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The *Candida albicans* AAA ATPase homologue of *Saccharomyces cerevisiae Rix7p* (YLL034c) is essential for proper morphology, biofilm formation and activity of secreted aspartyl proteinases

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ABSTRACT. Proper morphology is essential for the ability of *Candida albicans* to switch between yeast and hyphae and thereby sustain its virulence. Here we identified, by differential screening, a novel *C. albicans* AAA ATPase encoding gene, *CaYLL34* (*RIX7*), with enhanced expression in hyphae. Phylogenetic analysis suggests that *CaYLL34* belongs to a "VCP-like" subgroup of AAA ATPases essential for yeast viability and contains a bipartite nuclear localization signal. Inactivation of one copy of *CaYLL34*, by the URA-Blaster method, generated the heterozygous mutant strain M61. This strain has severe phenotypic alterations, such as a highly increased vacuole, abnormal cell shape and reduced growth in different conditions. Also, major pathogenicity factors are affected in M61, for instance, a significant decrease of hypha formation (>90%), surface biofilm adhesion (86%) and secreted aspartyl proteinase activity (76.5%). Our results show that the partial impair-

ment of CaYll34p cellular levels is sufficient to affect the proper cellular morphology and pathogenicity factors and suggest that this protein is required for biogenesis of ribosomal subunits. Accordingly, we propose that the product of *CaYLL34* could be tested as a novel target for antifungal drugs.

Key words: AAA ATPases, *Candida albicans*, CottonPrep, *YLL34*, *RIX7*, Differential screening, Insertional mutagenesis

INTRODUCTION

The yeast *Candida albicans* is commensal in humans, where it predominantly colonizes the mucosal surfaces of the gastrointestinal tract. However, especially in immunocompromised patients, *C. albicans* develops into an opportunistic pathogen that can cause superficial as well as life-threatening disseminated infections (Odds, 1988; Fidel and Sobel, 1996). The opportunistic infections in immunocompromised hosts represent an increasingly common cause of mortality and morbidity (Fisher-Hoch and Hutwagner, 1995; Groll et al., 1998). *C. albicans* can switch its morphology from budding yeast to pseudohyphae (chains of elongated cells with visible constrictions at the sites of septa) and hyphae (linear filaments without visible constrictions at the septa) (Mitchell, 1998). The pathogenicity of *C. albicans* seems to be dependent on this capacity for yeast to hypha transition and also, the formation of biofilms and secretion of aspartyl proteinases.

Hyphae are considered the more invasive forms because they are frequently identified in infected tissues and because mutant strains defective in hyphal growth are avirulent (Leberer et al., 1996; Lo et al., 1997; Stoldt et al., 1997). Yeast to hyphae transition depends on environmental conditions and could be controlled by transcriptional regulators such as the products of genes *TUP1*, *CPH1* and *EFG1* (Braun and Johnson, 2000). *TUP1* represses genes responsible for carrying out filamentous growth and *EFG1* and *CPH1* (an orthologue of *Saccharomyces cerevisiae STE12*) are putative transcriptional activators of hyphae (Braun and Johnson, 2000). Under different growth conditions, a network of signalling pathways is employed to simultaneously assess the multiple nutrients available, cell density and other growth conditions. The integrated output of these pathways determines gene expression and dimorphic transition (Liu, 2001). Different signalling pathways and transcriptional factors seem to converge to regulate the transcription of a common set of hyphal-specific genes (Liu, 2001). Several genes have been identified whose expression is induced during hyphal growth. Such genes include *HYR1*, *ALS3*, *HWP1*, and *ECE1*, most of which encode cell wall proteins. *HYR1* is activated in response to hyphal development (Bailey et al., 1996). *ECE1* is associated with cell elongation (Birse et al., 1993). *ALS3* is a hyphal-specific gene and its feature suggests that Als3p is a cell surface glycoprotein (Hoyer et al., 1998). *HWP1* encodes a hyphal-specific surface protein that is implicated in adhesion to human oral epithelial cells (Staab et al., 1999).

Biofilm formation by *Candida* spp is a major pathogenicity factor and has gained considerable interest recently (Calderone and Gow, 2002). Infections by *Candida* involve biofilm formation on implanted devices such as indwelling catheters and prosthetic heart valves (Haw-

ser and Douglas, 1994; Douglas, 2003). Biofilms of *C. albicans* formed *in vitro* on catheter material consist of matrix-enclosed microcolonies of yeasts and hyphae, arranged in a bilayer structure. These biofilms seem to contribute to multiple resistance to drugs in current clinical use, including amphotericin B and fluconazole (Baillie and Douglas, 1999). The extent of resistance varies according to the *Candida* species and the nature of the material on which the biofilm is formed, and apparently, the bloodstream is constantly seeded with cells from within the biofilm formed on implanted devices.

Another important pathogenicity factor of *C. albicans* is the extracellular proteolylic activity of the secreted aspartyl proteinases (SAPs) which are encoded by at least 10 genes whose expression varies with morphogenesis and environment (Hube et al., 1994; Monod et al., 1998; Naglik et al., 1999). These SAPs have different pH optima and specificity for distinct substrates and participate in host protein digestion for nutritional supplement, escape from immunity, adhesion and tissue degradation during invasion (Hube et al., 1998; Koelsch et al., 2000). Inactivation of *SAP1* to *SAP3* genes influences the course of superficial infections, while *SAP4* to *SAP6* are important for systemic infections (Sanglard et al., 1997; De Bernardis et al., 1999; Schaller et al., 1999; Felk et al., 2002). These proteinases are expressed in distinct stages of systemic infections and are responsible for the damage of different tissues and organs (Felk et al., 2002). Although all factors that regulate the expression of each specific proteinase have not yet been identified, these authors propose that impaired proteinase expression is responsible for the decreased virulence of hypha defective mutants.

Differential screening has been used to search for differentially expressed genes in distinct phases of organismal development (Van De Loo et al., 1995; Thipyapong et al., 1997). Also, antifungal drugs currently used are based on a relatively small number of targets, and therefore, the characterization of novel, fungal-specific functions, preferentially related to fungal pathogenicity, could contribute to the discovery of new drug targets (Whiteway, 2000). Here, to discover novel genes differentially expressed in *C. albicans* hyphae, we used a differential screening method and characterized *CaYLL34*, a gene with enhanced expression in hyphae of *C. albicans* and a putative member of the AAA ATPase (ATPase associated with various cellular activities) family. Insertional inactivation of this gene leads to impairment of several cellular functions related to morphology and pathogenicity.

MATERIAL AND METHODS

Candida albicans genomic library construction

Candida albicans ATCC 90029 strain was grown on a rotary shaker at 30°C in YPD medium (1% yeast extract, 2% dextrose, 2% peptone) overnight. Total DNA was extracted using the small scale and quick protocol (Wash et al., 1994) and fragmented by sonication. Fragments from 1 to 4 kb were isolated by preparative agarose electrophoresis, ligated in pUC18 vector and used to transform *E. coli* DH5-α. DNA from 552 clones was extracted using a "CottonPrep" method, a modification of the MiniPrep protocol (Sambrook et al., 1989) adapted for 96-well plates. Briefly, after growth for 16 h at 37ºC, lysis in a 200-µL solution II (0.2 N NaOH, 1% SDS) and neutralization in a 200-µL solution III (3 M potassium, 5 M potassium acetate), the plates were incubated for 30 min on ice. A small amount of hydrophobic cotton was added to each well and the plates were centrifuged for 45 min at 4,000 rpm at 10°C and 250

µL of the supernatant was collected, transferred to other 96-well plates (1 mL capacity) containing 200 µL cold isopropanol and briefly homogenized. After plate centrifugation at 4,000 rpm for 20 min at 10°C, the supernatant was discarded and the plate inverted on absorbent paper. Pellets were washed with 200 µL cold ethanol, centrifuged for 15 min at 4,000 rpm at 10°C and dried at 60°C for 15 min, and plasmids were suspended in 20 µL sterile water and homogenized by vortexing for 3 min.

Differential screening

Plasmids from individual clones of the genomic library described above were spotted on six Hybond-N+ membranes using a dot-blot vacuum system. These membranes contained as controls an actin gene clone, a plasmid with no inserts (pUC18) and a dot with no plasmid. Radiolabeled yeast and hyphal cDNA probes were prepared from total RNA by RT-PCR (described below) in which dCTP was substituted by [α-^{32}P]-dCTP. The six library membranes were incubated with 20 mL hybridization solution (6X SSC, 50% formamide, 5X Denhardt's solution, 1% SDS, and 1.5 mg salmon sperm DNA) at 42°C for 2 h. Yeast cDNA probe was added and the membranes were hybridized overnight. Membranes were washed three times for 10 min at room temperature with 1X SSC/1% SDS and exposed to X-ray film for 48 h. The second hybridization, using hyphal cDNA probe, was performed after the removal of yeast probe checked by autoradiography. Autoradiograms were scanned and analyzed by the ImageQuant program (Molecular Dynamics) and data processed using Excel (Microsoft).

Probes for library screening were prepared from yeast and hyphal cDNA. For this, *C. albicans* ATCC 90029 was grown on YPD medium overnight at 30ºC with constant agitation to produce yeast cells. To obtain hyphae, yeasts were diluted to 1.0 x 10^7 cells/mL in fetal bovine serum containing 5 mg/mL dextrose and incubated at 37°C with agitation for 3 h. Hyphal induction was 80% (germ tube formation). RNA was extracted from 1.5 mL of yeast or hyphal culture where cells were treated with 200 µL 1 M sorbitol/Zymoliase (20,000 IU) and incubated at 37°C for 120 min with mild agitation. After centrifugation the pellets were resuspended in 1 mL Trizol™ (Life technologies) and the RNA extracted according to the manufacturer. After checking integrity by agarose gel electrophoresis, RNA samples were treated with DNAse-RNAse free (Promega).

Yeast and hyphal cDNA were produced using Superscript™ II enzyme (Life Technologies) following the manufacturer's protocol. Each reaction contained 1 µg of total RNA, 1 µL oligo $dT_{12\text{-}18}$, 50 mM and 1 µL 10 mM dNTP in 10 µL final volume. Reactions were incubated at 65°C for 5 min and cooled on ice. To each reaction tube, 10 µL of the following mixture was added: 4 µL of 5X first-strand buffer, 2 µL 10 mM $MgCl_2$, 2 µL 0.1 M DTT, 1.4 µL RNAguard™ (Amersham Pharmacia Biotech), and 1 µL Superscrip™ II. Reactions were incubated at 42°C for 50 min and then at 70°C for 15 min, and the resulting cDNAs stored at -20°C.

Reverse transcriptase-polymerase chain reaction

Primers for amplification of *CaYLL34* 3' region were YLL34F (5'-ACG CCG TGT ATT ATT TTC TTT G) and YLL34R (5'-ATT TCT TGT ATT TTT GGG TTT GG), and for amplification of *ACT1* (*C. albicans* actin gene) primers ACT1F (5'-AGA ATT GAT TTG GCT GGT AGA GAC) and ACT2R (5'-AGA AGA TGG AGC CAA AGC AGT AAT) were used.

RT-PCRs were performed in a total volume of 25 µL with 1 µL cDNA (approximately 2.5 ng for radiolabeling and 0.06 ng for expression analysis), 1 U *Taq* DNA pol (Gibco), 25 ρmol of each primer, 1X *Taq* buffer, 0.8 mM each dNTP and 4 mM $MgCl_2$. Cycling conditions were as followed: 94°C for 5 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1:30 min, and final extension at 72°C for 5 min. Products of these reactions were separated by ethidium bromide/agarose gel electrophoresis and gel images were scanned. Amplicons were quantified using the ImageQuant (Molecular Dynamics) and Excel (Microsoft) programs and band intensities normalized using actin amplification as control.

DNA sequencing and contig assembly

For sequencing, clones of interest were subcloned because the average insert sizes were above 2,000 bp. Clone plasmids were extracted and fragmented by sonication. Fragments between 300 and 1,000 bp were isolated from preparative 1% agarose gels, ligated into pUC18 and cloned in *E. coli* DH5-α. Inserts were sequenced using dideoxy-nucleotide BigDye Terminators (Applied Biosystems) in an ABI377/96 automated sequencer. Contigs were assembled using Phred-Phrap-Consed programs (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

Computational sequence analysis

Nucleotide sequences were submitted to BLAST analysis (Blastn and Blastx) at the NCBI - National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and *Candida* Stanford Genome - Stanford Genome Technology Center (http://sequence-www.stanford.edu/group/candida/search.html) sites. To search for conserved functional motifs, protein sequences were analyzed at the Pfam website, a database of protein families and HMMs, Washington University in St. Louis (http://pfam.wustl.edu/hmmsearch.shtml), and to search for transmembrane domains, sequences were submitted to CBS, Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark (http://www.cbs.dtu.dk/services/TMHMM). The amino acid sequence of the CaYLL34 protein was obtained using EditSeq program (DNAstar, Madison, WI, USA) and the standard codon usage except for CTG, which encodes serine instead of leucine in *C. albicans* (Santos and Tuite, 1995). Sequences were aligned using MEGALIGN program (DNAstar) and the Clustal method (Higgins and Sharp, 1989). The alignments and the corresponding accession number tables are available upon request to: marcelo@ecb.epm.br.

Phylogenetic inference

The amino acid sequence of CaYLL34p (GenBank accession No. AAR84642) was used as query on TBLASTN search. The amino acid sequences of the 100 best hits were selected and aligned by eye using SEAVIEW (Galtier et al., 1996). Removal of sequences with large number of gaps resulted in the alignment of 505 positions from 75 sequences, excluding gaps. Phylogenetic tree was inferred using MrBayes program (Huelsenbeck and Ronquist, 2001) with the JTT model (Jones et al., 1992) for amino acid substitution with gamma distribution (Yang, 1994), alpha shape parameter = 0.899821 and 95% confidence interval of 0.794562-

1.020755. The chain length was 100,000 and four simultaneous chains were run and sampled every 100 generations. A consensus tree was built from 600 trees corresponding to 60,000 generations for which the likelihood scores converged to a stable value. Bootstrap values were obtained from 100 pseudo-replicates generated by the neighbor-joining method in PAUP 4.0. (Swofford, 1998).

Candida albicans transformation and isolation and characterization of mutants

Transformation was performed using a slight modification of the lithium acetate method described elsewhere (Fonzi and Irwin, 1993). Briefly, *C. albicans* CAI4 (*ura3*Δ::*imm434*/*ura3*Δ::*imm434*) was incubated in YPD-uridine at 30°C for 48 h. A single colony was isolated and used to inoculate 2 mL YPD-uridine. After growth overnight at 30°C, 0.5 mL of this suspension were transferred to 50 mL YPD-uridine and incubated at 30°C for 6 h. When the absorbance (600 nm) increased 4-fold, cells were pelleted by centrifugation, washed in sterile water and resuspended in 0.5 mL TE/LiOAc. For transformation, 5 to 10 µL cassette DNA and 10 µg salmon sperm DNA were added to 0.1 mL of competent cells which were prepared as described above, and then incubated for 30 min at room temperature. After addition of 0.7 mL PLATE mix the suspension was incubated for 16 h at room temperature. The suspension was incubated at 42°C for 1 h and cells pelleted (30 s) were resuspended in 0.1 mL sterile water, plated on SD-agar and incubated for 5 days. After replating for 48 h, the DNA of transformants was extracted.

Southern blot analysis was performed using genomic DNA of ATCC 90029, CAI4 and M61 that was digested with *Bam*HI and *Bgl*II. After separation by agarose gel electrophoresis, fragments of the digestion products were transferred to a nylon membrane as described elsewhere (Sambrook et al., 1989). A sequence of *CaYLL34* 3' region labeled with [α-^{32}P]-dCTP using Random Primers DNA Labeling System (Life Technologies) was used as a probe (555 bp) (Figure 6A). This sequence was also present in cassette construction to *CaYLL34* inactivation, and therefore hybridizes to the cassette and the wild-type gene.

Growth curves of the ATCC 90029, CAI4, L296, and M61 strains were determined for cultures in YPD agar at 30°C for 72 h and then incubated in 2X YPD overnight in a shaker at 200 rpm at 37°C. The cultures were diluted with fresh medium and the cell suspensions were set to 0.5 optical density (OD) at 600 nm. An inoculum of 5 mL of each strain was added to 45 mL of 2X YPD, in 2 replicates. Afterward, one replicate was incubated at 30°C and the other at 37°C in a shaker at 200 rpm. To estimate the doubling time of strains in both growth conditions, the cultures were measured (OD_{600}) every 3 h in the first 30 h. After that, the cultures were measured at 36 and 48 h.

Counter-selection was used to select Ura3$^-$ auxotrophs as described elsewhere [Fonzi and Irwin, 1993 #82], on minimal medium containing 5-fluoroorotic acid (5-FOA, 625 µg/mL) and uridine (100 µg/mL). Prior to selection, M61 mutant was plated on YPD medium supplemented with uridine and incubated for 48 h at 30°C. One individual colony was taken from the plate and suspended in H_2O (1 mL). Dilutions of the suspension were spread on SD with uridine to determine the number of colony forming units present and portions were spread on 5-FOA medium to select Ura3$^-$ cells. The 5-FOA plates were scored after 3-4 days of incubation at 30°C. DNA of some colonies was extracted and submitted to PCR with primers URA3-F and URA3-R to confirm *URA3* split.

Microscopy analysis and phenotypic tests for species identification

To analyze the phenotypic changes of M61 mutant strain, CAI4 (parental strain) and M61 were grown in YPD, SD 50 (minimum medium containing 50 mM glucose), and fetal bovine serum (FBS), overnight at 37°C in a shaker. The morphology was examined by phase-contrast microscopy (BX 60 System Microscope, Olympus Optical Co., Tokyo, Japan) and photographed (PM 30 Automatic Photomicrographic System, Olympus Optical Co.). Micro-morphology analysis was used to confirm species identification, ATCC 90029, CAI4 and M61 strains were cultured for 48 h in Sabouraud-Dextrose agar (Difco Laboratories, Detroit, MI, USA). One colony of each strain was striated on cornmeal agar Tween-80, covered with sterilized coverslips and incubated at 25-28°C for 48 to 96 h. The cultures were examined daily with an optical microscope (phase-contrast) (BX 60 System Microscope, Olympus Optical Co.) using 10X and 40X lenses to observe blastoconidia, artroconidia, hyphae, pseudohyphae, and chlamydoconidia. The presence of chlamydoconidia in all three cultures was confirmatory for *C. albicans* identification. Species identification was further confirmed for these three strains by culturing on CHROMagar Candida medium (CHROMagar, Paris, France), and all of them produced green colonies, which is diagnostic of *C. albicans.* Classical biochemical analysis of sugar assimilation and fermentation also confirmed the *C. albicans* profile in all three strains.

Biofilm production measurement by crystal violet staining and XTT reduction assays

The growth conditions and biofilm formation for crystal violet staining and XTT (tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reduction assay used in this study were adapted from that described elsewhere (Jin et al., 2003). The ATCC 90029, CAI4, L296, and M61 strains were cultured in YPD agar at 30°C for 48 h and then incubated in SD 110 mM glucose broth overnight on a shaker at 200 rpm for 18 h at 37°C. The cultures were harvested and washed twice with PBS, the cell suspensions were set to an absorbance of 0.4 at 520 nm, and 100 µL of these suspensions were tranferred to a 96-well cell culture plate. No cells were added to one row to be used as control. The plate was incubated at 37°C for 1 h 30 min at 75 rpm (adhesion period). The wells were washed twice with PBS and 100 µL of SD 110 mM glucose was added. The plate was incubated at 37°C for 66 h at 75 rpm (biofilm formation).

For the crystal violet staining, each well was washed twice with PBS and dried for 1 h at room temperature. Next, each of the washed wells was stained with 110 µL of 0.4% aqueous crystal violet solution for 45 min. Afterwards, the wells were washed four times with 200 µL MilliQ sterile water and destained with 200 µL of 95% ethanol. After 45 min, 100 µL of destaining solution of each sample was transferred to a new plate that was measured with a microtiter plate reader (Multiskan MS, Labsystems, Finland) at 540 nm. The absorbance values of the controls were subtracted from the values of the test wells to minimize background interference. Two independent experiments were performed with 7 replicates for each strain (a total of 14 replicates).

For the XTT reduction assay, an XTT solution was prepared (1 mg/mL in PBS) and filter-sterilized through a 0.22-µm pore size filter. Menadione solution (0.4 mM in acetone) was filtered and mixed with XTT solution at a ratio of 1 to 5 by volume before the assay. After biofilm formation, the wells were washed three times with 200 µL PBS. To each well, 200 µL of PBS and 12 µL of the XTT-menadione solution were added. The culture plate was incubated in

the dark for 2 h at 37°C, and then, 100 µL of solution of each sample was transferred to a new plate and measured with a microtiter plate reader at 492 nm. The absorbance values of the controls were subtracted from the values of the test. The experiment was performed using 7 replicates for each strain.

Aspartyl proteinase assays

The growth conditions for biofilm induction was modified from that described by Hawser and Douglas (1994). The ATCC 90029, CAI4, L296, and M61 strains were cultured in SD 50 mM (YNB medium supplemented with 50 mM glucose) plates at 30°C for 72 h. A loopful was inoculated in 20 mL of SD 50 mM and cultured on a shaker at 37°C for 24 h at 70 rpm. The cells were washed twice with PBS and suspended to an absorbance of 0.8 at 520 nm. One milliliter of each cell suspension was added to the wells of 24-well tissue culture plates and incubated for 1 h 30 min on a shaker at 37°C (adhesion period) at 70 rpm. The wells were washed once with 2.5 mL PBS and 1 mL of SD 50 mM was added. For the CAI4 strain, the medium was supplemented with 0.1 M uridine. The plates were incubated in a shaker at 37°C for 48 h at 70 rpm to allow biofilm formation. The medium was changed after 24 h. The medium was removed and 100 µL PBS was added to resuspend the biofilms. The biofilms of 8 wells of each stain were transferred to an Eppendorf tube and centrifuged briefly. Fifty microliters of the supernatant was used in the aspartyl proteinase assays.

The internally quenched fluorogenic peptide Abz-AIKFFARQ-EDDnp flanked by *ortho*-aminobenzoic acid (Abz) and N-[2,4-dinitrophenyl] ethylenediamine (EDDnp) was used to test the aspartyl proteinase activity following the method previously described (Pimenta et al., 2001). The peptide was synthesized by solid-phase in an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system, Shimadzu, Japan) as described elsewhere (Hirata et al., 1994). The molecular weight and purity of the synthesized peptide was checked by amino acid analysis and MALDI-TOF mass spectrometry, using a TofSpec-E from Micromass, Manchester, UK. Stock solutions of the substrate were prepared in DMSO, and the concentration was measured spectrophotometrically using the molar extinction coefficient of 17,300 M^{-1} cm^{-1} at 365 nm. Abz-AIKFFARQ-EDDnp (5 µM) was assayed in 50 mM sodium citrate buffer, pH 4.0, at 37°C. The reaction was followed by measuring the fluorescence at λ_{ex} = 320 nm and λ_{em} = 420 nm continuously in a Hitachi F-2500 spectrofluorometer. The slope was converted into nmols of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of peptide. For inhibition studies, 2 µM of pepstatin was added to the reaction.

RESULTS

Differential screening and clone selection

To identify genes that are differentially expressed in yeast-to-hypha transition, we used a differential screening method where six Hybond-N+ membranes were spotted with 540 clones of *C. albicans* genomic library and screened sequentially with radiolabeled yeast and hyphal cDNA probes as previously described (Melo et al., 2003). Hybridization signals were quantified by scanning of the autoradiogram and analysis by the ImageQuant program (Molecular Dynamics) with signal normalization using actin gene sequences. Putative differentially expressed clones

were blotted in another membrane for confirmatory hybridization which revealed that three clones, G3, D4 and E4, had 1.7-, 1.5- and 3.1-fold increased signals with the hyphal probe, respectively (Figure 1). Clone E4 was selected for further analysis because it was the only clone of our set to show an estimated hyphal expression level above 2-fold compared to yeast forms. The full-length sequence of clone E4 insert is 2274 bp and was determined by shotgun sequencing. The contig of clone E4 was assembled using 208 reads ranging between 500 and 600 bases and yielding a high-quality contig sequence (phred score >40 as calculated by Phred-Phrap-Consed programs) (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998).

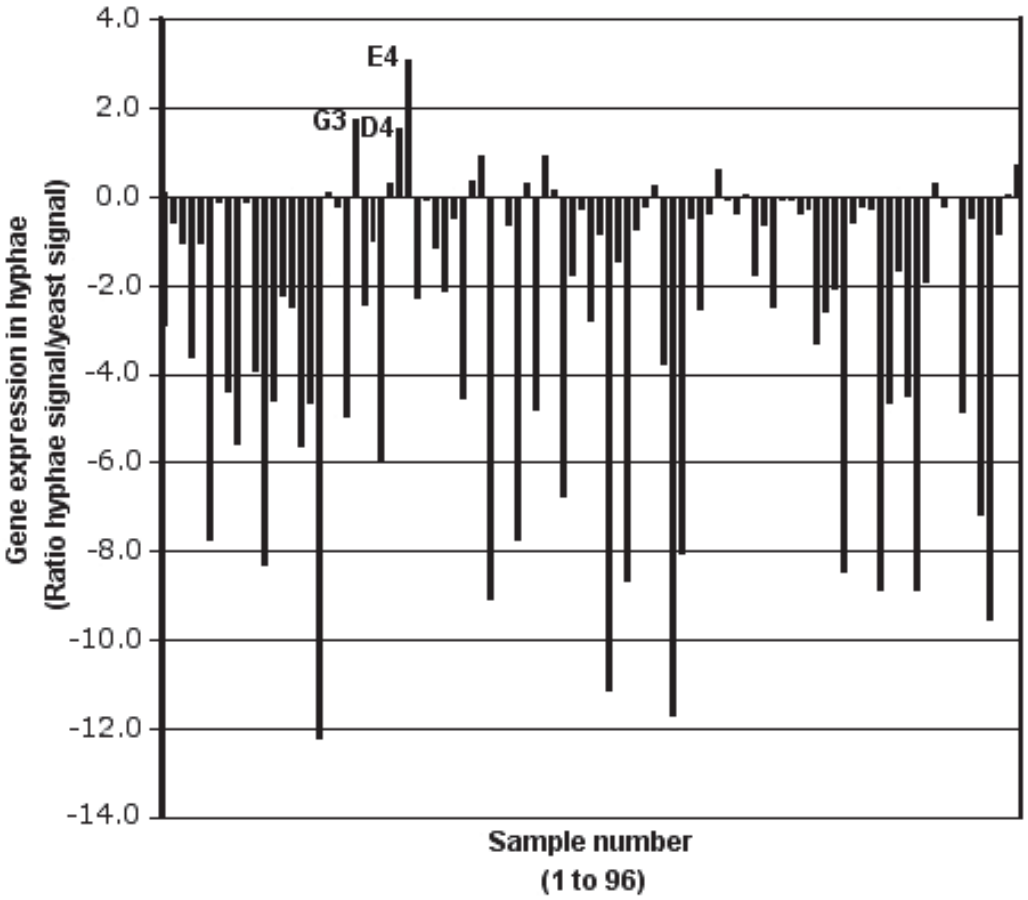

Figure 1. Differential gene expression of *Candida albicans* calculated from membrane VII used for differential screening. The intensity of the two images produced by membrane VII (A and B) was analyzed by ImageQuant (Molecular Dynamics) program, using actin gene expression as reference. Positive values on scale indicate 2- and 4-fold increases in gene expression in hyphae and negative values indicate increased gene expression in yeast forms. Clones G3, D4 and E4 contain genes with increased expression in hyphae.

To identify genes contained in clone E4, potential open-reading frames (ORF) were sought using the "Find ORF" tool in the NCBI website. Two ORFs were found in clone E4 (frame -3). The first ORF, E4.1, corresponded to 507 bp in the central region (positions 1142 to 1648) and another ORF, E4.2', was in the contig end, positions 1 to 376 (Figure 2). Blastx

analysis of ORF E4.1 showed no significant similarities to GenBank whereas ORF E4.2' showed a significant similarity with the *Saccharomyces cerevisiae* YLL034c, which encodes for a protein that belongs to the AAA ATPase family and is required for cell viability (e-value = 4×10^{-62}, 71% identity). ORF 4.2' also matched with contig6-2158 of the Stanford University *Candida albicans* Genome Project (SUCGP), which contained the preliminarily annotated *YLL34* gene. These results suggest that ORF 4.2' corresponds to a part (376 bp) of gene *YLL34* included in contig6-2158 (SUCGP).

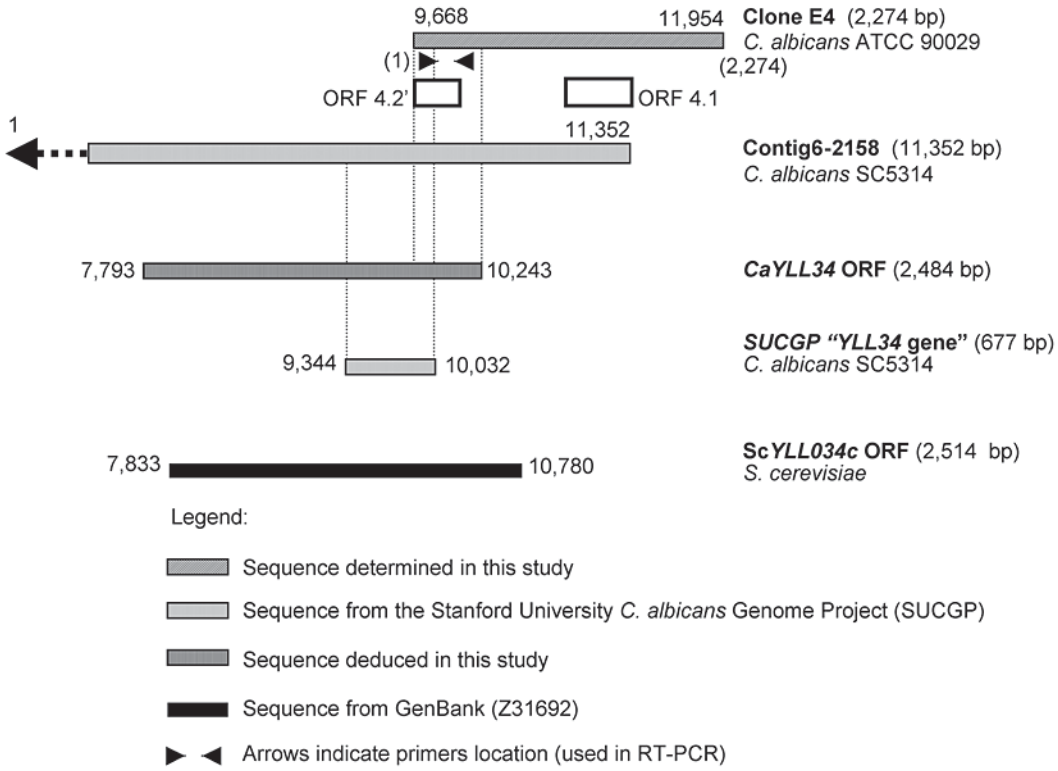

Figure 2. Diagram of assembly by alignment of several different clones containing *YLL34* gene sequences. These sequences were obtained from two strains of *Candida albicans* ATCC 90029 (this study) and *C. albicans* SC5314 (Stanford University *Candida* Genome Project, SUCGP), and *S. cerevisiae YLL034c* open-reading frame (ORF). The SUCGP "YLL34 gene" at the time of assembly 6 preliminary annotation.

Subsequently, the complete *YLL34* ORF was obtained from analysis of contig6-2158 of SUCGP (11,352 bp) using the "Find ORF" program. Five ORFs were found at frame +2. ORFs 1, 2 and 3 were not significant (less than 100 codons) and ORF 4 (positions 743 to 2347) had 1605 bp but was of no interest because it did not overlap clone E4. ORF 5 had 2484 bp (positions 7877 to 10,360) encoding a putative peptide of 827 amino acids and overlapping with 100% identity the 575 bp at the 5' end of clone E4, containing ORF E4.2'. The complete *YLL34* ORF was therefore assembled using the high quality sequence of clone E4 and the contig6-2158. This ORF corresponds to the gene we named *CaYLL34* and has 2484 bp (Figure 2). The diagram in Figure 2 also depicts the alignment between (1) Clone E4, (2) ORFs E4.1 and E4.2', (3) contig6-

2158 (SUCGP), (4) our inferred *CaYLL34* ORF, (5) the *YLL34* gene annotated by SUCGP, and (6) *ScYLL034c* ORF (GenBank Z31692). This result shows that the *CaYLL34* ORF is fully located in contig6-2158.

Characterization of the putative CaYLL34 gene product

The *CaYLL34* ORF (GenBank accession No. AY493662) encodes a putative protein of 827 amino acids and 91.8 kDa (Figure 3A) and was compared to similar proteins that belong to the AAA ATPase family, namely, protein ScYLL034cp (837 amino acids, from GenBank Z731339 and NP013066), *S. cerevisiae* valosin-containing protein (VCP) (NP010157), *Homo sapiens* VCP (NP009057) and *Schizosaccharomyces pombe* AAA ATPase (NP596710) (Figure 3B). The pairwise identities between CaYll34p and these proteins were: 58.3% to ScYLL034cp, 37.2% to *S. cerevisiae* VCP, 36.7% to *Homo sapiens* VCP, and 29.3% to *S. pombe* AAA ATPase. The alignment depicted in Figure 3B also suggests that the AAA ATPase family has at least two groups of members containing two AAA motifs: one group containing the CDC48 domain (proteins 4 and 5, Figure 3B) and the other without a CDC48 domain (proteins 1, 2 and 3, Figure 3B). This analysis suggests that CaYll34p and ScYLL034cp are encoded by orthologous genes and that CaYll34p does not belong to the CDC48 plus, valosin group.

Analysis of CaYll34p using Pfam indicates the presence of two domains common to the AAA ATPase family (P-loop) (Iyer et al., 2003). The scores of each domain were $E = 2.7 \times 10^{-85}$ and $E = 5.5 \times 10^{-89}$, respectively. These domains were localized between residues 229-420 and 558-746 (Figure 3A). Other proteins similar to CaYLL34p were also submitted to Pfam analysis. The VCP proteins contain two AAA ATPase domains and one CDC48 (cell division protein 48) domain (Figure 3A). These results indicate that all of these proteins belong to the AAA ATPase family.

Because the AAA ATPases are a vast family of very ancient and related proteins we investigated whether CaYLL34p belongs to a subgroup within this family with a more specific or defined cell role. One hundred members of AAA ATPases were considered in the analysis from which 75 provided high-quality alignment with CaYll34p (available upon request to marcelo@ecb.epm.br). This dataset was used as input for phylogeny inference using a Bayesian method. In Figure 4, the resulting phylogenetic tree depicts 9 major groups clustered by function. At least two types, or groups, of plant, mammalian and nematode AAA ATPase are observed while only one group of yeasts is present. The yeast group is most closely related to the mammalian nuclear group, thus suggesting that CaYll34p might be located in the nucleus. So far, no functions or properties have been attributed to this group of AAA ATPases in *C. albicans* although the deletion of the *S. cerevisiae* homolog ScYLL034cp encoding gene is lethal in haploid yeasts (El-Moghazy et al., 2000). In addition, the *S. cerevisiae* mutant *rix7-1* is complemented by *YLL034c*, and therefore, *YLL34* and *RIX7* are synonymous (http://db.yeastgenome.org/cgi-bin/locus.pl?locus=Rix7, accessed December 10, 2004) (Cherry et al., 1998). Gene *RIX7* encodes a member of the AAA ATPase family, which contains a bipartite nuclear localization signal and which is required for biogenesis and nuclear export of 60S ribosomal subunits (Gadal et al., 2001). Accordingly, CaYll34p has a potential bipartite nuclear localization signal (http://bo.expasy.org/prosite/) in residues 165-182 at the N-terminus (Prosite sequence PS00015) (Hulo et al., 2004) (Figure 3B). Our results suggest that *CaYLL34* is an essential gene encoding an AAA ATPase functionally related to “Mammalian type 2” and “Nematode type 2” (Figure 4)

A) Sequence of putative protein CaYll34p

MSKSRTVTGSLDQKIYNLIHDLLQEQTEENKRAATADEEHDPFSCMASSHNLTVSQVLAY
VQMKDFQLKRMKKNYLEKSIAASLKVIRQDETEEFGDRENGIGDESEAENDKECENDLME
VKDLNAINKSVVSLWNQEKTDGESDGVNNEEGQQADTTELLKGNKRKAKGLAKTQLKKQK
RKIDYSTPNIDLSSLGGVESVTTQLLEIIGLPILHPEIYSSTGVEPPRGVLLYGPPGCGK
TTIANALAGELKVPFINISAPSVVSGMSGESEKKLREIFEEAKQIAPCLIFMDEIDAITP
KRDGGAQREMEKRIVAQLLTLMDELTLEKTGGKPVVVIGATNRPDSLDSALRRAGRFDRE
ICLNVPNEEQRISILKAMTKNIKLENGEHFNYRELSKLTPGYVGADLKSLVTAAGISAIK
RIFETMSELQEESHLVKDDSMDADPVSLDANKEDMIKKFEQKSEAEKLSTIKKFLNMHPD
PLNQEQLAPLAITYQDFVNALPSVQPSAKREGFATIPDVTWQNVGALFKIRMELHMCIVQ
PIKKPELYLKVGIAAPAGVLMWGPPGCGKTLLAKAVANESRANFISIKGPELLNKYVGES
EKAVRQVFQRARASTPCIIFFDELDALVPRRDTSMSESSSRVVNTLLTELDGLNDRKGVF
VIGATNRPDMIDPAMLRPGRLDKTLYIELPTPEERLEILKTLVRTSNSPLHANVDLNAIS
RDSRCGNFSGADLSSLVKEAGVWALKKRFFQNQKIQELDSSGFYEDSIGEDDISITAEDF
DHALSSIRPSVSDRDRMRYEKLNKKLGWNIISDKEPDDAGSTSDKND

B) Conserved domains in putative protein CaYll34p

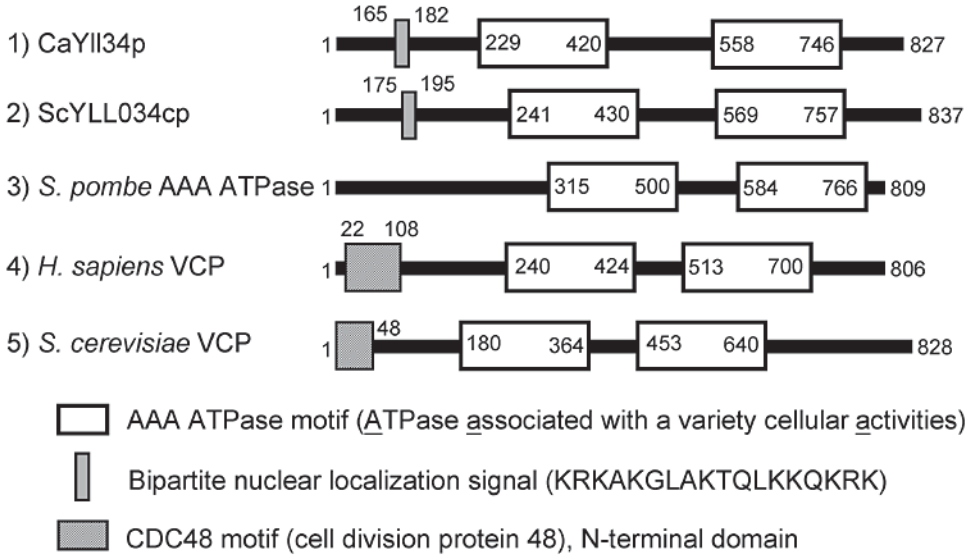

Figure 3. Sequence analysis of the putative CaYll34p protein. In **A** the two AAA ATPase domains of CaYll34p (216 amino acids each) are underlined. In **B** depiction of Pfam HMM search result of 1) CaYll34p, 2) ScYLL034cp, 3) *S. pombe* AAA ATPase (gi|19113502|ref|NP_596710.1), 4) *Homo sapiens* valosin containing protein (VCP) (gi|6005942|ref|NP_009057.1) and 5) *S. cerevisiae* VCP (gi|6320077||NP_010157.1). In all of these proteins, two AAA ATPase motifs of 216 amino acids were found. The e-value of these matches were e<1.1 x 10^{-82}. CDC48 motif matched only to VCP proteins. Bipartite nuclear localization signals are present only in CaYll34p and ScYLL034cp (*RIX7*) as determined using Prosite search (http://bo.expasy.org/prosite/). The sequences of CaYll34p nuclear localization signals (KRKAKGLAKT QLKKQKR) and the corresponding ScYLL034cp (*RIX7*) (KKSKKRSKEGTCKVKRQKIK) are indicated in the corresponding domains.

that may have a function related to *RIX7* in ribosome biogenesis. Because CaYll34p has only 30-40% identity to human VCP-like AAA ATPases, it may be possible in the long run to find drugs that will interfere with CaYll34p function but not with the human homologs.

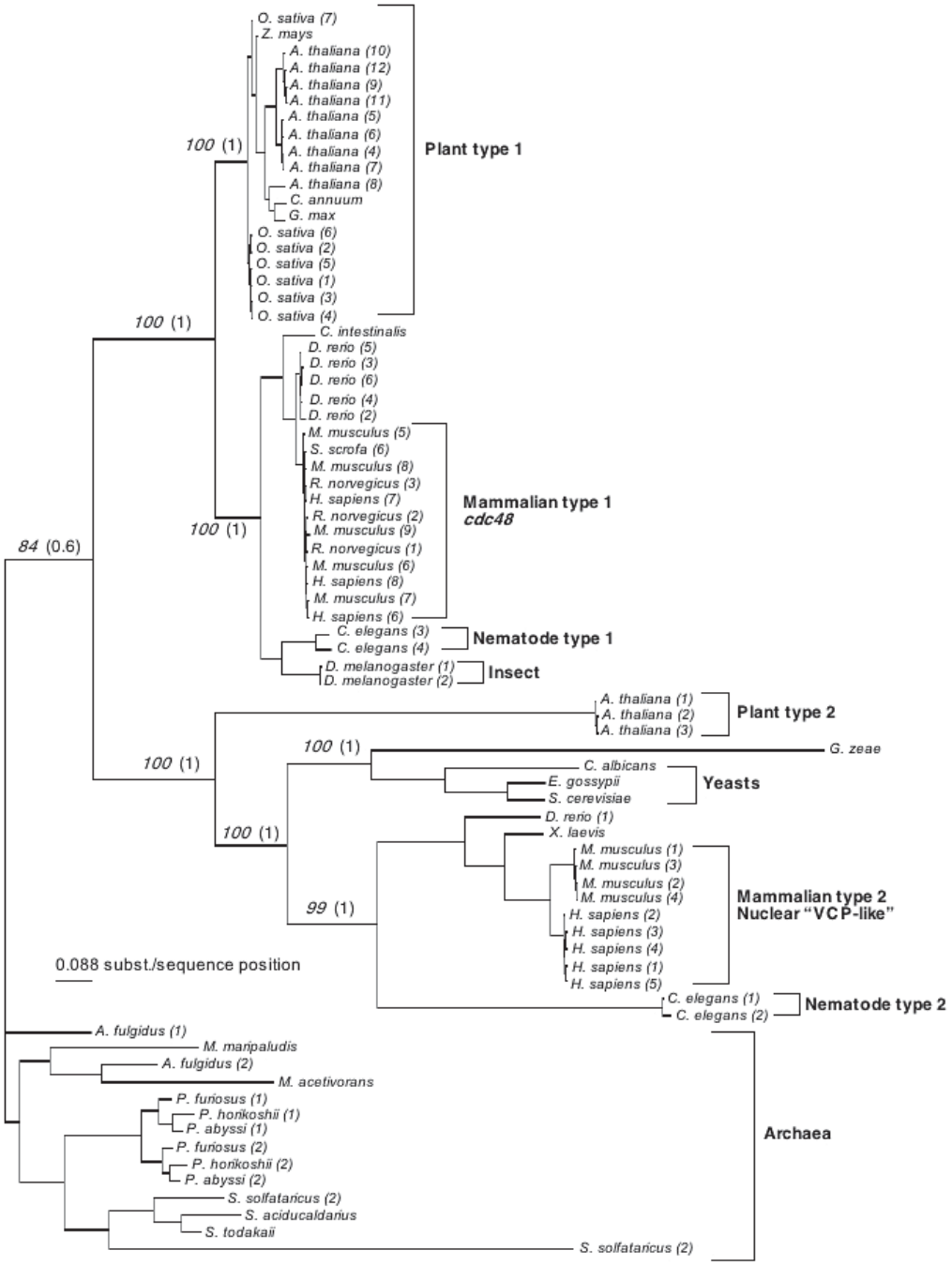

Figure 4. Phylogenetic relationship of *CaYLL34* to other members of AAA ATPases. Phylogenetic analysis based on amino acid sequences of 75 members of the AAA ATPases identifies 9 subtypes, namely: plant types 1 and 2, nematode types 1 and 2, mammalian types 1 and 2, insect, yeasts and Archaea. These subtypes represent clusters by function. Numbers in italics indicate the bootstrap frequency in 100 pseudo-replicates and numbers in parentheses indicate the posterior probabilities obtained by Bayesian analysis using MrBayes program (Huelsenbeck and Ronquist, 2001). The phylogeny was inferred using amino acid sequences and 505 aligned positions, excluding gaps, and JTT amino acid substitution model (Jones et al., 1992). VCP = valosin containing protein.

Differential expression of CaYLL34

The increased expression level of *CaYLL34* in hyphae was confirmed by RT-PCR. Specific primers for *CaYLL34* were designed based on the regions for this gene contained in clone E4 (ORF 4.2') (Figure 2), yielding an expected amplicon of 432 bp, using yeast and hyphal cDNAs as templates and actin cDNA amplification as positive control (*ACT1*, 443 bp) (Figure 5). We observed that the expression of *CaYLL34* is 3.6-fold increased in hyphae compared to yeast forms, as estimated by densitometry in 14 independent experiments. The typical amplicons used are shown in Figure 5A and the densitometry was performed using the ImageQuant program and normalization with actin amplicons. This result confirmed the analysis shown in Figure 1 and shows the consistency between estimates obtained by autoradiogram densitometry and RT-PCR. The cDNA concentration used in this RT-PCR (approximately 0.06 ng) was standardized by preliminary assays (data not shown) in order to yield amplicons that are in the linear range of the PCR and therefore will not saturate the signal and still be visible in the ethidium bromide/agarose gel electrophoresis. In Figure 5B cDNA control with mock RT reaction shows that bands in Figure 5A are mRNA derived and not due to genomic DNA contamination.

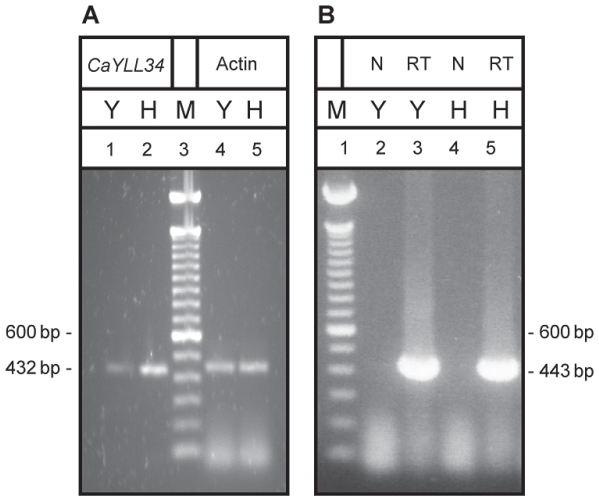

Figure 5. Semi-quantitative RT-PCR of *CaYLL34* gene visualized on agarose gel. Yeast and hypha cDNA of *C. albicans* ATCC 90029 were diluted 1:400 and 1 µL was used in each sample as indicated above the numbers. Actin gene amplification was used to normalize the signal (in A, *lanes* 4 and 5). In panel **A**, primers YLL34F and YLL34R amplified part of *CaYLL34* gene in yeasts (Y) and hyphae (H) (*lanes* 1 and 2) and primers ACT1 and ACT2 amplified part of actin gene in yeasts (Y) and hyphae (H) (*lanes* 4 and 5), and *lane* 3 (M) is 100-bp ladder. In panel **B**, ACT1 and ACT2 amplified part of actin gene in an RT-dependent reaction only (RT) and not in mock reactions without RT (N) (*lanes* 2 to 5, *lane* 1 is 100-bp ladder) to show that the RNA preparations of yeast (Y) and hyphae (H) used in RT-PCR analysis are not affected by contamination with genomic DNA.

Partial disruption of CaYLL34

To investigate the role of *CaYLL34* in *C. albicans*, we inactivated one allele of the gene in the diploid parental strain *C. albicans* CAI4 (Ura$^-$), consequently generating the mutant

strain M61. Inactivation was carried out using the "URA-Blaster" method (De Backer et al., 2000). For this we generated a *URA3-HisG-CaYLL34* cassette in two steps (Figure 6A). Firstly, a fragment corresponding to 420 bp of the 5' end of the *CaYLL24* gene was amplified (from *C. albicans* genomic DNA), digested with *Bgl*II and *Bam*HI and ligated to *Bgl*II digested plasmid p5941 (Fonzi and Irwin, 1993), thus generating plasmid pYLL34-2. Secondly, a fragment corresponding to 570 bp of the 3' end of *CaYLL34* was amplified (from *C. albicans* genomic DNA), digested with *Bgl*II and *Bam*HI and ligated to *Bam*HI digested pYLL34-2, thus generating plasmid pYLL34-4. Digestion of pYLL34-4 with *Bgl*II and *Bam*HI released the HUH-*CaYLL34* cassette containing respectively the 250 bp 5' end region of *CaYLL34*, *HisG*, *Ura3* gene, *HisG*, and 555 bp of the 3' end region of *CaYLL34*.

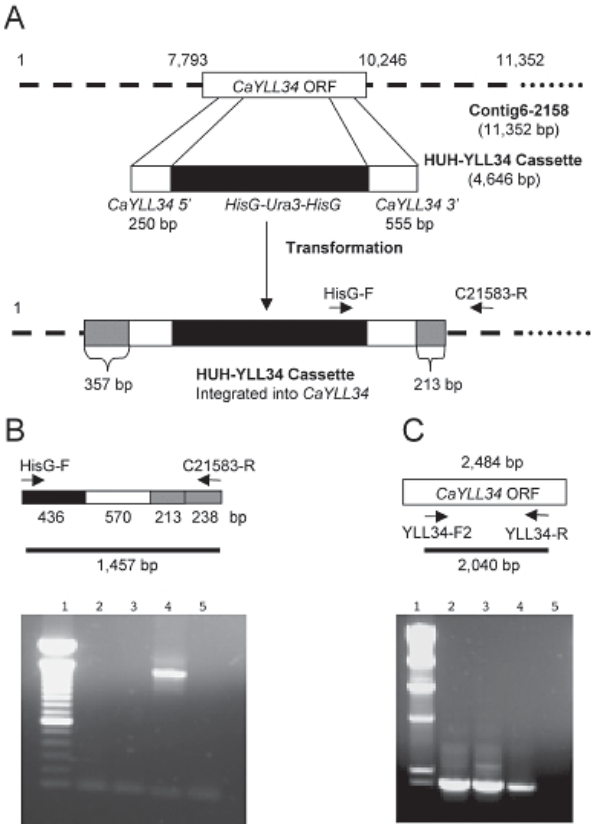

Figure 6. Schematics of the insertional inactivation of *CaYLL34* and verification of mutant genotype. In panel **A**, construction of HUH-YLL34 cassette with resulting expected integration and location of primers HisG-F and C21583-R. Numbering is relative to the 5' end of contig6-2158 of the Stanford University *Candida* Genome Project. In panel **B**, amplicon using primers HisG-F and C21583-R in genomic DNA of *Candida albicans* strains where HUH-YLL34 integrated correctly. The resulting amplicon, 1457 bp, was completely sequenced confirming the structure. Gel *lanes* are 1 - 100-bp ladder (Gibco); 2 - ATCC 90029; 3 - CAI4; 4 - M61, and 5 - negative control (blank). Amplification was observed only in M61 thus confirming that M61 has one copy inactivated by HUH-YLL34 cassette. PCR was from 40 ng of genomic DNA in panels **B** and **C**. In panel **C**, amplification of the wild-type YLL34 allele in M61, ATCC90029 and CAI4. Gel *lanes* are 1 - lambda-*Hin*dIII; 2 - ATCC 90029; 3 - CAI4; 4 - M61, and 5 - negative control (blank). Amplification was observed in all strains, although approximately half in M61 confirming that it has only one wild-type allele as opposed to two observed in ATCC90029 and CAI4.

The HUH-YLL34 cassette was used to transform the *C. albicans* CAI4 (Ura$^-$) strain using the lithium acetate method and three different concentrations of DNA (18, 10 and 5 µg). After five days of incubation, 98 Ura$^+$ colonies were observed. Transformant colonies were analyzed by PCR using primers HisG-F and C21583-R, which amplified a fragment of 1457 bp in the mutant containing the correctly integrated cassette (Figure 6B). The 1457-bp amplicon from mutant M61 (*yll34*Δ::*hisG-CaURA3-hisG*/*YLL34/ura3*Δ::*imm434*/*ura3*Δ::*imm434*) was sequenced and the expected structure, generated by homologous recombination, was confirmed. Amplification of *CaYLL34* shows that strain M61 is a heterozygote. Specific primers for *CaYLL34* were used for PCR from M61 strain genomic DNA, generating the 2040-bp amplicon (Figure 6C). The amplification signal in M61 as compared to CAI4 suggests that the number of copies of *CaYLL34* per diploid genome is approximately half in M61. Also, Southern blot analysis shows that the HUH-YLL34 cassette integrated only one the *CaYLL34* allele (Figure 7A) and in no other part of the genome, thereby confirming the results obtained by PCR (Figure 6C) and showing that no other genes were disrupted. The 6.1-kb band in the *Bam*HI/*Bgl*II digest of M61 corresponds to the CaYLL34 allele with the HUH-YLL34 integrated and the 2.4-kb band in CAI4, M61 and ATCC90029 represents the wild-type allele (Figure 7A).

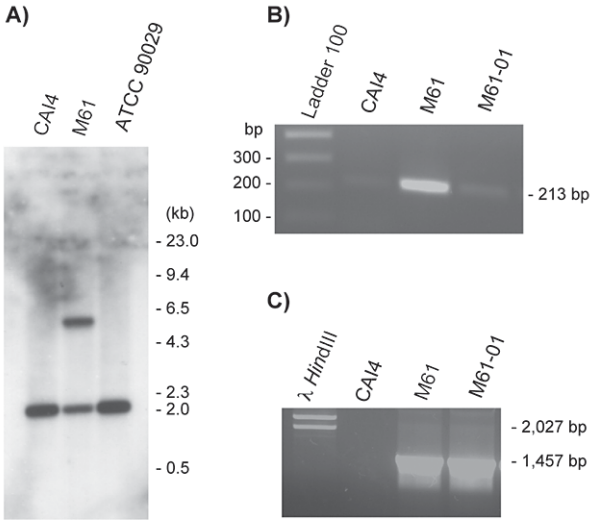

Figure 7. Genotype analysis on mutant M61 and M61-01 by Southern blot and PCR. In **A** Southern blot of digested DNA of CAI4, M61 and ATCC 90029 with *Bam*HI and *Bgl*II. The probe was *CaYLL34* 3' (555 bp) region labeled with [α-^{32}P]-dCTP. This probe hybridized with *CaYLL34* (~2000 bp) present in all strains. In M61 the ~6500-bp fragment corresponds to the cassette inserted in *CaYLL34* gene. The cassette size is 4648 bp. PCR of genomic DNA of CAI4, M61 and M61-01 (URA3$^-$). In **B** amplicon of 213 bp of *URA3* gene using primers URA3-F and URA3-R showing that *URA3* was not present in M61-01 mutant. In **C** amplicon of 1457 bp using primers HisG-F and C21583-R showing that *HisG* sequence interrupts *CaYLL34* in M61-01 mutant.

M61 was then used to generate another mutant, M61-01, by counter-selection with 5-FOA rendering M61-01 Ura minus but still having one allele of *CaYLL34* inactivated by intervening *HisG* sequence (Figure 7B) (Fonzi and Irwin, 1993). In all the phenotypic analysis discussed below we did not observe differences between M61 and M61-01, and therefore, the phenotypic alterations in M61 do not seem to be a consequence of the "Ura effect" (Lay et al., 1998; Brand et al., 2004).

Phenotypic analysis of M61 mutant strain

To analyze the morphologic changes that occurred in M61-01 due to insertional inactivation of *CaYLL34*, this strain was examined by phase-contrast microscopy and compared to its parental strain CAI4 under different growth conditions (Figure 8). The comparison between CAI4 (Ura^-) and M61-01 (Ura^-) in SD medium at 37ºC is striking and shows that failure to induce filamentation is not caused by any effects involving the Ura^+ marker and strongly suggests that inactivation of only one allele of *CaYLL34* is sufficient to impair hyphal formation (Figure 8). There was no difference in morphology when M61-01 (Ura^-) and M61 (Ura^+) were compared. In YPD and SD50 media (no hyphal inducers) CAI4 exhibited blastoconidia and minor hyphal formation while M61 just showed blastoconidia and no hyphae at all. In FBS medium (hyphal inducer) CAI4 showed abundant hypha formation and few blastoconidia while M61 formed very short hyphae, pseudohyphae and many blastoconidia. Also, M61 blastoconidia have a very large central vacuole. These cells showed a significant increase relative to the parental phenotype suggesting an abnormal vacuolar accumulation. These enlarged cells have buds with identical morphology in all growth conditions tested.

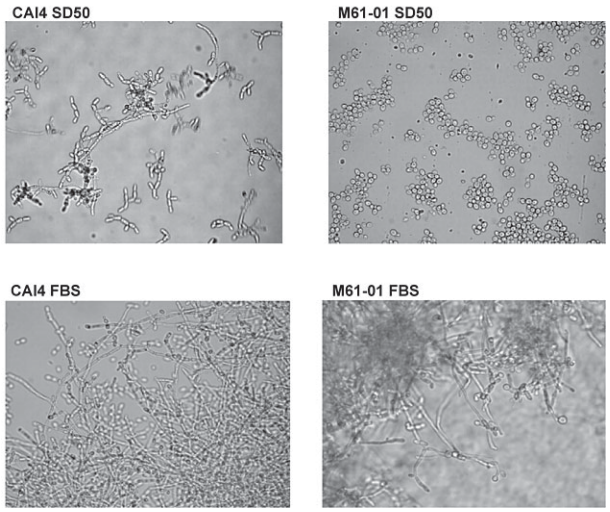

Figure 8. Micromorphology of CAI4 (parental strain) and M61-01 ($URA3^-$ mutant) cultured in SD 50 (50 mM glucose) and FBS (fetal bovine serum), after incubation at 37°C, 70 rpm for 24 h. Photographs with phase-contrast microscopy at 400X magnification.

Strains ATCC90029, CAI4 and M61 were subjected to species identification tests such as micromorphologic analysis, culture on CHROMagar Candida, sugar assimilation and fermentation, and RAPD which readily identified them as *C. albicans* (Melo et al., 1998), thus ruling out any possible contamination with other fungal species (data not shown).

Growth curves of the mutant M61, the parental strain CAI4, the *C. albicans* reference strain ATCC 90029, and the L296 strain (clinical isolate from hemoculture) were also compared (data not shown). The exponential phase of the curves at 30°C suggests that M61 growth is

slower than in CAI4, ATCC and L296. The exponential growth phase at 37°C indicates that CAI4 and M61 grow at approximately the same rates and much more slowly than ATCC and L296.

The expression level of *CaYLL34* (*Rix7*) mRNA in the mutant strains M61 and M61-01 is approximately half of the expression level in the CAI4 strain (Figure 9) as expected because of the deletion of one of the copies. This shows that the effects observed in the mutant are due to the decreased level of *CaYLL34* (*Rix7*) expression.

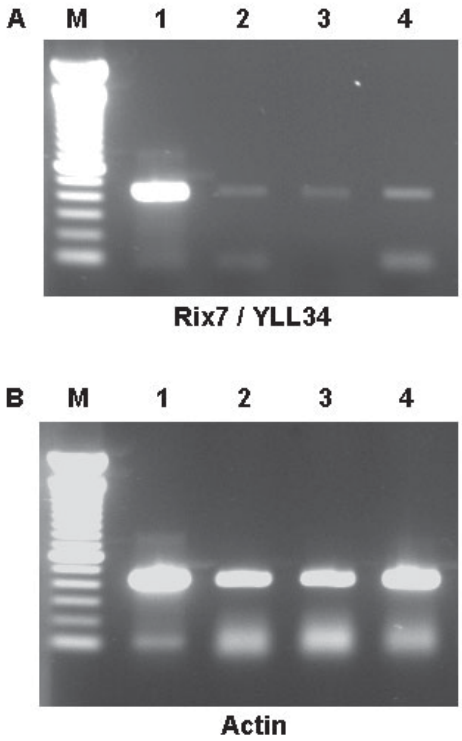

Figure 9. Steady-state levels of *CaYLL34* (*Rix7*) mRNA in parental and mutant strains M61 and M61-01. Panel **A**, total RNA (approximately 200 ng) was reverse transcribed and used for amplification with CaYLL34-specific primers. *Lane* 1, genomic DNA, *lane* 2, M61 total RNA, *lane* 3, M61-01 total RNA, and *lane* 4, CAI4 total RNA. Panel **B** shows the same samples as in **A** where actin-specific primers were used for amplification. As confirmed by densitometry the band intensity in *lane* 3, panel A is half the intensity in *lane* 4, which indicates that the partial deletion inactivated one copy and that the other remains fully functional.

A significant difference in ribosomal numbers per cell would explain at least in part the effects in the *Rix7* mutants here described, because *Rix7* is involved in ribosomal export. We observed that there was no significant difference in the total cellular rRNA levels of CAI4 and the M61 and M61-01 mutants. However, this does not imply that the number of ribosomes in the cytoplasm of *Rix7* mutant cells and CAI4 is identical.

Biofilm production and aspartyl proteinase activity of mutant M61

Biofilm production and aspartyl proteinase activity are known pathogenicity factors of

C. albicans and therefore were compared in M61, CAI4, ATCC 90029, and L296 strains (Figure 10). Biofilm production was quantified by the violet crystal assay which indicated that M61 has significantly reduced (86%) biofilm production as compared to CAI4 (Figure 10A) which has in turn the same level as compared to an authenticated pathogenic strain isolated from a patient.

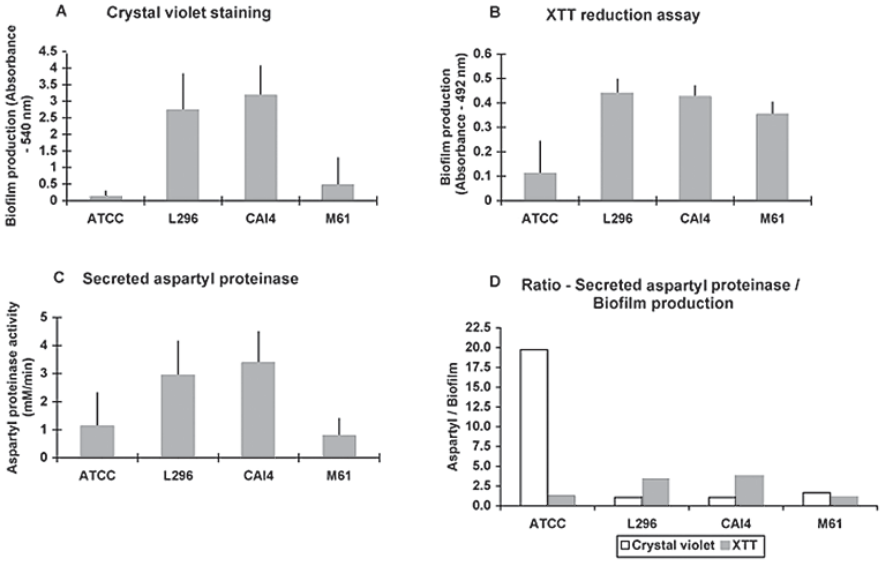

Figure 10. Biofilm and aspartyl-proteinase activity in *Candida albicans* strains ATCC 90029, L296, CAI4, and M61. In panel **A**, biofilm production was quantified using crystal violet staining. In panel **B**, biofilm production was quantified using the XTT reduction assay. Bars indicate the average of 4 measurements and error bars the standard deviation. In panel **C**, aspartyl-proteinase activity was determined using fluorogenic peptides as described in experimental procedures. In panel **D**, rate of aspartyl-proteinase activity/biofilm production quantified by crystal violet staining and XTT reduction assay.

Strain M61 also has a significant decrease in aspartyl proteinase activity in biofilms as compared to CAI4 (76.5%) and L296 (72.4%) strains, suggesting that M61 may be less pathogenic (Figure 10C). We hypothesize that strain ATCC 90029 has lower levels of biofilm production and aspartyl protease activity because of its long-term storage and *in vitro* culturing.

The difference in biofilm quantitation using crystal violet staining and XTT reduction methods can be explained by the fact that the cristal violet stains the whole cell mass in the biofilm, while XTT reduction, where XTT is converted to formazan by cell metabolism, corresponds only to living cells (Figure 10). Therefore, we demonstrated that the secretion of aspartyl proteinases is reduced in M61 mutant when compared to the parental CAI4 as shown by XTT reduction data, which indicates that only living cells would be producing and secreting aspartyl proteinase.

DISCUSSION

Here, we characterized a novel *C. albicans* gene, *CaYLL34*, and a corresponding subtype of Ascomycota AAA ATPases. AAA ATPases define a very broad category of pro-

teins which comprise the P-loop NTPases, a very abundant class which accounts for 10-18% of the predicted gene products in the sequenced prokaryotic and eukaryotic genomes (Iyer et al., 2003). CaYll34p contains two AAA domains and belongs to the "Cell Division/Centrosome/ER Homotypic Fusion" group according to the Fröhlich classification (Fröhlich, 2001), or the "D1 and D2" group according to the Frickey and Lupas classification (Frickey and Lupas, 2004). Also, CaYll34p is not related to the SEC18, a secretion AAA ATPase previously characterized in *C. albicans* (Nieto et al., 1993), because it has only 32% similarity in the 254 carboxyl-terminal residues while the remainder is completely different. The closest relative of *CaYLL34* with a known function is the *C. elegans* MAC-1 gene encoding a cell survival CED-4-interacting protein (NM064413) (Wu et al., 1999) located in the "Nematode type 2, *Caenorhabditis elegans (1)*" cluster in our phylogeny (Figure 4). Our phylogenetic analysis indicates that CaYll34p is not closely related to the *cdc48* group and is probably functionally related to the mammalian nuclear VCP-like AAA ATPases (Figure 4). Accordingly, CaYll34p has a bipartite nuclear localization signal in the N-terminus, which further supports the phylogenetic pattern that groups this gene product with other nuclear related family members (Figure 3). Although CaYll34p is similar to this subset of mammalian ATPases (30-40% identity), there are still sequence differences that can be explored to design specific inhibitors for these *C. albicans* ATPases, which could be tested in antifungal therapy. The closest relatives of CaYll34p are other fungal proteins forming a robust clade (>95% bootstrap frequency and posterior probability = 1) of "Ascomycota *cdc48* minus" AAA ATPases including the *S. cerevisiae* ScYLL034c, *Eremothecium gossypii* (Saccharomycetales) and *Gibberella zeae* (Hypocreales). This group was until now uncharacterized by functional data other than cell viability.

The identity of *CaYLL34* ORF with *S. cerevisiae YLL034c* (58.3%) suggests that they are homologous (Figure 3). These genes encode a protein similar to the VCP (member of the AAA ATPase family) (Dai and Li, 2001). VCP is highly conserved and the orthologues in yeast, pig and human have already been identified. This protein is involved in seemingly unrelated functions. It was reported to associate with clathrin, a structural protein of coated membranes involved in receptor-mediated endocytosis (Pleasure et al., 1993). VCP and co-factor p47 have been shown to mediate the regrowth of Golgi cisternae from mitotic Golgi fragments, and is likely to mediate other membrane fusion reactions (Dai and Li, 2001). VCP is required for ubiquitin-proteasome (Ub-Pr) degradation (Ghislain et al., 1996; Dai et al., 1998; Meyer et al., 2000; Dai and Li, 2001). In these reactions, VCP is proposed to have a chaperone role in modifying protein conformation and/or disassembling protein complexes to facilitate their translocation into the proteasome (Dai and Li, 2001). *ScYLL034c* is essential for cell growth and along with *CaYLL34*, its putative homolog, is probably necessary for membrane fusion and protein degradation. Hyphae of *C. albicans* have a great number of vacuoles that contain proteinases necessary for tissue invasion, and CaYll34p may have an important role in their fusion and ubiquitin-proteasome degradation.

The CaYll34p is probably a nuclear protein involved in ribosome biogenesis. The homologue of *CaYLL34* in *S. cerevisiae* complements defective mutants of *RIX7* (Brown, 2001; Gadal et al., 2001). Also, in *S. cerevisiae*, YLL034c/Rix7p interacts with Apc9c, as shown by results of a large-scale two hybrid study (Hazbun et al., 2003). Therefore, the analogy between CaYll34p and Rix7p functions and protein-protein interactions constitute an interesting working hypothesis for investigation in order to understand the cellular role of CaYll34p. Indeed, if CaYll34p participates in the biogenesis and export of 60S ribosomal subunits this would explain the drastic

impairment and pleiotropic effects due to the inactivation of only one *CaYLL34* allele as observed in mutant M61 (and M61-01 accordingly).

Hyphal over-expression of *CaYLL34* observed in the initial screening (Figure 1) was confirmed by semi-quantitative RT-PCR (Figure 5A). Although, it is not possible from our results to determine which hypha activation pathway controls *CaYLL34* expression and which transcriptional factors are involved in its expression, we show that *CaYLL34* is essential for proper hypha formation in different inducing media (Figure 8). Characterization of regulatory sequences and transcriptional regulation of *CaYLL34* could therefore provide new insights into yeast to hyphae transition. Analysis of the differential screening result indicates that the great majority of genes spotted are expressed more in the yeast form (the negative bars in Figure 1), and when yeast to hyphae transition occurs, few genes show increased expression while the expression of the majority is reduced. Although it was not our aim to propose a general model for yeast stage gene repression with concomitant increased expression of few hyphal genes, it seems that our results support, at least in part, models suggesting that filamentation genes are under negative control by genes such as *TUP1* and *RBF1* (Magee, 1997; Braun and Johnson, 2000; Whiteway, 2000).

The insertional inactivation of one copy of *CaYLL34* generated the heterozygous mutant M61 and M61-01 after counter-selection to eliminate the Ura^+ marker (Figures 6 and 7). This mutant has much slower growth and very limited hyphal induction ability (Figures 8 and 9). As observed in *S. cerevisiae*, this gene is essential, and therefore, inactivation of all genomic copies (null allele homozygous) is lethal (El-Moghazy et al., 2000). Despite the fact that M61 has one wild-type allele, it has severe phenotypic alterations in morphology and impairment in biofilm production and aspartyl proteinase activity, which are major pathogenicity factors (Figure 10). Interestingly, our study shows for the first time a quantitative analysis of aspartyl proteinases in biofilms of *C. albicans*. These results indicate that even a partial effect on gene dose is sufficient to affect *C. albicans* growth, morphological changes and pathogenicity.

As far as we know, this is the first description of an AAA ATPase gene expressed differentially in hyphae. Also, our phylogenetic analysis suggests that *CaYLL34* is an important gene subfamily of Ascomycota (yeasts). Genes of this subfamily may be involved in processes with major implications in morphological changes associated, more specifically in *C. albicans*, with pathogenicity. Therefore, because CaYll34p is necessary for the switching of yeast to hyphal form, it would be a candidate target for development of antifungal drugs (Whiteway, 2000). Cellular localization and biochemical characterization of this essential protein (CaYll34p) is a future perspective. Our results on the pleiotropic effects of inactivation of *CaYLL34* and its homology with *RIX7* suggest that CaYll34p has a nuclear localization, is required for biogenesis and export of 60S ribosomal subunits and interacts with protein Apc9c. Testing of this hypothesis could elucidate the specific role of this AAA ATPase in *C. albicans* survival and pathogenicity and contribute in the long-term to providing a novel target for antifungal therapy.

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