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The *Histoplasma capsulatum* Vacuolar ATPase is Required for Iron Homeostasis, Intracellular Replication in Macrophages, and Virulence in a Murine Model of Histoplasmosis

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Summary

Histoplasma capsulatum is a dimorphic fungal pathogen that survives and replicates within macrophages (M ϕ). To identify specific genes required for intracellular survival, we utilized *Agrobacterium tumefaciens*-mediated mutagenesis, and screened for *H. capsulatum* insertional mutants that were unable to survive in human M ϕ . One colony was identified that had an insertion within *VMA1*, the catalytic subunit A of the vacuolar ATPase (V-ATPase). The *vma1* mutant (*vma1::HPH*) grew normally on iron replete medium, but not on iron deficient media. On iron deficient medium, the growth of the *vma1* mutant was restored in the presence of wild type (WT) *H. capsulatum* yeasts, or the hydroxamate siderophore, rhodotorulic acid. However, the inability to replicate within M ϕ was only partially restored by the addition of exogenous iron. The *vma1::HPH* mutant also did not grow as a mold at 28°C. Complementation of the mutant (*vma1/VMA1*) restored its ability to replicate in M ϕ , grow on iron poor medium, and grow as a mold at 28°C. The *vma1::HPH* mutant was avirulent in a mouse model of histoplasmosis, whereas the *vma1/VMA1* strain was as pathogenic as WT yeasts. These studies demonstrate the importance of V-ATPase function in the pathogenicity of *H. capsulatum*, in iron homeostasis, and in fungal dimorphism.

Introduction

Histoplasma capsulatum is a facultative intracellular pathogen endemic to the Ohio and Mississippi river valley. Typically *H. capsulatum* causes asymptomatic infections or mild influenza-like illnesses in immunocompetent hosts. In rare cases, immunocompetent individuals that inhale a large inoculum of the saprophytic conidia develop potentially lethal disseminated infections. In contrast, *H. capsulatum* infection of immunocompromised hosts causes serious morbidity, and often progresses into a life threatening disseminated disease in individuals with AIDS, transplant recipients, and patients receiving corticosteroids or anti-TNF α therapy (Wheat *et al.*, 1982)(Wheat *et al.*, 1983)(Antinori *et al.*, 2006)(de Francesco Daher *et al.*, 2006)(Wood *et al.*, 2003)(Jain *et al.*, 2006). After an individual inhales *H. capsulatum* conidia, the conidia are phagocytosed by alveolar macrophages (M ϕ), convert into the pathogenic yeast phase, and then the yeasts replicate

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within a modified phagosome. Activation of T-cell mediated immunity is required to restrict fungal proliferation and clear the infection (Newman, 2001).

Survival and replication of *H. capsulatum* yeasts within M ϕ is essential for the pathogenesis of histoplasmosis. Upon ingestion by M ϕ , yeasts must make new proteins, rapidly modify the phagosomal environment, and obtain nutrients, particularly iron, for intracellular survival (Newman *et al.*, 1992)(Newman *et al.*, 1994). In human M ϕ , *H. capsulatum* yeasts inhibit phagolysosomal fusion (PL-fusion) and regulate intraphagosomal pH as part of their survival strategy (Newman *et al.*, 2006). To date, three genes or factors have been identified that play a role in the virulence of *H. capsulatum*, but the mechanism(s) by which they work are unclear. These are *CBP1* that encodes for a calcium-binding protein (Sebghati *et al.*, 2000), the yeast phase-specific cell wall polysaccharide α -(1,3)-glucan (Rappleye *et al.*, 2004), and the yeast phase-specific gene *YPS3* that encodes for a cell surface and secreted protein (Bohse and Woods, 2007).

Although, the reverse genetics approach is useful when promising virulence genes have been identified, gene targeting in *H. capsulatum* is tedious, and the deletion of a single gene does not always result in a clear phenotype. Recently, the development of an efficient insertional mutation technique for *H. capsulatum* using *Agrobacterium tumefaciens*-mediated transformation has made it possible to use a forward genetic screen to identify genes required for a specific phenotype. Characterization of *Agrobacterium*-mediated transformants in *H. capsulatum* yeasts demonstrate that approximately 90% have a single unique insertion with no chromosomal rearrangements or deletions (Sullivan *et al.*, 2002). Recently, a forward genetic screen of 50,000 *H. capsulatum* insertional mutants was used successfully to identify novel genes required for the synthesis of alpha-1,3-glucan (Marion *et al.*, 2006).

In the present study, we applied a forward genetic screen using an *A. tumefaciens*-mediated insertional mutant library of *H. capsulatum* yeasts to identify genes required for intracellular survival in human M ϕ . Of 2,000 colonies screened, we identified a mutant that was unable to grow in human M ϕ , that had an insertion within the gene encoding for the V-ATPase catalytic subunit A (*VMA1*). Herein evidence is presented that expression of *VMA1* is required for iron homeostasis, yeast to mold transition, intracellular growth in human and murine M ϕ , and virulence in a murine model of pulmonary histoplasmosis.

Results

Insertional mutagenesis screen

To identify gene products required for the intracellular growth of *H. capsulatum*, we screened approximately 2000 *A. tumefaciens*-mediated insertional mutant colonies of *H. capsulatum* for their ability to replicate in human M ϕ . *H. capsulatum* mutants were grown in 96-well plates for 24 h, diluted 1/20, and approximately 10^4 yeasts were transferred onto M ϕ monolayers. After 24 h monolayers were visually examined by phase-contrast microscopy to verify the intracellular localization of all yeasts. The monolayers then were cultured for 10-14 days, and the monolayers were monitored for the presence of extracellular yeasts. We identified eight *H. capsulatum* insertional mutants that were unable to grow in M ϕ in the initial screen. These colonies were re-screened for intracellular growth in human M ϕ . One colony, designated 3d1, demonstrated no visible growth within human M ϕ after two weeks, while 6 colonies showed some growth within M ϕ , but the monolayers still remained intact. These results remained consistent after a third round of screening.

TAIL-PCR then was used to identify the disrupted gene in the *H. capsulatum* mutant deficient for intracellular growth in M ϕ . The mutant had an insertion within the V-ATPase

catalytic subunit A, *VMA1* (Fig 1B). Figure 1C shows the results of RT-PCR to confirm the loss of *VMA1* in the 3d1 mutant (column 3), and the re-expression of *VMA1* in the complemented mutant strain, *vma1/VMA1* (column 4). Although the *vma1::HPH* mutant could not replicate in M ϕ , they grew normally in standard medium at 37°C at a wide range of pH (Fig 2). Only at pH 7.5 was the rate of growth of the *vma1* mutant decreased compared to WT and *vma1/VMA1* yeasts, and the delay was only for 24 hr.

***vma1::HPH* mutant yeasts are unable to convert to a mold**

One of the characteristics of *vma* mutants is that they have severe developmental defects. Fungal *vma* mutants that exhibit this phenotype include *Saccharomyces cerevisiae* (Enyenihi and Saunders, 2003), *Neurospora crassa* (Bowman *et al.*, 2000), *Candida albicans* (Poltermann *et al.*, 2005), and *Ashbya gossypii* (Forster *et al.*, 1999). To determine if the developmental transition of yeast to mold was affected in *H. capsulatum vma1* mutants, yeasts were streaked on HMM plates at pH 5.5 or 7.5, and cultured for 14 days at 28°C. At a pH of 7.5, the *vma1::HPH* mutant was unable to form mycelia at 28°C, whereas the wild type (WT) and *vma1/VMA1* strains formed mycelia normally (Fig 3). At pH 5.5, a few hyphae were produced by the *vma1* mutant but these were clearly stunted in their growth compared to WT and complemented yeasts.

***vma1::HPH* mutants do not grow on iron limited medium**

Vacuolar acidification has been implicated in iron acquisition, storage, and homeostasis. For example, the *S. cerevisiae* V-ATPase knockout is unable to grow on iron limited media (Bode *et al.*, 1995). As acquisition of iron is essential for the intracellular survival and growth of *H. capsulatum* in M ϕ (Newman *et al.*, 1994)(Newman *et al.*, 1995), we sought to determine if iron acquisition or usage is disrupted in *H. capsulatum vma1::HPH* mutants. WT, *vma1::HPH*, and *vma1/VMA1* strains were streaked onto HMM plates containing 5 :M apotransferrin (HMM/Apotf), and cultured for 10-14 days at 37°C. *vma1::HPH* mutant yeasts were unable to grow on the iron limited media, while WT and *vma1/VMA1* yeasts grew normally (Fig 4, bottom panels).

At pH 7.5, two molecules of ferric iron are tightly bound to transferrin (Princiotta and Zapolski, 1975), whereas at pH 6.5 transferrin only is half-saturated. Therefore, we sought to determine if the *vma1* mutant would grow on HMM/Apotf medium at pH 6.5. The data in Fig 5A show that at the lower pH, the *vma* mutant grew normally, whereas at pH 7.5 (Fig 5B), the mutant did not grow.

The ability of *vma1::HPH* mutants to grow on iron deficient medium at pH 6.5 suggests that the mutant can obtain and utilize free iron, but not iron bound to transferrin. In *S. cerevisiae*, V-ATPase activity is required for loading of Cu onto the FET3 protein, and for the function of the FET3/FTR1 high affinity iron uptake pathway. However, the genome of the G217B strain of *H. capsulatum* does not contain the *FET3* or *FTR1* genes (results of BLAST search of the *H. capsulatum* Genome Project, Washington Univ., St. Louis, MO). However, *H. capsulatum* has other iron acquisition systems that might be disrupted in *vma1* mutant yeasts. Therefore, we next sought to determine if WT yeasts might secrete a component(s) that would restore the growth of *vma1::HPH* mutant yeasts on iron restricted medium. *vma1::HPH* mutant yeasts were spread onto a HMM/Apotf plate and WT yeasts were spotted onto the center of the plate. Figure 5C shows that WT *H. capsulatum* yeasts complemented the growth of *vma1::HPH* mutant yeasts. This complementation presumably was mediated by a factor(s) that diffused outward from the WT yeasts to the *vma1::HPH* mutant yeasts, and was utilized by the mutant yeasts to promote normal growth.

H. capsulatum yeasts secrete hydroxamate siderophores under iron limited conditions (Howard *et al.*, 2000), and *H. capsulatum* yeasts are able to chelate iron from holotransferrin (Timmerman and Woods, 1999). Therefore, we hypothesized that WT yeasts might secrete siderophores to complement the growth of *vma1::HPH* mutants. To determine if siderophores would complement the growth of *vma1::HPH* mutants under iron poor conditions, *vma1::HPH* mutant yeasts were spread onto HMM/Apotf plates, and rhodotorulic acid was spotted onto the edge of the plate. In the presence of rhodotorulic acid the *vma1::HPH* mutants grew normally (Fig 5D).

We then sought to determine if *vma1::HPH* yeasts were deficient in siderophore synthesis. WT and mutant yeasts first were grown in iron replete HMM media to mid log phase. The yeasts were harvested, resuspended to 5×10^7 /ml in RPMI (without phenolphthalein) containing 1 μ M apotransferrin and 18 g/l of glucose, and then cultured for an additional 7 days. Supernatant was harvested and then tested in the Atkin's Assay (Atkin *et al.*, 1970) (Payne, 1994). Remarkably, *vma1::HPH* mutant yeasts produced the same amount of siderophores as the WT yeasts (331 μ M for WT yeasts and 337 μ M for *vma1::HPH* mutant yeasts; mean of three experiments).

To confirm that *vma1::HPH* mutants produced siderophores, the supernates were tested for their ability to stimulate the growth of *Microbacteria flavescens* JG-9 (Payne, 1994). As shown in the top panel of Fig 6, *M. flavescens* does not grow in the presence of free iron, but has an absolute requirement for siderophores. The pictures in the bottom panel of Fig 6, demonstrate again that the *vma1::HPH* mutant produced siderophores as well as WT and *vma1/VMA1* yeasts.

Treatment of WT G217B yeasts with bafilomycin recapitulates the *vma1* mutant phenotype

Bafilomycin A is a specific inhibitor of V-ATPases (Bowman *et al.*, 1988). We hypothesized, therefore, that co-culture of WT yeasts with bafilomycin would mimic the *vma1::HPH* mutant phenotype. To test this hypothesis, WT yeasts were streaked onto either standard HMM plates, or HMM plates containing 5 μ M apotransferrin. The plates also were prepared with and without various concentrations of bafilomycin. On standard HMM plates, 5 μ M bafilomycin partially inhibited the growth of WT yeasts, and 30 μ M bafilomycin almost completely inhibited the growth of yeasts (Fig 7, top panel). On iron poor medium, the results were even more striking, in that 5 μ M bafilomycin completely inhibited the growth of the yeasts (Fig 7, bottom panel).

Free iron only partially restores the growth of *vma1::HPH* mutants in M ϕ

Our initial phenotypic screen demonstrated that *vma1::HPH* mutants do not replicate in human M ϕ . Since the mutant yeasts do not grow under iron poor conditions, and iron acquisition is an absolute necessity for *H. capsulatum* to survive and multiply in M ϕ (Newman *et al.*, 1994)(Newman *et al.*, 1995), we next sought to determine if the inability to grow in M ϕ could be reversed by the addition of exogenous iron. For these experiments, both holotransferrin and FeNTA were used as a source of iron. However, only the data with FeNTA is shown as the holotransferrin did not enhance the growth of the *vma1* mutant. As shown in figure 8, WT and *vma1/VMA1* yeasts grew equally well in both human (top panel) and murine M ϕ (bottom panel). In contrast, the *vma1::HPH* mutant did not grow in either cell type as expected. The cpm obtained with the *vma1::HPH* mutant are essentially background counts, and are the same as the cpm obtained with uninfected M ϕ (data not shown). Further, although the addition of FeNTA to cultures containing *vma1::HPH* mutant yeasts significantly enhanced mutant growth in M ϕ , the amount of growth still was minimal compared to WT and *vma1/VMA1* yeasts.

PL-fusion occurs in Mφ that have ingested *vma1::HPH* mutant yeasts

H. capsulatum yeasts inhibit PL-fusion in human Mφ as part of their strategy for intracellular survival (Newman *et al.*, 2006). As *vma1* mutant yeasts were unable to grow in human Mφ, we hypothesized that these mutants would not inhibit PL-fusion. Therefore, we quantified PL-fusion in Mφ that had phagocytosed WT or *vma1::HPH* mutant yeasts using LysoTracker Red (Newman *et al.*, 2006). After 2 and 4 h, Mφ that had ingested *vma1::HPH* yeasts demonstrated 58% and 64% phagolysosomal fusion, respectively (mean of two experiments). Mφ that had ingested WT yeasts demonstrated only 5% PL-fusion fusion at both time points.

Pathogenicity of *vma1* mutant in a murine model of histoplasmosis

Finally, we sought to determine if *vma1::HPH* mutants were pathogenic in a murine model of histoplasmosis. In the first experiment, a sublethal inoculum of WT, *vma1::HPH*, and *vma1/VMA1* yeasts were instilled intranasally into mice, and cfu were quantified in the lung and spleen at one week post-infection. The data in figure 9 show that one week after infection, no *vma1::HPH* mutant yeasts were cultured from either organ. In contrast, WT and *vma1/VMA1* yeasts replicated to a similar degree in the lung and spleen. In the second experiment, a lethal inoculum of yeasts was instilled intranasally, and the mice were observed for mortality. Mice infected with WT or *vma1/VMA1* yeasts all died by day 13 (Fig 10). In contrast, all the mice that were infected with *vma1::HPH* mutant yeasts survived out to 30 days. Further, none of the mice ever appeared to be sick. At this point we sought to determine if the mice that had been infected with the *vma1::HPH* mutants would be immune to a lethal inoculum of WT yeasts. However, all of the mice died by 15 days post-infection.

Discussion

Identifying the genes and gene products that promote the intracellular survival of *H. capsulatum* in Mφ is essential to understanding the pathogenesis of histoplasmosis. In the present study, a forward genetics approach, *A. tumefaciens*-mediated transformation, was used to identify genes required for the intracellular survival of *H. capsulatum* strain G217B in human Mφ. A *vma1* insertional mutant was identified that did not replicate in Mφ. Additional screening of transformants has identified other genes that are required for intracellular growth in Mφ. For the 3d1 mutant that was used in the present studies, it is unknown why an insertion in the 3' UTR should result in the loss of *VMA1* gene expression. Most likely this area of the 3' UTR contains a regulatory sequence that is critical for *VMA1* transcription.

Although the ³H-leucine assay for intracellular growth only quantifies fungistasis, the fact that no yeast growth was observed after two weeks in culture with Mφ, and that PL-fusion occurred in Mφ containing the *vma1* mutant, suggests not only that growth of the yeasts was inhibited, but that a majority of the yeasts were killed. The lack of *VMA1* expression in the mutant probably indirectly permits PL-fusion to occur. Thus, iron homeostasis and other metabolic processes are disrupted in the *vma1* mutant, and this may affect the ability of the yeasts to produce element(s) that are responsible for inhibiting PL-fusion. Alternatively, PL-fusion may simply have occurred after the yeasts had died or become metabolically inactive.

The *vma1* mutant also appeared to be killed upon intranasal infection of mice. Thus, one week after infection, *vma1* mutant yeasts could not be cultured from either the lung or the spleen, suggesting that these organs were sterile. The one caveat here is that our limit of detection for the culture of *H. capsulatum* yeasts is about 100. At the very least, it is clear that the *vma1* mutant is avirulent in a murine model of pulmonary histoplasmosis.

Eukaryotic V-ATPases are proton pumps, and in fungi and most other eukaryotic cells their primary role is the ATP-driven transport of protons from the cytosol into acidic organelles. These acidic organelles include lysosomes, early and late endosomes, and the late golgi apparatus (Mellman *et al.*, 1986). Organelle acidification appears to be involved in protein sorting in biosynthetic and endocytic pathways, proteolytic activation of zymogen precursors, and transmembrane transport of viral contents and toxins (Mellman *et al.*, 1986) (Stevens and Forgac, 1997) (Nishi and Forgac, 2002). In plants and fungi, the lysosome-like vacuole is involved in the storage of metabolic building blocks, calcium and metal ion homeostasis, and osmotic control (Klionsky *et al.*, 1990), and vacuolar acidification is critical for these functions.

V-ATPases are multi-subunit enzymes composed of a peripheral complex, V_1 , attached to a membrane-bound complex, V_0 (Nishi and Forgac, 2002). The V_1 sector contains three copies of the catalytic subunit A, and, in the present study, it is the gene for this subunit that was disrupted by the insertion of the *HPH* gene. Several interesting yeast phenotypes have been described that arise from the loss of V-ATPase function. In *S. cerevisiae*, the loss of V-ATPase function leads to a pH-dependent lethality in which mutants can not grow at pH 7 or above, but are able to grow at pH 5.0-5.5 (Nelson and Nelson, 1990). Other fungi, including *N. crassa*, *C. albicans*, and *S. pombe* also show a pH-dependent growth phenotype upon disruption of V-ATPase activity (Bowman *et al.*, 2000) (Poltermann *et al.*, 2005) (Iwaki *et al.*, 2004). In standard HMM media the *H. capsulatum vma1* mutant also demonstrated a pH-dependent growth phenotype. However, this phenotype did not appear to be as severe as reported for other fungi. Thus, the growth rate of *vma1::HPH* was only modestly effected at pH 7.5, and was normal at pH 6.5 and 5.5.

Another characteristic of *S. cerevisiae vma* mutants is that they have severe defects in sporulation and germination (Enyenihi and Saunders, 2003), but the germination defect can be suppressed at low pH. Other fungal mutants with defects in V-ATPase function that demonstrate aberrant hyphal growth include *N. crassa*, *A. nidulans*, and *C. albicans* (Bowman *et al.*, 2000) (Melin *et al.*, 2004) (Poltermann *et al.*, 2005). Similarly, the *H. capsulatum vma1* mutant also demonstrated an inability to produce hyphae when grown at 28°C, but this was not reversed by culturing the mutant yeasts at pH 5.5.

A third characteristic of yeast *vma* mutants is that they are defective in many aspects of metal ion homeostasis (Eide *et al.*, 2005). Some of these defects may be caused an inability to store and detoxify metal ions (Eide *et al.*, 1993), whereas other defects are more complex. Of particular interest in regards to the pathogenesis of *H. capsulatum* is that vacuoles (that contain V-ATPases) in *S. cerevisiae* are known to be involved in iron storage and metabolism, and protect the yeasts from the oxidative effects of iron (Eide *et al.*, 1993) (Li *et al.*, 2001). *S. cerevisiae* yeasts deficient in V-ATPase activity are disrupted in iron acquisition due to improper loading of copper onto the FET3 multicopper oxidase (Davis-Kaplan *et al.*, 2006). Although, the G217B strain of *H. capsulatum* does not contain the FET3/FTR1 iron capture pathway (results of BLAST search of the *H. capsulatum* Genome Project, Washington Univ., St. Louis, MO), *vma1::HPH* mutant yeasts were unable to grow on iron poor medium, demonstrating that the *H. capsulatum* V-ATPase is involved in iron homeostasis. This idea was reinforced by the demonstration that bafilomycin, an inhibitor of the V-ATPase (Bowman *et al.*, 1988), partially blocked the growth of WT G217B on iron replete medium, and completely blocked the growth of WT yeasts on iron poor medium. Thus, treatment of WT yeasts with bafilomycin recapitulated the *vma1::HPH* mutant phenotype. A link between iron homeostasis and the V-ATPase also has been observed in *A. nidulans*. In this fungus *sidC* is required for the synthesis of the iron storage siderophore, ferricrocin, and disruption of *sidC* results in a phenotype similar to an *A. nidulans* V-ATPase knockout (Eisendle *et al.*, 2003; Melin *et al.*, 2004).

The inability of the *vma1::HPH* mutants to grow on iron poor medium could be reversed by the simultaneous presence of WT yeasts, or the addition of the siderophore rhodotorulic acid. A logical interpretation of this data is that the *vma1* mutant is defective in siderophore production, and WT yeasts enhanced mutant growth by secreting siderophores into the medium. To our surprise, however, under iron poor conditions the *vma1::HPH* mutant was able to synthesize and secrete siderophores. Thus, we hypothesize that under iron restrictive conditions, the *vma1* mutant is able to produce siderophores, but is unable to produce an additional molecule(s) that is required for iron acquisition and/or usage. Although the *vma1* mutant makes siderophores, it is unclear if the mutant still is unable to take up iron, or whether it is unable to use iron. Experiments with radiolabeled iron should address this question.

In contrast, the addition of exogenous free ferric to *vma1::HPH* mutant-infected M ϕ only partially restored intracellular growth, but the additional growth was miniscule compared to the intracellular growth of WT or *vma1/VMA1* yeasts. As human M ϕ produce H₂O₂ upon ingestion of *H. capsulatum* yeasts (Bullock and Wright, 1987), this toxic oxygen radical probably contributed to the demise of the *vma1* mutant. Supporting this idea is the known sensitivity to oxidant stress of yeast mutants lacking a functional V-ATPase (Thorpe *et al.*, 2004)(Milgrom *et al.*, 2007).

As numerous physiological processes depend on the activity of V-ATPases, drugs that specifically target fungal V-ATPase may serve as a new class of drugs for treatment. Currently, three classes of natural products act as specific and potent inhibitors of V-ATPases. The macrocyclic lactones, bafilomycin and concanamycin inhibit eukaryotic V-ATPases from animals, plants and fungi. The benzolactone enamides, including salicylilalamides and lobatamides preferentially inhibit V-ATPases from mammalian sources (Bowman and Bowman, 2005). The recently discovered chondropsins show preference for the *N. crassa* V-ATPase (Bowman *et al.*, 2003). Thus, chondropsins could lead to novel anti-fungal agents (Bowman and Bowman, 2005).

Experimental Procedures

Reagents

Apotransferrin, holotransferrin, nitriloacetate, ferric chloride, and gentamicin were purchased from Sigma-Aldrich (St. Louis, MO). Rhodotorulic acid, cefotaxime, hygromycin, HAMS F12, and RPMI 1640 were purchased from Fisher Scientific (Hampton, NH). Bafilomycin was a generous gift from K. Altendorf (Univeristat Osnabruck, Osnabruck, Germany). Iron nitriloacetate (FeNTA) and its control, NTA, were prepared fresh for each experiment as described previously (Newman *et al.*, 1994). NTA was prepared in distilled water and mixed 1:1 with ferric chloride in 1 N HCl to generate FeNTA. The pH of the solution was adjusted to 7.0 with 1 N NaOH.

Yeasts

H. capsulatum strains G217B, G217B *vma1* mutants, and G217B *vma1/VMA1* were maintained as previously described (Newman *et al.*, 1990). For experiments, yeasts were grown in *Histoplasma* M ϕ medium (HMM) (Klimpel and Goldman, 1987) at 37°C with orbital shaking at 150 rpm. After 48 h, yeast were harvested by centrifugation, washed three times with HBSS containing 20 mM Hepes and 10 μ g/ml of gentamicin (HBSSh), and resuspended to 30 ml in HBSSh. Large clumps of yeasts were removed by centrifugation at 200 $\times$ g for 5 min at 4°C. The top 5 ml was removed, and the suspension was standardized to 1 $\times$ 10⁵/ml in RPMI 1640 containing 5% heat-inactivated FCS and 10 μ g/ml of gentamicin.

Agrobacterium tumefaciens-mediated transformation

A. tumefaciens transformed with the Ti plasmid and the pCB301-HYG binary vector plasmid (Fig 1A) was grown for 48 h at 25°C on LC media containing 250 µg/ml of spectinomycin to select for the Ti plasmid, and 100 µg/ml of kanamycin to select for the pCB301 plasmid. A swab of *A. tumefaciens* containing the plasmid vector was used to inoculate 10 ml of liquid LC culture containing the same antibiotics and grown at 25°C on an orbital shaker at 125 rpm overnight or until the culture reached stationary phase. Two ml of the culture were washed once with induction media (IM), and used to inoculate a second 10 ml culture in IM supplemented with 200 µM of the inducer acetosyringone and antibiotics, that was incubated overnight. *H. capsulatum* strain G217B grown on HMM plates for 4 days was harvested in 5 ml of HMM. The yeasts were pelleted by centrifugation and resuspended in 5 ml of HMM. A 1:1 volume of the *A. tumefaciens* liquid culture and *H. capsulatum* suspension were mixed together, and 400 µl were plated on uncharged nylon membranes (Biodyne A 60102; Pall, East Hills, NY) on solid IM media containing inducer and antibiotics. Co-cultures were incubated at 25°C for 3 days and then the membranes were transferred to HMM plates supplemented with 200 µg/ml hygromycin and 200 µM cefotaxime. The HMM plates were incubated at 37°C for 10 days or until colony growth was evident. The plates were stored at 4°C for up to 6 months (Sullivan *et al.*, 2002).

Phenotypic screen

A. tumefaciens transformed *H. capsulatum* colonies were picked with sterile toothpicks and were inoculated into 200 µl of RPMI 1640 in flat bottom 96 well plates. After 24 h of culture at 37°C, the wells were resuspended and diluted 1:20 by transferring 10 µl of yeasts into a new 96 well plate with 200 µl of RPMI 1640 per well. Ten µl of yeasts then were used to inoculate human ϕ monolayers. ϕ were incubated at 37°C in 5% CO₂ for up to 10 days and screened by phase-contrast microscopy for wells in which the yeasts did not lyse the ϕ monolayer. Generally, after 4-5 days of culture, most monolayers had been destroyed by replicating yeasts. *H. capsulatum* colonies negative for growth during this initial screen were streaked out on solid HMM media and a single colony was re-screened. Mutants that did not replicate in ϕ after three rounds of screening were selected for further study.

TAIL PCR

To identify the disrupted gene, thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was performed as previously described (Singer and Burke, 2003). A DNA miniprep from a single *H. capsulatum* colony was used to prepare the DNA (Tian and Shearer, 2002). The mixture AD1,2,3,6 and LB1,2,3 primers were used for the PCR reactions. An agarose gel was used to verify the fidelity of the PCR reactions. The tertiary TAIL PCR product was cloned into pCR2.1 TOPO/TA (Invitrogen, Carlsbad, CA) and sequenced using M13F and M13R primers. The resulting sequence was BLAST searched against the *H. capsulatum* G217B genomic database (Washington Univ., St. Louis, MO).

Complementation of the *vma1::HPH* mutant

The *H. capsulatum* *VMA1* gene was amplified by PCR using the sense primer, VMAS' GAGACCTGCAGAAAGAGAAGGAATGAAA, and the antisense primer VMAA' CAAAAGCGGCGAGAAGCAAGC with a high fidelity polymerase (Phusion; Finnzymes, Findland). The sequence of the PCR product was from 1.5 Kb upstream of the coding region to 1.5Kb downstream of the coding region. Thus, the insert contained the endogenous promoter and terminator for the *VMA1* gene. The PCR product was cloned into pCR8/GW/TOPO TA (Invitrogen, Carlsbad, CA), and verified by restriction digestion, and sequencing. The insert then was cloned into the R1-R2 site of pCB301-bleo-R1-R2 using the LR Clonase enzyme (Invitrogen, Carlsbad, CA). The gateway vector pCB301-bleo-R1-R2 was generated

by the insertion of the Gateway RfB cassette, a bleo resistance cassette containing the *Aspergillus nidulans* GPD regulator sequences, the *Streptoalloteichus hindustanus* *BLE* gene, and the *A. nidulans* TrpC terminator sequence, into the pCB301 backbone. The resulting pCB301-bleo-*VMA1* plasmid was transformed into *A. tumefaciens* by electroporation, and then transformed into *H. capsulatum* as previously described. Complementation was verified by RT-PCR (Fig 1C) and by Western Blot (not shown).

RT-PCR

H. capsulatum yeasts were grown in HMM media for 48 h and RNA was extracted from a pellet of 1×10^8 yeasts using the Master Pure Yeast RNA purification system (Epicentre, Madison, WI). cDNA was made from 1 µg of RNA using random hexamer primers with the Superscript First Strand for RT-PCR system (Invitrogen). The cDNA was amplified by PCR using the *VMA1* primers rtVMA-1S, GTGCGTTTGGTTGCGGGAA and rtVMA-1A, GCATCTCTCCCAA-ACGTCCTGA, and the *gapdh* primers rtGAPDHS, ATTGGGCGTATTGTCTTCC, and rtGAPDHA, TTGAGCATGTAGGCAGCATA. The PCR products were analyzed by gel electrophoresis on a 2% agarose gel.

Human and Murine Mφ

Human mononuclear cells were purified from blood obtained from the Hoxworth Blood Center, Cincinnati OH, by dextran sedimentation and Ficoll-Hypaque (LKB Biotechnology Inc., Piscataway, NJ) centrifugation as previously described (Newman *et al.*, 1980). The mononuclear cells were washed in HBSSh and suspended to 5×10^6 /ml in HBSSh containing 0.1% autologous serum. One-tenth ml aliquots of cells were plated in 96-well tissue culture plates, and the monocytes were allowed to adhere for 1 h at 37°C in 5% CO₂. Adherent monocytes were washed twice with HBSS to remove lymphocytes, and then were cultured for 7 days in M199 containing 10% autologous serum and 10 µg/ml of gentamicin to allow for differentiation into Mφ. Alternatively, monocytes were separated from lymphocytes using an EasySep CD14 positive selection kit (Stem Cell Technologies, Vancouver, BC, Canada), following the manufacturer's instructions. Mφ were obtained by culture of monocytes at 1×10^6 /ml in Teflon beakers with RPMI 1640 containing 15% pooled human serum, and 10 µg/ml gentamicin. Mφ were harvested after 5-7 days in culture.

Mouse peritoneal Mφ (PM) were harvested from 6-10 week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) by peritoneal lavage. Ten ml of HBSS was injected into the peritoneal cavity of euthanized mice with an 18-ga needle and the peritoneal fluid was withdrawn. Cells were standardized to 1×10^6 /ml in RPMI 1640 containing 10% FCS and 10 µg/ml gentamicin and 0.1-ml volumes of cells were adhered in 96-well plates and cultured for 24 h before use.

Quantitation of the intracellular growth of *H. capsulatum* yeasts in Mφ

The intracellular replication of *H. capsulatum* yeasts in Mφ was quantified by ³H-leucine incorporation as previously described (Newman and Gootee, 1992). Culture media was removed from Mφ monolayers and 100 µl of *H. capsulatum* yeasts (1×10^5 /ml) in RPMI 1640 containing 5% FCS and 10 µg/ml gentamicin were added to each well. The yeasts and Mφ were cultured for 24 h at 37°C. The plates then were centrifuged at 1500 rpm, the supernatant was carefully aspirated through a 27-ga needle, and 50 µl of ³H-leucine (153 Ci/mmol; New England Nuclear, Boston, MA) in sterile water (1.0 µCi) and 5 µl of 10X yeast nitrogen broth (Difco Laboratories, Detroit, MI) were added to each well. The plates then were incubated for an additional 24 h at 37°C. Finally, 50 µl of L-leucine (10 mg/ml) and 50 µl of sodium hypochlorite were added to each well, and the contents of the wells were harvested onto glass fiber filters using an automated harvester (Skatron, Sterling, VA). The filters were placed into scintillation vials, 1 ml of scintillation fluid was added, and the vials

were counted in a Beckman LS 7000 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, CA). Results are presented as the mean cpm $\pm$ SEM. All experimental points were quantified in triplicate and at least three independent experiments were performed.

Quantitation of Phagolysosomal Fusion (PL-fusion)

Teflon beaker-cultured M ϕ were adhered in 24-well tissue culture plates to 12 mm round glass coverslips. Monolayers then were incubated with 200 nm LysoTracker Red (Invitrogen-Molecular Probes, Eugene, OR) in RPMI 1640 containing 5% FCS for 2 h to label lysosomes. After two washes, the M ϕ were infected with viable *H. capsulatum* yeasts (2×10^6) for 1 h at 37°C. After an additional two washes, the M ϕ were cultured in 200 nm LysoTracker for an additional 2 h. After washing, the monolayers were fixed in 3.75% paraformaldehyde for 20 min at 25°C. The M ϕ then were covered in Dulbecco's PBS containing 5% glucose. Coverslips were mounted cell-side-down in 90% glycerol in PBS onto microscope slides, and one-hundred yeast-containing phagosomes were counted and scored for lysosomal fusion or no fusion (Newman *et al.*, 2006).

Detection of siderophores

H. capsulatum yeasts were grown to mid-log phase in HMM, washed once with HBSS, and then resuspended to 5×10^7 /ml in 50 ml RPMI (without phenolphthalein) supplemented with 18 g/l of dextrose and 1 μ M of apotransferrin. The cultures then were incubated at 37°C with orbital shaking at 150 rpm for 7 days. The yeasts were centrifuged, and the supernatants were filter sterilized. For determination of siderophore production by the Atkin's Assay (Atkin *et al.*, 1970), 0.5 ml of culture supernatant was mixed with ferric perchlorate agent. After 5 min at room temperature, the absorbance was quantified at 480 nm. Uninoculated RPMI medium served as the blank. A standard curve with ferrichrome c was used to calculate the μ M of siderophores released by the yeasts. The standard curve was linear ($R^2 = 0.9994$) from 10 μ M to 727 μ M.

Alternatively, *Microbacterium flavescens* JG-9 (ATCC 25091) was maintained on ATCC 424 *Arthobacter* medium containing (per liter) 10 g of peptone, 10 g of yeast extract, 2 g of K_2HPO_4 , 20 μ g of rhodotorulic acid (34nM), and 15 g of bacto agar. The assay medium was same medium used to maintain growth, but rhodotorulic acid was omitted and a bacterial suspension was added to the molten medium. Approximately 5 to 10 ml/liter of a bacterial suspension were added until the medium just started to turn cloudy. Wells were cut in the plates using the end of a sterile glass pipette, 50 μ l of *Histoplasma* culture supernatant was placed in each well, and the plates were incubated at 30°C for 2 days (Payne, 1994).

Murine Model of Histoplasmosis

To produce infection in naïve mice, six to eight-week old male C57BL/6 mice were anesthetized by isoflurane and infected intranasally with 2×10^6 (sublethal) or 2×10^7 (lethal) yeasts in a 30- μ l volume of HBSS. Animals were sacrificed on day 7 and lungs and spleens were homogenized in HBSS, serially diluted, and dispensed (100 μ l) onto HMM plates containing gentamicin. Plates were incubated at 37°C in a humidified incubator, for 7 to 10 days before counting cfu per organ. Differences in fungal burden in organs were analyzed by using the Wilcoxon rank sum test. Animals receiving a lethal dose of *H. capsulatum* were monitored twice daily. Mice with ruffled fur, labored breathing, and inactivity were euthanized. Differences in survival were analyzed by the log-rank test.

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Figure 1.

Identification of an *H. capsulatum* *vma1::HPH* mutant after *Agrobacterium*-mediated insertional mutagenesis. (A) Schematic of the binary plasmid used to randomly insert the *HPH* cassette into the *H. capsulatum* genome. The *Aspergillus fumigatus* *GAPDH* promoter was used to drive *HPH* expression and the *A. fumigatus* *trpC* was used as the terminator. (B) Schematic showing the predicted insertional site of the *HPH* cassette within the *VMA1* gene of the isolated *vma1::HPH* mutant *H. capsulatum* strain. (C) RT-PCR analysis of *VMA1* (lanes 2 to 4) and *GAPDH* (lanes 5 to 7). Expression of WT (lanes 2 and 5), 3d1 *vma1::HPH* mutant (lanes 3 and 6), and *vma1/VMA1* (lanes 4 and 7) were performed after 48 h growth in HMM media.

Figure 2.

VMA1 expression is not required for yeast growth at 37°C. Yeasts were grown in HMM media at 37°C with orbital shaking at 150 rpm and the turbidity of 0.5 ml aliquots were quantified at OD₆₀₀. Growth was monitored in HMM media made to pH 5.5, 6.5, and 7.5 as shown in the different panels.

Figure 3.

VMA1 expression is required for the yeast to mycelia phase transition. WT, *vma1::HPH* mutant, and *vma1/VMA1* yeasts were streaked onto HMM plates made to pH 7.5 (left panel) and pH 5.5 (right panel), incubated at 28°C for 14 days. Pictures of representative individual colonies were photographed using an inverted microscope. WT and *vma1/VMA1* yeasts produced abundant mycelia at either pH. The *vma1::HPH* mutant produced only a few short branched mycelia at pH 5.5, and virtually no mycelia at pH 7.5.

Figure 4.

VMA1 expression is required for growth of *H. capsulatum* on iron restricted media. WT, *vma1::HPH* mutant, and *vma1/VMA1* yeasts were streaked onto HMM plates with (lower panel) and without (upper panel) 5 μ M apotransferrin and incubated for 10-14 days at 37°C. Only the *vma1::HPH* mutant yeasts were unable to grow on iron restricted medium (middle pane, lower panel).

Figure 5.

Rescue of the growth of *vma1::HPH* mutant yeasts on iron restricted medium. *vma1::HPH* mutant yeasts were streaked onto HMM plates containing 5 μ M apotransferrin at either pH 6.5 (A) or pH 7.5 (B) and incubated for 7 days at 37°C. The mutants were able to grow at pH 6.5, but not pH 7.5. In panels C and D, 1×10^6 *vma1::HPH* mutant yeasts in 100 μ l HBSS were spread onto HMM plates containing 5 μ M apotransferrin. Next, 10 μ l of WT yeasts (1×10^8 /ml) was spotted onto the center of the plate (C) or 10 μ l of 1 mg/ml apo-rhodotorulic acid (RA) was spotted onto the edge of the plate (D), and the cultures were incubated at 37°C for 7 days. Both WT yeasts and rhodotorulic acid rescued the growth of the *vma1::HPH* mutant yeasts.

Figure 6.

vma1::HPH mutant yeasts produce siderophores under iron starvation conditions. *Microbacteria flavescens* JG-9 was dispensed into ATCC 424 *Arthobacter* medium as described in Experimental Procedures. Fifty microliters of supernatant from WT, *vma1::HPH* mutant, and *vma1/VMA1* yeasts grown for 7 days under iron starvation conditions, were added to the center wells and the plates were incubated for 48 h at 30°C (lower panel). Fifty microliters of FeNTA (1 mg/ml), was added as a negative control, and 50 µl of rhodotorulic acid (1 mg/ml) was added as a positive control (upper panel). All three *H. capsulatum* strains produced siderophores as evidenced by the growth of *M. flavescens* that can only grow in the presence of siderophores. No growth was observed with plates supplemented with FeNTA.

Figure 7.

Incubation of WT G217B yeasts with bafilomycin recapitulates the *vma1* mutant phenotype. WT yeasts were streaked onto HMM or HMM/Apotf plates that contained 5 – 30 μ M bafilomycin (Baf) or plates that did not contain bafilomycin as a control (Co). All plates then were incubated for 7 days at 37°C. Under iron sufficient conditions, 30 μ M bafilomycin completely inhibited the growth of WT yeasts (top panel), whereas under iron restricted conditions, only 5 μ M bafilomycin was necessary to completely inhibit growth.

Figure 8.

Exogenous iron partially restores intracellular growth of *vma1::HPH* mutants in human and mouse M ϕ . M ϕ were infected with 1×10^4 yeasts and cultured for 48 h. FeNTA at 75 μ g/ml was added as indicated 1 h after infection was initiated. The data presented are the means $\pm$ SEM of 4 experiments performed in triplicate. *, $p < 0.001$ compared to WT and *vma1/VMA1*; **, $p = 0.001$ compared to *vma1::HPH* mutant. There was no difference in the intracellular growth between WT and *vma1/VMA1* yeasts.

Figure 9.

VMA1 expression is required for colonization of mouse lungs and spleens. Male C57/BL6 mice were intranasally infected with 2×10^6 yeasts. Six mice were inoculated with each of the three strains of *H. capsulatum*, and the lungs and spleens were removed after 7 days and cfu quantified. The data are presented as the Mean $\pm$ SD. *, No colonies were recovered from mice infected with *vma1::HPH* mutant yeasts; $p = 0.002$ compared to WT and *vma1/VMA1* *H. capsulatum* yeasts. The limit of detection is 100 cfu.

Figure 10.

The *H. capsulatum* vacuolar ATPase is required for virulence in a pulmonary model of histoplasmosis. Ten mice per group were intranasally infected with 2×10^7 *H. capsulatum* yeasts. All of the mice infected with WT and *vma1/VMA1* *H. capsulatum* yeasts died by 13 days post-infection. In contrast, none of the mice infected with the *vma1::HPH* mutant yeasts died.