Candida albicans adhesin Als3p is dispensable for virulence in the mouse model of disseminated candidiasis

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The presence of specific proteins, including Ece1p, Hwp1p and Als3p, distinguishes the Candida albicans hyphal cell wall from that of yeast-form cells. These proteins are thought to be important for the ability of C. albicans cells to adhere to living and non-living surfaces and for the cell-to-cell adhesion necessary for biofilm formation, and also to be pivotal in mediating C. albicans interactions with endothelial cells. Using an in vitro flow adhesion assay, we previously observed that yeast cells bind in greater numbers to human microvascular endothelial cells than do hyphal or pseudohyphal cells. This is consistent with previous observations that, in a murine model of disseminated candidiasis, cells locked in the yeast form can efficiently escape the bloodstream and invade host tissues. To more precisely explore the role of Als3p in adhesion and virulence, we deleted both copies of ALS3 in a wild-type C. albicans strain. In agreement with previous studies, our als3Δ null strain formed hyphae normally but was defective in biofilm formation. Whilst ALS3 was not expressed in our null strain, hypha-specific genes such as ECE1 and HWP1 were still induced appropriately. Both the yeast form and the hyphal form of the als3Δ strain adhered to microvascular endothelial cells to the same extent as a wild-type strain under conditions of flow, indicating that Als3p is not a significant mediator of the initial interaction between fungal cells and the endothelium. Finally, in a murine model of haematogenously disseminated candidiasis the mutant als3Δ remained as virulent as the wild-type parent strain.

INTRODUCTION

The ability of the opportunistic fungal pathogen Candida albicans to interact with host cells is an important component of systemic infections, which have mortality rates ranging from 30 to 50% (Viudes et al., 2002; Wey et al., 1988). The use of implanted medical devices provides a substrate upon which C. albicans can form biofilms, complex structures which consist of a mixture of yeast, pseudohyphae and hyphae embedded in an extracellular matrix (Ramage et al., 2005). Fungal cell wall proteins are believed to be important for the ability of C. albicans cells to adhere to living and non-living surfaces and for the cell-to-cell adhesion necessary for biofilm formation, and also to be pivotal in mediating C. albicans interactions with endothelial cells. It had been thought that filamentation was necessary to penetrate the vascular endothelium in order to enter tissues, but in a murine model of systemic candidiasis, C. albicans cells can rapidly escape the bloodstream after infection, with fewer than 10% of introduced cells recoverable from blood 10 min after injection (MacCallum & Odds, 2005). Further, although strains that are locked in the yeast form are avirulent in the murine model of systemic candidiasis (Lo et al., 1997; Saville et al., 2003; Stoldt et al., 1997), it has been demonstrated that yeast cells can effectively escape the bloodstream and penetrate organs (Bendel et al., 2003; Chen et al., 2006; Saville et al., 2003; Spellberg et al., 2003). The importance of yeast morphology for dissemination is further supported by our recent
observation that yeast-form cells adhere better than hyphal cells to endothelial cells under conditions of flow (Grubb et al., 2009). Although cells locked in the filamentous state also display reduced virulence (Braun & Johnson, 1997; Braun et al., 2000; Murad et al., 2001), the ability to form hyphae is clearly important for C. albicans to cause pathology after dissemination: cells locked in the yeast form remain avirulent until they are permitted to form hyphae, after which point, mice succumb to the infection (Saville et al., 2003). To better understand the capacity of C. albicans to cause disease, previous studies have examined not only the regulation of hypha formation, but also the importance of specific cell wall proteins in C. albicans virulence.

In C. albicans, the agglutinin-like sequence (ALS) genes encode a family of cell wall proteins involved in adhesion (Hoyer et al., 2008). The cell surface glycoprotein Als3p is a major component of the hyphal but not the yeast cell wall (Argimón et al., 2007; Coleman et al., 2009; Zhao et al., 2004). Strains lacking Als3p can still form hyphae (Nobile et al., 2006; Zhao et al., 2004), but biofilm formation in vitro is impaired (Nobile et al., 2006; Zhao et al., 2006). In the absence of Als3p, hyphal cells grow in parallel orientation rather than in the tangled arrangement observed with wild-type cells (Zhao et al., 2006). This results in a weaker structure and reduced biofilm mass. Conversely, although overexpression of ALS3 does not restore the ability of the efg1Δ mutant strain to form hyphae, it does lead to an increase in biofilm mass (Zhao et al., 2006). Biofilms formed in vivo exist in polymicrobial environments and Als3p appears to be important for these interactions as well. Without Als3p, C. albicans hyphae are impaired in their ability to adhere to the human oral cavity bacterium Streptococcus gordonii and form a biofilm (Silverman et al., 2010). Although Als3p is clearly important, it is not the sole determinant of biofilm formation. An als3Δ mutant is able to form an extensive biofilm in vitro under hypoxic conditions (Stichternoth & Ernst, 2009) and in vivo using a rat venous catheter model (Nobile et al., 2006), whilst overexpression of HWP1, but not ALS3, effectively restores the ability of a bcr1Δ null strain to form biofilms on murine mucosal surfaces (Dwivedi et al., 2011).

Previous studies have demonstrated that a lack of Als3p impairs the ability of C. albicans to adhere to human buccal epithelial cell (BEC) monolayers, and that incubation with specific anti-Als3p monoclonal antibodies blocks C. albicans adhesion to human BEC or human umbilical vascular endothelial cell (HUVEC) monolayers (Coleman et al., 2009). Without Als3p, C. albicans has reduced ability to damage TR146 oral squamous cell carcinoma cells cultured in vitro either as a reconstituted human epithelium model (Zhao et al., 2004) or as monolayers (Almeida et al., 2008), and is endocytosed less efficiently by HUVEC or oral epithelial cells (Phan et al., 2007). This endocytosis has been shown to be mediated by C. albicans Als3p interactions with E-cadherin on oral epithelial cells and N-cadherin on endothelial cells. These observations have led to the supposition that Als3p might play an important role during disseminated infections, although no study using the haematogenously disseminated murine model to test this hypothesis has yet been published. During a disseminated C. albicans infection, fungal cells must form the initial attachments to host tissues not under static conditions, but subject to the stresses of fluid moving through the blood vessels. Our recent data show that, in contrast to the situation under static conditions, yeast-form cells adhere better than hyphae under conditions of flow (Grubb et al., 2009). We therefore sought to test the ability of an als3Δ null strain to adhere to endothelial cells under these conditions and, since it has not previously been reported, to test the capacity for virulence of the mutant strain in the widely used murine model of haematogenously disseminated candidiasis.

METHODS

Strains and media. The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Strains were routinely maintained as ~80 °C frozen stocks and grown on yeast extract-peptone-dextrose (YPD). For filamentation assays in liquid medium, strains were grown overnight at 28 °C, washed in sterile PBS, diluted 1:20 into fresh medium such as RPMI 1640 supplemented with L-glutamine and buffered with MOPS (Angus Buffers and Chemicals), and incubated with shaking at 37 °C. Biofilm formation was determined as described by Nobile & Mitchell (2005). Briefly, cells from an overnight culture were diluted to OD600 0.5 in 2 ml Spider medium and added to a well of a 12-well plate containing a 15 mm × 15 mm square cut from a silicone sheet (Bentec Medical) and pretreated overnight in fetal bovine serum (FBS) (Lonza). The plate was incubated at 37 °C for gentle shaking for 90 min and then the squares were washed with sterile PBS to remove non-adherent cells. Incubation was continued with fresh Spider medium for 48 h and the wells were photographed. For the flow adhesion assay, C. albicans was grown as described previously (Grubb et al., 2009). Briefly, cultures were grown overnight at 25 °C in YPD broth. For yeast-form growth, cells were diluted 1:10 in fresh YPD and grown with shaking at 25 °C for 3 h, while, to induce hypha formation, cells were diluted 1:10 in fresh YPD supplemented with 5% FBS and grown with shaking at 37 °C for 1 h. Microscopic examination of the cells confirmed the morphology and that they remained separated with no evidence of significant clumping. After culture, C. albicans cells were washed once in HEPES-buffered Hank’s balanced salt solution (HBSS) and resuspended at 2 × 106 c.f.u. ml−1 in HBSS (Sigma). All plasmid manipulations were performed with Escherichia coli strain DH5α with selection on Luria–Bertani plates containing 100 μg ampicillin ml−1 when necessary.

Strain construction. Both copies of ALS3 were sequentially deleted from wild-type strain SC5314 using the SAT1 flipper (Reuss et al., 2004). First, regions flanking the ALS3 coding sequence (designated LHF and RHFooter and RHFinner, respectively) were PCR-amplified using the primer pairs ALS3_LHF_UPS with ALS3_LHF_DS, ALS3_RHF_outer_UPS with ALS3_RHF_outer_DS and ALS3_RHF_ inner_UPS with ALS3_RHF_inner_DS (Table 3). The amplification products were ligated into the Smal site of plasmid pMT3000. The LHF region was liberated from the plasmid by digestion with Apal and Xhol at the sites engineered into the primers and ligated between the Apal and Xhol sites of the pSF52 plasmid (Reuss et al., 2004) to form plasmid pALHFS. The inner and outer RHF regions were liberated from the plasmids by digestion with SacI and SacII and...
ligated between the SacI and SacI sites in pALHFS to form plasmids pASinner and pASouter, respectively. To replace the first copy of ALS3, the entire deletion cassette of pASouter was liberated as a KpnI–SacI fragment and transformed into C. albicans strain SC5314 using a modified electroporation transformation method (Köhler et al., 1997). Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 μg nourseothricin ml⁻¹ (Werner Bioagents), as described by Reuss et al. (2004). Genomic DNA was prepared from several of the nourseothricin-resistant transformants obtained using the MasterPure Yeast DNA Extraction kit (Epicentre Biotechnologies), digested with BglII, transferred to a positively charged Nytran membrane (Whatman) and subjected to Southern blot analysis as described by Church & Gilbert (1984). The LHF PCR product (described above) was labelled with [³²P]dCTP using Ready-To-Go DNA labelling beads (GE Healthcare) and used as a probe. Strains in which the cassette had correctly integrated into one allele of ALS3 were grown in yeast extract-peptone-maltose to activate the FLP recombinase and remove the deletion cassette. The resulting nourseothricin-sensitive transformants were selected on YPD agar plates containing 20 μg nourseothricin ml⁻¹ (Werner Bioagents), as described by Reuss et al. (2004). The transformation and verification process was repeated using the deletion cassette from pASinner to isolate homozygous deletion strains. Two independent homozygous deletion isolates, 17322 and 17323, were subjected to a battery of in vitro tests and found to behave identically. Therefore, isolate 17322 was analysed in all of the subsequent experiments.

**Quantitative PCR.** RNA was isolated from C. albicans cells using the MasterPure Yeast RNA Extraction kit (Epicentre Biotechnologies). RNA was treated with amplification grade DNase I (Invitrogen) and used for cDNA synthesis with the Masterscript kit (5 PRIME). The primer pairs (Table 3) were used in conjunction with SYBR Green PCR Master Mix (Applied Biosystems) and tweek tec real-time 96-well PCR plates (Eppendorf) in an ABI 7300 Real-Time PCR system (Applied Biosystems). Dissociation curves were analysed for all reactions to verify single peaks/products. Expression levels were analysed using ABI 7300 System SDS software (Applied Biosystems).

**Flow adhesion assay.** The flow adhesion assay was performed as described previously (Grubb et al., 2009) using the immortalized human microvascular endothelial cell line HMEC-1 (Ades et al., 1992) (provided by F. I. Candal, Centers for Disease Control and Prevention, Atlanta, GA, USA). Briefly, HMEC-1 cells were cultured on sterilized glass slides in four-well plates for 72 h until confluent monolayers had formed. Any non-adherent cells were removed by washing with HBSS. The slides were then mounted in a parallel plate flow chamber (GlycoTech) and placed onto a 37 °C stage in an environmental chamber also maintained at 37 °C. C. albicans cells at 2 × 10⁶ ml⁻¹ were perfused through the flow chamber and over the endothelial cell monolayers using an automated syringe pump (Harvard Apparatus) for 15 min at a shear stress of 1.5 dynes cm⁻² (0.15 N m⁻²). Adhesion of C. albicans to endothelial cells was visualized using an integrated high-resolution digital camera (AxioCam MRm, Zeiss) with AxioVision 4.6 software (Imaging Associates) attached to a Zeiss Axiovert 200M inverted microscope. Images of at least three random fields of view (× 20 objective; area of the field analysed=0.15 mm²) were taken after 15 min of flow. Each experiment was performed three times and at least three images were recorded per slide. Differences between samples were analysed using Student’s t test.

**Murine virulence assays.** For injection, cultures of the als3Δ strain and a wild-type strain were grown overnight at 28 °C in YPD. Cells were harvested by centrifugation and washed three times in sterile pyrogen-free saline. Cells were counted using a haemocytometer, and appropriate dilutions were made so that the required dosage of cells could be injected in a final volume of 200 μl into the lateral tail veins of 6–8-week-old female BALB/c mice. Confirmation of the number and viability of cells present in the infecting inocula was performed by plate counts. Groups of five mice were used for each condition. Days on which the animals died were recorded; severely moribund animals were humanely sacrificed to minimize suffering and recorded as having died the following day. In all experiments, one kidney was processed for histopathology, whereas the other kidney, brain and spleen were homogenized and fungal loads determined by plating dilutions onto Sabouraud agar plates. All experiments were performed in accordance with institutional regulations in place at the University of Texas at San Antonio. Mice were allowed a 1 week acclimatization period before experiments were started.

The competition experiment was based on that described by Wu et al. (2007) and was performed using the GFP-labelled wild-type

### Table 1. Strains used in this study

<table>
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<th>Parent</th>
<th>Genotype</th>
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<td>Wild-type</td>
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<td>RPS1::RPS1::GFP</td>
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<td>173</td>
<td>SC5314</td>
<td>ALS3::als3A::SAT1-FLIP*</td>
<td>This study</td>
</tr>
<tr>
<td>1732</td>
<td>173</td>
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<td>This study</td>
</tr>
<tr>
<td>17322</td>
<td>173</td>
<td>als3A::FR1::als3A::SAT1-FLIP</td>
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<td>17323</td>
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</tr>
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</tr>
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<td>173232</td>
<td>17322</td>
<td>als3A::FR1::als3A::FRT</td>
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</table>

*SAT1-FLIP denotes the SAT1 flipper cassette.

### Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Reference or source</th>
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<tr>
<td>pSFS2</td>
<td>Reuss et al. (2004)</td>
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<tr>
<td>pMT3000</td>
<td>Paget et al. (1994)</td>
</tr>
<tr>
<td>pALHFS</td>
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</tr>
<tr>
<td>pARHFFouter</td>
<td>This study</td>
</tr>
<tr>
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<td>This study</td>
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<td>pALHFS</td>
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<td>pASouter</td>
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<tr>
<td>pASinner</td>
<td>This study</td>
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</table>
containing 100 Sabouraud agar plates to obtain the total fungal burden. Colonies the cells to be injected or homogenized tissues were plated onto sacrifice time point (3, 6 or 24 h post-injection). To assess the relative using a haemocytometer, and equal numbers from each strain were
were fixed in 10 % buffered formalin and stored at 4 C until
microscopic evaluation. (Grocott, 1955) or haematoxylin and eosin (H&E) stain prior to
Histopathology. Kidneys excised from deceased or sacrificed mice
were cut and stained with Grocott–Gomori methenamine-silver (GMS) (Bassilana et al., 2004; Correia et al., 2010). To avoid this potential
pitfall, we used the flipper method to delete both copies of Als3p in the wild-type strain SC5314. To verify
als3 null strain matched
D reported for previous als3Δ mutants, our null strain was
defective in its ability to form a biofilm on an elastomer surface in vitro (Fig. 1a) even though it was able to form hyphae. Furthermore, when material was removed from the well inoculated with the wild-type strain and examined microscopically the hyphal cells were densely packed and intertwined, whereas the als3Δ hyphae were more loosely associated and spread out in an open network (Fig. 1b). The heterozygote generally resembled the wild-type. These observations were in agreement with the phenotypes reported for deletion strains constructed by other groups using different methodologies (Nobile et al., 2006; Zhao et al., 2004).

To confirm that there was no ALS3 expression in the mutant, RNA was isolated from filamentous cultures and used for quantitative real-time PCR analysis. This showed that hyphae formed by the null strain lacked ALS3 transcript, while in the wild-type strain, ALS3 transcript was present in the heterozygote. We also examined the expression of two other hypha-specific genes, ECE1 and HWP1. Transcription of these genes in the mutant strain was not markedly different from that seen in the wild-type strain or the ALS3 heterozygote, thus confirming that, except for an absence of ALS3, the hyphae formed by the null strain induced other hyphal genes as expected (Fig. 2). It has been shown elsewhere that Als1p and Als3p have some functional redundancy during biofilm formation in an intravenous rat catheter model (Nobile et al., 2008), while Als1p, Als3p and Als5p all contain amino acid sequences predicted to allow amyloid formation (Otoo et al., 2008). Since it was possible that deletion of ALS3 resulted in compensatory elevated

### Table 3. Oligonucleotides used in this study

Underlined sequences indicate restriction enzyme recognition sites engineered into the oligonucleotides.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
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<td>5’-GGGCCCATGAGCAAACAATCCGAAG-3’</td>
<td>This study</td>
</tr>
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<td>ALS3_LHF_DS</td>
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<td>This study</td>
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<tr>
<td>ALS3_RHF_outer_UPS</td>
<td>5’-CCGCGGTTGCTACTAAGTTTGAAGGGTG-3’</td>
<td>This study</td>
</tr>
<tr>
<td>ALS3_RHF_outer_UPS</td>
<td>5’-GAGCTCAGCCGAACACCTAATCAC-3’</td>
<td>This study</td>
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<tr>
<td>ALS3_RHF_inner_UPS</td>
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<td>ALS3_RHF_inner_UPS</td>
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</tr>
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<td>ACT1-A</td>
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<tr>
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</tr>
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<td>Cleary et al. (2010)</td>
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<td>ALS5_5</td>
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<td>5’-TTGATACTGGTTATTATCTGAGGAGAAA-3’</td>
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(Sc3314-GFP) and als3Δ mutant (173221) strains. Cells were counted using a haemocytometer, and equal numbers from each strain were mixed prior to injection. Groups of five mice were used for each sacrifice time point (3, 6 or 24 h post-injection). To assess the relative proportions of the two strains before and after infection, dilutions of the cells to be injected or homogenized tissues were plated onto Sabouraud agar plates to obtain the total fungal burden. Colonies from these plates were then replica-plated onto YPD agar plates containing 100 μg nourseothricin ml−1 (Werner Bioagents) to identify the proportion of recovered cells that were nourseothricin-resistant (Sc3314-GFP).

**RESULTS AND DISCUSSION**

**Phenotypic analysis of the deletion strain**

Positional effects which alter URA3 expression have a profound influence on the virulence of *C. albicans* (Brand et al., 2004; Correia et al., 2010). To avoid this potential pitfall, we used the SAT1 flipper method to delete both copies of ALS3 in the wild-type strain SC5314. To verify that the in vitro phenotype of our als3Δ null strain matched that reported previously, we grew it under hypha-inducing conditions and compared its growth with that of the wild-type parental strain. As expected, the wild-type, heterozygote and deletion strains all formed hyphae under inducing conditions such as growth in RPMI 1640 at 37 °C or on solid Spider medium (data not shown). As reported for previous als3Δ mutants, our null strain was defective in its ability to form a biofilm on an elastomer surface in vitro (Fig. 1a) even though it was able to form hyphae. Furthermore, when material was removed from the well inoculated with the wild-type strain and examined microscopically the hyphal cells were densely packed and intertwined, whereas the als3Δ hyphae were more loosely associated and spread out in an open network (Fig. 1b). The heterozygote generally resembled the wild-type. These observations were in agreement with the phenotypes reported for deletion strains constructed by other groups using different methodologies (Nobile et al., 2006; Zhao et al., 2004).

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**Histopathology.** Kidneys excised from deceased or sacrificed mice were fixed in 10% buffered formalin and stored at 4°C until required. Kidneys were embedded in paraffin, and tissue slices were cut and stained with Grocott–Gomori methenamine-silver (GMS) (Grocott, 1955) or haematoxylin and eosin (H&E) stain prior to microscopic evaluation.
expression of other ALS family members, particularly \textit{ALS1} and \textit{ALS5}, we examined transcription of these two genes in the \textit{ALS3/als3}\(\Delta\) heterozygote and the \textit{als3}\(\Delta\) null strain. There was some variation in the expression of \textit{ALS1} and \textit{ALS5} between the different replicate hyphal cultures but there was clearly no trend of \textit{ALS1} or \textit{ALS5} transcriptional upregulation to compensate for decreasing levels of \textit{ALS3} transcript in the heterozygote and \textit{als3}\(\Delta\) null strains (Fig. 2). This suggests that the expression of these genes is not upregulated to compensate for the loss of Als3p.

\textbf{Adhesion to endothelial cells under conditions of flow}

Adhesion of \textit{C. albicans} to both itself and host cells is critical for the establishment of biofilms and fungal...
infection. We previously tested the influence of morphology on the ability of *C. albicans* to adhere to human endothelial cells under flow, and observed that yeast cells adhere in greater numbers to these host cells than hyphae or pseudohyphae (Grubb *et al.*, 2009), consistent with our results showing that yeast cells are able to escape the bloodstream and invade deep tissues (Saville *et al.*, 2003). We used microvascular-derived endothelial cells (HMEC-1) in our study because it is the microvascular endothelium at which most adhesion and extravasation occur *in vivo* (Fiebig *et al.*, 1991). During a disseminated infection, Candida cells are most likely to encounter cell surface molecules expressed by the microvascular endothelium, and so we felt that this model represented the best system to analyse *C. albicans* adherence. Since Als3p is not found on the surface of yeast cells (Coleman *et al.*, 2009), it was not expected that a lack of Als3p would affect the ability of the cells to adhere to microvascular endothelial cells under flow. This proved to be the case (Fig. 3), with no significant difference in the adhesion of wild-type and als3Δ yeast cells (*P* = 0.172). The abundance of Als3p in the hyphal cell wall (Coleman *et al.*, 2009), and its importance for the formation of normal biofilms *in vitro* (Nobile *et al.*, 2006; Zhao *et al.*, 2006), suggested that cells lacking Als3p might be deficient in their ability to adhere to the endothelial cells. However, in our flow model, the hyphae of the als3Δ strain adhered as well as their wild-type counterparts (*P* = 0.251), although in both strains hyphae adhered less well than yeast cells (Fig. 3). These results reinforce our previous observations that yeast cells adhere more efficiently than hyphae to endothelial cells.

**Virulence of the als3Δ null strain in a murine model of systemic candidiasis**

The role of Als3p in virulence has been examined using a variety of *in vitro* methods. Strains lacking Als3p have been found to be impaired in their ability to damage different cell types grown in culture (Almeida *et al.*, 2008; Zhao *et al.*, 2004). Furthermore, als3Δ strains have been reported to be deficient in ferritin binding *in vitro* (Almeida *et al.*, 2008) and to show decreased endocytosis by human cells (Phan *et al.*, 2007). The results of these assays have prompted some researchers to suggest that Als3p plays a key role in *C. albicans* virulence. However, direct examination of the virulence of a strain lacking Als3p *in vivo* using the systemic infection model has yet to be reported. We therefore tested our als3Δ null strain in the commonly used murine model of haematogenously disseminated candidiasis. Contrary to the implications of earlier studies, deletion of ALS3 did not impair the virulence of the mutant strain compared with its wild-type parent (Fig. 4a). This does not rule out the possibility that Als3p plays a role in the damage caused to host cells, as previous *in vitro* tests suggest, but it is clearly not a major determinant in the outcome of a systemic infection.

To assess the phenotype of the strain growing within the tissues, we performed a histological analysis of infected kidneys retrieved from the deceased mice. The mutant strain was able to form abundant hyphae within the kidneys (Fig. 4b) and showed no deficiency in its ability to invade or to damage host tissue. Moreover, neutrophil infiltration was readily apparent in tissues infected with either the wild-type or the als3Δ strain (Fig. 4c). In agreement with recent results using a neonatal intraperitoneal infection model (Tsai *et al.*, 2011), we found that infections with the wild-type or mutant strain resulted in efficient dissemination, with similar fungal burdens in the brain, kidney and spleen (data not shown). After earlier studies showing that yeast-form cells can disseminate, and our finding that als3Δ yeast or hyphae can adhere to endothelial cells under flow as efficiently as a wild-type strain, it was perhaps not surprising to find abundant *C. albicans* cells in the kidneys. However, if as previously reported, als3Δ cells are deficient for ferritin assimilation *in vivo*, this does not appear to impair their ability to proliferate within host tissues.

In order to examine the kinetics of the infection process more closely, we performed a competition assay. Equal numbers of wild-type and als3Δ null yeast cells were injected into mice, and the proportions of each strain residing in the kidney, spleen and brain were measured at 3, 6 and 24 h post-infection. The relative proportions of the wild-type and als3Δ cells recovered from the organs matched those present in the infecting inoculum (Fig. 5). Only the 24 h spleen sample showed a significant deviation from the inoculum (*P* < 0.05), with a greater proportion of als3Δ cells being recovered from the infected tissues. These results reinforce our observations that the als3Δ strain is
equally able to disseminate from the bloodstream as a wild-type strain and is equally able to cause disease.

Several earlier studies have examined the interactions of strains lacking Als3p with human cells in vitro and found that als3Δ null strains are defective in their ability to invade and damage reconstituted tissue culture models and cell monolayers (Almeida et al., 2008; Zhao et al., 2004). However, our results indicate that Als3p is not required for C. albicans cells to leave the bloodstream, invade the deep organs or cause a lethal disease in mice. The fact that the survival curves produced by the wild-type and the als3Δ mutant strains are indistinguishable suggests that Als3p plays a much more subtle role in pathogenesis than might be inferred from studies focused on the ability of fungal cells to damage human cells in a static environment. Interestingly, Als3p and several other cell wall proteins have recently been shown not to be required for C. albicans to be taken up by murine macrophages in vitro, nor does a lack of Als3p impair the ability of the fungus to kill these macrophages (McKenzie et al., 2010). Examining the various models and conditions used to characterize the

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**Fig. 4.** Virulence of the als3Δ null strain. (a) Approximately 2.8×10⁵ C. albicans yeast cells of the wild-type SC5314 and 2.7×10⁵ yeast cells of the als3Δ strain (left panel) or 3.7×10⁵ C. albicans yeast cells of the wild-type SC5314 and 3.2×10⁵ yeast cells of the als3Δ strain (right panel) were injected into the lateral tail veins of 6–8-week-old female BALB/c mice. Deletion of ALS3 did not change the virulence properties of the parental wild-type strain using this model (P=0.696 or P=0.570). Examination of kidneys retrieved from infected mice revealed (b) extensive C. albicans growth containing hyphal cells in infections by both the wild-type control and the als3Δ deletion strain (GMS staining), and (c) infiltration of numerous neutrophils (H&E staining).
role of Als3p, it is apparent that this protein is important in \textit{C. albicans} adhesion; however, it is equally clear that its role is not general in nature but particular to specific types of adhesion. Under flow conditions, it appears that Als3p is not required for \textit{C. albicans} hyphae to bind to endothelial cells, but it is vital for the formation of normal biofilms in which hyphae intertwine in a complex 3D structure (Zhao et al., 2006). Therefore, it seems likely that the most important role for Als3p is in the adhesion of \textit{C. albicans} cells to other \textit{C. albicans} cells, particularly during biofilm formation. In fact, it has recently been suggested that cell wall adhesins may have evolved from a mating agglutination system (Nobile \textit{et al.}, 2008; Soll, 2008) and that biofilm formation by white cells in response to \(x\)-pheromone is an important component of \textit{C. albicans} mating (Daniels \textit{et al.}, 2006; Sahni \textit{et al.}, 2009, 2010). \textit{C. albicans} can inhabit many niches in the host, and thus encounters a variety of cell types and environmental conditions. Whilst \textit{in vitro} models recapitulating some of these conditions have implied a broad role for Als3p in \textit{C. albicans} virulence.

\textbf{Fig. 5.} Competitive fitness of the \textit{als3\Delta} null strain. (a) A total inoculum of \(5.7 \times 10^6\) \textit{C. albicans} yeast cells (3 and 6 h groups) or \(2.5 \times 10^6\) yeast cells (24 h group) was injected into the lateral tail veins of 6–8-week-old female BALB/c mice for timed sacrifice competition experiments. Results are presented as the mean proportion of recovered cells that were nourseothricin-resistant. Inocula consisted of roughly equal proportions of wild-type (light-grey bars) and \textit{als3\Delta} (dark-grey bars) cells. Cells recovered from homogenized tissues were plated onto Sabouraud agar plates to obtain the total fungal burden. Colonies (\(n=849–1788\)) from these plates were then replicated onto YPD agar plates containing 100 \(\mu\)g nourseothricin ml\(^{-1}\) to identify the proportion that were resistant. Error bars, SD. Significance was assessed by the chi-squared test. A \(P\) value of \(<0.05\) was considered significant. (b) In each group, fungal burden is clustered around the median. Overall fungal burden in the organs was similar between time points, with the exception of the 24 h kidney sample. However, this sample still fell within the range of burdens seen at the same time point in previous experiments (Cleary \textit{et al.}, 2010).
albicans virulence, our results indicate that its role may be more specific than general, since the deletion strain showed no defect in virulence in the widely used haematogenously disseminated murine infection model.

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Als3p is dispensable for C. albicans virulence


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