The *Candida albicans* pH-regulated *KER1* gene encodes a lysine/glutamic-acid-rich plasma-membrane protein that is involved in cell aggregation

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Immunoscreening of a *Candida albicans* cDNA library with a polyclonal germ-tube-specific antibody (pAb anti-gt) resulted in the isolation of a gene encoding a lysine/glutamic-acid-rich protein, which was consequently designated *KER1*. The nucleotide and deduced amino acid sequences of this gene displayed no significant homology with any other known sequence. *KER1* encodes a 134 kDa lysine (14±5 %)/glutamic acid (16±7 %) protein (Ker1p) that contains two potential transmembrane segments. *KER1* was expressed in a pH-conditional manner, with maximal expression at alkaline pH and lower expression at pH 4±0, and was regulated by *RIM101*. A Δ*ker1/*Δ*ker1* null mutant grew normally but was hyperfloculent under germ-tube-inducing conditions, yet this behaviour was also observed in stationary-phase cells grown under other incubation conditions. Western blotting analysis of different subcellular fractions, using as a probe a monospecific polyclonal antibody raised against a highly antigenic domain of Ker1p (pAb anti-Ker1p), revealed the presence of a 134 kDa band in the purified plasma-membrane fraction from the wild-type strain that was absent in the homologous preparation from Δ*ker1/*Δ*ker1* mutant. The pattern of cell-wall protein and mannoprotein species released by digestion with β-glucanases, reactive towards pAbs anti-gt and anti-Ker1p, as well as against concanavalin A, was also different in the Δ*ker1/*Δ*ker1* mutant. Mutant strains also displayed an increased cell-surface hydrophobicity and sensitivity to Congo red and Calcofluor white. Overall, these findings indicate that the mutant strain was affected in cell-wall composition and/or structure. The fact that the *ker1* mutant had attenuated virulence in systemic mouse infections suggests that this surface protein is also important in host–fungus interactions.

**INTRODUCTION**

Many of the genes required for virulence in bacterial pathogens are regulated in response to environmental signals (pH, temperature, osmotic pressure, and iron and calcium ion concentrations) indigenous to the host niche (Brown & Gow, 1999; Mekalanos, 1992). In the dimorphic, opportunistic fungal pathogen *Candida albicans*, the ability to respond to ambient pH appears to play a critical role in growth and virulence (for a review see Peñalva & Arst, 2002). It is known that external pH and temperature influence, at least in vitro, the yeast-to-mycelium transition of *C. albicans*, which is one of the virulence traits of this organism (Buffo et al., 1984; Odds, 1988).

An acidic pH generally encourages growth in the yeast form, whereas a neutral pH promotes hyphal development. The pH response in *C. albicans* involves the differential expression of at least three genes, *PHR1*, *PHR2* and *PRA1* (Mühlschlegel & Fonzi, 1997; Saporito-Irwin et al., 1995; Sentandreu et al., 1998). *PHR1* is expressed at a pH above 5±5 and is required for normal morphology at these pH levels. The gene product plays a key role in systemic infections. *PHR2* is expressed at an acidic pH, is required...
for normal morphology at these pH values, and contributes to virulence in the more acidic vaginal environment (De Bernardis et al., 1998; Mühlschlegel & Fonzi, 1997). 

**Methods**

**Micro-organisms and growth conditions.** The *C. albicans* strains used in this study are listed in Table 1. Cells were routinely grown in YPD or SD media at 28°C with shaking. The effects of acid or alkaline growth conditions were tested in YPD, modified Lee’s medium (Lee et al., 1975) containing 0.5 g proline per litre but lacking other amino acids, and medium 199 (Gibco) and adjusted to pH 4 or 7.5 with 155 mM HEPES. Different conditions were used to induce germ-tube formation: (i) addition of 10% (v/v) fetal calf serum to YPD medium (Gow & Gooday, 1982) or to water, (ii) starvation-stimulated dimorphism as described elsewhere (Casanova et al., 1989), or (iii) changes in pH and temperature (pH 4 to 7.5 and 25 to 37°C) in modified Lee’s medium (Porta et al., 1999). Media were supplemented with 25 μg uridine ml⁻¹ when appropriate. For sensitivity assays, plates containing solid (1.5% agar) YPD, Spiders and modified Lee’s media were supplemented with Congo red (50, 100 and 200 μg ml⁻¹), Calcofluor white (5, 20 and 25 μg ml⁻¹) and SDS (0.01%, 0.025% and 0.05%) and incubated at 30 or 37°C.

**Screening of libraries and cloning of KER1 gene.** A cDNA library of *C. albicans* (strain ATCC 26555) germ-tube-specific mRNA in the expression vector *gt11* (Manuel et al., 1996) was used for immunoscreening with a germ-tube-specific polyclonal antibody (pAb anti-gt), previously obtained by our group (Casanova et al., 1989) at a 1:1000 dilution by standard methods (Ausubel et al., 1994). cDNAs from immunoreactive clones were amplified and sequenced and blasted at the *S. cerevisiae* genome. A Δker1 null mutant was constructed and phenotypic analysis and virulence tests were subsequently conducted. Experimental evidence reported here on the subcellular location and potential functions of KER1 gene suggest that it may be involved in the integrated pH-response pathway, cell-wall biogenesis, and virulence.

Table 1. Strains of *C. albicans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Parental strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>26555</td>
<td>Δura3::Limm434/URA3</td>
<td>Wild-type</td>
<td>ATCC*</td>
</tr>
<tr>
<td>SC5314</td>
<td>Δura3::Limm434</td>
<td>Wild-type</td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>CAF2</td>
<td>Δura3::Limm434</td>
<td>SC5314</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAI4</td>
<td>Δura3::Limm434/Δura3::Limm434</td>
<td>SC5314</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>CAI4-URA3</td>
<td>Δura3::Limm434/Δura3::Limm434 RP10::URA3</td>
<td>CAI4</td>
<td>This work</td>
</tr>
<tr>
<td>C1H</td>
<td>Δura3::Limm434/Δura3::Limm434 Δker1::hisG/KER1</td>
<td>CAI4</td>
<td>This work</td>
</tr>
<tr>
<td>C1N7</td>
<td>Δura3::Limm434/Δura3::Limm434 Δker1::hisG/Δker1::hisG</td>
<td>C1H</td>
<td>This work</td>
</tr>
<tr>
<td>CAC1</td>
<td>Δura3::Limm434/Δura3::Limm434 Δker1::hisG/Δker1::hisG, RP10::URA3</td>
<td>C1N7</td>
<td>This work</td>
</tr>
<tr>
<td>CAR2</td>
<td>Δura3::Limm434/Δura3::Limm434 Δapr2::hisG/Δapr2::hisG/URA3::hisG</td>
<td>CAR14</td>
<td>Ramón et al. (1999)</td>
</tr>
</tbody>
</table>

*American Type Culture Collection.*
used to transform *E. coli* following standard plasmids (Ausubel et al., 1992). Positive transformants were checked by plasmid purification, enzyme digestion and PCR, and sequenced with an Applied Biosystems model 370A automated sequencer.

**Nucleic acid manipulation and analysis.** Genomic DNA preparation from *C. albicans* (Rose et al., 1990), digestion with restriction enzymes, and Southern blotting to nylon filters were carried out by standard protocols (Ausubel et al., 1992). The digoxigenin detection kit was used for DNA hybridization and probe labelling (Roche). Total RNA from *C. albicans* was prepared basically according to the procedure described by Hube et al. (1994). mRNA from total RNA was isolated using the Dynabeads mRNA Purification Kit (Dynal Biotech ASA). For cDNA synthesis (RT), mRNA was annealed with Oligo d(T)15 primer (Gibco-BRL) at 70°C for 10 min and cooled on ice for 1 min. The final reaction volume was made up to 40 μl with first-strand buffer, 0.5 mM dNTPs, 80 U RNaseOUT (Gibco-BRL) and 400 U Superscript II RNase H (Invitrogen). RT-PCR amplifications were carried out with primers PFC1 (primer PFC1), SpH1 (primers PRC1 and F1C1F), PstI (F1C1R), BglII (F2C1F), and KpnI (F2C1R) are underlined.

**Table 2. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’→3’)</th>
<th>Purpose†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFC1</td>
<td>GGCCTCTAGAGCTCTAGCTGCGGCAAAAGAGCTA</td>
<td>pUCA construction</td>
</tr>
<tr>
<td>PRC1</td>
<td>GGCCTCGATGCTCCGCTGCTCTATGATTATC</td>
<td>pUCA construction</td>
</tr>
<tr>
<td>F1C1F</td>
<td>GGCCTCGTCAATTCTCATTCTTATTTTTTAACCG</td>
<td>pACA construction</td>
</tr>
<tr>
<td>F1C1R</td>
<td>GGCAGATTCGCCGATATACTGACACATTAG</td>
<td>pACA construction</td>
</tr>
<tr>
<td>F2C1F</td>
<td>GGCAGTACCCGAGAGAAGTCTGACATTCC</td>
<td>pUCA constructionSouthern analysis</td>
</tr>
<tr>
<td>F2C1R</td>
<td>GGCAGGTACCAGAGAAGTCTGACATTCC</td>
<td>pUCA constructionSouthern analysis</td>
</tr>
<tr>
<td>AC1F</td>
<td>GATGGTAATGATTCTTATGGTGAAAGA</td>
<td>Strain constructions</td>
</tr>
<tr>
<td>SC1F</td>
<td>CGGAGATTTTCTCATAAAGGACCCAC</td>
<td>Expression analysis</td>
</tr>
<tr>
<td>SC1R</td>
<td>AGTCAATCTGTGCTCCCTTGCG</td>
<td>Expression analysis</td>
</tr>
<tr>
<td>HisG1</td>
<td>GGGCGTGGGCGGATGACATGTGCTCG</td>
<td>pACA and strain constructions</td>
</tr>
<tr>
<td>HisG2</td>
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</tr>
<tr>
<td>FURA</td>
<td>GACAGTCAACATCAAGGCTATAG</td>
<td>Strain constructions</td>
</tr>
<tr>
<td>RURA</td>
<td>CCAACAGCATTCTTATACC</td>
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</tr>
<tr>
<td>EFB1F</td>
<td>ATTAGACGATTCTGGTGCTAC</td>
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<tr>
<td>EFB1R</td>
<td>CATCCTCTTCAAACAGCAACGCTTG</td>
<td>Expression analysis</td>
</tr>
</tbody>
</table>

*Engineered restriction sites for XbaI (primer PFC1), SpH1 (primers PRC1 and F1C1F), PstI (F1C1R), BglII (F2C1F), and KpnI (F2C1R) are underlined.
†Relevant use of primer in this study.

For RNA expression analysis, semiquantitative RT-PCR was carried out. Appropriate primer pairs to generate unique cDNA amplifications were made using information from the literature, gene databases or sequences for the *KER1* and *EFB1* genes (Table 2). PCR reaction mixtures were carried out with 1 μg total nucleic acids. Reactions were run for one cycle of 10 s at 94°C, 28 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, and the content in tubes was analysed at cycle number 20 for comparative expression determinations. Differential expression levels were analysed by Molecular Analyst-Bio-Rad software (Bio-Rad). Semiquantitative RT-PCR assays were performed in triplicate to assess its reliability.

**Plasmid and strain construction for disruption of the *KER1* gene.** The technique of Fonzi & Irwin (1993) to disrupt genes in *C. albicans* was used with minor modifications. DNA sequences flanking the gene were obtained by PCR using as template plasmid DNA obtained from KER1 cloning, and primers F1C1F, F1C1R amplifying for 360 bp (F1C1), and F2C1F and F2C1R amplifying for 732 bp (F2C1) (see Table 2). These fragments were cut with the enzymes matching their respective ends and cloned into pBBS10 (Braun & Johnson, 2000) to get the pACA construction. pACA was cut with HindIII and Asp718 to linearize the 5 kb fragment, including F2C1, the hisG–ura3–hisG cassette and F2C1, and transformed into *C. albicans* CAI4 strain basically according to the lithium acetate procedure described by Gietz et al. (1995). Transformed cells were selected as URA+ on SD medium. One spontaneous URA+ derivative from a URA+-independent clone was selected on SD medium containing 5′-fluoroorotic acid (5′-FOA) and uridine, and used to delete the second allele of *KER1*. The disruption transformation was repeated to generate a null mutant. Both strains were verified by PCR and Southern blot analysis. For further phenotypic analysis, CAI4 and CIN7 strains were transformed with Clp10 integrating vector using the RP10 locus for the URA3 gene integration (Murad et al., 2000) to obtain the URA+ CAI4-URA3 and CAC1 strains.

**Subcellular fractionation and plasma membrane isolation.** Cells grown in Lee’s liquid medium at 30°C overnight were collected by centrifugation (4000 g, 10 min) and washed twice with chilled 1 mM PMSF in 10 mM Tris buffer (pH 7.2) (buffer A) and broken by shaking glass beads (425–600 μm, Sigma). The cell walls were sedimented from the cell-free homogenate, washed four times with buffer A, boiled for 5 min with 2% SDS to remove non-covalently bound proteins, and finally washed four more times with buffer A. The purified cell walls were digested in buffer A containing 0.5 mg Zymolyase 20T ml⁻¹ (ICN Biomedicals) for 3 h at 28°C. After treatment, the wall residue was removed by centrifugation and the solubilized material was concentrated by freeze-drying. The supernatant fluid obtained subsequently to cell breakage was centrifuged at 40 000 g for 40 min to obtain a mixed membrane fraction (P40), and the resulting supernatant was then centrifuged at 100 000 g for 1 h to obtain a microsomal fraction (P100). Plasma-membrane fraction was isolated according to the procedure described by Serrano (1988), slightly modified. The cell homogenate was centrifuged for 10 min at 700 g to remove large debris and the supernatant was further centrifuged for 40 min at 20 000 g. This
second pellet, enriched in plasma membranes, was resuspended with about 14 ml 20% (v/v) glycerol and 0·1 ml 100 mM PMSF and applied to a discontinuous gradient made of 8 ml 53% (w/w) and 16 ml 43% (w/w) sucrose solutions in distilled water. Purified plasma membranes were recovered at the interface between the two sucrose solutions after centrifugation for 6 h at 25 000 r.p.m. in a Beckman SW28 rotor. The interface band was diluted in water and pelleted by centrifugation for 20 min at 25 000 r.p.m. The purified membranes were resuspended in 20% glycerol. The total sugar content in the cell-wall digests was determined by the method of Dubois et al. (1956); whereas the protein content in the other samples (P40 and P100) was determined by the Lowry method.

**Antibodies.** An antibody recognizing Ker1p protein was prepared by Sigma-Genosys, by using a synthetic peptide selected from the deduced amino acid sequence from KER1 gene. PAb anti-Ker1p was raised against a 15-mer residue (HKVPYKFSYHPTLE) derived from the N-terminal domain of the protein. The polyclonal antibody germ-tube-specific (pAb anti-gt) against purified walls from mycelial cells of C. albicans was obtained as described previously (Casanova et al., 1989).

**SDS-PAGE and Western blotting.** SDS-PAGE under denaturing conditions was performed basically as described by Laemmli (1970) using slab gradient (4–20% or 4–10%) gels. Electrophoretic transfer to nitrocellulose paper was carried out as described previously (Casanova et al., 1989). Blotted proteins were immunodetected by using the primary specific antibodies (PAb anti-gt and PAb anti-Ker1p; see above) diluted (1:1000 and 1:500 respectively) in 0·01 M Tris/HCl buffer (pH 7·4), containing 0·9% NaCl, 0·05% Tween 20 and 3% bovine serum albumin as a blocking agent. Peroxidase-conjugated secondary antibodies (Bio-Rad) were used at 1:2000 dilution, with 4-chloro-1-naphthol as the chromogenic reagent. Concanavalin A (Con A) staining of nitrocellulose blots was conducted as described elsewhere (Casanova et al., 1989).

**Flow cytometry analysis.** For flow cytometric determination of cell aggregation, liquid cultures of each strain were filtered through a 30 μm diameter nylon mesh and analysed immediately in an EPICS XL-MCL flow cytometer (Beckman-Coulter). Cell aggregation was estimated from the measurement of forward-angle light scatter (FS Log), an indicator of particle size, and 90° side light scatter (SS Log), an indicator of particle complexity (Hewitt & Nebe-von-Caron, 2001) in 10 000 individual cells.

**Cell-surface hydrophobicity (CSH).** CSH of individual cells was determined by light microscopy observations, following attachment of latex-poly styrene microspheres (0·760 μm diameter; Sigma), according to the method described by López-Ribot et al. (1991). According to the criterion of Hazen & Hazen (1987), cells with three or more attached microspheres were considered to be positively hydrophobic. CSH of cell populations was determined by an aqueous-hydrocarbon biphasic hydrophobicity assay by mixing l·2 ml cell samples (OD_{600} of 1·000) with 0·3 ml of cyclohexane and vigorous vortexing for 3 min. The phases were then allowed to separate, and the percentage change in OD_{600} of the aqueous phase was considered the hydrophobicity value of the cell population (Hazen & Hazen, 1987).

**Virulence tests.** Strains CAF2 and CAC1 (Table 1) were grown in SDGY medium (4% glucose, 1% neopeptone, 0·1% yeast extract, 10% glycerol; pH 3·5) overnight in a shaking incubator at 30°C. Harvested cells were washed twice in water, and resuspended in saline solution to give an inoculum of approximately 500 c.f.u. (g mouse body weight)⁻¹ in a final volume of 100 μl. Five DBA/2 mice (Harlan Laboratories) were inoculated intravenously with the CAF2 strain and six DBA/2 mice with the CAC1 strain. Mice weighed approximately 20 g. Survival was monitored twice daily. Animals that became seriously ill, showing hunched posture, ruffled fur and reduced mobility, were humanely terminated and their deaths recorded as occurring on the following day. For viable cell counting, the left kidney and brain of dead mice were removed aseptically post-mortem, weighed and homogenized with an UltraTurrax apparatus in 0·5 ml sterile distilled water. Dilutions of the organ homogenates were plated on Sabouraud agar, containing 5 g chloramphenicol l⁻¹ and 2 g gentamicin sulphate l⁻¹ to determine tissue burdens of C. albicans in each organ.

**RESULTS**

**Isolation, cloning and characterization of the KER1 gene**

Immunoscreening of an expression library with a germ-tube-specific polyclonal antibody led to the isolation of a cDNA clone that when blasted against the sequence databases showed a novel incomplete ORF contained in the contig 4-3030. A genomic clone of the gene was obtained by PCR using two primers deduced from the contig sequence, and engineered with the appropriate restriction sites (Table 2) for cloning into pUC19. The resulting construction was confirmed by sequencing. The ORF encodes a putative polypeptide of 1197 amino acids with a deduced molecular mass of 134 027 Da and a pl of 5·11, which is rich in lysine (14·5%) and glutamic acid (16·7%). For this reason it was called Ker1p (for lysine/glutamic-acid-rich).

BLAST or FASTA comparisons of the translated amino acid sequence of Ker1p with the translated GenBank databases revealed homology with several proteins known or predicted to encode coiled-coil domains. These include conventional and non-conventional myosins, laminin, caldesmon and intermediate filaments. Best matches were found with a 200 kDa diagnostic antigen of Babesia bigemina (Tebele et al., 2000) and with the liver-stage antigen (LSA-1) of Plasmodium falciparum (Kun et al., 1999). In general, the homologous regions were found throughout the central segment of Ker1p (amino acids 407–830) with unique sequences in the N- and C-terminal regions flanking the large central region containing the predicted coiled-coil domains.

A search for sequence similarities in the S. cerevisiae protein databases (SGD and MIPS) revealed that there was no obvious homologue of Ker1p in this organism. Best alignments were found with Uso1p (Nakajima et al., 1991), Mlp1p (Strambio-de-Castillia et al., 1999a), Slk19p (Strambio-de-Castilla et al., 1999b) and Imh1p (Kjer-Nielsen et al., 1999) proteins with amino acid identities of approximately 20%, distributed randomly over the sequences. All these proteins have in common a high content in K and E amino acids and predominantly z-helical structure over the entire length of the protein, which is indicative of structural analogy rather than true homology. Similar rates of identity were observed with ScMnn4p, the product of the MNN4 gene, which regulates mannosyl phosphorylation in S. cerevisiae (Jigami & Odani, 1999). The
Table 3. Secondary-structure analysis (predicted topology)
The predicted α-helix content is 66%. The amino acid positions of predicted coiled coils and transmembrane domains are shown.

<table>
<thead>
<tr>
<th>Coiled coils</th>
<th>Transmembrane domains</th>
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<tbody>
<tr>
<td>70–124</td>
<td>913–930</td>
</tr>
<tr>
<td>366–393</td>
<td>532–554</td>
</tr>
<tr>
<td>419–513</td>
<td></td>
</tr>
<tr>
<td>575–696</td>
<td></td>
</tr>
<tr>
<td>708–815</td>
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amino acid sequence analysis of ScMnn4p revealed a striking lysine/glutamic acid repeat region and also predicted the presence of x-helical conformation. Secondary-structure analysis of Ker1p (Geourjeon & Deléage, 1995), coiled-coil structures (Lupas et al., 1991) and transmembrane domains (Hofmann & Stoffel, 1992) prediction according to Expasy analysis (see Table 3) predicted a transmembrane localization.

Regulation of KER1 expression
Differential expression of KER1 was assessed by semiquantiative RT-PCR using conditions favouring yeast or hyphal development. As shown in Fig. 1(a), maximum expression was found in modified Lee’s medium in both yeast and hyphal cultures. However, KER1 expression was down-regulated during growth in rich culture media. Interestingly, KER1 expression was also down-regulated when cells were grown in Lee’s medium at pH 4, suggesting that KER1 expression may also be pH-dependent. To explore this possibility, the effect of media at different pH and incubation temperatures on KER1 expression was assessed (Fig. 1b). The highest transcription levels were observed when cells were grown at pH 7.5 in Lee’s or M199 medium regardless of the growth temperature employed. In C. albicans, it has been demonstrated that the RIM101 pathway is required for some alkaline responses (Davis et al., 2000). Therefore, expression of KER1 was analysed in Δrim101/Δrim101 mutants that are deficient in a transcription factor regulating pH responses. Since the ratio KER1/EFB1 is higher at pH 4 than at pH 7.5 (Fig. 1b), rim101p could be controlling KER1 by repressing its expression at an acidic pH. These results demonstrate that RIM101 is required for the expression of KER1.

Construction of a KER1 null mutant
Disruption of KER1 gene was achieved by the ura-blaster technique (Fonzi & Irwin, 1993). A disruption cassette (see Methods) was used to replace a 3587 kb fragment of KER1 which included the entire ORF (Fig. 2a). The resultant C1H heterozygous and C1N7 null mutant strains (Table 1) were confirmed by PCR and Southern analysis (Fig. 2b and c, respectively). To confirm knockout of both KER1 gene alleles in the null mutant, RT-PCR was performed using specific primers for KER1 (SC1F-SC1R; see Table 2). The intron-containing gene that encodes the elongation factor EF-1β (EFB1) of C. albicans (Maneu et al., 1996) was included as internal control for possible contamination by genomic DNA. Results shown in Fig. 2(d) indicated the absence of KER1 transcripts under both growth conditions.

Subcellular localization of Ker1p
Different subcellular fractions (cell-wall extract, P40 and P100) obtained from the wild-type and CAC1 null mutant strains were analysed by SDS-PAGE and Western immunoblotting using pAb anti-gt (Fig. 3a) and pAb anti-Ker1p (Fig. 3b) as probes. An immunoreactive 134 kDa species was detected in the P40 fraction (mixed membranes), whereas no immunoreactive bands were detected in the P40 obtained from the null mutant strain, suggesting a...
Fig. 2. (a) Schematic representation of the construction, integration and selection of the cassette used to disrupt the KER1 gene. Methionine and stop codons are represented by checkered boxes. Significant primers for the disruption strategy are indicated in bold. (b) Ethidium-bromide-stained agarose (0-8 %) gel of PCR products obtained from C. albicans wild-type strain CAI4 (lanes 1 and 2), heterozygous strain C1H (lanes 3 and 4) and homozygous strain C1N7 (lanes 5 and 6). PCR was performed using whole cells as template and AC1F-SC1R (lanes 1, 3 and 5) and AC1F-HisG1 (lanes 2, 4 and 6) as primers amplifying for 1140 bp and 760 bp respectively. (c) Southern blot analysis of representative Δker1 mutants obtained during the disruption process. Genomic DNA (10 μg) from C. albicans CAI4 (lanes 1 and 4), C1H (lanes 2 and 5) and C1N7 (lanes 3 and 6) strains was digested with EcoRV (lanes 1–3) and with EcoRV–BamHI combination (lanes 4–6), subjected to electrophoresis, blotted and hybridized with F2C1 probe obtained by PCR and labelled with digoxigenin-dUTP, under high-stringency conditions. F2C1 probe detected a 9.5 kb and a 4.5 kb fragment for each restriction enzyme combination respectively for the wild-type strain, and a 3.3 kb fragment for the null mutant strain. (d) Qualitative analysis of KER1 expression. cDNA from strains CAI4 (lanes 1 and 2) and C1N7 (lanes 3 and 4), incubated under conditions to induce growing as budding yeast (lanes 1 and 3) and as germ tubes (lanes 2 and 4), was amplified by multiplex PCR using specific primers for KER1 (SC1F-SC1R; see panel a and Table 2) and EFB1 as internal control (Table 2).
membrane localization for the Ker1p protein. The null mutant also displayed an altered pattern of immunoreactive bands in Zymolyase cell-wall digests with respect to the wild-type strain (compare lanes 3 and 4 in Fig. 3a and b, respectively). Further SDS-PAGE and Western immunoblotting analysis of the purified plasma-membrane fraction revealed the presence of a 134 kDa polypeptide band only in the parent CAI4-URA3 strain (Fig. 3c, arrow).

Phenotypic analysis of the Δker1 mutant

The effect of the KER1 gene deletion on the phenotype of the Δker1 mutant was studied under conditions that normally promote both yeast and hyphal growth in C. albicans. The CAC1 mutant and the control CAI4-URA3 strains were grown in modified Lee’s medium at 25 °C (pH 4) and 37 °C (pH 7.5). Under incubation conditions that promote hyphal growth, cells of the CAC1 strain tended to aggregate. Aggregation was most dramatic at pH 7.5 and 37 °C after 3 h incubation, as assessed by macroscopic and light microscopy observations and flow cytometry analysis (data not shown). Overall, late-stationary-phase cells grown under different incubation conditions (i.e. in Lee’s medium at 28 °C or YPD medium at 30 °C) also tended to aggregate (not shown).

Western blotting analysis revealed that Con A-reactive mannoprotein species present in the cell-wall Zymolyase digests from CAC1 mutant strain exhibited different electrophoretic mobilities and a greater polydispersity when compared to their counterparts in the homologous extracts from CAI4-URA3 parental wild-type strain (not shown), thus suggesting that mannosylation of cell-wall glycoproteins could be affected by loss of KER1 function. Besides, Ker1p appears to be involved in cell surface hydrophobicity (CSH), a biological property considered to be an important virulence trait in C. albicans, and that appears to be associated with the glycosylation levels of cell-wall glycoproteins (Masuoka & Hazen, 1997). CSH determined by an aqueous-hydrocarbon biphasic partition assay (see Methods), showed that 92.5 ± 4.2 % of cells in the cultures of CAC1 strain displayed CSH, whereas only 61.3 ± 2.9 % of the cells in the cultures of the parental strain were found to be hydrophobic (values are the mean of three independent experiments carried out in duplicate ± standard deviations). Attachment of latex-polystyrene microspheres (see Methods), confirmed the previous results.

Finally, the sensitivity of Δker1 mutants to substances that interfered with cell-wall assembly was examined. No significant differences in sensitivity to Congo red, SDS and Calcofluor white were found between mutant and parental strains when incubated either at 30 °C or at 37 °C in several rich media (data not shown). However, when CAC1 cells were grown in solid Lee’s medium at 37 °C, sensitivities to Calcofluor white and Congo red increased with respect to those of the parental strain at alkaline pH but not at acidic pH (Fig. 4). These observations are in agreement with results from the KER1 expression analysis, since the highest level of KER1 expression was observed at pH 7.5 in Lee’s medium.

![Fig. 3. Western immunoblotting analysis of subcellular fractions from C. albicans strains grown at 30 °C in modified Lee’s liquid medium. Slab gradient polyacrylamide gels (4–20 % in a, and 4–10 % in b and c) were loaded with P40 fraction (P40) (a and b, lanes 1 and 2; 50 μg of material expressed as total protein content per well), with cell-wall Zymolyase digests (Zy) (lanes 3 and 4; 200 μg of material expressed as total sugar content per well), and the purified plasma-membrane fraction (PM) (c, 50 μg of material expressed as total protein content per well) from strains CAI4-URA3 (lanes 1 and 3) and CAC1 (lanes 2 and 4). After SDS-PAGE, polypeptides were transferred to nitrocellulose sheets and immunodetected with pAb anti-gt in (a) and with pAb anti-Ker1p in (b) and (c).](http://mic.sgmjournals.org)
Effect of KER1 deletion on virulence

The virulence of the Ura+ parental and disrupted strains was studied in a murine model of disseminated *C. albicans* infection. Prior to animal studies, two factors that might also affect the virulence of the mutant strains, the generation time, which was found to be the same in both strains, and the effect of URA3 gene disruption, were evaluated. To ensure full URA3 expression in the null mutant, strain C1N7 was transformed to strain CAC1 with the Clp10 integrating vector. Mice infected with the parental control strain (CAF2) succumbed to infection within 5 days of challenge, while all the mice infected with CAC1 survived to day 10. Two of the six animals challenged with CAC1 were still alive 28 days after challenge (Fig. 5). As shown in Table 4, tissue burdens of *C. albicans* recovered from organs post-mortem also showed a lower severity of infection by CAC1 as compared to CAF2. For all CAF2-infected mice, samples from the left kidney and brain were culture-positive, with mean burdens of 6.0 ± 0.2 and 4.9 ± 0.4 log10 (c.f.u. g⁻¹), respectively. For the six CAC1-infected mice, one kidney homogenate and two brain homogenates were culture-negative for *C. albicans*. Mean burdens for positive organs were significantly lower than for CAF2-infected mice at 5.5 ± 0.3 and 3.8 ± 0.5 log10 (c.f.u. g⁻¹), respectively (P < 0.05, Student’s t-test).

**DISCUSSION**

Molecular genetic approaches have identified several genes encoding hypha-specific cell-surface proteins that may contribute to differences in cell-wall structure or function in *C. albicans* (Hoyer et al., 1995; Staab et al., 1996). In this context, immunoscreening of a cDNA library with a germ-tube-specific polyclonal antibody (Casanova et al., 1989) led to the isolation of a novel gene, designated KER1, encoding a putative polypeptide of 1197 amino acids, rich in lysine (14.5 %) and glutamic acid (16.7 %). A search for sequence similarities in the current protein databases revealed no obvious homologue of Ker1p in other organisms.

Although the polyclonal antiserum used in the immunoscreening was mostly directed towards cell-wall components of *C. albicans* (Casanova et al., 1989), bioinformatic and Western immunoblotting analysis suggest that KER1 encodes an integral membrane protein rather than a cell-wall protein. These findings are not unusual since it has already been reported that antibodies raised against purified cell-wall preparations may also cross-react with non-wall cell components released during the isolation and purification processes, and that may be present as contaminants in the isolated walls (Eroles et al., 1997).

Environmental pH strongly influences morphogenesis in *C. albicans*. The RIM101-dependent pH signalling pathway plays a central role in the control of pH responses, morphogenesis and niche-specific responses during *C. albicans* infections (De Bernardis et al., 1998). In the absence of RIM101, KER1 gene was no longer expressed under alkaline conditions, suggesting that KER1 is a component of the same pH response induced at an alkaline pH (Davis et al., 2000). However, ambient pH was not the sole factor influencing its expression, since KER1 mRNA was also detected at low levels in cells grown in YNB, YPD medium supplemented with serum, or YPD buffered at
interaction of hyphal cell surfaces in the media under a variety of conditions, particularly in media sources, on both rich and minimal culture media, and at various temperatures. The most obvious consequence of KER1 deletion was that null mutant cells flocculated extensively under a variety of conditions, particularly in media that encourage germ-tube formation, which is consistent with the fact that aggregation may occur primarily by interaction of hyphal cell surfaces in the Δker1 null mutant, as already suggested for other null mutants of C. albicans (Calera & Calderone, 1999). In addition, Δker1 null mutant cells in stationary phase tended to aggregate under growing conditions that do not promote KER1 expression.

C. albicans mutants defective in N-linked mannosylation, like those strains lacking CaSRB1, the gene encoding GDP-mannose pyrophosphorylase (Warit et al., 2000), and MNN9, encoding the mannosyltransferase (Southard et al., 1999), were also found to flocculate, thus suggesting that aggregation phenotype could be due, at least partly, to an impairment in the N-mannosylation pathway of cell-wall mannoproteins. On the other hand, CSH which is considered to play an important role in host–parasite interaction and virulence of C. albicans (Chaffin et al., 1998; Hazen, 1990) has also been related to properties that may be essential in the interaction of the fungal cell and the environment through adhesion phenomena and modulation of the immune response (Chaffin et al., 1998; Martínez et al., 1998), membrane proteins also play an essential role in fungal physiology because they are involved in nutrient transport, energy generation, and signal transduction pathways, ultimately leading to growth and host adaptation (Monteoliva et al., 2002). It has been estimated in S. cerevisiae that about 1200 genes may be involved in cell-wall construction as deletion of these genes resulted in an altered cell wall (De Groot et al., 2001). In this context, KER1 could be added to the growing list of Candida genes involved in cell-wall structure that when mutated are uniformly impaired to some degree in morphogenesis. These include the genes required for synthesis or assembly of glucan (e.g. CaKRE9, PHR1 and PHR2) (Fonzi, 1999; Lussier et al., 1998), chitin (e.g. CHS1) (Munro et al., 2001) and mannan (e.g. MNN9, MNT1, SRB1, PMT1 and PMT6) (Buurman et al., 1998; Southard et al., 1999; Timpel et al., 1998, 2000; Warit et al., 2000). Overall, our results indicate that deletion of the KER1 gene clearly resulted in a cascade of pleiotropic effects, mostly affecting cell-surface-related properties that may be essential in the interaction of the fungal cells with the environment.

### ACKNOWLEDGEMENTS

This work was supported in part by grant BMC2001-2975 from the Programa Nacional de Promoción General del Conocimiento, Ministerio de Ciencia y Tecnología, Spain (to J. P. M.), grant CTIDIB/2002/3 from the Subsecretaría de la Oficina de Ciencia y Tecnología de la Generalitat Valenciana, Valencia, Spain (to M. C.) and grants 063204 and 056847 (to N. A. R. G. and F. C. O.) from the Wellcome Trust. A. G. is the recipient of a predoctoral fellowship from the Conselleria de Cultura, Educación y Ciencia, Generalitat Valenciana.
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