Microbiology (2004), **150**, 3341–3354 DOI 10.1099/mic.0.27320-0

The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*

Raquel Martinez-Lopez, Lucia Monteoliva, Rosalia Diez-Orejas, César Nombela and Concha Gil

Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, Spain

Correspondence
Concha Gil
conchagil@farm.ucm.es

Received 11 May 2004
Revised 7 June 2004
Accepted 19 July 2004

Ecm33p is a widely distributed fungal protein with functional relevance, clearly demonstrated by *ecm33*Δ mutant phenotypes, mainly related to the cell wall. Homology searches with *Saccharomyces cerevisiae* genes identified *Candida albicans* Ecm33p, as well as the two other proteins of its family: Pst1p and the product of *YCL048w*. *C. albicans* Ecm33p is a 423 aa protein which has the typical features of cell-surface GPI proteins and is able to complement *S. cerevisiae ecm33*Δ cell wall defects. Heterozygous (RML1) and homozygous (RML2) mutants of *CaECM33* were obtained, as well as a single and a double reintegrant (RML3 and RML4, respectively). *Caecm33* mutant strains displayed an aberrant morphology, being more rounded and bigger than the wild-type, suggesting morphogenetic defects. They also exhibited cell wall defects, with enhanced sensitivity to different compounds that interfere in polymerization of cell wall components (Calcofluor white, Congo red and hygromycin B) and a marked tendency to flocculate extensively. In addition, CaEcm33p is required for normal *C. albicans* yeast-to-hyphae transition *in vitro*. In liquid medium (5 % serum), the transition was delayed in *Caecm33* mutants, and after 24 h the culture contained very abnormal large and rounded cells. On solid medium (10 % serum, Spider or SLADH) RML2 failed to produce hyphae and media invasiveness. *CaECM33* showed a gene dosage effect, demonstrated by the intermediate phenotype of the heterozygous mutants RML1 and confirmed by Northern blot analysis. Furthermore, CaEcm33p is also involved in *C. albicans* virulence. In a murine systemic model of infection, 100 % mouse survival and no kidney or brain colonization were obtained 30 days after infection with 10^6 *Candida* cells of any homozygous or heterozygous *Caecm33*Δ mutant tested. In contrast, all mice infected with parental or RML4 (two Ca*ECM33* copies reintegrated) strains died in a few days, showing that, in these conditions, two *CaECM33* copies were required for virulence.

INTRODUCTION

Candida albicans is the major fungal pathogen in humans, particularly in immunocompromised patients (Vincent *et al.*, 1998). It is a polymorphic fungus that grows either in yeast form or as hyphae. Both types of morphology may be present in infected tissue, and therefore both may possibly play important roles in pathogenesis (Gow, 1997; Mitchell, 1998).

The cell wall, as the outermost cellular structure, determines the shape of the fungal cell and represents the initial point of interaction between the host and pathogen. In addition, given that mammalian cells lack a cell wall, this cellular compartment could be a promising molecular target site in searches for new specific antifungal drugs (Groll *et al.*, 1998; Odds, 2003; Gimeno *et al.*, 1992; Liu *et al.*, 1994). A better knowledge of *C. albicans* cell wall structure and composition, and functional analysis of proteins of unknown function, may contribute to understanding the involvement of the wall in fungal morphogenesis and pathogenesis as well as to the discovery of novel antifungal therapies.

Fungal cell wall structure has been studied most extensively in *Saccharomyces cerevisiae* (Klis *et al.*, 2002; Martin *et al.*, 2000; Pardo *et al.*, 2000; Lipke & Ovalle, 1998; Orlean, 1997). However, several reports (Kapteyn *et al.*, 1994, 1995a, b; 2000; Sanjuan *et al.*, 1995) concerning the cell wall organization of *C. albicans* have demonstrated that a similar model is also valid for this pathogenic fungus (Chaffin *et al.*, 1998; Klis *et al.*, 2001). The *C. albicans* cell wall is mainly composed of three components interconnected by covalent bonds: 1,3-β- and 1,6-β-glucans (50–60 %), mannoproteins

Abbreviations: CWP, cell wall protein; 5-FOA, 5-fluoro-orotic acid; GPI, glycosylphosphatidylinositol.

(30–40 %) and chitin (0·6–9 %) (Chaffin *et al.*, 1998). Cell wall proteins (CWPs) can be coupled to cell wall components in different ways (Klis *et al.*, 2001). The total number and functions of CWPs are still poorly known. Several chemical and/or enzymic strategies for their isolation, both from intact cells (Casanova *et al.*, 1992; Lopez-Ribot *et al.*, 1996) and from isolated cell walls after cell breakage (Kapteyn *et al.*, 1994; Elorza *et al.*, 1985; Mormeneo *et al.*, 1996; Ruiz-Herrera *et al.*, 1994) have been described. Different proteomic approaches have also been used in order to obtain a comprehensive and integrated view of the cell wall proteome in both *C. albicans* and *S. cerevisiae* (Pardo *et al.*, 2000; Pitarch *et al.*, 2002, 2003; Urban *et al.*, 2003). In one of these approaches, which involved the analysis of proteins secreted into the medium when *S. cerevisiae* protoplasts were regenerating their cell walls, the gene product of ORF *YDR055W* was identified (Pardo *et al.*, 1999, 2000) and named Pst1p (**P**rotoplast-**s**secre**t**ed protein). The *C. albicans* Pst1p homologue has been identified not only by *in silico* analysis, but also as a functional secretory protein in a heterologous genome-wide screening (Monteoliva *et al.*, 2002). There are three other *S. cerevisiae* proteins that show a significant degree of similarity to Pst1p and display similar characteristics: the *ECM33*/*YBR078W*, *SPS2*/*YDL052C* and *YCL048W* gene products. These four proteins have been grouped in the so-called SPS2 family (Caro *et al.*, 1997), named after the first-described member. These proteins have the typical features of GPI (glycosylphosphatidylinositol)-anchored proteins, with a signal peptide, serine- and threonine-rich region and a potential C-terminal domain for GPI anchor attachment (Caro *et al.*, 1997; De Groot *et al.*, 2003). *PST1* has been reported to be induced in different cell wall mutants or in response to transient cell wall damage (Jung & Levin, 1999; Garcia *et al.*, 2004), as it acts in the compensatory mechanism triggered by the Slt2p-MAP kinase cascade responsible for cell wall integrity (Martin *et al.*, 1993). However, the *S. cerevisiae* mutant strain *pst1*Δ did not show any cell wall defect while deletion of *ECM33* led to a weakened cell wall. This defect was aggravated by simultaneous deletion of *PST1* (Pardo *et al.*, 2004). Ecm33p is therefore important for correct ultrastructural organization of cell wall polymers (glucan and chitin) and, furthermore, for the correct assembly of the mannoprotein outer layer of the cell wall (Pardo *et al.*, 2004). Because of the relevant role of Ecm33p in cell wall integrity, we have undertaken to characterize *C. albicans* Ecm33p. In the work described here we obtained a *C. albicans* deletant mutant strain (*Caecm33*Δ) and analysed the role of Ecm33p in morphogenesis, cell wall integrity and virulence of the fungus.

METHODS

Strains, media and growth conditions. The strains of *Escherichia coli*, *S. cerevisiae* and *C. albicans* used in this study are listed in Table 1.

For *C. albicans*, cells were routinely grown in either YPD medium (1 % yeast extract, 2 % bacto peptone, 2 % glucose) or SD Ura$^-$ (SD lacking uridine and uracil) (Ausubel *et al.*, 1993). YPD medium was supplemented with 60 μg uridine ml^{-1} for growth of Ura$^-$ strains. For selection of Ura$^-$ clones, SD Ura$^-$ medium was supplemented with 60 μg uridine ml^{-1} and 1 mg 5-fluoroorotic acid (5-FOA) ml^{-1} (Boeke *et al.*, 1984). *E. coli* strains were cultured in LB medium or on LB plates with ampicillin added to 100 μg ml^{-1}.

For phenotypic analysis of mutants, YPD plates were supplemented with different concentrations of Calcofluor white (25–28 μg ml^{-1}), Congo red (100–250 μg ml^{-1}), or hygromycin B (75–200 μg ml^{-1}).

Table 1. Strains

Strain	Relevant characteristics	Source or reference
E. coli		
DH5αF′	K12 Δ(*lacZYA–argF*)*U169 supE44 thi-1 recA1 endA1 hsdR17 gyrA relA1* (*ϕ*80*lacZ*ΔM15)F	Hanahan (1983)
S. cerevisiae		
FY1679-28C	*MATα ura3-52 trp1Δ63 leu2Δ1 his3 Δ200* YGSC	Berkeley
FBEHo41-01A(AL) (*ecm33*Δ)	*MATα ura3-52 HIS3 leu2Δ1 LYS2 trp1Δ63 ybr078w*::Δ*kanMX4*	EUROSCARF
C. albicans		
SC5314	Parental strain	Gillum *et al.* (1984)
CAF2	*URA3*/*ura3*Δ::*imm43*	Fonzi & Irwin (1993)
CAI-4	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	Fonzi & Irwin (1993)
RML1	*CaECM33*/*Caecm33*Δ::*hisG-CaURA3-hisG*	This study
RML1a	*CaECM33*/*Caecm33*Δ::*hisG*	This study
RML2	*Caecm33*Δ::*hisG*/*Caecm33*Δ::*hisG-CaURA3-hisG*	This study
RML2a	*Caecm33*Δ::*hisG*/*Caecm33*Δ::*hisG*	This study
RML3	*Caecm33*Δ::*hisG*/*CaECM33-clz-URA3-clz-hisG*	This study
RML3a	*Caecm33*Δ::*hisG*/*CaECM33-clz-hisG*	This study
RML4	*CaECM33-clz-hisG*/*CaECM33-clz-URA3-clz-hisG*	This study

A total of 5 % or 10 % fetal bovine serum was added to liquid or solid YPD respectively for filamentation tests. Plates of Spider and SLADH media (Gimeno *et al.*, 1992; Liu *et al.*, 1994) were used for the morphological studies.

DNA and RNA manipulation methods. PCR, restriction digestion and gel electrophoresis were performed by standard methods (Sambrook *et al.*, 1989). Bacterial plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). All DNA fragments for cloning were gel-purified with the QIAquick Gel Extraction Kit (Qiagen). Yeast genomic DNA was isolated according to Ausubel *et al.* (1993). All DNA-modifying enzymes were provided by Roche and used according to the manufacturer's recommendations. *CaECM33* expression was detected by Northern blotting. Exponentially growing cells were harvested by centrifugation, and total RNA was isolated by the 'mechanical disruption protocol' using the RNeasy MIDI kit (Qiagen), following the manufacturer's instructions. RNA concentrations were determined by measuring absorbance at 260 nm. Northern blots were prepared according to the protocol of Hube *et al.* (1994). A 2639 bp fragment corresponding to positions −366 to +513 with respect to the start codon of the *CaECM33* ORF was amplified and radiolabelled with [α-^{32}P]dCTP by PCR using the specific oligos PROBE1 (5′-taggacgtgacaagatacaggatcgca-3′) and PROBE2 (5′-aaaacaatgttcttagcactgctc-3′) to give the *ECM33*-specific probe. The hybridization conditions described by McCreath *et al.* (1995) were used. Uniformity of RNA loading was determined by ethidium bromide staining. For Southern blotting, genomic DNA was digested by *Xba*I and *Eco*RI and separated on a 0·8 % agarose gel prior to transfer to nitrocellulose and probing. The probe was generated by PCR using the Nonradioactive Labelling and Detection Kit (Boehringer Mannheim) and the specific oligonuleotides PROBE1 and PROBE2.

For the RT-PCR study, *C. albicans* cDNA was synthesized from mRNA with an oligo d(T)15 primer using the Promega RT-PCR kit. The oligonuclotides RNAUPPER (5′-ctgccaacatcaactttg-3′) and RNALOWER (5′-tgaaagcactacaagacaat-3′) were used to define the intron sequence. The oligo RNAUPPER comprises 9 bp just before the 5′ splice site (underlined) and 11 bp just after the 3′ splice site (double-underlined) so at the annealing temperature selected it is supposed to hybridize only with the cDNA in which the intron region has been deleted but not with the DNA.

Analysis of *CaECM33*. The searches for homologous sequences were carried out using the tBLASTn and BLASTn programs of the Stanford database (www-sequence.stanford.edu/group/candida).

The DNA analysis for the signal peptide search was done using the SignalP program in the SignalP V1.1 Worldwide Web Prediction Server (www.cbs.dtu.dk/services/SignalP) (Nielsen *et al.*, 1997). The program GPI-Predictor GPI Modification Site Prediction (http://mendel.imp.univie.ac.at/gpi_server.html) (Eisenhaber *et al.*, 1998) was used to define the GPI signal of the proteins.

Construction of plasmid YEPCaECM33. A 3 kb amplicon containing the *CaECM33* gene sequence was obtained by PCR using the sense primer ECM0 (5′-gagcgagctctggctctacttgtctgaa-3′) and the antisense primer ECM4 (5′-tgcaagctttgtcaccttccggtccca-3′), containing engineered restriction sites *Sac*I and *Hin*dIII (underlined), respectively. This amplicon was subcloned into the pGEMT vector, rendering plasmid pGECM04. This was then digested with *Sna*BI and *Sca*I to obtain a 4054 bp fragment comprising the *CaECM33* gene. This fragment was then treated with Klenow enzyme and subcloned into the *Sma*I-linearized YEP352 plasmid. The resulting plasmid was named YEPCaECM33; it included the entire *CaECM33* gene from position −330 to +2650 with respect to the start codon.

Plasmid construction for disruption of the *CaECM33* gene. The *CaECM33* gene was disrupted by replacing the entire ORF with a *hisG-URA3-hisG* cassette (Fonzi & Irwin, 1993). The disruption cassette was constructed by two consecutive PCR amplifications with genomic CAF2 DNA as template. In the first step, an amplicon of 1127 bp was obtained from the genomic DNA using the sense primer 5UPPER (5′-gttgagctcttgacgggaacaaagaat-3′) and the antisense primer 5LOWER (5′-tgcactagttggcagttaatagcaagaa-3′), containing engineered *Sac*I and S*pe*I restriction sites (underlined), respectively. The amplicon obtained was digested with *Sac*I and S*pe*I and then subcloned into plasmid pSkh-ura-h. This plasmid was previously obtained by subcloning the 4 kb *Bam*HI–*Bgl*II fragment from PUCK6B1, which contains the *hisG-URA3-hisG* cassette, into pBluescriptSK previously digested with *Bam*HI. The resulting plasmid was named skpcr5; it contained the 5′ region upstream of the gene (position −1095 to +10 with respect to the start codon) and the *hisG-URA3-hisG* cassette.

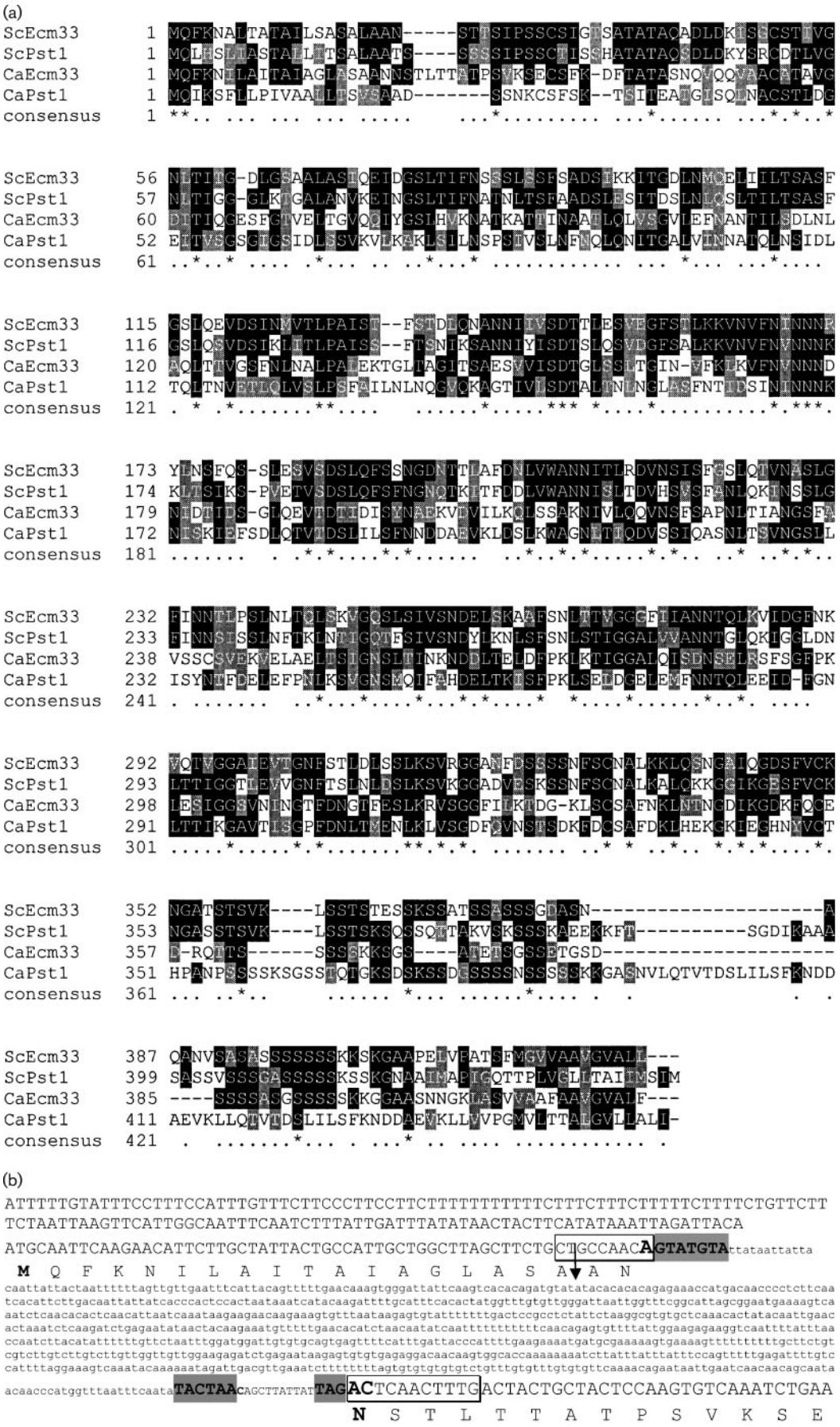
In the second step, an amplicon containing the 500 bp downstream of the non-coding region was obtained using the sense primer ECM3 (5′-gttctgcagaggaaccaacacaaagaa-3′) and the antisense primer ECM4 (5′-tgcaagctttgtcaccttccggtccca-3′), containing engineered *Pst*I and *Hin*dIII restriction sites (underlined), respectively. The amplicon obtained (pcr3) was then digested with *Pst*I and *Hin*dIII, and subsequently ligated into skpcr5 (previously digested with the same restriction enzymes just mentioned) to create plasmid pSkECMhuh, in which the *CaECM33* upstream and downstream DNA regions were flanking the *hisG-URA3-hisG* disruption cassette.

Plasmid construction for reintegration of the *CaECM33* gene. To reintroduce the *CaECM33* gene, the integration plasmid pD1ECM was constructed as follows. Plasmid pD1, which contains the *C. albicans URA3* gene flanked by direct repeats of the chloramphenicol acetyltransferase gene (*cat*) was kindly provided by Dr Blanca Eisman (Facultad de Farmacia, Dpto Microbiología II, Universidad Complutense de Madrid).

A 3 kb fragment containing the intact *CaECM33* gene that had been obtained by *Sac*I digestion of plasmid pGECM0-4 was ligated into the unique *Sac*I site of plasmid pD1. The resulting plasmid was named pD1ECM; it contained the complete *CaECM33* ORF gene (from position −330 to +2650 with respect to the start codon) and the *URA3* gene flanked by direct repeats of the *cat* gene.

Isolation of the *CaECM33* null mutant. *CaECM33* disruption was achieved as described by Fonzi & Irwin (1993). CAI4 cells were transformed to Ura$^+$ prototrophy with 10 µg of a *Sac–Acc*I fragment from the plasmid pSkECMhuh. Transformants were selected as Ura$^+$ in SD minimal medium lacking uridine and checked for integration of the cassette at the *CaECM33* locus by Southern blot analysis. One of the heterozygous disruptants recovered (designated *C. albicans* RML1) was used to select spontaneous Ura$^-$ derivatives in SD minimal medium containing 5-FOA. These clones were analysed by Southern blot hybridization to identify those that had undergone intrachromosomal recombination between the *hisG* repeats. One of these Ura$^-$ derivatives (termed RML1a) was used to replace the second *CaECM33* allele in a similar way, using the *Sac*I–*Acc*I fragment from pSKECMhuh. Transformed cells were selected as null mutant RML2 once the correct allele had been verified by Southern blot analysis.

Reintroduction of the *C. albicans ECM33* gene. The integration plasmid pD1ECM (constructed as described above), containing the functional *CaECM33* and *URA3* genes, was used to transform the null mutant strain in order to reintroduce these two genes. pD1ECM was digested with *Sna*BI, which has a single recognition site in the *CaECM33* sequence but not in the *URA3* or vector regions. This linearized plasmid was then transformed into the Ura$^-$


(a)
ScEcm33 1 MQFKNALTATAILSASALAAN-----STTSIPSSCSIGTSATATAQADLDKISGCSTIVG
ScPst1 1 MQLHSLIASTALLTSALAATS----SSSIPSSCTISSHATATAQSDLDKYSRCDTLVG
CaEcm33 1 MQFKNILAITAIAGLASAANNSTLTTATPSVKSECSFK-DFTATASNQVQQVAACATAVG
CaPst1 1 MQIKSFLLPIVAALLTSVSAAD-------SSNKCSFSK--TSITEATGISQLNACSTLDG
consensus 1 **.. ... ...*..... ...*..... ...*.*..*
ScEcm33 56 NLTITG-DLGSAALASIQEIDGSLTIFNSSSLSSFSADSIKKITGDLNMQELIILTSASF
ScPst1 57 NLTIGG-GLKTGALANVKEINGSLTIFNATNLTSFAADSLESITDSLNLQSLTILTSASF
CaEcm33 60 DITIQGESFGTVELTGVQQIYGSLHVKNATKATTINAATLQLVSGVLEFNANTILSDLNL
CaPst1 52 EITVSGSGIGSIDLSSVKVLKAKLSILNSPSIVSLNFNQLQNITGALVINNATQLNSIDL
consensus 61 ..*..* .... .*... .. ..*...* .... *... ...*....
ScEcm33 115 GSLQEVDSINMVTLPAIST--FSTDLQNANNIIVSDTTLESVEGFSTLKKVNVFNINNNR
ScPst1 116 GSLQSVDSIKLITLPAISS--FTSNIKSANNIYISDTSLQSVDGFSALKKVNVFNVNNNK
CaEcm33 120 AQLTTVGSFNLNALPALEKTGLIAGITSAESVVISDTGLSSLTGIN-VFKLKVFNVNNND
CaPst1 112 TQLTNVETLQLVSLPSFAILNLNQGVQKAGTIVLSDTALTNLNGLASFNTIDSININNNK
consensus 121 . * .*.....**.... ......*.....*** *....*.....*.***.
ScEcm33 173 YLNSFQS-SLESVSDSLQFSSNGDNTTLAFDNLVWANNITLRDVNSISFGSLQTVNASLG
ScPst1 174 KLTSIKS-PVETVSDSLQFSFNGNQTKITFDDLVWANNISLTDVHSVSFANLQKINSSLG
CaEcm33 179 NIDTIDS-GIQEVTDTIDISYNAEKVDVILKQLSSAKNIVLQQVNSFSAPNLTIANGSFA
CaPst1 172 NISKIEFSDLQTVTDSLILSFNNDDAEVKLDSLKWAGNLTIQDVSSIQASNLTSVNGSLL
consensus 181 ....*.*....*.*..... ..*..*.*.....*.*.. ..* ..*.*..
ScEcm33 232 FINNTLPSLNLTQLSKVGQSLSIVSNDELSKAAFSNLTTVGGGFIIANNTQLKVIDGFNK
ScPst1 233 FINNSISSLNFTKLNTIGQTFSIVSNDYLKNLSFSNLSTIGGALVVANNTGLQKIGGLDN
CaEcm33 238 VSSCSVEKVELAELTSIGNSLTINKNDDLTELDFPKLKTIGGALQISDNSELRSFSGFPK
CaPst1 232 ISYNTFDELEFPNLKSVGNSMQIFAHDELTKISFPKLSELDGELEMFNNTQLEEID-FGN
consensus 241 . ...*...*....*...*.*....* *....*.....*..*. ....
ScEcm33 292 VQTVGGAIEVTGNFSTLDLSSLKSVRGGANFDSSSSNFSCNALKKLQSNGAIQGDSFVCK
ScPst1 293 LTTIGGTLEVVGNFTSLNLDSLKSVKGGADVESKSSNFSCNALKALQKKGGIKGESFVCK
CaEcm33 298 LESIGGSVNINGTFDNGTFESLKRVSGGFILKTDG-KLSCSAFNKLNTNGDIKGDKFQCE
CaPst1 291 LTTIKGAVTISGPFDNLTMENLKLVSGDFQVNSTSDKFDCSAFDKLHEKGKIEGHNYVCT
consensus 301 .....*.....*.*.....**.*.*. ....* * ..*.. *.*.*....*.
ScEcm33 352 NGATSTSVK----LSSTSTESSKSSATSSASSSGDASN----------A
ScPst1 353 NGASSTSVK----LSSTSKSQSSQLTAKVSKSSSKAEEKKFT----------SGDIKAAA
CaEcm33 357 D-RQITS------SSSGKKSGS----ATETSGSSETGSD----------
CaPst1 351 HPANPSSSSKSGSSTQTGKSDSKSSDGSSSSNSSSSSKKGASNVLQTVTDSLILSFKNDD
consensus 361 ... ..*.. ..... *... .....*.... . . .
ScEcm33 387 QANVSASASSSSSSSKKSKGAAPELVPATSFMGVVAAVGVALL---
ScPst1 399 SASSVSSSGASSSSSKSSKGNAAIMAPIGQTTPLVGLLTAIIMSIM
CaEcm33 385 ----SSSSASGSSSSSKKGGAASNNGKLASVVAAFAAVGVALF---
CaPst1 411 AEVKLLQIVIDSLILSFKNDDAEVKLLVVPGMVLTTALGVLLALI-
consensus 421 . .....*... . ...* .. ..
(b)
ATTTTTGTATTTCCTTTCCATTTGTTTCTTCCCTTCCTTCTTTTTTTTTTTCTTTCTTTCTTTTTCTTTTCTGTTCTT
TCTAATTAAGTTCATTGGCAATTTCAATCTTTATTGATTTATATAACTACTTCATATAAATTAGATTACA
ATGCAATTCAAGAACATTCTTGCTATTACTGCCATTGCTGGCTTAGCTTCTGCTGCCAACAGTATGTAttataattatta
M Q F K N I L A I T A I A G L A S A A N
caattattactaatttttagttgttgaatttcattacagtttttgaacaaagtgggattattcaagtcacacagatgtatatacacacacagagaaaccatgacaacccctcttcaa
tcacattcttgacaattattatcacccactccactaataaatcatacaagattttgcatttcacactatggtttgtgttgggattaattggtttcggcattagcggaatgaaaagtca
aatctcaacacactcaacattaatcaaataagaagaacaagaaagtgtttaataagagtgtatttttttgactccgcctctattctaaggcgtgtgctcaaacactatacaattgaac
actaaatctcaagatctgagaatataactacaagaaatgtttttgaacacatctaacaatatcaatttttttttttcaacagagtgttttattggaagagaaggtcaatttttatttaa
ccaatcttacatatttttttgttctaatttggatggattgtgtgcagtgagttttcatttgattacccattttgaagaaaatgatgcgaaaaagtgaaaagttttttttttgcttctgt
cgtcttgtcttgtcttgttggttgttggaagagatctgagaataagagtgtgtgagaggacaacaagtggcaccaaaaaaaatcttatttatttatttccagttttgagattttgtc
cattttaggaaagtcaaatacaaaaaatagattgacgttgaaatctttttttttagtgtgtgtgtctgtttgtgtttgtgtgttcaaaacagaataattgaatcaacaacagcaata
acaacccatggtttaatttcaataTACTAACAGCTTATTATTAGACTCAACTTTGGACTACTGCTACTCCAAGTGTCAAATCTGAA
N S T L T T A T P S V K S E

strain RML2a (derived from RML2 in SD minimal medium containing 5-FOA and checked by Southern blotting to confirm intrachromosomal recombination between the *hisG* genes) using a lithium-acetate-based transformation protocol (Walther & Wendland, 2003). Ura^+ prototrophs were selected on minimal medium lacking uridine. Insertion of the functional *CaECM33* gene into the null mutant as a result of spontaneous recombination was confirmed by Southern blot analysis. One of the heterozygous reintegrants recovered (designated *C. albicans* RML3) was used to select spontaneous Ura^- derivatives in SD minimal medium containing 5-FOA. These clones were analysed by Southern blot hybridization to identify those that had undergone intrachromosomal recombination between the chloramphenicol resistance gene repeats. One of these Ura^- derivatives (termed RML3a) was used for reintegration of the second *CaECM33* allele in a similar way using the *Sac*I-linearized pD1ECM plasmid. Transformed cells (RML4) selected as Ura^+ carried two functional *CaECM33* copies reintegrated at the *CaECM33* genome locus.

Phenotypic analysis of mutants. Calcofluor white (CFW), Congo red (CR) and hygromycin B (HB) sensitivities were tested by streaking cells onto plates following the method described by Van der Vaart *et al.* (1995). Aliquots (5 µl) of serial 1/10 dilutions of cells that had been grown overnight and adjusted to an OD_{600} of 0·7 were deposited on the surface of YPD plates containing different concentrations of CFW (25–28 µg ml^{-1}), CR (100–250 µg ml^{-1}) and HB (75–200 µg ml^{-1}). These samples were then grown at 30 °C and monitored for 2 days.

For filamentation tests, *C. albicans* strains were grown overnight in YPD at 30 °C and then subcultured at an OD_{600} of 0·05 into 5 % serum prewarmed to 37 °C. For tests in solid media, cells were counted and 30 cells were plated on 10 % serum YPD, Spider or SLADH media plates.

Staining and fluorescent image analysis. A 100 µl volume of Calcofluor white (0·3 g l^{-1}) (Sigma F-6259) was added to 1 ml diluted sample in a 1·5 ml Eppendorf vial covered with aluminium foil. Samples were mixed and incubated at room temperature for 5 min. A few drops of the solution were placed on a glass slide and covered with a coverslip for analysis. The dye fluoresces when bound to chitin and glucans, and thus stains cell walls and septa. Images were obtained by fluorescence microscopy.

Murine model of disseminated candidiasis. Female BALB/c mice were obtained from Harlan France. Groups of 10 female mice ranging in age from 6 to 8 weeks, with a weight of about 20 g, were used. *C. albicans* cells were harvested from YED agar plates, washed twice with PBS and diluted to the desired density in the same buffer prior to injection into the lateral tail vein of mice in a volume of 0·5 ml (10^6 blastospores). Survival experiments were carried out in groups of 10 mice and mortality was monitored for 30 days.

At day 30, the fungal burden of kidneys and brain was determined. For this purpose kidneys and brain were removed, homogenized and quantitatively cultured on Sabouraud dextrose agar containing 10 chloramphenicol mg l^{-1}.

RESULTS

Identification of CaEcm33p and its homologues

The presence of proteins homologous to Ecm33p or to other members of its family has been described in various fungal species. In order to identify them in *C. albicans*, we screened *C. albicans* genome sequences in the Stanford database (http://sequence-www.stanford.edu/group/candida/search.html). *C. albicans* Ecm33p BLAST searches using the *S. cerevisiae ECM33* sequence identified a DNA sequence with high homology within contig-6-2398. This DNA sequence encoded an ORF without a defined ATG. In the *C. albicans* database, CandidaDB (htpp://genolist.pasteur.fr/CandidaDB), two different and incomplete sequence entries were annotated as *ECM33* (*ECM33.1* and *ECM33.3*) and, by contrast, there was no *PST1* entry. We defined the entire *ECM33* sequence by protein BLAST homology with *S. cerevisiae* Ecm33p, which displayed 54·37 % homology with the predicted *C. albicans* protein (Fig. 1a). We then cloned the *CaECM33* DNA sequence and tested its functional homology by complementation of *S. cerevisiae ecm33*Δ cell wall defects. For this purpose, we transformed the plasmid YEPCaECM33, which included the complete *CaECM33* gene sequence from positions −330 bp to +2650 bp with respect to the start codon, into *S. cerevisiae ecm33*Δ. The *CaECM33* gene was able to complement the sensitivity of *S. cerevisiae ecm33*Δ to Calcofluor white and Congo red (data not shown). In this way, we clearly identified that the ECM33.3 entry (CandidaDB) corresponded to the C-terminal region of the *C. albicans* Ecm33p functional homologue, and ECM33.1 was CaPstIp, previously cloned as a secretory protein (Monteoliva *et al.*, 2002). The *C. albicans* genome included a third protein of this family, IPF13972 at CandidaDB, which appears to be the counterpart to the *S. cerevisiae YCL048w* gene product, although there was no *SPS2* homologue. Thus, in *C. albicans* this family of GPI-anchored proteins contains only three members.

Features of *C. albicans ECM33* and Ecm33p

Once we had determined the exact sequence of *CaECM33*, we observed that it seemed to include an intron, as occurs in its *S. cerevisiae* homologue, since we found the theoretical intron splicing sequences 5′ splice site (AG/GTATGT) and 3′ splice site (TACTAAC....TAG) described for *S. cerevisiae* (Rymond & Rosbash, 1985, 1986) within the *CaECM33*

Fig. 1. (a) Sequence alignment of *C. albicans* and *S. cerevisiae* Ecm33p and PstIp. Identical amino acids among two or more sequences are shaded in black while amino acids that are equivalent are shaded in grey; 100 % consensus between four of the proteins is marked with an asterisk. (b) DNA sequence analysis and amino acid sequence of the 5′ region of *CaECM33* comprising the intron (annotated in GenBank as accession no. AY630439). The intron comprises the nucleotides between numbers 10724350 and 10725240 of contig-6-2398 (in the Stanford database). ATG starts at number 10724289 of the same contig. The Ala-18/Ala-19 N-terminal signal peptide cleavage site is indicated by an arrow. 5′ (**GTATGTA**) and 3′ (**TACTAAC**AGCTTATTAT**TAG**) splicing sites are shaded in grey. The 9 bp before the 5′ splicing site and the 11 bp after the 3′ splicing site which constitute the RNAUPPER oligo used to amplify the cDNA are boxed. The adenine just before the 5′ splicing site and the adenine and cytosine just after the 3′ splicing site encode an asparagine residue.

DNA sequence. These 5′ and 3′ splicing sequences were localized at positions +60 and +929, respectively, with respect to the start codon (Fig. 1b). To check this possibility, we carried out a RT-PCR using mRNA from the wild-type strain CAF2 as template and the oligos RNAUPPER and RNALOWER designed as described in Methods. Sequencing of the RT-PCR product and alignment of this sequence with the *CaECM33* DNA confirmed the presence of an intron of 950 bp just after the previously defined 5′ splice region (Fig. 1b).

Protein translation after mRNA splicing releases CaEcm33p, a 423 aa protein which includes two highly hydrophobic regions. The first one corresponds to the N-terminal signal peptide, which includes the first 18 aa and is eventually removed by cleavage between Ala-18 and Ala-19 according to the bioinformatic analysis (SignalP). The second hydrophobic region is composed of the last 22 aa and comprises a GPI anchor putative signal. The ω site (processing site where the glycosidylinositol group will be anchored to the protein; Eisenhaber *et al.*, 1998) would correspond to the Gly-401. Thus, CaEcm33p has the typical features of fungal GPI-anchored proteins and, as in the case of the yeast homologue counterpart (Terashima *et al.*, 2003), it might be located at the plasma membrane.

Construction of *Caecm33* mutants and reintegration strains

To investigate the function of CaEcm33p, null mutants were constructed by targeted gene disruption and analysis of the resulting phenotype.

Disruption of the *CaEM33* gene was performed by following the strategy described by Fonzi & Irwin (1993), in which a cassette consisting of the *C. albicans URA3* gene flanked by direct repeats of the *Salmonella typhimurium hisG* gene is used (Fig. 2a). This cassette was used to replace the entire *CaECM33* ORF. A linear *Sac*I–*Acc*I fragment including the cassette flanked by *CaECM33* upstream and downstream regions was used to transform *C. albicans* CAI4. Southern blot analysis of a representative isolate, *C. albicans* RML1, after digestion with *Xba*I and *Eco*RI, revealed that the cassette had integrated in the allele properly, giving rise to a fragment of 2607 bp. The 1369 bp and 1838 bp fragments corresponding to the other wild-type allele were still present in the strain (Fig. 2b). Ura$^-$ segregants of *C. albicans* RML1 were selected in medium containing 5-FOA (Boeke *et al.*, 1984) and examined by Southern blot analysis. Ten of the fifteen independent segregants examined had undergone intrachromosomal recombination between the *hisG* repeats, resulting in excision of the *URA3* marker and one copy of *hisG*. One of these Ura$^-$ segregants, named RML1a, was used to generate the homozygous *Caecm33* null mutant (RML2) by transformation of *C. albicans* RML1a with the same disruption cassette. Three of the ten Ura$^+$ transformants exhibited a hybridization pattern consistent with targeting of the previously undisrupted allele in which the parental 1369 bp and 1838 bp *Xba*I–*Eco*RI fragments were absent and the 2607 bp fragment corresponding to the cassette integration appeared instead, indicating a correct integration (Fig. 2b). Northern blot analysis demonstrated that no *CaECM33* mRNA was present in RNA samples from the null mutant C. *albicans* RML2. To

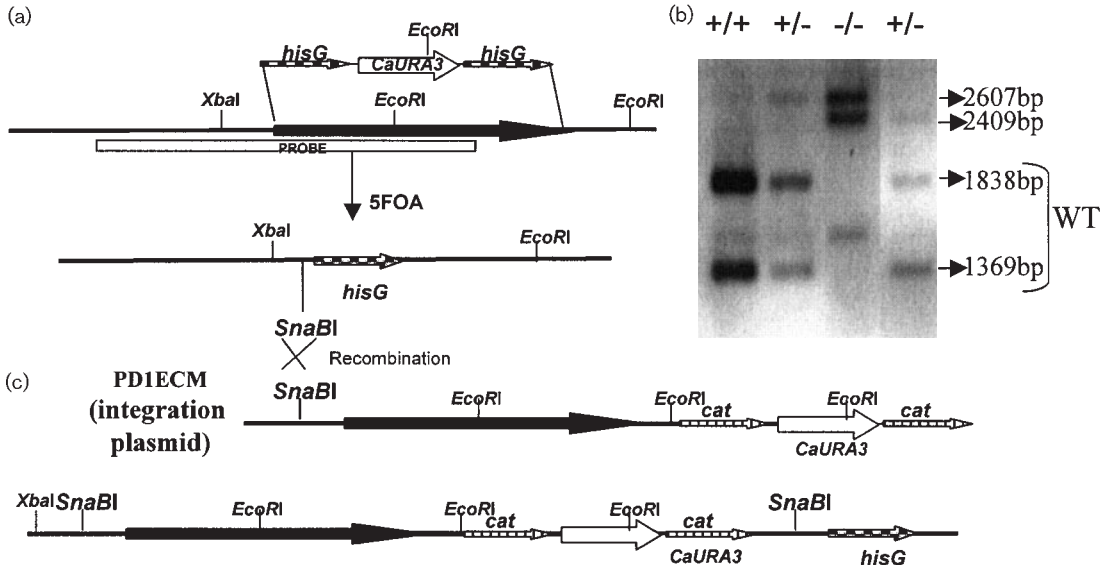


Fig. 2. Construction of the *Caecm33*Δ mutant and single reintegrant strains. (a) The entire *CaECM33* ORF was substituted by the *hisG-URA3-hisG* cassette. (b) Southern blot analysis demonstrating generation of RML1 (*ECM33*/*ecm33*::*hisG-URA-hisG*), RML2 (*ecm33*::*hisG*/*ecm33*::*hisG-URA-hisG*) and RML3 (*ecm33*::*hisG*/*ecm33*::*hisG-PD1ECM*). (c) Construction of the cassette (PD1ECM *cat-URA3-cat*) used to reintroduce *CaECM33* at its original locus.

confirm that the phenotypes displayed by the mutant strains were due to *CaECM33* depletion, reintegration strains were obtained.

Strain RML2a (the Ura$^-$ derivative of the *Caecm33* null mutant) was selected as the recipient for transformation with the *Sac*I-digested integration plasmid pD1ECM containing a functional *CaECM33* gene (Fig. 2c). Southern blot analysis of a representative isolate, *C. albicans* RML3, after digestion with *Xba*I and *Eco*RI, revealed that the linearized integrative plasmid had integrated in the allele properly, giving rise to the 1369 bp and 1838 bp parental fragments (Fig. 2b). Once we had tested by Southern blotting that the RML3 Ura$^-$ derivative selected (RML3a) had undergone intrachromosomal recombination between the chloramphenicol resistance gene repeats, we transformed this strain with the same integrative plasmid. Two independent transformants (RML4) carrying two copies of the *CaECM33* gene were obtained and checked by Southern blotting.

CaEcm33 mutant strains display several morphological surface and cell wall defects

C. albicans RML1 and RML2 mutants had an aberrant morphology, which varied depending on the growth conditions and medium. On solid media, *ecm33*Δ cells seemed to be rounder and larger than in liquid media. This rounder and larger shape of mutant cells was even more marked in cultures in the stationary phase. These results are consistent with the results of Bidlingmaier & Snyder (2002) implicating *ECM33* in the apical growth of the yeast *S. cerevisiae*, using a novel transposon-based mutagenesis system. In their screening, the elongated bud morphology of the *cdc34-2* mutant was altered when the transposon was inserted in the *ECM33* allele. After staining with Calcofluor white, a compound that binds to chitin or glucan polymer of the cell wall, the null mutant also showed large aggregates of Calcofluor-white-stained material; the composition of this material is currently being studied (Fig. 3a).

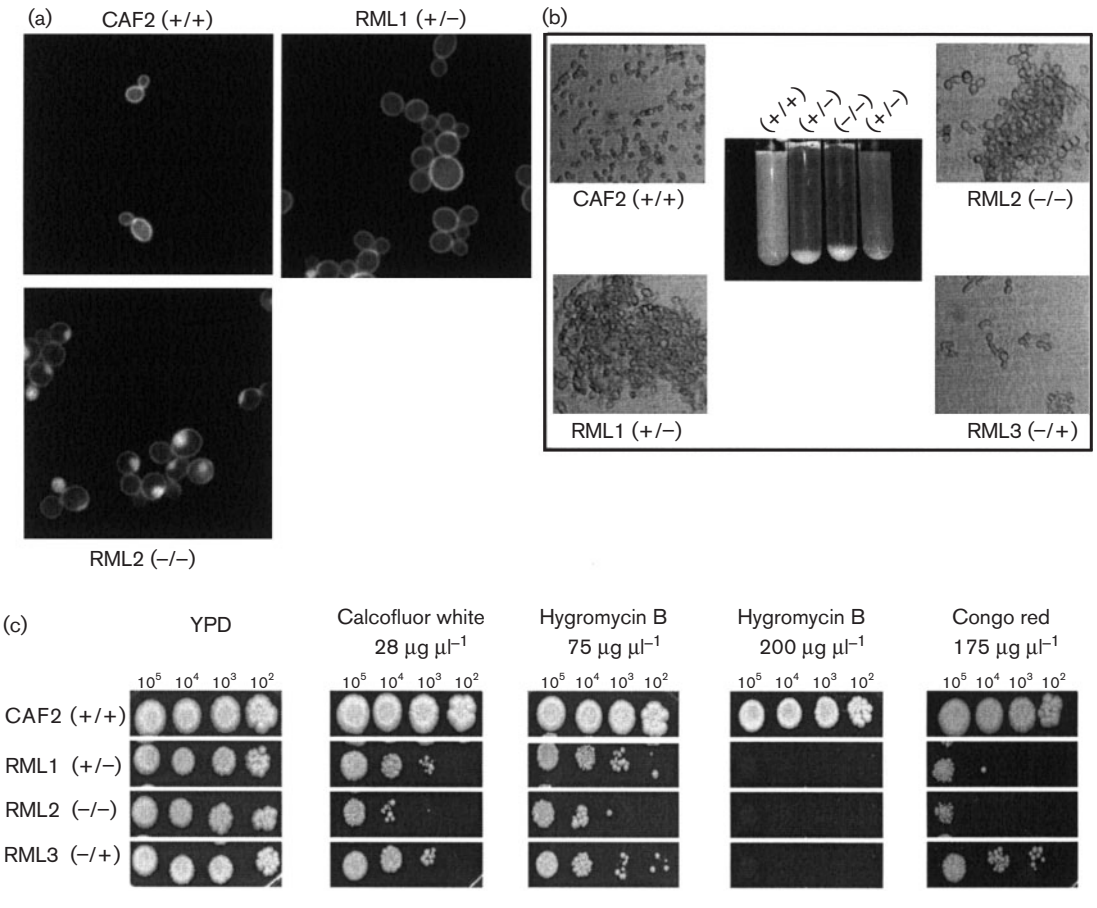


Fig. 3. Phenotypic analysis of *Caecm33* mutants. (a) Microscopy of Calcofluor-white-stained *C. albicans* cells (wild-type, heterozygous and homozygous mutants). Cells were grown in SD medium at 30 °C for 24 h. (b) Deletion of *CaECM33* induces flocculation of *C. albicans*. Photograph of test tubes containing stationary-phase blastospores of wild-type (+/+), or RML1 (+/–), RML2 (–/–) or RML3 (–/+) mutants after growth in YPD at 30 °C, and photomicrographs of the corresponding cultures. (c) Sensitivity to Calcofluor white, Congo red and hygromycin B of the wild-type strain (Caf2) and *Caecm33* heterozygous, homozygous and single reintegrant mutant strains (RML1, RML2 and RML3, respectively).

We also observed a marked tendency of RML1 and RML2 cells to flocculate extensively, forming large aggregates of cells that rapidly sedimented to the bottom of the tube when growing in YPD liquid cultures at 30 °C with gentle shaking (Fig. 3b). These aggregates were not due to a deficient cell wall separation of the cells since they were easily dispersed by 10 s sonication. This suggested that the formation of these aggregates might be caused by alterations in the superficial layers of the cell wall. Interestingly, this flocculation effect was only observed when cells were growing in YPD medium but not in YNB, where the cells exhibited a larger and rounder shape than in YPD.

We found that, as occurred in *S. cerevisiae ecm33*Δ, *C. albicans ecm33*Δ mutants exhibited great sensitivity to various compounds that interfere in the cross-linking of cell wall components (Pardo *et al.*, 2004). We tested the sensitivity of the mutants to Calcofluor white, Congo red and hygromycin B. Interestingly, the heterozygous mutant (RML1) already exhibited great sensitivity to all three of these compounds at all concentrations tested compared to the parental strain (Fig. 3c). This high sensitivity was even more dramatic in the case of the null mutant (RML2) and the effect was reversed when a wild-type copy of *CaECM33* was reintegrated at its locus (RML3). These results could suggest that the amounts of the different components of the cell wall have been modified in the mutant strains.

Ecm33p is required for normal filamentation of *C. albicans in vitro*

The presence of numerous E-box motifs within the promoter region of the *CaECM33* gene suggested a role for Ecm33p in the filamentation process. To explore this hypothesis, we studied the filamentation phenotype of *Caecm33*Δ mutants. When growing in liquid YPD supplemented with 5 % serum at 37 °C, the null mutant RML2 exhibited a slightly delayed filamentation. At 24 h growth the homozygous mutant (RML2) showed a high number of large round cells and far fewer hyphal aggregates than the parental strain. Upon closer microscopic observation the mutant filaments were found to be thicker (Fig. 4a). The germ tubes of *Caecm33*Δ mutants were wider than those of the parental strain: the CAF2 germ tube mean width was 1·9 μm (SD 0·09, $n=50$) while the RML1 and RML2 mutants exhibited a mean width of 2·10 μm (SD 0·1, $n=50$) and 3·25 μm (SD 0·14, $n=50$), respectively. The number of blastospores present in the culture medium of the RML2 mutant after 24 h was also markedly lower than for the parental strain. This suggested that once converted to hyphae, *Caecm33*Δ cells could not efficiently revert to the yeast form in liquid medium.

We also tested the effect of 10 % serum in solid media. In this case the differences between RML1 and RML2 mutants and the parental strain were more dramatic. The colonies of the parental strain had a very wrinkled morphology, while the *Caecm33*Δ mutant had a completely smooth colony morphology showing a severe lack of yeast-to-hypha transition (Fig. 4b). Upon microscopic observation, we found that the wild-type strain presented large hyphal aggregates while the heterozygous mutant (RML1) showed the same hyphal pattern as observed in 5 % liquid serum, with thicker and shorter hyphae. In contrast, homozygous mutants exhibited an elongated cell shape with no hyphal growth (Fig. 4b).

The RML2 mutant also failed to form filaments after 7 days incubation on Spider medium, showing smooth colony morphology, whereas the wild-type produced abundant filaments at this point with a typical invasive phenotype. The behaviour of the heterozygous mutant (RML1) and the single reintegrant (RML3) was intermediate, with different regions of the colony periphery invading the agar plate (Fig. 4c). Similar results were observed in SLADH medium (data not shown).

CaECM33 shows a gene dosage effect

All the phenotypic analyses suggested a gene dosage effect for *CaECM33*, since the heterozygous mutant RML1 already showed defects in cell wall maintenance, invasiveness, yeast-to-hypha transition and morphology. To check this possibility, we tested the expression levels of *CaECM33* in the current set of strains by Northern blot analysis. As the probe, we used almost the complete *CaECM33* sequence which had been amplified by PCR using the sense primer PROBE1 and the antisense primer PROBE2. One hybridization product corresponding to *CaECM33* mRNA was detected in all the strains except for the homozygous mutant RML2. As we expected, both the heterozygous and the single reintegrant showed approximately half the amount of *CaECM33* mRNA exhibited by the parental strain, suggesting that both *CaECM33* alleles are transcribed and contribute to total mRNA levels (Fig. 5a).

CaECM33 is involved in virulence

To examine the role of *CaECM33* in virulence, a murine model of systemic infection was used. Eight mice per strain were injected intravenously through the tail vein with the following strains: CAF2 (wild-type), the heterozygous mutant (RML1), the homozygous mutant (RML2), the single reintegrant (RML3), and the double reintegrant carrying two copies of *CaECM33* at its original locus (RML4). The mice were monitored for survival and for fungal infection in the kidney, which is known to have a linear relationship with median survival times in mice (Hurtrel *et al.*, 1980; Odds, 1988). Two days after infection with 10^6 *Candida* cells, mice injected with both the wild-type and the double reintegrant RML4 strains showed signs of systemic disease, including weight loss. By day 5, there were no survivors among the mice injected with cells containing both copies of the wild-type *CaECM33* gene (Fig. 6). In contrast, 100 % of mice injected with an equal inoculum of RML2 cells as well as those containing a single copy of the *CaECM33* gene (heterozygous and single

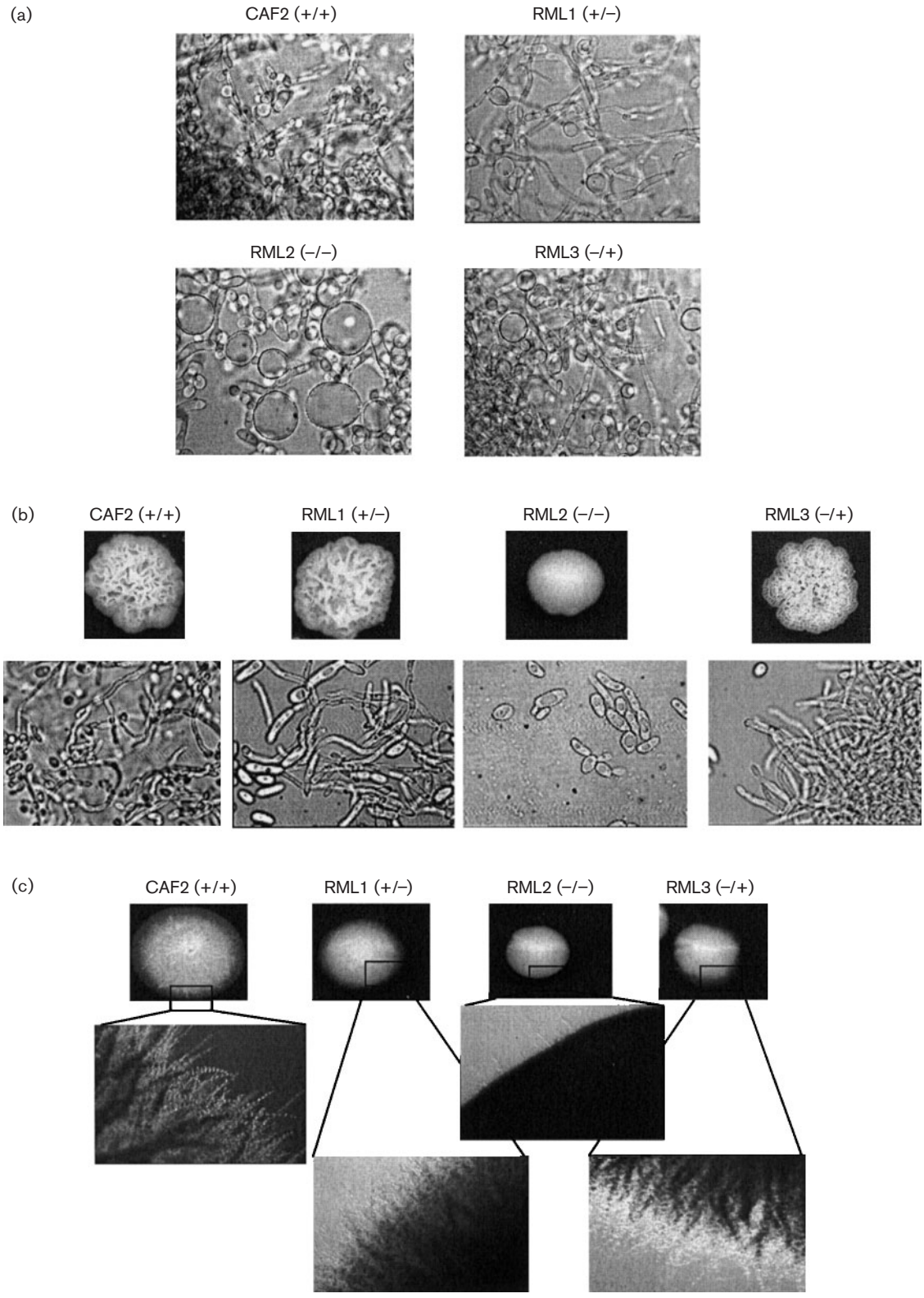


Fig. 4. Yeast-to-hypha transition in *Caecm33*Δ mutants. Wild-type strain CAF2, the heterozygous strain (RML1), the isogenic disrupted strain RML2, and the reintegrant strain carrying the *CaECM33* coding sequence (RML3) were tested in (a) liquid YPD supplemented with 5 % serum, (b) YPD containing 10 % serum, or (c) Spider plates. Spider and YPD + 10 % serum plates were incubated at 37 °C for 7 days. Liquid YPD + 5 % serum was incubated at 37 °C for 24 h.

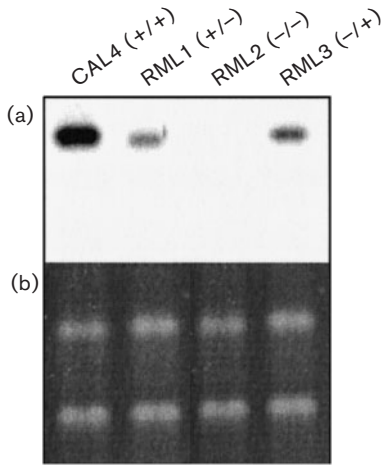


Fig. 5. (a) Northern blot analysis showing expression of *CaECM33* in strains containing heterozygous and homozygous mutants (RML1 and RML2 respectively) as well as the single reintegrant strain (RML3) and the wild-type CAF2. (b) Ethidium-bromide-stained gel showing the equal loading of samples.

reintegrant strains; RML1 and RML3 respectively) survived for the observed period of 30 days. *C. albicans* cells were not found in either the kidneys or the brains of the mice infected with RML2 or RML3, showing a complete lack of colonization. Taken together, these results suggest the importance of the presence of both intact copies of *CaECM33* gene for the mortality and kidney colonization produced by *C. albicans* cells in a murine systemic model.

DISCUSSION

The 'Ecm33p family' in *C. albicans*

Because of the important role played by proteins included in the so-called SPS2 family (Sps2p, Ecm33p, Pst1p and the gene product of *YCL048*w; Caro *et al.*, 1997) in *S. cerevisiae* cell wall maintenance, we investigated if they were also present in *C. albicans* and were involved in similar roles. After homology searches in the Stanford database (http://sequence-www.stanford.edu/group/candida/search.html) we identified the *C. albicans* homologues to *ECM33*, *PST1* (previously reported to be expressed and secreted; Monteoliva *et al.*, 2002) and *YCL048W*. The absence of the *SPS2* homologue was in concordance with the lack of meiosis in this organism. Like its *S. cerevisiae* counterpart, the *CaECM33* sequence contains an intron which changes the ORF of the protein, preventing the correct annotation of this gene in the Stanford database. *CaECM33* mRNA '*in vivo*' splicing sites were defined by RT-PCR experiments. We confirmed that CaEcm33p (a 423 aa protein) was the counterpart of *S. cerevisiae ECM33* since the expression of the *CaECM33* gene from the episomic plasmid YEPCaECM was able to complement the cell wall defects displayed by a *S. cerevisiae ecm33*Δ mutant. This family was classically the so-called SPS2 family because Sps2p was the first member of the family described. However, because in *C. albicans* this family of GPI-anchored proteins lacks Sps2, and Ecm33p has been identified in different fungal species – *Aspergillus fumigatus* (Bruneau *et al.*, 2001), *Kluyveromyces lactis*, *Candida tropicalis*, *Pichia farinosa*, *Saccharomyces kluyveri*, *Saccharomyces bayanus* and *Zygosaccharomyces rouxii* (Souciet *et al.*, 2000) – and its functional relevance has been clearly demonstrated by the drastic phenotypes displayed by *ecm33* mutants, we propose to call it the 'Ecm33 family'.

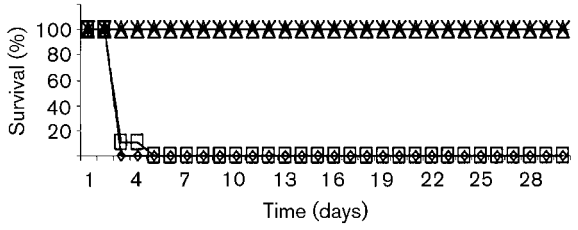


Fig. 6. Virulence study of *Caecm33* mutants. Female BALB/c mice were injected with 0·5 ml saline solution containing 10^6 c.f.u. of one of the following *C. albicans* strains: wild-type strain CAF2 (□), the heterozygous strain RML1 (×), the isogenic disrupted strain RML2 (△), and the reintegrant strains carrying one copy (RML3, ●) or both copies (RML4, ◇) of the *CaECM33* coding sequence.

Both *C. albicans* and *S. cerevisiae* Ecm33p-related proteins show features of GPI-anchored proteins, having a signal peptide, a serine- and threonine-rich region and a GPI anchor signal.

ScECM33 and *CaECM33* share a high degree of homology (53·67 %); therefore it seems quite possible that CaEcm33p localizes at the membrane like its *S. cerevisiae* counterpart (Terashima *et al.*, 2003).

CaECM33 is involved in cell wall integrity and morphogenesis

As we have demonstrated in this study, Ecm33p plays an important role in very different processes of *C. albicans*. The high sensitivity to Calcofluor white and Congo red displayed by the RML2 mutant shows that the cell wall is affected (Roncero & Duran, 1985) since these compounds interact with polysaccharides, interfering in the assembly of the chitin and 1,3-β-glucan (Cabib & Bowers, 1971). The presence of a weak cell wall requires the cells to induce the cell wall integrity pathway to survive (Carotti *et al.*, 2002). When the cell wall integrity pathway is activated, the so-called 'compensatory mechanism' is triggered. This cell wall salvage response involves: (i) a marked increase in the chitin content; (ii) changes in the association between cell wall polymers (while only 2 % of CWPs are linked directly to chitin in a wild-type cell, this linkage is 20-fold

more abundant in *gas1* cells); (iii) an increase in the bulk of CWPs; and (iv) a transient redistribution of the 1,3-β-glucan synthase complex throughout the cell. In the light of this, we think that an increase in the amount of cell wall material (mostly CWPs and chitin) that is not being efficiently distributed all around the cell wall in the null mutant strain could explain the Calcofluor-white-stained aggregates seen in the RML2 mutant. Similarly, the presence in the null mutant of an inadequate distribution and composition of the cell wall net could lead to a greater exposure of the cell surface flocculins (Teunissen & Steensma, 1995a; Teunissen *et al.*, 1995b, c), leading to flocculation promoted by interactions among the CWPs of different cells.

The lack of Ecm33p also leads to morphogenetic defects, with a complete lack of yeast-to-hypha transition on solid media such as Spider, SLADH and YPD supplemented with 10 % serum while, on the contrary, we only found a slightly delayed filamentation when cells were growing in liquid media. We hypothesize that this difference may occur because different sensors involved in the signal pathway might be altered or mislocalized in the *Caecm33* mutant cell wall and these alterations may be enhanced by the stronger physical constraints suffered by the cells in solid media. Different phenotypes on solid and liquid media have also been observed in strains harbouring mutations in *CPH1* filamentation pathway genes (Kohler & Fink, 1996; Leberer *et al.*, 1996; Csank *et al.*, 1998). It is interesting to note the presence of a cph1 sequence recognition signal in the 5′ upstream region of the *CaECM33* gene localized at position −2·8 kb with respect to the start codon. Although it seems to be located too far away to belong to the *CaECM33* promoter, there is no other gene annotated in the DNA region between the cph1 signal and *CaECM33*. Four E-boxes have also been identified in this promoter region (positions −130 bp, −280 bp, −750 bp, −1296 bp with respect to the ATG start codon) that can also influence the yeast-to-hypha defects observed in the mutants. These E-box domains (consensus sequence, 5′-CANNTG-3′) (Massari & Murre, 2000; Robinson & Lopes, 2000) are known to bind bHLH transcription factors related to Efg1p, and *EFG1* has been described as a major regulator of cell wall dynamics in *C. albicans* (Sohn *et al.*, 2003). Furthermore, activation of some hypha-specific genes depends upon Efg1 (Braun & Johnson, 2000; Sharkey *et al.*, 1999).

Another phenomenon to mention is the presence of large rounded cells in the RML2 serum culture after the 24 h incubation period. These cells are mainly located at the terminal region of the filaments and could correspond to hyphal cells in which the cell wall has been almost lost and then behave as protoplasts. In fact, some of these rounded cells seemed to have exploded, as occurs when protoplasts are exposed to osmotic stress.

It is evident from phenotypic analysis of *Caecm33*Δ that both intact alleles of *CaECM33* are necessary for all the processes that this protein has been shown to be implicated in. Generally, heterozygous mutants do not display any defect, even in the case of some essential genes (Monteoliva *et al.*, 1996), and the mutant phenotype is only patent when both copies are deleted. However, a gene dose effect has been described for other genes such as *CST20* and *HST7* (Kohler & Fink, 1996) and also for proteins located at the plasma membrane such as the amino acid permease Cagap1 (Biswas *et al.*, 2003). As we have shown in this study, the presence of a single copy of the *CaECM33* gene leads to defects in cell wall organization, morphogenesis and yeast-to-hypha transition, as well as in the virulence of the fungus. These defects were enhanced in the case of the null mutant, suggesting that the null alleles are not recessive.

An alternative possibility is that the partial dominance of the null mutation in the heterozygote is a reflection of a more complex mechanism such as defective pairing between the normal and the deleted allele (Aramayo & Metzenberg, 1996).

Both *CaECM33* alleles are required for virulence of *C. albicans* in a murine model of systemic candidosis

Our results demonstrate that Ecm33p plays an important role in *C. albicans* virulence. The cell wall is the first fungal structure in contact with the host environment. It is directly involved in different virulence factors such as adhesion, being able to modulate the immunological response against the infection. Different cell wall genes have been isolated and their roles in virulence have been addressed (Navarro-Garcia *et al.*, 2001). Although there are a number of reports in which the deletion of biosynthetic cell wall enzymes did not result in a dramatic reduction in virulence, such as for example Bgl2 (Sarthy *et al.*, 1997), Chs2 (Gow *et al.*, 1994) and Xog1 (Gonzalez *et al.*, 1997), there are other studies that clearly implicate some CWPs in virulence, such as Hwp1p (Staab *et al.*, 1999), Int1 (Kinneberg *et al.*, 1999), Phr2 (De Bernardis *et al.*, 1998) and Mnt1 (Buurman *et al.*, 1998). As we have shown, the Ecm33 GPI protein plays an important role not only in the maintenance of cell wall integrity but also in the correct yeast-to-hypha transition that also has been demonstrated to be indispensable for a complete virulence response. The presence of both intact copies of the *CaECM33* gene was necessary for both mouse mortality and kidney and brain colonization of *C. albicans* cells in a murine systemic model. When only a single copy of *CaECM33* was present, as in the case of the heterozygous and single reintegrant mutants, there was 100 % mouse survival at the inoculated dose of 10^6 *C. albicans* cells, which differs from the gene dosage effect observed in other phenotypes described previously. However, we are now carrying out other systemic murine infection analyses in which we modify the number of cells inoculated, in an attempt to define the differences between the heterozygous and homozygous mutants.

ACKNOWLEDGEMENTS

We thank the Centro de Secuenciación Automatizada of the Universidad Complutense de Madrid for expert assistance in DNA sequence analysis. Raquel Martinez was the recipient of a fellowship from the Ministerio de Educación y Ciencia de España. This work was supported by grants BIO 2003-00030 from the Comisión Interministerial de Ciencia y Tecnología (CYCIT, Spain), CPGE 1010/2000 for Strategic Groups from Comunidad Autonoma de Madrid and EU QLK3-CT2000-01537 (Eurocellwall).

REFERENCES

Aramayo, R. & Metzenberg, R. L. (1996). Meiotic transvection in fungi. *Cell* **86**, 103–113.

Ausubel, F. M., Kingston, R. E., Brent, R., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993). *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates & Wiley Interscience.

Bidlingmaier, S. & Snyder, M. (2002). Large-scale identification of genes important for apical growth in *Saccharomyces cerevisiae* by directed allele replacement technology (DART) screening. *Funct Integr Genomics* **1**, 345–356.

Biswas, S., Roy, M. & Datta, A. (2003). *N*-Acetylglucosamine-inducible *CaGAP1* encodes a general amino acid permease which coordinates external nitrogen source response and morphogenesis in *Candida albicans*. *Microbiology* **149**, 2597–2608.

Boeke, J. D., LaCroute, F. & Fink, G. R. (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.

Braun, B. R. & Johnson, A. D. (2000). *TUP1*, *CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. *Genetics* **155**, 57–67.

Bruneau, J. M., Magnin, T., Tagat, E., Legrand, R., Bernard, M., Diaquin, M., Fudali, C. & Latge, J. P. (2001). Proteome analysis of *Aspergillus fumigatus* identifies glycosylphosphatidylinositol-anchored proteins associated to the cell wall biosynthesis. *Electrophoresis* **22**, 2812–2823.

Buurman, E. T., Westwater, C., Hube, B., Brown, A. J., Odds, F. C. & Gow, N. A. (1998). Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of *Candida albicans*. *Proc Natl Acad Sci U S A* **95**, 7670–7675.

Cabib, E. & Bowers, B. (1971). Chitin and yeast budding. Localization of chitin in yeast bud scars. *J Biol Chem* **246**, 152–159.

Caro, L. H., Tettelin, H., Vossen, J. H., Ram, A. F., van den, E. H. & Klis, F. M. (1997). In silicio identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast* **13**, 1477–1489.

Carotti, C., Ferrario, L., Roncero, C., Valdivieso, M. H., Duran, A. & Popolo, L. (2002). Maintenance of cell integrity in the gas1 mutant of *Saccharomyces cerevisiae* requires the Chs3p-targeting and activation pathway and involves an unusual Chs3p localization. *Yeast* **19**, 1113–1124.

Casanova, M., Lopez-Ribot, J. L., Martinez, J. P. & Sentandreu, R. (1992). Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labelling with biotin: comparison with other techniques. *Infect Immun* **60**, 4898–4906.

Chaffin, W. L., Lopez-Ribot, J. L., Casanova, M., Gozalbo, D. & Martinez, J. P. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol Mol Biol Rev* **62**, 130–180.

Csank, C., Schroppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D. Y. & Whiteway, M. (1998). Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and candidiasis. *Infect Immun* **66**, 2713–2721.

De Bernardis, F., Muhlschlegel, F. A., Cassone, A. & Fonzi, W. A. (1998). The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* **66**, 3317–3325.

De Groot, P. W., Hellingwerf, K. J. & Klis, F. M. (2003). Genome-wide identification of fungal GPI proteins. *Yeast* **20**, 781–796.

Eisenhaber, B., Bork, P. & Eisenhaber, F. (1998). Sequence properties of GPI-anchored proteins near the omega-site: constraints for the polypeptide binding site of the putative transamidase. *Protein Eng* **11**, 1155–1161.

Elorza, M. V., Murgui, A. & Sentandreu, R. (1985). Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell walls. *J Gen Microbiol* **131**, 2209–2216.

Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.

Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Peña, J. M., Francois, J., Nombela, C. & Arroyo, J. (2004). The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. *J Biol Chem* **279**, 15183–15195.

Gillum, A. M., Tsay, E. Y. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* **198**, 179–182.

Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**, 1077–1090.

Gonzalez, M. M., Diez-Orejas, R., Molero, G., Alvarez, A. M., Pla, J., Nombela, C. & Sanchez-Perez, M. (1997). Phenotypic characterization of a *Candida albicans* strain deficient in its major exoglucanase. *Microbiology* **143**, 3023–3032.

Gow, N. A. (1997). Germ tube growth of *Candida albicans*. *Curr Top Med Mycol* **8**, 43–55.

Gow, N. A., Robbins, P. W., Lester, J. W., Brown, A. J., Fonzi, W. A., Chapman, T. & Kinsman, O. S. (1994). A hyphal-specific chitin synthase gene (*CHS2*) is not essential for growth, dimorphism, or virulence of *Candida albicans*. *Proc Natl Acad Sci U S A* **91**, 6216–6220.

Groll, A. H., De Lucca, A. J. & Walsh, T. J. (1998). Emerging targets for the development of novel antifungal therapeutics. *Trends Microbiol* **6**, 117–124.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Hube, B., Monod, M., Schofield, D. A., Brown, A. J. & Gow, N. A. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol Microbiol* **14**, 87–99.

Hurtrel, B., Lagrange, P. H. & Michel, J. C. (1980). Systemic candidiasis in mice. I. Correlation between kidney infection and mortality rate. *Ann Immunol* **131C**, 93–104.

Jung, U. S. & Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol Microbiol* **34**, 1049–1057.

Kapteyn, J. C., Montijn, R. C., Dijkgraaf, G. J. & Klis, F. M. (1994). Identification of beta-1,6-glucosylated cell wall proteins in yeast and hyphal forms of *Candida albicans*. *Eur J Cell Biol* **65**, 402–407.

Kapteyn, J. C., Montijn, R. C., Dijkgraaf, G. J., van den, E. H. & Klis, F. M. (1995a). Covalent association of beta-1,3-glucan with

beta-1,6-glucosylated mannoproteins in cell walls of *Candida albicans*. *J Bacteriol* **177**, 3788–3792.

Kapteyn, J. C., Dijkgraaf, G. J., Montijn, R. C. & Klis, F. M. (1995b). Glucosylation of cell wall proteins in regenerating spheroplasts of *Candida albicans*. *FEMS Microbiol Lett* **128**, 271–277.

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Muller, W. H., Andel, A. Verkleij A. J., Makarow, M., van den, E. H. & Klis, F. M. (2000). The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol Microbiol* **35**, 601–611.

Kinneberg, K. M., Bendel, C. M., Jechorek, R. P., Cebelinski, E. A., Gale, C. A., Berman, J. G., Erlandsen, S. L., Hostetter, M. K. & Wells, C. L. (1999). Effect of *INT1* gene on *Candida albicans* murine intestinal colonization. *J Surg Res* **87**, 245–251.

Klis, F. M., De Groot, P. & Hellingwerf, K. (2001). Molecular organization of the cell wall of *Candida albicans*. *Med Mycol* **39**, Suppl 1, 1–8.

Klis, F. M., Mol, P., Hellingwerf, K. & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **26**, 239–256.

Kohler, J. R. & Fink, G. R. (1996). *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. *Proc Natl Acad Sci U S A* **93**, 13223–13228.

Leberer, E., Harcus, D., Broadbent, I. D. & 7 other authors (1996). Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proc Natl Acad Sci U S A* **93**, 13217–13222.

Lipke, P. N. & Ovalle, R. (1998). Cell wall architecture in yeast: new structure and new challenges. *J Bacteriol* **180**, 3735–3740.

Liu, H., Kohler, J. & Fink, G. R. (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. *Science* **266**, 1723–1726.

Lopez-Ribot, J. L., Casanova, M., Gil, M. L. & Martinez, J. P. (1996). Common and form-specific cell wall antigens of *Candida albicans* as released by chemical and enzymatic treatments. *Mycopathologia* **134**, 13–20.

Martin, H., Arroyo, J., Sanchez, M., Molina, M. & Nombela, C. (1993). Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37 degrees C. *Mol Gen Genet* **241**, 177–184.

Martin, H., Rodriguez-Pachon, J. M., Ruiz, C., Nombela, C. & Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **275**, 1511–1519.

Massari, M. E. & Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* **20**, 429–440.

McCreath, K. J., Specht, C. A. & Robbins, P. W. (1995). Molecular cloning and characterization of chitinase genes from *Candida albicans*. *Proc Natl Acad Sci U S A* **92**, 2544–2588.

Mitchell, A. P. (1998). Dimorphism and virulence in *Candida albicans*. *Curr Opin Microbiol* **1**, 687–692.

Monteoliva, L., Sánchez, M., Pla, J., Gil, C. & Nombela, C. (1996). Cloning of *Candida albicans SEC14* gene homologue coding for a putative essential function. *Yeast* **12**, 1097–1105.

Monteoliva, L., Matas, M. L., Gil, C., Nombela, C. & Pla, J. (2002). Large-scale identification of putative exported proteins in *Candida albicans* by genetic selection. *Eukaryot Cell* **1**, 514–525.

Mormeneo, S., Rico, H., Iranzo, M., Aguado, C. & Sentandreu, R. (1996). Study of supramolecular structures released from the cell wall of *Candida albicans* by ethylenediamine treatment. *Arch Microbiol* **166**, 327–335.

Navarro-Garcia, F., Sanchez, M., Nombela, C. & Pla, J. (2001). Virulence genes in the pathogenic yeast *Candida albicans*. *FEMS Microbiol Rev* **25**, 245–268.

Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Int J Neural Syst* **8**, 581–599.

Odds, F. C. (1988). *Candida and Candidosis*. London: Baillière Tindall.

Odds, F. C. (2003). Reflections on the question: what does molecular mycology have to do with the clinician treating the patient? *Med Mycol* **41**, 1–6.

Orlean, P. (1997). Biogenesis of yeast cell wall and surface components. In *The Molecular and Cellular Biology of the Yeast Saccharomyces. Cell Cycle and Biology*, pp. 229–362. Edited by J. R. Pringle, J. R. Broach & E. W. Jones. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Pardo, M., Monteoliva, L., Pla, J., Sanchez, M., Gil, C. & Nombela, C. (1999). Two-dimensional analysis of proteins secreted by *Saccharomyces cerevisiae* regenerating protoplasts: a novel approach to study the cell wall. *Yeast* **15**, 459–472.

Pardo, M., Ward, M., Bains, S., Molina, M., Blackstock, W., Gil, C. & Nombela, C. (2000). A proteomic approach for the study of *Saccharomyces cerevisiae* cell wall biogenesis. *Electrophoresis* **21**, 3396–3410.

Pardo, M., Monteoliva, L., Vázquez, P., Martínez, R., Molero, G., Nombela, C. & Gil, C. (2004). *PST1* and *ECM33* encode two yeast cell surface GPI proteins important for cell wall integrity. *Microbiology* **150** (in press).

Pitarch, A., Sanchez, M., Nombela, C. & Gil, C. (2002). Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus *Candida albicans* cell wall proteome. *Mol Cell Proteomics* **1**, 967–982.

Pitarch, A., Sanchez, M., Nombela, C. & Gil, C. (2003). Analysis of the *Candida albicans* proteome. I. Strategies and applications. *J Chromatogr B Analyt Technol Biomed Life Sci* **787**, 101–128.

Robinson, K. A. & Lopes, J. M. (2000). *Saccharomyces cerevisiae* basic helix-loop-helix proteins regulate diverse biological processes. *Nucleic Acids Res* **28**, 1499–1505.

Roncero, C. & Duran, A. (1985). Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: in vivo activation of chitin polymerization. *J Bacteriol* **163**, 1180–1185.

Ruiz-Herrera, J., Mormeneo, S., Vanaclocha, P., Font-de-Mora, J., Iranzo, M., Puertes, I. & Sentandreu, R. (1994). Structural organization of the components of the cell wall from *Candida albicans*. *Microbiology* **140**, 1513–1523.

Rymond, B. C. & Rosbash, M. (1985). Cleavage of 5′ splice site and lariat formation are independent of 3′ splice site in yeast mRNA splicing. *Nature* **317**, 735–737.

Rymond, B. C. & Rosbash, M. (1986). Differential nuclease sensitivity identifies tight contacts between yeast pre-mRNA and spliceosomes. *EMBO J* **20**, 3517–3523.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanjuan, R., Zueco, J., Stock, R., Font, d. M. & Sentandreu, R. (1995). Identification of glucan-mannoprotein complexes in the cell wall of *Candida albicans* using a monoclonal antibody that reacts with a (1,6)-beta-glucan epitope. *Microbiology* **141**, 1545–1551.

Sarthy, A. V., McGonigal, T., Coen, M., Frost, D. J., Meulbroek, J. A. & Goldman, R. C. (1997). Phenotype in *Candida albicans* of a disruption of the *BGL2* gene encoding a 1,3-beta-glucosyltransferase. *Microbiology* **143**, 367–376.

Sharkey, L. L., McNemar, M. D., Saporito-Irwin, S. M., Sypherd, P. S. & Fonzi, W. A. (1999). *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1*, and *RBF1*. *J Bacteriol* **181**, 5273–5279.

Sohn, K., Urban, C., Brunner, H. & Rupp, S. (2003). *EFG1* is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Mol Microbiol* **47**, 89–102.

Souciet, J., Aigle, M., Artiguenave, F. & 21 other authors (2000). Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies. *FEBS Lett* **487**, 3–12.

Staab, J. F., Bradway, S. D., Fidel, P. L. & Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283**, 1535–1538.

Terashima, H., Hamada, K. & Kitada, K. (2003). The localization change of Ybr078w/Ecm33, a yeast GPI-associated protein, from the plasma membrane to the cell wall, affecting the cellular function. *FEMS Microbiol Lett* **218**, 175–180.

Teunissen, A. W. & Steensma, H. Y. (1995a). Review: the dominant flocculation genes of *Saccharomyces cerevisiae* constitute a new subtelomeric gene family. *Yeast* **11**, 1001–1013.

Teunissen, A. W., van den Berg, J. A. & Steensma, H. Y. (1995b). Localization of the dominant flocculation genes *FLO5* and *FLO8* of *Saccharomyces cerevisiae*. *Yeast* **11**, 735–745.

Teunissen, A. W., van den Berg, J. A. & Steensma, H. Y. (1995c). Transcriptional regulation of flocculation genes in *Saccharomyces cerevisiae*. *Yeast* **11**, 435–446.

Urban, C., Sohn, K., Lottspeich, F., Brunner, H. & Rupp, S. (2003). Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell. *FEBS Lett* **544**, 228–235.

van der Vaart, J. M., Caro, L. H., Chapman, J. W., Klis, F. M. & Verrips, C. T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *J Bacteriol* **177**, 3104–3110.

Vincent, J. L., Anaissie, E., Bruining, H. & 12 other authors (1998). Epidemiology, diagnosis and treatment of systemic *Candida* infection in surgical patients under intensive care. *Intensive Care Med* **24**, 206–216.

Walther, A. & Wendland, J. (2003). An improved transformation protocol for the human fungal pathogen *Candida albicans*. *Curr Genet* **42**, 339–343.