The putative vacuolar ATPase subunit Vma7p of Candida albicans is involved in vacuole acidification, hyphal development and virulence

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The vacuolar H\(^{+}\)-ATPase (V-ATPase) component Vma7p of the human-pathogenic yeast Candida albicans regulates hyphal growth induced by serum and Spider medium and is essential for virulence. In order to characterize the functions of the putative V-ATPase subunit Vma7p of C. albicans, null mutants were generated. The resulting mutants showed reduced vacuole acidification, which correlated with defective growth at alkaline pH. In addition, defects in degradation of intravacuolar putative endosomal structures were observed. vma7 null mutants were sensitive towards the presence of metal ions. It is concluded that the sequestration of toxic ions in the vacuole via a H\(^{+}\) gradient generated by the V-ATPase is affected. The vma7 null mutant strains were avirulent in a mouse model of systemic candidiasis. In addition, C. albicans vma7 null mutants and the null mutant strain of the Vma7p-interacting phosphatidylinositol 3-kinase Vps34p showed similar phenotypes. In summary, the V-ATPase subunit Vma7p is involved in vacuolar ion transport and this transport is required for hyphal growth and virulence of C. albicans.

INTRODUCTION

Candida albicans is the major human fungal pathogen (Odds, 1994). This dimorphic yeast can cause life-threatening infections in immunocompromised patients and mucosal infections in healthy individuals. Recent experimental data suggest that virulence of C. albicans depends on several properties, including the ability to switch between different morphogenetic forms, recognition and adhesion to host epithelial and endothelial cells and also the secretion of proteinases and phospholipases (Cutler, 1991; Köhler & Fink, 1996). A number of virulence factors of C. albicans have been characterized; however, the mechanisms that enable the opportunistic fungus to become pathogenic have not yet been unravelled.

The fungal vacuole is an acidic compartment which is involved in hydrolysis, storage, osmoregulation, homeostasis and detoxification (Teter & Klionsky, 2000). In C. albicans, transport pathways into the vacuole are linked with hyphal growth and virulence (Bruckmann et al., 2000; Palmer et al., 2003; Theiss et al., 2002). The vacuolar H\(^{+}\)-ATPase (V-ATPase) is required for vacuole proton transport and is necessary for vacuole acidification.

The V-ATPase is located in endomembranes of eukaryotic cells and the amino acid sequences are highly conserved between yeasts, mammals and plants. Saccharomyces cerevisiae serves as a model organism for vacuolar transport. In S. cerevisiae, the V-ATPase regulates transport of small molecules and ions across the vacuolar membrane and provides an acidic environment that is required for protein degradation in the vacuole and intracellular targeting of vacuolar proteins (Forgac, 2000). The V-ATPase of S. cerevisiae comprises a multiprotein complex which is formed by two subunits with 13 proteins altogether; the soluble, cytoplasmic V\(_i\) subunit is composed of eight proteins and the membrane-bound V\(_0\) subunit contains five proteins (Graham et al., 2000). The V-ATPase component Vma7p controls the assembly of the V\(_0\) and V\(_i\) subunits and the targeting of the V-ATPase to the vacuolar membrane (Graham et al., 1994, 2000; Nelson et al., 1994; Tomashek et al., 1996).

The yeast vacuole acts as a storage and sequestration site of...
metal ions and regulates ion homeostasis. The balance between storage and sequestration is controlled by a $\text{H}^+$ gradient that is generated by the V-ATPase (MacDiarmid et al., 2002). Toxic metal ions are inactivated directly in the vacuole (e.g. $\text{Mn}^{2+}$, $\text{Ni}^{2+}$, $\text{Zn}^{2+}$, $\text{Co}^{2+}$) or in the cytosol (e.g. $\text{Cu}^{2+}$, $\text{Cd}^{2+}$). In addition, the V-ATPase controls the distribution of plasma membrane ATPase, as demonstrated for S. cerevisiae (Perzov et al., 2000). In C. albicans, a plasma membrane ATPase mediates high copper tolerance (Weissman et al., 2000). Therefore, it is possible that the V-ATPase influences indirectly the tolerance to copper ions through regulation of the plasma membrane ATPase assembly.

We have recently shown that the phosphatidylinositol (PI) 3-kinase Vps34p, a key enzyme of vacuolar protein transport, interacts physically with Vma7p, a component of the Candida V-ATPase complex (Eck et al., 2005). The PI 3-kinase Vps34p controls virulence of C. albicans. The vps34 null mutant strain is avirulent in a mouse model of systemic candidiasis, unable to form hyphae on different solid media, shows delayed yeast-to-hyphae transition in liquid media, is hypersensitive to high temperature and hyperosmotic stress and exhibits reduced adherence to human cells (Eck et al., 2000; Bruckmann et al., 2000). In addition, the vps34 mutant displays enlarged and electron-transparent vacuoles (Bruckmann et al., 2001).

Given the formation of a complex between the V-ATPase component Vma7p and the virulence-regulating Vps34p, we were interested to characterize the function of Vma7p in C. albicans. C. albicans vma7 null mutants were generated and these mutant strains were assayed for vacuole acidification, detoxification, endosomal transport, pH-dependent growth, hyphal development and virulence.

### METHODS

#### Strains and growth conditions.

The C. albicans strains used in this study are listed in Table 1. Yeast strains were grown either in YPD medium [2% (w/v) glucose, 2% (w/v) peptone, 1% (w/v) yeast extract], YNB [0.7% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose] or Sabouraud medium [2% (w/v) glucose, 1% (w/v) peptone (casein)] at 30°C. Growth was monitored by counting cell numbers with a haemocytometer. Hyphal growth was induced on solid medium, cells were allowed to grow overnight in YPD at 30°C and were then washed with 0.9 M NaCl, diluted and spread either on Spider plates [1% (w/v) nutrient broth, 0.2% (w/v) K$_2$HPO$_4$, 1.35% (w/v) agar, 1% (w/v) mannitol; Lee et al., 1975] or on YPD plates supplemented with 15% (w/v) fetal calf serum (FCS) at a density of 20–100 cells per plate. Plates were incubated at 37°C for at least 7 days. Hyphal growth in liquid media was induced by diluting late-exponential-phase cultures grown at 30°C tenfold either into fresh YPD supplemented with 10% (w/v) FCS or into Spider medium at 37°C. Sensitivities of the mutants to various metal ions were assayed on YPD plates supplemented with the appropriate salts. The effects of alkaline and acid growth conditions were tested with solid Sabouraud medium [2% (w/v) glucose, 1% (w/v) peptone (casein), 2% (w/v) agar] containing 100 mM Tris base. This medium was adjusted to pH 4.0, 6.0 and 8.0 with citric acid. In addition, the Sabouraud medium was adjusted to pH 3.0 with citric acid and then supplemented with Tris base to achieve pH 4.0, 6.0 and 8.0. The vma7 mutant strains were selected on CSM (complete supplement mixture) medium (Qbiogene).

#### Deletion of the VMA7 gene by PCR-based gene targeting.

Primer used in this study are listed in Table 2. The method was performed according to Gola et al. (2003). Marker genes were amplified by primers S1 [positions −98 (upstream) to +3] and S2 [−97 (downstream) to +355]. To confirm proper deletion of the

### Table 1. Strains used in this study

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
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<td>E. coli XL-1 Blue</td>
<td>∆ara3::imm434/Δara3::imm434; Δhis1::hisG/Δhis1::hisG; Δarg4::hisG/Δarg4::hisG; Δarg5/Δarg5; HIS1</td>
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<td>C. albicans SC5314</td>
<td>Wild-type</td>
<td>Fonzi &amp; Irwin (1993)</td>
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<td>Wilson et al. (1999)</td>
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<td>CNC44</td>
<td>∆ara3::imm434/Δara3::imm434; Δhis1::hisG/Δhis1::hisG; Δarg4/Δarg4::hisG; HIS1::URA3-hisG/Δarg5/Δarg5</td>
<td>Negredo et al. (1997)</td>
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<td>CAV3</td>
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<td>VU</td>
<td>∆ara3::imm434/Δara3::imm434; Δhis1::hisG/Δhis1::hisG; Δarg4/Δarg4::hisG; Δvma7::URA3/VMAY7</td>
<td>Bruckmann et al. (2000)</td>
</tr>
<tr>
<td>VH</td>
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<td>This work</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>VUR</td>
<td>∆ara3::imm434/Δara3::imm434; Δhis1::hisG/Δhis1::hisG; Δarg4/Δarg4::hisG; Δvma7::URA3/Δvma7::VMAY7</td>
<td>This work</td>
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Table 2. Primers used in this study

Non-chromosomal sequences in primers S1 and S2 are in lower case.

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<tr>
<td>I1</td>
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<td>I2</td>
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<tr>
<td>S1</td>
<td>CACCTTACCAGCTCCGGTTATCATGTGTGGAAAAAAGGGTTTGCAACAAACGCTATCAGATCTCAATCTGAGCAGGAAGCTGATGAGCTACGTTGTAGATGAGGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTG</td>
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<td>S2</td>
<td>GTGGTACCTGAAATGCGACATCCCGTATCAGGTCGAAAAAACAAACTTTCACGACAACCAAACATCAGATCTCAATCTGAGCAGGAAGCTGATGAGCTACGTTGTAGATGAGGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTGAGTTG</td>
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complete VMA7 ORF [357 bp, 118 amino acids, Stanford’s C. albicans sequencing project Assembly 19, ORF19.806 on Contig76; http://sequence-www.stanford.edu/group/candida/index.html], gene-specific (G1 and G4) and marker-gene-specific primers (U2 and U3 for URA3, H2 and H3 for HIS1) were used. Internal primers (I1, I2) were used to verify the presence of the VMA7 coding region. In addition, the correct deletion of VMA7 was confirmed by Southern analysis according to standard protocols. The probe was amplified by PCR using primers SB1 (positions −1420 to −1399, upstream of VMA7) and SB2 (−395 to −416, upstream of VMA7).

Reintegration of VMA7. The primers G1/G4 were used to amplify the VMA7 gene of C. albicans SC5314. The PCR product representing the complete VMA7 ORF was transformed into the vma7 null mutant strain VUH. The PCR fragment integrates into the original locus. Reintegrants were selected on plates supplemented with 10 mM MnCl₂, as the vma7 null mutants are sensitive to 10 mM MnCl₂. Proper integration of VMA7 was confirmed by PCR with the primer pairs I2/G1, I1/G4, I1/I2, U2/G1, U3/G4, H2/G1 and H3/G4. Control experiments indicated that the rescued wild-type strain did not result from a spontaneous second-site suppressor mutation: firstly, transformation of the null mutant strains without DNA did not result in spontaneous suppressor mutations, and secondly, transformation with the unrelated ARG4 marker resulted in Arg⁺ clones, which indicate that the mutant strains can be transformed.

Fluorescent labelling and microscopy. Quinacrine staining was performed as described by Augsten et al. (2002). Images were acquired using a fluorescence microscope (Olympus BX51TF) equipped with a BP450-490 excitation filter (U-MWBSA), BA520 beam splitter and DM500 emission filter. FM4-64 staining was carried out essentially as described previously (Vida & Emr, 1995). For microscopy, cells were placed on slides covered with a thin 1% agarose film. Transport of FM4-64 was viewed using a BP510-550 excitation filter (U-MWG2), BA590 beam splitter and DM570 emission filter (Olympus BX51TF).

Virulence studies. Six-week-old male outbred NMRI mice (Harlan-Winkelmann) were housed five per cage and checked daily. The various strains of C. albicans were grown in Sabouraud dextrose broth at 30 °C until late exponential phase. Cells were washed three times and resuspended in 0.9% NaCl. Aliquots (200 µl) of suspensions containing 5 × 10⁶ or 5 × 10⁶ cells were used to infect immunocompetent mice by intravenous injection into the lateral tail vein. Survival was monitored for 20 days. For comparison of survival curves, the log-rank test was used (Peto et al., 1977). A P value ≤0.05 was considered as significant.

RESULTS

Disruption of the VMA7 gene encoding a V-ATPase subunit in C. albicans

vma7 null mutant strains of C. albicans BWP17 were generated using a PCR-based approach to characterize the role of the putative V-ATPase component Vma7p. In a first step, two separate heterozygous vma7 mutants were generated which contained either the URA3 (VU) or the HIS1 (VH) genes as selectable markers. As the absence of a URA3 allele complicates the interpretation of mutant phenotypes (Lay et al., 1998; Cheng et al., 2003), the URA3-gene-containing mutant strain VU was used to assay the phenotypes of the heterozygous mutant strain VMA7/vma7.

In the heterozygous strain VU, the second VMA7 allele was replaced by the HIS1 gene resulting in VUH and in the VH strain by the URA3 gene resulting in VHU (Fig. 1a). Proper deletion of the VMA7 genes in all strains was confirmed by Southern analysis and by PCR (Fig. 1b, data not shown). Chromosomal DNA of the C. albicans strains SC5314 (wild-type), VU, VH, VUH and VHU was isolated and restricted with the restriction enzyme AflII. Following separation of the restricted DNA by agarose gel electrophoresis and Southern hybridization, the correct 8.45 kb wild-type fragment was identified. Replacement of one VMA7 allele by the URA3 gene (VU) or the HIS1 gene (VH) resulted in an additional fragment of 9.45 kb. The replacement of the second VMA7 allele by the HIS1 or URA3 gene led to two vma7 null mutants (VUH, VHU). The loss of the 8.45 kb fragment is consistent with the replacement of the second allele (Fig. 1a, b).
A VMA7 reintegrant strain VUR was generated by replacing the HIS1 gene in the vma7 null mutant strain VUH with the VMA7 gene (Fig. 1c). Proper integration was confirmed by PCR (Fig. 1d). The PCR products obtained with the primer pairs I2/G1 and I1/G4 show the correct localization of reintegrated VMA7 gene. The primer pairs U2/G1, U3/G4, H2/G1 and H3/G4 result in PCR products which show that the HIS1 gene was replaced by the VMA7 gene and that the URA3 gene remains.

**vma7 null mutant strains show defective vacuolar acidification and pH-dependent growth**

The acidic environment in the vacuoles of the vma7 mutants was tested by staining with the fluorescent dye quinacrine, which is a marker for acidic pH (Roberts *et al*., 1991). Both vma7 null mutants (VUH and VHU) lacked vacuolar staining, thus indicating an alkaline vacuolar milieu. The wild-type strain SC5314, the Arg3 strain CNC44 and the heterozygous mutant strain VU showed stained vacuoles, indicative of an acidic vacuolar pH (Fig. 2). *C. albicans* CNC44 was used as an additional control as this strain, similar to the vma7 null mutants, contains a reintegrated URA3 and HIS1 gene and both alleles of the ARG genes are deleted. In addition, the vps34 null mutant strain CAV3 also showed alkaline vacuolar pH, thus indicating similar roles for Vma7p and Vps34p in vacuolar acidification.

The correlation of defective vacuole acidification and growth at different pH values of the vma7 null mutants was tested. Growth of both null mutant strains, VUH and VHU, was clearly inhibited at alkaline pH 8·0, reduced at pH 6·0 and was not affected at pH 4·0 (Fig. 3a). The same effect was observed and was even more pronounced for the vps34 null mutant strain CAV3. In order to control that the reduced growth was independent of the concentration of citric acid, the pH was also adjusted with Tris; identical results were obtained (data not shown). Growth of both the *C. albicans* vma7 and vps34 null mutant strains is affected by alkaline pH, thus indicating related functions of the two proteins. In contrast, assaying temperature sensitivity, clear differences were observed between the vma7 mutants and the vps34 mutant. The deletion of VMA7 did not result in increased sensitivity to high temperatures (Fig. 3b), but the vma7 null mutant strains showed a growth defect at 22 °C (data not shown).

**vma7 null mutants show defects in the degradation of intravacuolar structures**

Endocytotic uptake and vesicle-mediated transport to the vacuole in cells of the vma7 null mutants were analysed by using the lipophilic styryl dye FM4-64. This dye intercalates into the plasma membrane and is transported into the vacuole by the endocytic pathway (Vida *et al*., 1993; Vida & Emr, 1995). Endocytotic transport of FM4-64 to the vacuole

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**Fig. 1.** Disruption of the VMA7 genes in *C. albicans*. (a) Strategy for the replacement of chromosomal VMA7 genes by URA3 and HIS1 genes. Thick arrows show the VMA7 genes; the positions of PCR primers (Table 2) are indicated by thin arrows. pFA-URA3 and pFA-HIS1 are plasmids used for PCR with primers S1 and S2 (Table 1). AflII sites used in Southern analysis are shown. (b) Southern analysis of AflII-digested chromosomal DNA from *C. albicans* strains. Lanes: 1, parental strain BWP17; 2, VU mutant strain (heterozygous VMA7/vma7 mutant strain with integrated URA3 gene); 3, VUH mutant strain (homozygous vma7 null mutant strain); 4, VH mutant strain (heterozygous VMA7/vma7 mutant strain with integrated HIS1 gene); 5, VHU mutant strain (homozygous vma7 null mutant strain). The blot was hybridized with non-radioactively labelled PCR product obtained with primers SB1 and SB2. (c) Reintegration of the VMA7 gene resulting in *C. albicans* revertant strain VUR. (d) Confirmation of correct integration of VMA7 by PCR. DNA fragments obtained by PCR with the indicated primer pairs were separated on agarose gel.
was monitored by fluorescence microscopy (Wendland et al., 1996).

After an initial pulse and a chase of 60 min, FM4-64 was present predominantly in the vacuole in *C. albicans* wild-type strains and the heterozygous *vma7* mutant strain VU, *vma7* null mutant strains VUH and VHU and *vps34* null mutant strain CAV3. *v*, Vacuoles. Bar, 10 μm (applies to all images).

**Fig. 2.** *C. albicans* Vma7p and Vps34p are involved in vacuolar acidification. Fluorescence staining with quinacrine (which stains acidic compartments specifically) and DIC microscopy are shown of *C. albicans* wild-type strain SC5314, Arg<sup>-</sup> strain CNC44, heterozygous mutant strain VU, *vma7* null mutant strains VUH and VHU and *vps34* null mutant strain CAV3. *v*, Vacuoles. Bar, 10 μm (applies to all images).

The role of Vma7p in metal ion homeostasis

A major function of the V-ATPase is sequestration of toxic ions via a H<sup>+</sup> gradient. In order to assay whether the putative V-ATPase component Vma7p affects ion homeostasis, we tested whether and how metal ions influence growth of the *C. albicans* *vma7* null mutant strains VUH and VHU. The *C. albicans* strains were grown on plates supplemented with Co<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> or Cu<sup>2+</sup>. The *vma7* null mutants showed increased sensitivity toward high concentrations of tested metal ions, thus demonstrating a role of Vma7p in ion detoxification (Fig. 5).

Growth of the *vma7* mutants was inhibited by CoCl<sub>2</sub> and MnCl<sub>2</sub> at a concentration of 1 mM, by NiSO<sub>4</sub> at 2 mM, by ZnCl<sub>2</sub> at 500 μM and by CuCl<sub>2</sub> at 8 mM. In addition, the sensitivity of the *vps34* null mutant strain CAV3 was tested. In general, the sensitivity of the *vma7* mutants towards metal ions was higher than that of the *vps34* mutant. The sensitivity towards Cu<sup>2+</sup> was similar (Fig. 5). In summary, the growth of both *vma7* and *vps34* mutants is sensitive towards the presence of metal ions, thus indicating a role of Vma7p and similarly of Vps34p in metal ion detoxification.

**Fig. 3.** Growth inhibition of *C. albicans* *vma7* and *vps34* null mutants by alkaline pH. *C. albicans* wild-type strain SC5314, Arg<sup>-</sup> strain CNC44, heterozygous mutant strain VU, *vma7* null mutant strains VUH and VHU and *vps34* null mutant strain CAV3 were plated on solid agar at the indicated pH values (a) and temperatures (b).

**Role of Vma7p in metal ion homeostasis**

Vacuolar transport is involved in germ tube formation (Palmer et al., 2003). To this end, we tested the *vma7* null mutants for hyphal development using different conditions for induction (Fig. 6). Hyphal development was induced

**Vma7p is involved in serum- and mannitol (Spider medium)-regulated induction of filamentous growth**

Vacuolar transport is involved in germ tube formation (Palmer et al., 2003). To this end, we tested the *vma7* null mutants for hyphal development using different conditions for induction (Fig. 6). Hyphal development was induced
with serum and in Spider medium with mannitol as sole carbon source. Both \(vma7\) mutant strains VHU and VUH did not form hyphae in either liquid medium supplemented with serum or in liquid Spider medium (Fig. 6a). However, both mutants showed filament development when grown on solid Spider medium, but not on solid medium supplemented with serum (Fig. 6b). These results indicate that the V-ATPase component Vma7p regulates hyphal growth upon induction by either serum or liquid medium with mannitol (Spider medium), but not by solid Spider medium.

To rule out the possibility that defective hyphal formation was due to a growth defect, growth of the null mutant strains was tested in different media. In YPD medium, the \(vma7\) mutants showed comparable growth to the \(C.\ albicans\) wild-type strain SC5314. In Sabouraud medium, the \(vma7\) null mutants exhibited the same division rate and both showed delayed growth compared with the wild-type strain. In liquid Spider medium and YPD medium with serum, the \(vma7\) null mutant strains exhibited nearly identical growth compared with YPD medium.

**Vma7p is required for virulence of \(C.\ albicans\)**

The \(vma7\) null mutants show defective hyphal development. Therefore, the virulence of the \(vma7\) heterozygous and homozygous mutants (VU, VUH and VHU) and the VMA7 null mutants (VUH, VHU) reveal staining of invavacuolar structures (ivs). Cells lacking Vps34p (CAV3) accumulate FM4-64 in presumptive pre-vacuolar compartments (pvc). v, Vacuoles. Bar, 10 \(\mu\)m (applies to all images).

**Fig. 4.** Distribution of the fluorescent endocytic marker FM4-64 in \(C.\ albicans\) \(vma7\) null mutants. Cells were pulsed for 30 min with FM4-64 and then chased for 60 min. Fluorescence microscopy and DIC microscopy were used to view the cells. Wild-type cells (SC5314 and CNC44) and the heterozygous mutant cells (VU) show the typical ring-staining pattern of the vacuole membrane. The \(vma7\) null mutants (VUH, VHU) reveal staining of intravacuolar structures (ivs). Cells lacking Vps34p (CAV3) accumulate FM4-64 in presumptive pre-vacuolar compartments (pvc). v, Vacuoles. Bar, 10 \(\mu\)m (applies to all images).
revertant strain (VUR) was tested in a mouse model of systemic candidiasis. Both \textit{vma7} null mutants were avirulent in this animal model. All mice infected with $5 \times 10^6$ or $5 \times 10^5$ mutated \textit{Candida} cells survived during the complete course of the experiment. In contrast, the heterozygous \textit{vma7} mutant strain VU and the \textit{VMA7} revertant strain VUR showed nearly the same, high virulence as the two control strains SC5314 and CNC44 (Fig. 7).

The \textit{VMA7} reintegrant strain VUR shows wild-type phenotype

The identical phenotypes of the two independently constructed \textit{vma7} null mutants indicated that the modified phenotypes can be correlated to the absence of the \textit{VMA7} alleles. This was confirmed by generation and characterization of a \textit{VMA7} reintegrant strain, VUR. Reintegration of the \textit{VMA7} gene into the \textit{vma7} null mutant strain VUH resulted in reconstitution of the wild-type phenotype, thus indicating that deletion of the \textit{VMA7} genes causes the observed phenotypes. The \textit{VMA7} revertant strain VUR was characterized on plates supplemented with different metals at concentrations that are toxic for the \textit{vma7} null mutant strains and under acidic and alkaline pH. The \textit{VMA7} revertant strain showed resistance against high metal ion concentrations and alkaline pH, as did the wild-type strains SC5314 and CNC44 and the heterozygous \textit{vma7} mutant strain VU (data not shown). In addition, the revertant strain showed high virulence in the mouse model, similar to the wild-type strain (Fig. 7).

\section*{DISCUSSION}

We have demonstrated that the V-ATPase of the human-pathogenic yeast \textit{C. albicans} acts as a multifunctional intracellular protein that regulates vacuole acidification, endosomal transport, pH-dependent growth, metal ion detoxification, hyphal growth and virulence.

\textit{C. albicans} \textit{vma7} null mutants were generated and showed...
defective proton transport into the vacuole, as indicated by the alkaline vacuolar pH. This demonstrates a decisive role of Vma7p in the functioning of C. albicans V-ATPase. This effect is in agreement with data obtained from the apathogenic yeast S. cerevisiae, where Vma7p affects both V0 and V1 subunits, reflecting a role in bridging the subunits to form a functional V-ATPase complex (Graham et al., 2000).

The C. albicans vma7 null mutants also showed defective endosomal protein sorting. In the first step of the endocytotic pathway, transmembrane proteins and lipids are sorted into vesicles. These vesicles are invaginated into the lumen of the early endosomes. The endosomal vesicles are transported into multivesicular bodies. Upon fusion of multivesicular bodies with the vacuole, the vesicles and their contents are degraded by hydrolases and lipases (Katzmann et al., 2002). In the C. albicans vma7 null mutant cells, the vacuoles accumulated putative endosomal compartments. This accumulation indicates defective degradation of intravacuolar structures. The alkaline vacuolar lumen may prevent the degradation of intravacuolar structures by hydrolases and lipases, which are only active in an acidic vacuolar milieu.

Copper resistance is most likely a prerequisite for the survival of C. albicans in the digestive tract of its host, as Cu²⁺ concentrations, at approximately 10 μM, are relatively high in the stomach and duodenum (Underwood, 1977). Copper tolerance in C. albicans is mediated by a plasma membrane ATPase (Weissman et al., 2000). The influence on the targeting of plasma membrane ATPase of the V-ATPase has been shown in S. cerevisiae (Perzov et al.,

**Fig. 6.** vma7 null mutants show defects in filamentous growth in liquid and on solid hyphae-inducing media. (a) Hyphal formation of C. albicans wild-type strain SC5314, Arg⁻ strain CNC44, heterozygous mutant strain VU, vma7 null mutant strains VUH and VHU and vps34 null mutant strain CAV3 in liquid Spider medium or medium supplemented with 10 % serum. Data show one representative experiment; 100 % hyphae means that 100 % of yeast cells had formed true hyphae or pseudohyphae. (b) Filamentous development of C. albicans wild-type strain SC5314, Arg⁻ strain CNC44, heterozygous mutant strain VU and vma7 null mutant strains VUH and VHU on solid medium containing mannitol as carbon source (Spider) or 15 % serum. Strains were grown for 7 days at 37 °C. Bars, 1 mm.
We demonstrated that the wild-type *C. albicans* strain SC5314 tolerates Cu$^{2+}$ concentrations of 8 mM. The same concentration was toxic for the *vma7* null mutants, which tolerated lower concentrations, of 4 mM. The increased Cu$^{2+}$ sensitivity of the *vma7* null mutants may cause the avirulent phenotype, as the toxicity of copper is increased under the acid, anaerobic conditions which exist in the digestive tract. We hypothesize that a defective V-ATPase affects the distribution of plasma membrane ATPase in the *vma7* null mutants and thus leads to decreased copper resistance.

The *vma7* null mutants showed increased sensitivities to other metal ions (Zn$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{2+}$), linking Vma7p activity to the detoxification of metal ions. Detoxification depends on the proton gradient of the vacuole membrane, which is defective in the *vma7* null mutant strains. Thus, defective detoxification is likely caused by the low proton concentration in the vacuole.

In addition, the *vma7* null mutants showed no hyphal growth in liquid medium. Defective hyphal formation of the *vma7* null mutant strains may cause the avirulent phenotype, as the transition from the ellipsoidal yeast form to the filamentous form is important for virulence of *C. albicans*. Both the mitogen-activated protein (MAP) kinase pathway and the cAMP-regulated cascade are major signalling pathways of hyphal induction (Köhler & Fink, 1996; Sonneborn et al., 2000). The defective hyphal development of the *vma7* null mutants in both serum and Spider medium is explained by a defective cAMP signalling pathway, because nutrient starvation in Spider medium induces hyphal development via the MAP kinase and cAMP pathways whereas serum-induced hyphal growth is controlled exclusively by the cAMP pathway (Ernst, 2000). The *vma7* null mutants show defective hyphal growth in liquid Spider medium, but not on solid Spider medium. This effect is explained by derepression of hyphal development in the *vma7* null mutants by specific growth conditions on solid Spider medium which activate a so-far uncharacterized pathway of hyphal induction.

The altered position of the *URA3* gene or deletion of one *URA3* allele may complicate the interpretation of mutant phenotypes (Lay et al., 1998; Cheng et al., 2003). Therefore, the *URA3* gene was integrated at the same locus in the heterozygous and null mutant strains. The related phenotypes of the heterozygous mutant strain and of the wild-type strain argue against a *URA3*-position effect in the *vma7* null mutant strain. In addition, after supplementation of

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**Fig. 7.** Vma7p is involved in pathogenicity of *C. albicans*. *C. albicans* wild-type strain SC5314, Arg$^-$ strain CNC44, heterozygous mutant strain VU, *vma7* null mutant strains VUH and VHU and revertant strain VUR were tested in a mouse model of systemic candidiasis. Survival of mice infected with $5 \times 10^6$ (●) or $5 \times 10^5$ (▼) cells was monitored for 20 days ($n=10$). $P$ values $<$ 0.05 were estimated as significant (Peto et al., 1977).
medium with uridine, the mutant phenotypes did not revert (data not shown), which confirms that the null mutant phenotypes do not result from a URA3-position effect.

Recently, we have shown that Vma7p interacts physically with the PI 3-kinase Vps34p, a key enzyme of vacuolar protein transport (Eck et al., 2005). In addition, the C. albicans vma7 and vps34 null mutants show the same defects in vacuolar acidification and detoxification of metal ions. The physical and functional interaction between Vma7p and Vps34p links vacuolar proton transport and vacuole acidification with vacuolar protein transport. Vps34p may control the assembly of the V-ATPase subcomplexes by directly interaction with Vma7p. This assumption is supported by results obtained in human cells and in S. cerevisiae. In human cells, the assembly of V-ATPase subunits Vp0 and V1 depends on PI 3-kinases (Sautin et al., 2005) and, in S. cerevisiae, the V-ATPase is involved in intracellular protein transport to the vacuole (Munn & Riezman, 1994; Stevens & Forgac, 1997; Bonangelino et al., 2002; Perzov et al., 2002).

In this study, we show that the putative V-ATPase component Vma7p of C. albicans influences cellular functions which are connected to the acidification of the vacuole and transport processes into the vacuole. These cellular functions influence hyphal growth and virulence of C. albicans.

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REFERENCES


