The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects; the MAD2 adhesin enables attachment to plants

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Running title: *Metarhizium anisopliae* adhesins

Data deposition: GenBank accession numbers: *Mad1* mRNA (DQ338437); *Mad2* mRNA (DQ338438) and genomic DNA (DQ338439).
ABSTRACT

Metarhizium anisopliae is a fungus of considerable metabolic and ecological versatility, being a potent insect pathogen that can also colonize plant roots. The mechanistic details of these interactions are unresolved. We provide evidence that M. anisopliae adheres to insects and plants using two different proteins, MAD1 and MAD2 that are differentially induced in insect hemolymph and plant root exudates, respectively, and produce regional localization of adhesive conidial surfaces. Expression of Mad1 in Saccharomyces cerevisiae allowed this yeast to adhere to insect cuticle. Expression of Mad2 caused yeast cells to adhere to a plant surface. Our study demonstrated that as well as allowing adhesion to insects, MAD1 at the surface of M. anisopliae conidia or blastospores is required to orientate the cytoskeleton and stimulate expression of genes involved in the cell cycle. Consequently, disruption of Mad1 in M. anisopliae delayed germination, suppressed blastospore formation and greatly reduced virulence to caterpillars. Disruption of Mad2 blocked adhesion of M. anisopliae to plant epidermis, but had no effects on fungal differentiation and entomopathogenicity. Thus regulation, localization and specificity control the functional distinction between Mad1 and Mad2, and enable M. anisopliae cells to adapt their adhesive properties to different habitats.

INTRODUCTION

As judged by species number the ascomycetes are the most successful fungi, comprising some 75% of known species (30). They include many pathogens of plants and animals, and ascomycetes are also the principal inhabitants of soils containing solid resources such as plant and animal remains. Many economically important pathogenic fungi have the ability to be free living in soil and elsewhere but there is limited knowledge of the range of ecological niches they can occupy in the absence of a host (16). For example, the commercially important biocontrol agent Metarhizium anisopliae is a ubiquitous pathogen of insects and a well established model organism for the study of insect-microbe interactions (2, 26). However, only recently was it reported to colonize the rhizosphere (the layer of soil influenced...
by root metabolism) and adhere to plant root surfaces (15). *M. anisopliae* is believed to exert a considerable influence within this ecological niche by repelling and killing soil insects (15).

A major goal of evolutionary and ecological genetics is to identify genes that adapt organisms to their ecological niches and to understand the interaction between their products and the environment. However, genes directly involved in ecological attributes are hard to identify (4). Most forms of fungal adaptation at the molecular level, such as circadian rhythms (20), have focused on the traditional model systems used for genetic analysis such as yeasts and *Neurospora crassa*. However, many ecologically important life styles such as pathogenicity are not represented by these models. Acquiring a detailed understanding of each species in every ecosystem is unrealistic, but *M. anisopliae* provides a genetically tractable model to address important questions as to the ecological genetics and molecular mechanisms of two different interspecific interactions. In particular what genes make a fungus an entomopathogen, and what genes competent to colonize plant roots. In addition, strains of *M. anisopliae* have an extremely wide distribution within vastly different climates (artic to true desert) and ecological settings. Hence many important environmental adaptations made by fungi may be displayed by strains of *M. anisopliae*. Finally, *M. anisopliae* produces many different cell types for developmental studies including conidia, hyphae, appressoria (pre-penetration swellings produced by many plant and insect pathogens), uni-cellular blastospores (budding yeast-like form) and multi-cellular hyphal bodies of various sizes and shapes that differentiate from blastospores in insect hemolymph (24).

Current ecological knowledge of the conditions in which *M. anisopliae* lives has allowed us to consider gene function in laboratory conditions that model these environments in order to identify genes underlying ecologically relevant traits. Our previous EST analysis had identified two very high frequency contigs, CN808227 (4.08% of all clones in a cDNA library of *M. anisopliae* genes expressed in insect hemolymph) and CN809626 (8.9% of clones in a cDNA library of *M. anisopliae* genes expressed in root exudate) that had unknown functions (31). This study greatly extends our
understanding of how *M. anisopliae* interacts with insects and plants by establishing that CN808227 (designated *Mad1* for *Metarhizium* adhesin-like protein 1) and CN809626 (designated *Mad2*) are responsible for the anchoring capacity to insect and plant surfaces that enable *M. anisopliae* to effectively persist and colonize these different environments.

**MATERIALS AND METHODS**

**Gene cloning and knock-out.** Full DNA sequences of *Mad1* (based on the sequence of CN808227) and *Mad2* (CN809626) were obtained by primer walking (DNA Walking Speedup kit, Seegene). Strains lacking either *Mad1* or *Mad2* were constructed using the plasmid pGPS3Bar as described before (32). The 5′ region of the target gene *Mad1* was amplified using the primers Mad1-1U (GACTAGTTTCGTTTCTTCCGACTACCATT, 75) and Mad1-1L (GACTAGTTCTCTTCCAGTGTTAG, 1269). The 3′ region was amplified with Mad1-2U (ATTTGCGGCCGCCTCTTACCGACCTCTAC, 1410) and Mad1-2L (ATTTGCGGCCGCCTCTATTACCGACCTCTAC, 2678). The two PCR products were digested with the enzymes *SpeI* and *NotI*, respectively, and then inserted into the *SpeI* and *NotI* sites of pGPS3Bar using two steps subcloning to generate the disruption cassette pBarMad1. Similarly, the primers Mad2-1U (CCTGGATCCAGGAGAAGCCCGAAGTGAC) and Mad2-1L (CCTGGATCCGGCATGGTAGCAGGAACAGT), Mad2-2U (ATTTGCGGCCGAAGCTCGGGTCCCGGTACTGT) and Mad2-2L (ATTTGCGGCCGCAACTGGTTGTTGAGCATGTGTC, 2678) were used to amplify the 5′ and 3′ regions of *Mad2*. The PCR products were digested with *BamHI* and *NotI* and then inserted into pGPS3Bar to generate pBarMad2. Both constructs were linearized before being used to transform the blastospores of *M. anisopliae*. Putative transformants were verified by PCR and RT-PCR. At least three verified transformants of Mad1 or Mad2 were used for parallel functional analysis to compare with the wild type.
Adherence assays. Suspensions of WT, ΔMad1 and ΔMad2 conidia were prepared in 0.05% Tween-20 at a concentration of $2 \times 10^7$ conidia/ml, washed twice with water and re-suspended in water for use. Locust hind wings, fly wings and epidermis peeled from pieces of onion or celery (1 × 0.5 cm) were sterilized in 37% H$_2$O$_2$ for 5 min, washed twice in water, immersed in spore suspensions for 20 sec and placed on 0.7% water agar. After incubation for 8 hours (to induce spore swelling and initiation of germination), the number of conidia in 5 objective fields was counted under a light microscope before and after washing out the less adherent conidia in 0.05% Tween-20 for 30 sec. Adherence by WT and both mutants on the different materials was estimated using the average number of conidia per objective field after washing as compared to the average count before washing (always > 300 conidia per treatment). Experiments were done in triplicate and each experiment was repeated at least twice.

Yeast transformation and adherence assays. To confirm the binding properties of MAD1 and MAD2, the full cDNAs of Mad1 and Mad2 were amplified from correspondent EST clones (31). PCR was conducted using a Pfu Ultra polymerase (Stratagene) and the primers Mad1U (CCGGATCC CATTGCTGTCTTCGTCGTCT) and Mad1L (ATTTGCGGCCGC CATACCCGCTTAGCACAACA), Mad2U (CCGGATCC TCCAACCGCTCTCACCTTAT) and Mad2L (ATTTGCGGCCGCATATGCTGTGCGGTCAACAC) by introducing BamHI and NotI restriction sites (underlined). The digested product was inserted into BamHI/NotI digested pYes2 (Invitrogen) under the control of a Gal1 promoter to generate pYes2Mad1 and pYes2Mad2, respectively. Yeast transformation [using the Saccharomyces cerevisiae strain INVSc1 (Invitrogen)] and protein induction were performed following the manufacture’s instructions. Briefly, after 6 hrs incubation in galactose medium, potentially adherent yeast clones were screened by placing locust hind wings and peeled onion skin epidermis (see above) into the induction medium and incubating for up to
10 hours on a gyratory shaker (150 rpm). The control experiments were conducted by incubating wings or onion skins with un-transformed yeasts or yeast cells transformed with un-modified pYes2 plasmid. After incubation, the substrates were washed for 30 sec in 0.05 % Tween-20 to assay for cell binding abilities as above.

**Insect bioassays.** Virulence was assayed using conidia of the WT and mutant strains against newly emerged 5th instar larvae of *Manduca sexta* (28). Conidia were applied topically by immersing larvae for 20 sec in an aqueous suspension containing $2 \times 10^7$ conidia/ml. Each treatment had three replicates with 10 insects each and the experiments were repeated twice. Mortality was recorded every 12 hours. Additional infected insects were bled for microscopic observation of fungal development within the insect hemocoel.

**Reverse transcription PCR.** To determine the inductive effects of different growth conditions, 36 hour Sabouraud dextrose broth (SDB, Difco) cultures were harvested, washed and equal amounts (0.2 g wet weight) of mycelia inoculated into 10 ml of minimal medium (MM, glucose 10 g/l, NaNO$_3$ 6 g/l, KCl 0.52 g/l, MgSO$_4$.7H$_2$O 0.52 g/l, KH$_2$PO$_4$ 0.25 g/l), SDB, sterile water, 0.1% bean root exudate, 1% locust cuticle or *M. sexta* hemolymph for 6 hours. The root exudates, locust cuticle and *M. sexta* hemolymph were prepared as described (31). The RNA was extracted using a Qiagen RNeasy Plant Mini Kit, treated with DNase I and 1 µg was converted into single strand cDNA using an anchored oligo-dT primer (ABgene).

To monitor the influence of deleting *Mad1* or *Mad2* on gene expression, primers for various genes were designed (Table S1) and used for RT-PCR analyses of WT and mutants cultured in hemolymph or root exudate for 6 hours.
**Indirect immunofluorescence (IIF) assay.** Predicted antigenic sequences from the N-terminal domains of MAD1 [CESNFGKRGDIQGR (85-98 aa)] and MAD2 [CDDNDNDEWHYVHP (80-93 aa)] were synthesized commercially and used for raising antibodies in New England white rabbits (GeneScript). To localize MAD proteins, wild type conidia harvested from 20 day-old potato dextrose agar (PDA, Difco) plates were grown in SDB or 1% bean root exudates for 0-12 hours, fixed in 3.7% formaldehyde and used for IIF analysis using previously described protocols (32). Control samples were incubated without either the primary or secondary antibody. To explore the potential interactions between MAD1 and septin(s), an antibody against *Aspergillus nidulans* septin B (AspB) (33) was used to localize septin distribution on wild-type and mutant hyphal bodies.

**Fluorescent staining.** Cell actin distribution is visualized with fluorescein isothiocyanate labeled phalloidin (Sigma) at a final concentration of 0.05 mg/ml in PBS plus 1% DMSO. Cell walls and nuclei were stained with Calcofluor white and DAPI (Sigma), respectively.

**RESULTS**

**Molecular characteristics of MAD1 and MAD2.** The full ORF of *Mad1* cDNA encodes a protein of 717 amino acids (74.6 kDa with a predicted pI of 6.1). MAD2 comprises 306 amino acids (30.5 kDa with a pI of 6.0). They share a similar 3-domain structure to the *Candida albicans* cell wall ALS (Agglutinin-Like-Sequence) proteins (14, 27) that form rapid and extremely stable H-bond dependant associations with host proteins and peptides (9). Consistent with their being cell wall proteins, both MAD1 and MAD2 possess a hydrophobic signal peptide and a predicted glycosylphosphatidylinositol (GPI) cell wall anchor site (5) at their N- and C-terminal ends, respectively. Also similar to ALS adhesins (14, 29) (Fig. 1A), the MAD1 and MAD2 N-terminal regions (domain A) downstream of the signal peptides are predicted to be highly hydrophobic and the middle regions (domain B) contain Thr-rich tandem repeats. Sequence similarities between MAD1 and MAD2 largely exist within this middle
region (Fig. 1B). In MAD1 it contains 6 tandem repeats compromising 12 residues GKE\textit{T}TPAQQTTP while in MAD2 it has three repeats of TVPATMPG (Fig. 1B and 1C). The tandem repeat region of MAD1 also shows limited sequence similarities to the \textit{C. albicans} FLO11 (Fig. 1C and Fig. S1) which is required for yeast pseudohyphae formation (21). The working model for ALS proteins is that the tandem repeats are heavily glycosylated to produce a rigid elongated structure that holds the adhesive N-terminal domain at the cell surface (14, 25). Presumably, the smaller number of repeats in MAD2 means the distance between the cell surface and the N-terminal ligand binding region will be shorter as compared to MAD1.

Heterologous expression of \textit{M. anisopliae} genes in a non-adherent strain of \textit{S. cerevisiae}. Mad1 and Mad2 genes were expressed individually in yeast cells to determine if they conferred adhesive properties. Cells were transformed with pYes2 plasmid containing either Mad1 or Mad2 cDNA under the control of the tightly regulated \textit{Gal1} promoter. After 10 hours induction with 2\% galactose, the cells expressing MAD1 adhered to plastic so that they did not wash off. In contrast, the MAD2 expressing cells only adhered to each other (a process called flocculation in yeasts) (Fig. 2A). In contrast to \textit{Candida} adhesin INT1 (1), heterologous expressions of MAD1 or MAD2 in \textit{S. cerevisiae} did not result in the yeast producing filamentous growth.

Given that \textit{Mad1} and \textit{Mad2} are highly expressed in insect hemolymph and bean root exudates, respectively (31) and \textit{M. anisopliae} is an insect pathogen that also adheres to plant surfaces (15), additional adherence assays were conducted using locust wings and epidermal tissue peeled from onions. Yeast cells expressing MAD1 adhered to locust cuticle (92.2 \pm 6.1\% adherence) but not to onion epidermis, while the cells expressing MAD2 adhered to onion epidermis (85.5 \pm 7.3\%) but not to locust cuticle (Fig. 2B and 2E). Un-transformed yeast cells and cells transformed with un-modified pYes2 were non-adherent to either wing or onion surfaces.
Disruption of Mad1 and Mad2 blocks adhesion of M. anisopliae to insect and plant surfaces. To determine the contributions of MAD1 and MAD2 to conidial adherence, we used homologous replacement to obtain mutants of M. anisopliae disrupted in each gene. Adhesion assays showed that > 90% of the parental wild type (WT) conidia adhered to either locust cuticle or onion epidermis and could not be washed off with 0.05 % Tween-20. Only 5.3 ± 1.2 % of the ΔMad1 conidia adhered to locust cuticle but ΔMad2 conidia were as adherent as the WT (Fig. 2C and 2E). Conversely, ΔMad2 conidia showed very little adherence (4.8 ± 1.3 %) to onion epidermis while ΔMad1 conidia were as adherent as the WT (Fig. 2D and 2E). Very similar results were obtained when fly wings and celery epidermis were substituted for locust and onion surfaces (Fig. S2). Thus, MAD1 and MAD2 have unique (i.e. non substitutable) functions and are principally responsible for the ability of M. anisopliae to adhere to insect hosts and plant surfaces, respectively.

Expression and localization of MAD1 and MAD2. RT-PCR analyses were performed to measure expression of Mad1 and Mad2 in different media. Consistent with previous EST and microarray analyses (31), Mad1 and Mad2 were expressed at varying levels in all tested media. However, the Mad1 gene was up-regulated in nutrient rich media such as SDB and cell-free insect (Manduca sexta) hemolymph, while the Mad2 gene was up-regulated in bean root exudates and water (Fig. 3A). Time course studies showed that Mad1 and Mad2 transcripts are present at low levels in freshly collected conidia from 20 day-old PDA plates but transcripts started to accumulate within 2 hrs of incubation in SDB or root exudates (Fig. 3B).

For MAD1 and MAD2 to function as adhesins, they must be located on the cell surface. This was verified by using an indirect immunofluorescence (IIF) assay with antibodies raised against predicted antigenic amino acid sequences from the N-terminal regions of MAD1 (85-98 aa) or MAD2 (80-93 aa). Neither MAD1 nor MAD2 could be detected on freshly collected conidia suggesting an absence of surface antigens as antibodies do not penetrate the cell (32). Before germination, conidia absorb water
and swell. MAD1 was not detected in spores that had not swelled, irrespective of time point. However, coincident with swelling (6 to 8 hours for most spores in SDB), MAD1 localized at the ends of the bar-shaped *M. anisopliae* conidia (Fig. 3C). 100% of conidia germinating in SDB or successfully adhering to plastic or cuticle surfaces showed polar localization of MAD1 (Fig. 3C). Spores germinated in minimum medium or root exudates take longer (>12 hours) to produce detectable MAD1 but it was similarly localized. In contrast, MAD2 was preferentially localized in the middle of conidia when germinated in bean root exudates for 8 hours (Fig. 3D). Presumably this asymmetry results in regional localization of the adhesive surfaces related to the roles played by MAD1 and MAD2. The distribution patterns of MAD1 in ∆Mad2 mutant cells or MAD2 in ∆Mad1 cells were identical to WT cells. No immunofluorescence was observed when the ∆Mad1 and ∆Mad2 mutants were tested with antibodies to MAD1 and MAD2, respectively.

We also investigated the localization of adhesins on hyphal bodies harvested from insects infected with WT *M. anisopliae*. Consistent with RT-PCR analysis (Fig. 3A), MAD1, but not MAD2 was detected on the surfaces of hyphal bodies. MAD1 was produced irrespective of whether the hyphal bodies were unicellular (blastospores) or multicellular (Fig. 3E and 3F).

A punctuate distribution of MAD1 was detected on the surface of yeast cells expressing MAD1 (Fig. 3G) suggesting that MAD1 was heterogeneously and randomly distributed within the cell wall structure, while MAD2 expressed by yeast cells was distributed more uniformly on the surface of cells (Fig. 3H).

**Linkage of adhesion, germination, blastospore formation and the virulence of *M. anisopliae* to MAD1.** In addition to adhesion, MAD1, but not MAD2, affects conidial germination, blastospore formation and *in insecta* hyphal body differentiation (Fig. 4). Thus, 14 hours post-inoculation in SDB, 78.7 ± 2.3% of WT conidia and only 18.9 ± 3.6% of ∆Mad1 conidia have germinated (Fig. 4A and B). During 3 days culturing in isolated *M. sexta* hemolymph, the WT produced large numbers of
blastospores (Fig. 4C) while the ∆Mad1 mutant grew as long hyphae and produced very few blastospores (Fig. 4D). Likewise, the WT strain infecting 5th instar *M. sexta* larvae produced short variably shaped hyphal bodies composed of one to three cells (Fig. 4E). In contrast, 73.5 ± 4.32% of ∆Mad1 hyphal bodies were long (typically composed of more than 5 cells) and branched (Fig. 4F). However, the number of WT hyphal bodies in hemolymph (3.4 ± 1.5 × 10^6 cell per ml) was >20-fold more than the number produced by ∆Mad1 (1.5 ±1.1 × 10^5 cell per ml, n = 20 insects). Similar results were obtained using independently acquired ∆Mad1 mutants (Fig. S3).

To test whether deleting MAD1 or MAD2 influences fungal virulence, we bioassayed newly emerged 5th instar *M. sexta* caterpillars (Fig. 5). The LT\(_{50}\) values showed that the ∆Mad1 mutant takes a significantly longer time (5.35 ± 0.22 days) to kill insects than the WT does (3.92 ± 0.36 days) (*t*=24.76, *P* = 0.00081). In contrast, the difference between ∆Mad2 (4.11 ± 0.18 days) and the WT (3.92 ± 0.36 days) is not significant (*t*=2.92, *P*=0.12) showing that only MAD1 is a virulence factor.

**MAD1 is required for normal cytoskeleton organization and cell division.** Since we showed that disrupting the *Mad1* gene delays spore germination and results in big multicellular hyphal bodies (Fig. 4), experiments were performed to further explore the effects of MAD1 on the cell cycle. The initiation of spore germination in fungi is followed by actin polarization (alignment of actin to the poles of the conidia) and mitosis (18). Six hours post-inoculation in SDB, actin polymerization is evident in both WT (Fig. 6A) and ∆Mad1 conidia (Fig. 6B). However, it took 10 hrs for 90 % of ∆Mad1 spores to have polarized actin as compared to six hours for WT spores. The polymerized actin in ∆Mad1 conidia was scattered in clumps throughout the cytoplasm six hours post-inoculation (Fig. 6B). These results suggest that ∆Mad1 interferes with the organization of the cytoskeleton not due to a failure in assembly, but rather due to deficient interactions with proteins that regulate its distribution. This could be a direct interaction between MAD1 and actin, or indirect via a regulator such as septin.
Septins play crucial roles in cell division compartmentalization (3). For example, budding yeasts show a narrow mother-bud neck (the “septin-hourglass” shape). Mutants deficient in septins have very wide-necks providing a rapid diagnostic for septin defects (10). Wild type M. anisopliae budding hyphal bodies in hemolymph exhibit an hourglass shape almost indistinguishable from yeasts during septum formation (Fig. 6E). However, <10 % of dividing ∆Mad1 cells form an hourglass shape (Fig. 6F). Even where septa had formed, the actin cytoskeleton was usually less clearly delineated between daughter cells in ∆Mad1 as compared to the WT (Fig. 6C-F). Consistent with MAD1 being involved in the separation of cells after mitosis, 85.3 ± 9.8 % of ∆Mad1 hyphal bodies were multinucleate, whereas WT cells were invariably uninucleate (Fig. 6G-J). These results suggest that MAD1 is involved in achieving the “septin-hourglass” shape and subsequent separation of cells.

M. anisopliae EST AJ274373 is similar (E = 3 × 10^-34) to Aspergillus nidulans septin B (AspB). It is >2-fold up-regulated by WT cells in hemolymph (31). To label this septin we performed IIF assays using a polyclonal antibody against AspB (33). Fluorescent signals detected using the antibody demonstrated that septin rings are formed at the septa of WT but not ∆Mad1 hyphal bodies (Fig. 6K and 6L), confirming a role for MAD1 in septin organization. The dividing ∆Mad1 cell shown in Fig. 6L has an unusually well-staining septum for this mutation suggesting that septin B is a contributing factor but not a prerequisite for differentiation of the cell wall. The involvement of MAD1 in cytoskeletal organization and cell division was confirmed by testing additional MAD1 mutants (Fig. S4).

To test whether MAD1 effects gene expression, we performed RT-PCR analyses of selected genes involved in cell division (Table S1). Deletion of Mad1, but not Mad2, down-regulated conserved Metarhizium homologs (accession numbers in parenthesis) of an actin filament organization factor (CN809636) as well as septin B (AJ274373) (Fig. 7). Interestingly, although microscopy studies did not implicate MAD1 as important for nuclear division, Ami1 (AJ274250) involved in nuclear migration
and a G2/mitotic specific cyclin B (CN808863) were also down-regulated. However, diverse genes involved in carbohydrate metabolism (e.g., glucosidases AJ272748 and CN807981), proteolysis (e.g. subtilisin Pr1A CN808958, ubiquitin-specific proteinase AJ273678) and synthesis of secondary toxic metabolites (e.g. phenazine biosynthesis-like protein AJ273180, polyketide synthase AJ273296) did not show transcriptional differences between wild type and MAD-deletion mutants (data not shown).

**DISCUSSION**

Hundreds of fungal species are pathogenic to insects or can colonize plant roots. The ubiquitous soil fungus *M. anisopliae* however is remarkably versatile in being able to do both. This suggests that it must have evolved mechanisms to adhere to a variety of biological surfaces to initiate and maintain pathogenic and mutualistic interactions. Our study demonstrates that MAD1 and MAD2 are chiefly responsible for adhering conidia of *M. anisopliae* to insect and plant surfaces, respectively. Disruption of these genes produced approximately 90% reduction in adherence suggesting that *M. anisopliae* conidia possess little if any redundancy of adhesion molecules for the ligands present on plant and insect surfaces. We also constructed *S. cerevisiae* strains that express individual MAD proteins to determine if that confers the ability to adhere to insect or plant surfaces. The same method of heterologous expression has been used to characterize adhesins of *Candida* spp. (7), and has the advantage of guarding against any effect on adherence of additional adhesins in *M. anisopliae*.

Transformation of *Saccharomyces* with *Mad1* produced a strain capable of adhering to a plastic surface, as well as insect cuticle. By contrast, expression of *Mad2* causes yeast cells on plastic to aggregate (flocculation) due to cell-cell adhesion. Thus, although *Mad2* has little sequence similarity with the *Saccharomyces* flocculin gene family, it resembles them in promoting cell-cell adhesion.

It is not surprising that the deletion of *Mad1* significantly reduced the virulence of the mutant strain as it contributes to many of the characteristics that are associated with the pathogenicity of *M. anisopliae*. Attachment and adherence to the host surface are the key initial steps for colonization.
Passive hydrophobic interactions mediated by the hydrophobins are responsible for initial adherence of conidia to insect cuticle (13). As the spores swell preceding germination, the hydrophobin layer disintegrates and new cell wall materials are laid down (34). This is coincident with the appearance of MAD1 suggesting that its role in adhesion is to replace the hydrophobins with tighter more specific interactions. However, disrupting Mad1, but not Mad2, produced phenotypes that extended beyond adhesion to changes in germination and morphology. The formation of small hyphal bodies is a virulence strategy that facilitates rapid multiplication and dispersal of infectious propagules within the insect body (17, 32). It is likely therefore that the reduced number of large, and therefore presumably less mobile hyphal bodies produced by ∆Mad1 would contribute to diminished virulence.

Like MAD1, Candida adhesins Int1 (7), Als1 (6) and S. cerevisiae Flo11 (12) have also been reported to be involved in both adhesion and morphogenesis. Als1 and Flo11 may promote filamentation by mediating the effects of transcription factors (6, 12). However, the intermediate components involved in these pathways have not been determined. In this study, we demonstrated that knocking out MAD1 down regulated various genes, including a septin, involved in regulating the cytoskeleton and the cell cycle. A simple hypothesis to account for our findings would be that adhesin itself triggers gene expression. However, these changes were observed in hyphal bodies freely circulating in the hemolymph, suggesting that adhesins have more effects than previously realized. Consistent with the transcriptional changes, MAD1 influences the organization of the cytoskeleton. A delay in actin polarization correlates with the delay in ∆Mad1 spore germination. Likewise, dysfunctions of actin and septin in ∆Mad1 hyphal bodies could cause failures in cell division/compartamentalization. It is known that fungal cell compartmentalization is septin-dependent (22) and interactions between actin and septins occur in yeast (23) and A. nidulans (33). In yeasts, septins function in bud site selection, recruitment of proteins involved in budding, proper cell wall disposition and cytokinesis (10, 19). The intracellular domain of C. albicans adhesin Int1 is believed to
interact directly with septins Cdc11 and Cdc12 (8, 11). Co-localization of MAD1 and septin B was not observed in WT hyphal bodies in this study. Because MAD1 is localized outside the cell, it follows that the link with morphogenesis is likely to involve transmembrane signaling/interactions via its C-terminal tail, although we have not proven that this is the case.

The involvement of adhesin MAD1 in cytokinesis clearly merits further investigations. Attachment to insect surfaces and subsequent infection processes involves a variety of cellular structures and complicated patterns of gene expression and likely therefore requires several different signaling pathways to accomplish. The Mad genes themselves are differentially regulated by physiologically relevant conditions such as growth medium changes, morphological form and stage of growth. Thus, Mad1 transcripts are induced during spore swelling and during growth in hemolymph, two key stages of host invasion. There is overlap in regulation as Mad1 and Mad2 were produced at varying levels in all media tested, albeit sometimes at low levels. Further studies are needed to determine the effects of dosage of MAD proteins for the full expression of the adhesive phenotype. It is possible that low level constitutive production of MAD proteins may provide sensors that trigger decision processes in response to different subsets of environmental conditions such as insect and plant ligands. It will be interesting therefore to determine how Mad1 and Mad2 regulate, or are regulated, by other pathways involved in ecological adaptation. The results of this study could also be relevant to innovating new methods in agriculture. For example, the ability of MAD1 and MAD2 to cause yeast cells to differentially adhere to insect or plant surfaces suggests they could be used to target otherwise non-adherent pathogenic or symbiotic organisms to beneficials or pests.

ACKNOWLEDGEMENTS

This work was supported by an NSF grant MCB-0542904. The authors also highly appreciate Dr. Michelle Momany’s gift of the septin antibody.


**Figure legends:**

**Fig. 1.** Structural features of MAD1 and MAD2. A. Schematic structure of MAD1 and MAD2 showing glycine, threonine and proline-rich regions. SP, signal peptide; GPI, glycosylphosphatidylinositol-anchor site; HP, hydrophobic region. B. ClustalX alignment of the conserved region in MAD1 and MAD2 (*, indicates consensus sites). The MAD2 tandem repeats are shadowed. C. Conservation between tandem repeat regions (shadowed) in MAD1 and *Candida albicans* FLO11.

**Fig. 2.** Adherence assays. A. Binding of yeast cells to plastic inspite of washing with 0.05% Tween 20. Yeast cells were transformed with pYes2Mad1 or pYes2Mad2 and incubated for 10 hours in 2% raffinose (non-inducing) or galactose (inducing) media. In contrast to non-induced yeast cells, cells expressing MAD1 (Induced) became adherent to the plastic surface so that they did not wash off. In contrast, MAD2 expressing cells (Induced) were readily washed off plates but in the absence of washing adhered to each other (inset). B. Binding of yeast cells to locust wing cuticle and onion epidermis. Yeast cells expressing MAD1, but not MAD2, adhere to locust cuticle, while only yeast cells expressing MAD2 adhere to onion epidermis. C. Binding of *Metarhizium anisopliae* conidia incubated with locust wing cuticle for 8 hrs. *M. anisopliae* WT and ΔMad2 conidia adhere to locust cuticle but deleting Mad1 results in loss of adherence. D. Binding of *M. anisopliae* conidia incubated with onion epidermis for 8 hrs. Deletion of Mad2, but not Mad1 leads to the loss of conidial adherence to onion skin. E. Bar graph quantifying adherence of *M. anisopliae* conidia and yeast cells to locust wing cuticle and onion epidermis. Values represent means ± SD.

**Fig. 3.** Expression and localization of MAD1 and MAD2. A. RT-PCR analysis of Mad1 and Mad2 expression by wild type *Metarhizium anisopliae* transferred from SDB cultures to water, minimum medium (MM), 1% bean root exudate (RE), 1% *Manduca* larval cuticle (Cut) or cell free hemolymph (HE) for 6 hours. B. RT-PCR time course analysis of Mad1 and Mad2 expression. Conidia were
incubated in SDB (for Mad1 induction) or bean root exudate (for Mad2 induction) for up to 12 hours as indicated. C. Indirect immunofluorescence (IIF) with anti-MAD1 localizing MAD1 at the poles of a conidium incubated in SDB for 8 hours. D. IIF with anti-MAD2 of a conidium incubated in bean root exudates (8 hrs) demonstrating that MAD2 is more centrally localized than MAD1. E and F. IIF with anti-MAD1 demonstrating MAD1 production on unicellular (E) and multicellular (F) hyphal bodies harvested from infected M. sexta caterpillars. The panels on the right of E and F show the same cells stained with Calcofluor white. The arrow points to a septum. G and H. MAD1 and MAD2 production by transgenic yeast incubated with galactose for 10 hrs, demonstrating a patchy distribution of MAD1 (G) and an even distribution of MAD2 (H) on yeast cells. The panels on the right of C, D, G and H, show bright field microscopy of the same cells. Bar scale, 5 µm.

Fig. 4. Deletion of Mad1 affects conidial germination, blastospore formation and hyphal body differentiation. Conidial germination: 14 hours post inoculation in SDB medium, germination levels are higher for the wild type (A) than for ∆Mad1 (B). Blastospore formation: when cultured for 3 days in cell free insect hemolymph, the wild type buds off blastospores (C) while ∆Mad1 grows as hyphae (D). Hyphal body differentiation: Manduca sexta larvae were injected with conidia and bled at 10 hr intervals. At 50 hours the wild type (E) had formed variably shaped hyphal bodies with 1-3 cells but the ∆Mad1 mutant (F) had produced long and branched chains of cells (> 5 cells). HB, hyphal body; HC, insect hemocytes. Bar scale, 10 µm.

Fig. 5. Kinetics of insect survivorship in bioassays. Survival of Manduca larvae following topical application with $2 \times 10^7$ conidia/ml suspensions of wild-type, ∆Mad1 or ∆Mad2 strains (control insects were dipped in water). LT$_{50}$ values were 3.92 ± 0.36 days for WT, 5.35 ± 0.22 days for ∆Mad1 and
4.11 ± 0.18 days for ΔMad2. The difference between the wild type and ΔMad1 is significant (t=24.76, P=0.00081). The difference between the wild type and ΔMad2 is not significant (t=2.92, P=0.12).

**Fig. 6.** MAD1 is involved in cytoskeletal organization and cell division. A. FITC-phalloidin fluorescent staining of a wild type conidium incubated in SDB for 6 hrs showed polymerized actin aligning at the poles (the panel on the right shows bright-field microscopy of the same cell). B. Actin in conidia of ΔMad1 also polymerizes but is patchily distributed in the cytoplasm. C to F. Double staining hyphal bodies with FITC-phalloidin (the panels on the left of C to E) and Calcofluor (the panels on the right of C to E). Note that where septa form, the actin cytoskeleton is more clearly delineated between daughter cells in wild type M. anisopliae (C and E) as compared to the ΔMad1 mutant (D and F). G and H. Double staining cells of wild type hyphal bodies harvested from infected insects with DAPI and Calcofluor white showed that they are uninucleate whether comprised of one (G) or more cells (H). In contrast, ΔMad1 hyphal bodies are usually multinucleate, even when septa are formed (I and J). K and L. Immunofluorescent staining of hyphal bodies with an Aspergillus anti-septin B antibody shows the formation of a septin ring at the septum of a wild type cell (K) which is lacking in ΔMad1 cells (L) (the panels on the right show the same cells stained with Calcofluor). The septa are indicated by arrows. Bar scale, 5 µm.

**Fig. 7.** RT-PCR analysis of selected genes involved in cell division. 36-hr SDB cultures of wild type, ΔMad1 or ΔMad2 were transferred into Manduca sexta hemolymph (HE) or 1 % (w/v) root exudate (RE) for 6 hours. The mycelia were harvested for RNA extraction and 1 µg RNA was converted into cDNA for RT-PCR analysis. The selected genes include Metarhizium homologs of Ami1 (AJ274250) involved in nuclear migration, an actin filament organization factor (Act, CN809636), septin B (Sep, AJ274373) and cyclin B (Cyc, CN808863).