormeneo, H. Rico, and R. Sentandreu. 1991.

- Candida albicans* mycelial wall structure: supramolecular complexes released by zymolyase, chitinase and beta-mercaptoethanol. Arch. Microbiol. **155**: 312–319.
44. Marcilla, A., S. Mormeneo, M. V. Elorza, J. J. Manclus, and R. Sentandreu. 1993. Wall formation by *Candida albicans* yeast cells: synthesis, secretion and incorporation of two types of mannoproteins. J. Gen. Microbiol. **139**:2985–2993.
 45. Molinari, A., M. J. Gomez, P. Crateri, A. Torosantucci, A. Cassone, and G. Arancia. 1993. Differential cell surface expression of mannoprotein epitopes in yeast and mycelial forms of *Candida albicans*. Eur. J. Cell Biol. **60**:146–153.
 46. Murgui, A., M. V. Elorza, and R. Sentandreu. 1985. Effect of papulacandin B and calcofluor white on the incorporation of mannoproteins in the wall of *Candida albicans* blastospores. Biochim. Biophys. Acta **841**:215–222.
 47. Nakai, K., and M. Kanehisa. 1992. A knowledge base for predicting protein localization sites in eukaryotic cells. Genomics **14**:897–911.
 48. Navarro Garcia, F., M. Sanchez, J. Pla, and C. Nombela. 1995. Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. Mol. Cell. Biol. **15**:2197–2206.
 49. Nieto, A., P. Sanz, R. Sentandreu, and L. del Castillo Agudo. 1993. Cloning and characterization of the *SEC18* gene from *Candida albicans*. Yeast **9**:875–887.
 50. Odds, F. C. 1988. *Candida* and candidosis: a review and bibliography. Bailière Tindall, London, United Kingdom.
 51. Ollert, M. W., and R. A. Calderone. 1990. A monoclonal antibody that defines a surface antigen on *Candida albicans* hyphae cross-reacts with yeast cell protoplasts. Infect. Immun. **58**:625–631.
 52. Ponton, J., A. Marot Leblond, P. A. Ezkurra, B. Barturen, R. Robert, and J. M. Senet. 1993. Characterization of *Candida albicans* cell wall antigens with monoclonal antibodies. Infect. Immun. **61**:4842–4847.
 53. Posas, F., A. Casamayor, and J. Arino. 1993. The PPZ protein phosphatases are involved in the maintenance of osmotic stability of yeast cells. FEBS Lett. **318**:282–286.
 54. Rao, M. J. K., and P. Argos. 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta **869**:197–214.
 55. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 56. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
 57. Santos, E., J. R. Villanueva, and R. Sentandreu. 1978. The plasma membrane of *Saccharomyces cerevisiae*. Isolation and some properties. Biochim. Biophys. Acta **508**:39–54.
 58. Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi. 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. **15**:601–613.
 59. Scherer, S., and P. T. Magee. 1990. Genetics of *Candida albicans*. Microbiol. Rev. **54**:226–241.
 60. Scherer, S., and D. A. Stevens. 1988. A *Candida albicans* dispersed, repeated gene family and its epidemiologic applications. Proc. Natl. Acad. Sci. USA **85**:1452–1456.
 61. Sentandreu, M., M. V. Elorza, E. Valentin, R. Sentandreu, and D. Gozalbo. 1995. Cloning of cDNAs coding for *Candida albicans* cell surface proteins. J. Med. Vet. Mycol. **33**:105–111.
 62. Sherwood, J., N. A. Gow, G. W. Gooday, D. W. Gregory, and D. Marshall. 1992. Contact sensing in *Candida albicans*: a possible aid to epithelial penetration. J. Med. Vet. Mycol. **30**:461–469.
 63. Singer, S. J. 1990. The structure and insertion of integral proteins in membranes. Annu. Rev. Cell Biol. **6**:247–296.
 64. Soll, D. R. 1989. Dimorphism and high frequency switching in *Candida albicans*, p. 147–176. In D. R. Kirsch, R. Kelly, and M. B. Kurtz (ed.), The genetics of *Candida*. CRC Press, Boca Raton, Fla.
 65. Sudoh, M., S. Nagahashi, M. Doi, A. Ohta, M. Takagi, and M. Arisawa. 1993. Cloning of the chitin synthase 3 gene from *Candida albicans* and its expression during yeast-hyphal transition. Mol. Gen. Genet. **241**:351–358.
 66. Sundstrom, P. M., M. R. Tam, E. J. Nichols, and G. E. Kenny. 1988. Antigenic differences in the surface mannoproteins of *Candida albicans* as revealed by monoclonal antibodies. Infect. Immun. **56**:601–606.
 67. Torosantucci, A., M. Boccanera, I. Casalnuovo, G. Pellegrini, and A. Cassone. 1990. Differences in the antigenic expression of immunomodulatory mannoprotein constituents on yeast and mycelial forms of *Candida albicans*. J. Gen. Microbiol. **136**:1421–1428.
 68. Vermeulen, C. A., and J. G. Wessels. 1986. Chitin biosynthesis by a fungal membrane preparation. Evidence for a transient non-crystalline state of chitin. Eur. J. Biochem. **158**:411–415.
 69. von Heijne, G. 1986. A new method for predicting signal cleavage sites. Nucleic Acids Res. **14**:4683–4690.
 70. von Heijne, G. 1995. Membrane protein assembly: rules of the game. Bioessays **17**:25–30.