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VEA1 is required for cleistothecial formation and virulence in *Histoplasma capsulatum*

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ABSTRACT

Histoplasma capsulatum is a pathogenic fungus dependent on dimorphism for virulence. Among the four described Velvet family genes, two of them, Ryp2 and Ryp3, have been shown to be required for dimorphism. It is known that Velvet A (VeA) is necessary for sexual development and toxin production in *Aspergillus nidulans.* However, the role of the VeA ortholog in *H. capsulatum* has not yet been explored. Vea1, *H. capsulatum* homolog of VeA, was studied to determine its role in cleistothecial formation, dimorphism, and virulence. *H. capsulatum* Vea1 restores cleistothecial formation and partially restores sterigmatocystin production in an *A. nidulans veA* deletion strain. Furthermore, silencing *VEA1* in an *H. capsulatum* strain capable of forming cleistothecia abolishes cleistothecial formation. Silenced strains also switch to mycelial phase faster, and show impaired switching to the yeast phase once in mycelial phase. Virulence in mice and macrophages is attenuated in *VEA1* silenced strains and silenced strains demonstrate increased sensitivity during growth under acidic conditions. These results indicate that *H. capsulatum* Vea1 shares a similar role in development as VeA. *H. capsulatum* is also more susceptible to growth in acidic conditions when *VEA1* is silenced, which may contribute to the silenced strains' attenuated virulence in mice and macrophages.

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1. Introduction

Histoplasma capsulatum is a pathogenic fungus, causing pulmonary and systemic infection following inhalation. The severity and complications of histoplasmosis vary with the immune status of the individual and the number of infectious particles inhaled. Manifestations of the disease can range from asymptomatic to severe disseminated disease (Kauffman, 2007). The virulence of *H. capsulatum* is dependent upon dimorphism, which is the ability to switch between growth in yeast and mycelial phase responding to environmental cues, and organisms locked in mycelial phase are avirulent (Medoff et al., 1986). Genes required for dimorphism in *H. capsulatum* include the histidine kinase *DRK1* (Nemecek et al., 2006), and three genes required for yeast phase growth: *RYP1*, *RYP2* and *RYP3* (Nguyen and Sil, 2008; Webster and Sil, 2008). Ryp2 and Ryp3 are orthologs of VosA and VelB, respectively (Webster and Sil, 2008), that belong, together with VelC and VeA, to the Velvet family of proteins (reviewed by Calvo (2008)).

Proteins in the Velvet family are poised to affect virulence in *H. capsulatum*, not only because of their involvement in the dimorphic switch, but also because they regulate sexual development in other fungi (Kato et al., 2003; Kim et al., 2002; Calvo et al., 2004). *H. capsulatum* organisms of the (−) mating type are found in clinical samples more frequently than (+) types (Kwon-Chung et al., 1974; Kwon-Chung et al., 1984), indicating that genes involved in mating or mating type may affect virulence. This study focuses on Velvet A (VeA), a well-studied regulator of fruiting body/sclerotium development and secondary metabolism in other fungi such as *Aspergillus nidulans* (Kato et al., 2003; Kim et al., 2002), *Aspergillus parasiticus* (Calvo et al., 2004), *Aspergillus flavus* (Duran et al., 2007, 2009), and *Fusarium verticillioides* (Li et al., 2006; Myung et al., 2009, 2011) among others.

In *A. nidulans*, VeA forms a complex with light sensing proteins, FphA, LreA and LreB (Purschwitz et al., 2008), as well as with VelB and a putative methyl transferase called LaeA to activate sexual development and secondary metabolism in response to dark conditions (Bayram et al., 2008, 2010b). *A. nidulans* forms mating structures called cleistothecia in the dark, but this process is inhibited by light (Bayram et al., 2008; Calvo, 2008). In the absence of VeA or VelB, organisms no longer form cleistothecia (Kim et al., 2002; Bayram et al., 2008). LaeA regulates secondary metabolism in *A. nidulans* and deleting LaeA, VeA or VelB abolishes or reduces production of sterigmatocystin and penicillin (Kato et al., 2003; Bayram et al., 2008; Bok and Keller, 2004). Velvet proteins VelB and VosA also form a complex (Bayram et al., 2010a). VosA and

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VelB are also involved in spore viability (Ni and Yu, 2007; Bayram et al., 2008) and together they repress asexual spore formation (Bayram et al., 2010a).

Less is known about the Velvet family proteins in *H. capsulatum*. In this organism, Ryp2 and Ryp3 are orthologous to VosA and VelB, respectively (Webster and Sil, 2008). Based on studies mentioned above, *H. capsulatum* Velvet proteins would be expected to play a role in sexual development, conidiation, and/or sexual spore development, and indeed Ryp2 and Ryp3 mutant strains also have deficiencies in conidial viability (Webster and Sil, 2008). Additionally, Velvet family proteins affect thermal dimorphism in *H. capsulatum*: Ryp2 and Ryp3 mutants are locked in mycelial phase regardless of temperature (Webster and Sil, 2008), while *H. capsulatum* normally grows in mycelial phase at room temperature and switches to yeast phase at 37 °C (DeMonbreun, 1934). Homologs of Velvet family proteins VeA (Webster and Sil, 2008) and LaeA are also present in *H. capsulatum*; however their functions have not been studied at this time.

This study looks at the function of Vea1, the *H. capsulatum* homolog of VeA, in sexual development, morphology, and virulence of *H. capsulatum*. Vea1 was first studied by heterologous expression in an *A. nidulans* Δ*veA* strain, to determine the role of Vea1 in a facile mating system. It is difficult to study the effects of genetic manipulation on sexual development in *H. capsulatum* because the organisms rapidly lose the ability to mate in culture (Kwon-Chung et al., 1974). One *H. capsulatum* strain, UC26, has been described that stably forms empty cleistothecia with a mating partner (Laskowski and Smulian, 2010). We used this strain as a tool, and in the current study, silenced *VEA1* in the UC26 background to study the role of *VEA1* in mating and dimorphism. In *H. capsulatum*, *VEA1* silenced strains failed to produce cleistothecia, and displayed accelerated switching to the mycelial phase. *VEA1* silenced strains also showed impaired virulence in mice, indicating a link between *VEA1* and virulence in *H. capsulatum*. These studies also showed that *VEA1* silenced strains were more sensitive to acidic conditions and were less virulent in macrophages, indicating decreased virulence in macrophages as a mechanism for decreased virulence in the mouse model.

2. Materials and methods

2.1. Alignment of A. nidulans and H. capsulatum VeA homologs

Alignment was performed on *A. nidulans* (AAD42946.1) and *H. capsulatum* (ACB59235.1) VeA predicted amino acid sequences using ClustalX (2.0) software (www.clustal.org).

2.2. Complementation of A. nidulans ΔveA deletion strain

H. capsulatum VEA1 $Comp_{H.cap.\ veA}$ strains17, 26 and 27 were generated by transformation of RNKT3.3 (*biA1*, *pyroA4*, Δ*veA::argB*) with the plasmid pJLR8.11. pJLR8.11 was generated in the following manner: The 5′UTR region of *A. nidulans veA* was amplified from FGSC4 (wild type) genomic DNA using primers F: 5′-GAGC TGTTCATTCCGAAAC-3′ and R: 5′-GGGATAACACAAAATGCTCT-3′. The 5′UTR fragment was ligated into pJET1.2 (Clone Jet, Fermentas) to obtain pJLR1.5. pJLR1.5 and the plasmid pSM3 (*amp*^{R}, *pyroA*) were digested with *Bam*HI, and the 5′ UTR region was ligated into pSM3 (containing the *pyroA* auxotrophic marker gene) generating pJLR4.5. *H. capsulatum VEA1* cDNA was obtained by PCR using primers F: 5′-AAAAAACCCGGGATGGCTACAAAAGCGTCGTC-3′ and R: 5′-AAAAAACCCGGGCTACTCAGCAACAGCACACA-3′, which contain *Xma*I tails. The PCR product and pJLR4.5 were digested with *Xma*I, and ligated to generate pJLR5.1. The 3′ UTR region of *A. nidulans veA* was amplified from FGSC4 genomic DNA using primers F: 5′-TTTTTGGTACCGAATTCTGCCGGCGTTTATTTG-3′ and R: 5′-AAAAAAGGTACCCACAGGCATTCTACGGCATA-3′, which contain *Kpn*I tails. The obtained PCR product and pJLR5.1 were digested with *Kpn*I and ligated to generate pJLR8.11. Fungal transformation of RNKT3.3 with pJLR8.11 was carried out according to procedures previous described (Miller et al., 1985). Presence of the transformation cassette was verified by PCR with primers 5′-GAGCTGTT CATTCCGAAAC-3′ and 5′-AAAAAAGGTACCCACAGGCATT CTACGG-CATA-3′.

2.3. Quantification of A. nidulans conidial production

Approximately 1×10^6 spores from *A. nidulans* wild-type, Δ*veA*, and $Comp_{H.cap.\ veA}$ strains were inoculated into top-agar on 1% GMM plus biotin plates and incubated in the light or dark at 37 °C. After 4 days a 7.5 mm-diameter core was collected from each plate and homogenized in 1 ml sterile ddH_20. Spores were counted with a haemocytometer. The experiment included three replicates.

2.4. Visualization of A. nidulans cleistothecial production

Strains were inoculated as explained above for conidial quantification. A 7.5 mm diameter core was collected from each culture after 10 days of incubation at 37 °C. The core was then sprayed with 70% EtOH to improve visualization of cleistothecia. The experiment included three replicates.

2.5. Sterigmatocystin analysis

Presence of sterigmatocystin (ST) in the wild-type, Δ*veA*, and $Comp_{H.cap.\ veA}$ cultures was evaluated by Thin Layer Chromatography (TLC). Three 16 mm diameter cores were removed from each 1%GMM plus biotin top agar culture after 5 days of growth at 37 °C. Cores were extracted with chloroform, and extracts were evaporated overnight. Extracts were solubilized in chloroform. Samples were loaded onto a silica TLC plate with an ST standard. The metabolites were separated using a Toluene:Glacial Acetic Acid: Ethyl Acetate solvent system at a ratio of 80:10:10 [v:v:v]. After separation, plates were sprayed with aluminum chloride and baked at 80 °C for 10 min. The plates were then observed under UV light, at a wavelength of 257.3 nm.

2.6. H. capsulatum growth conditions

H. capsulatum yeast phase organisms were grown in liquid HMM media at 37 °C using an orbital shaker (200 rpm), or on sealed plates at 37 °C under 5% CO_2 in a humidified incubator. Mycelial phase organisms were grown at 25 °C on sealed HMM plates.

2.7. H. capsulatum strains used

VA6 is a clinical isolate obtained in Cincinnati, OH. G217B (ATCC 26032) was a generous gift from George Deepe (University of Cincinnati, Cincinnati, OH). UC26 is a hygromycin sensitive derivative of UC1, originally derived from the strain G217B; generation of UC1 (Smulian et al., 2007) and UC26 (Laskowski and Smulian, 2010) have previously been described.

2.8. H. capsulatum strains generated

VEA1 silenced strains V1, V2 and V6 and control strains C2 and C5 were generated by *Agrobacterium* transformation of UC26, using the integrative vector pRH19, or the control vector pVN69. *Agrobacterium* transformation was performed as previously described