Intracellular infection of tick cell lines by the entomopathogenic fungus *Metarhizium anisopliae*

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Several fungal pathogens are able to enter and persist within eukaryotic cells as part of their infectious life cycle. *Metarhizium anisopliae* is a saprophytic entomopathogenic fungus virulent towards numerous tick species, including those within the genera *Ixodes* and *Amblyomma*. Infection of the target organism by this fungus proceeds via several steps, including adhesion and penetration of the host cuticle, proliferation within tissues and the haemolymph, and eventual eruption through the host cadaver. To determine whether *M. anisopliae* could enter and persist within tick cells, we examined the uptake of wild-type and green fluorescent protein (GFP)-expressing fungal strains into two different tick cell lines, IDE12 and AAE2, derived from *Ixodes scapularis* and *Amblyomma americanum*, respectively. Uptake by tick cells was monitored by confocal fluorescent microscopy, as well as by scanning and transmission electron microscopy. *M. anisopliae*-specific antibodies were used to discriminate between extracellular and internalized fungal cells and to quantify the rate of uptake. Both cell lines internalized fungal conidia, and quantitative studies using the AAE2 cell line indicated that almost 70 % of the AAE2 cells contained internalized conidia after 6 h incubation. Internalization of conidia by AAE2 cells was time and temperature dependent and could be inhibited by 80 % with 1 mM cytochalasin D. Internalized conidia remained viable within the AAE2 cells, where they were able to germinate and grow, eventually erupting from the host cells. These data provide evidence that *M. anisopliae* conidia can be internalized, survive and grow within phagocytic cells *in vitro* and indicate that phagocytosis may serve as an alternative invasion route facilitating fungal nutrient acquisition, immune system evasion and dissemination throughout the host.

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INTRODUCTION

Ticks represent important reservoirs and vectors of animal and human infectious disease agents, including viruses, bacteria, fungi and protozoa (Parola & Raoult, 2001; Sonenshine, 1993). Obligate haematophagous arthropods, ticks parasitize almost every class of vertebrates and are found distributed in almost all ecosystems. *Metarhizium anisopliae* is an entomopathogenic fungus that displays a remarkably broad host range, spanning from insects to ticks and other members of the class Arachnida (Chandler *et al.*, 2000; Roberts & Leger, 2004). Infection by this fungus results from direct penetration of the cuticle, using a combination of enzymic and physical mechanisms, without any requirement for injection or specialized mode of entry. Strains of *M. anisopliae* have been shown to be pathogenic towards members of the 'soft tick' Argasidae

family such as the tick fowl *Argas persicargas*, as well as numerous members of the 'hard tick' Ixodidae family (Kaaya & Hassan, 2000; Polar *et al.*, 2005; Samish *et al.*, 2004; Sewify & Habib, 2001). These include tick species from a wide range of genera that are of economic, medical and veterinary importance such as *Amblyomma*, *Rhipicephalus* (*Boophilus*) and *Ixodes* (Fernandes *et al.*, 2003; Kirkland *et al.*, 2004b; Pirali-Kheirabadi *et al.*, 2007). Indeed, specific application methods have been developed for use of entomopathogenic fungi against *Ixodes scapularis*, the carrier of the Lyme-disease-causing spirochaete *Borrelia burgdorferi*, and various livestock ticks, such as *Rhipicephalus* (*Boophilus*) *microplus* and *Amblyomma variegatum* (Benjamin *et al.*, 2002; Bittencourt, 2000; Kaaya, 2000; Maranga *et al.*, 2006). Some tick species, such as *Dermacentor variabilis* and *Amblyomma americanum*, display a level of resistance to fungal infection that can be overcome by specific inoculation conditions that modify the chemical and structural composition of the cuticle (Kirkland *et al.*, 2004a, 2005).

The prevailing pathogenesis model of *M. anisopliae* involves (a) attachment of fungal spores (conidia) to

Abbreviations: SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Three video files of the dual-label time-lapse photography used to study internalization and growth of *M. anisopliae* conidia within tick cells are available with the online version of this paper.

arthropod cuticle, (b) penetration of cuticle via formation of specialized infectious structures known as appressoria and penetrant tubes followed by growth across the surface of the cuticle and within integumental tissues, (c) entry into the haemolymph, (d) reproduction within the haemolymph, producing cells (*in vivo* blastospores or hyphal bodies) that are able to evade the insect immune system and proliferate within the haemolymph, (d) hyphal growth within tissues and out from the arthropod host leading to development of new conidiogenous (spore-forming) cells on the surface of the cadaver, and (e) conidia formation and dispersal from the host. During infection *M. anisopliae* expresses and secretes a wide variety of compounds, including proteases, glycosidases, lipases, peptide mycotoxins and other secondary metabolites, all of which have been implicated as virulence factors (Diaz *et al.*, 2006; Hu & Leger, 2004; Pal *et al.*, 2007; St Leger *et al.*, 1997; Wang & St Leger, 2005, 2006)

Within this framework, an intracellular stage for entomopathogenic fungi has not been characterized. In this study, we demonstrate the uptake and survival of *M. anisopliae* conidia within *A. americanum* and *I. scapularis* tick cells using cell lines established from embryos (Kurtti *et al.*, 2005; Munderloh *et al.*, 1994). Internalization of conidia by *A. americanum* (line AAE2) was accompanied by cytoskeletal rearrangements in AAE2 cells, indicating that fungal cells exploited host phagocytic mechanisms to gain entry. These studies link the general arthropod fungal pathogen *M. anisopliae* to a growing number of pathogenic fungi that can gain entry and survive within eukaryotic cells.

METHODS

Fungal strains and cultures. Fungal strains were maintained on potato dextrose agar (Difco) at 26 °C. *M. anisopliae* 2575 and a green fluorescent protein (GFP) transformed isolate of *M. anisopliae* 2575 was obtained from Dr R. J. St Leger (University of Maryland, College Park, MD, USA).

Antibodies and chemical reagents. *M. anisopliae* mycelial and conidial cell wall extracts were used as antigens for the production of polyclonal antibodies that recognize conidia. Fernbach flasks containing 200 ml Sabouraud dextrose broth supplemented with 0.5 % yeast extract were inoculated with $5\text{--}10 \times 10^6$ conidia ml⁻¹ and incubated at 125 r.p.m. for 18–24 h at 26 °C. The fungal cells were harvested by centrifugation (5000 g, 20 min), washed once with distilled H₂O, and resuspended in 10 ml deionized H₂O. The cell suspension was then autoclaved for 15 min and the resultant suspension used for production of polyclonal antibodies in rabbits (antibodies were produced by Cocalico Biologicals). To test serum for the presence of antibodies recognizing *M. anisopliae*, conidia were added to 12-well microtitre plates and reacted with various dilutions of rabbit serum in PBS containing 10 % (v/v) goat serum. Bound primary antibody was detected by using Alexa Fluor 647 goat anti-rabbit (highly cross-absorbed) secondary antibody (Molecular Probes) diluted 1:200 in PBS-goat serum. Conidia were fixed with 2 % paraformaldehyde in PBS prior to microscopy.

Tick cell lines. Tick cell lines AAE2 and IDE12 (source of cell lines T. J. Kurtti) were derived from *Amblyomma americanum* and *Ixodes*

scapularis, respectively, and maintained as described previously (Kurtti *et al.*, 2005; Munderloh *et al.*, 1994). Cells were grown in either standard L15B or L15Bd medium (L15B diluted by one-fourth water/vol.) containing 5 % fetal bovine serum (Sigma), 5 % tryptose phosphate buffer (Difco) and 0.1 % lipoprotein (ICN) (referred to as complete L15B medium). Cells were kept in vented capped tissue culture flasks (typically 25 cm²) at 30 °C, 5 % CO₂ and split (typically 1:20) into fresh medium when confluent.

Uptake assay. AAE2 and IDE12 cells were seeded onto 12 mm diameter glass coverslips placed in microtitre plates (6-, 12- or 24-well; Falcon, Becton Dickinson) at $2\text{--}5 \times 10^5$ cells ml⁻¹ and grown for 12–16 h at 30 °C. Following cell growth, wells were blocked for 1 h with fresh L15B or L15Bd medium containing 0.1 % BSA. Cells were infected with spores diluted in complete L15B ($0.1\text{--}1 \times 10^8$ spores ml⁻¹) to the desired m.o.i. (m.o.i.: spore to tick cell ratio), ranging from 1:1 to 20:1. To prepare heat-killed spores, conidia in distilled H₂O were first autoclaved for 15 min at 121 °C. Internalization of fluorescent beads (1 µm, Fluorospheres polystyrene microspheres, red fluorescent, 580/605; Invitrogen-Molecular Probes) was used as an uptake/phagocytosis standard for the tick cell lines. To quantify the percentage of internalization, uptake assays were performed with samples (coverslips) removed at desired time points (0, 30 min, 1–8 h) and placed into PBS containing 2 % paraformaldehyde. External fungal cells were labelled with *M. anisopliae*-specific antibody reactions as described above. At least two replicates (coverslips) were examined per time point, with a minimum of three fields of view representing at least 100 host cells counted per coverslip. Each experiment was performed with at least three independent batches of cells.

Double labelling. LysoTracker Red DND-99 (Invitrogen) was used to stain tick cell lysosomal compartments. Briefly, the medium from wells containing cells to be treated was aspirated and replaced with fresh medium containing 100 nM LysoTracker Red. Cell uptake of the LysoTracker reagent was allowed to proceed at 30 °C for 30–60 min, after which the medium containing the dye was replaced with fresh medium (without the dye). Cells were used immediately for infection by (GFP-expressing) *M. anisopliae*.

Measurement of conidia survival in AAE2 cells. AAE2 cells were seeded at $1\text{--}5 \times 10^5$ cells ml⁻¹ in 24-well plates and grown for 12–16 h. Cells were infected (m.o.i.=2–5:1) with conidia harvested in complete L15B medium. After incubation at 30 °C for the desired time, unbound spores were removed by washing the wells three times with PBS/0.05 % Tween 20. Extracellular conidia were separated from internalized conidia by Centricoll density-gradient centrifugation. Infected tick cells were harvested and immediately applied on top of a step gradient of 25 and 50 % Centricoll (where 100 % Centricoll was defined by 9 volumes of pure Centricoll + 1 volume of 2.5 M sucrose, following the manufacturer's instructions). Centrifugation was performed in sterile 2 ml tubes, at 10 000 g for 10 min at 4 °C using a tabletop centrifuge. Tick cells (including those harbouring fungal conidia) were separated from external fungal cells and were collected within the top portion (25 % step) of the gradient (fungal conidia pelleted to the bottom of the tube). Host cells were lysed with 0.5 % Triton X-100 and serial dilutions of released conidia were plated onto either Sabouraud dextrose or potato dextrose agar. At least three replicates were plated per sample, with a minimum of 100 conidia counted per plate. Each experiment was performed with at least three independent batches of cells.

Microscopy. Live and fixed cell imaging was performed using either a Zeiss Axiovert Pascal LSM5 inverted microscope equipped with a laser scanning unit (for GFP, ex. 488 nm, em. 505 nm) or a Nikon Diaphot (Chroma EN GFP filter 41017) instrument. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed using Hitachi S400 and Zeiss EM10-CA

Fig. 1. Phase-contrast microscopic images of tick cell lines used in this study. (A) *Ixodes scapularis* cell line IDE12. (B) *Amblyomma americanum* cell line AAE2. Bars, 30 μ m.

electron microscopes, respectively. Time-lapse video was obtained using a TE2000-U inverted microscope (Nikon) using epifluorescent illumination and piezo-actuated z movement (Mad City Laboratories). Tick cells and GFP-expressing *M. anisopliae* were cultured in 35 mm glass bottom culture dishes (MatTek). A cascade 1K (Photometric) camera was used to collect monochrome serial z planes. Images collected from the red and green emission channels were processed by maximum projection of z planes and adjustment look-up table using Metamorph (version 7.1, Molecular Devices).

RESULTS

Tick cell lines derived from *I. scapularis* (IDE12) and *A. americanum* (AAE2) were cultured in complete L15B, and infected with conidia of a GFP-expressing strain of *M. anisopliae* (2575) suspended in the same medium. The tick cell lines adhered to glass or polystyrene and were motile (Fig. 1). These two cell lines derive from fragmented

Fig. 2. Internalization of *M. anisopliae* conidia by tick cell lines. Differential interference contrast (A, B), fluorescent (C, D), and merged (E, F) images of tick cell lines derived from AAE2 (A, C, E) and IDE12 (B, D, F) incubated with *M. anisopliae* GFP-expressing conidia at an m.o.i. of 3–4 : 1 (conidia : tick cells) for 2 h. Bars, 10 μ m.

Fig. 3. SEM images of internalization of *M. anisopliae* conidia by the AAE2 tick cell line 10 min (A) and 30 min (B) post-inoculation. Membrane ruffling can be noted around the area of conidium–tick cell contact (A). Small arrows (in B) indicate tick cell membrane enveloping conidial cells (large arrows). Bars, 1.2 μ m.

embryos isolated from developing tick eggs (Munderloh *et al.*, 1994); consequently their tissue of origin is unknown. We selected these lines because previous studies had demonstrated their ability to phagocytose tick-borne bacteria (Kurtti *et al.*, 2005; Mattila *et al.*, 2007) and because of the susceptibility of *I. scapularis* and *A. americanum* to infection by conidia of *M. anisopliae* (Kirkland *et al.*, 2004a, b).

Fluorescent microscopy indicated uptake of fungal conidia in both tick cell lines (Fig. 2). Superimposition of differential interference contrast and fluorescent microscopic images of the same sample showed co-localization of fungal cells with tick cells, with increasing numbers of fungal conidia co-localizing with the tick cells over time. Three-dimensional (Z-stack) imaging was consistent with internalization of the conidia within tick cells. Co-localization was apparent after 30 min incubation, and experiments using infection ratios ranging from 1 to 20 fungal conidia per tick cell gave essentially similar results, with greater numbers of internalized conidia per tick cell visible as the fungal to host cell ratio (m.o.i.) increased.

In order to prove the internal nature of the fungal cells, SEM and TEM were employed. SEM images of the interactions between fungal conidia and the *A. americanum* cells indicated initial adhesion and subsequent engulfment of the fungal spores by the host cells (Fig. 3). Host cells appeared to undergo membrane ruffling during the interaction with the fungal spores, and often engulfed multiple conidia. Initial contact and possible adhesion was noted (Fig. 3A) and the tick cell membrane appeared to extend and wrap around the conidia (Fig. 3B; small arrows). Treatment of tick cells with cytochalasin D resulted in altered tick cell morphology (contraction of pseudopodia and rounding), and few instances (less than 10 % relative to untreated control cells) of engulfment were observed by fluorescent microscopy and in SEM samples.

Internalization of fungal conidia by AAE2 cells was confirmed by TEM, in which adherent conidia were engulfed by the tick cells within 30 min after inoculation and the wrapping around of fungal conidia by tick cell membrane was noted (Fig. 4A). In most instances, internalized conidia appeared to be enclosed within an endosomal compartment, although this was not always apparent. Similar results were obtained using the IDE12 cell line. Qualitative analysis indicated that uptake increased over time, with tick cells often infected by multiple fungal conidia (Fig. 4B, C). Some tick cells were clearly in large vacuoles (Fig. 4C, large arrows), although a membrane surrounding all internalized fungal conidia was not always apparent (Fig. 4B, C, small arrows). Using an m.o.i. of 3:1, after 3 h, almost 40 % of the tick cells contained multiple conidia.

In order to determine whether internalized fungal cells could grow within the tick cells, uptake was allowed to proceed for 2–3 h before external fungal cells were removed by aspiration, the adherent cells were washed twice, and the medium replaced with fresh complete medium. At time points >3 h, it was noted that external *M. anisopliae* cells also appeared to adhere to the glass and/or polystyrene substrata. Washed tick cells harbouring internalized conidia were incubated for an additional 12–24 h at 30 °C. Fungal hyphae were observed having germinated within tick cells by TEM (Fig. 4D) and growing out from both the AAE2 and IDE12 tick cell lines by fluorescence microscopy (Fig. 5), and in several instances multiple hyphae were seen emerging from the same host cell. For some samples, extensive fungal growth was observed and the underlying tick cells were barely visible. Experiments in which the fungal antibiotics nystatin or amphotericin C were added to the washed cells (in order to minimize growth of any remaining external fungal conidia) gave essentially the same results.

In order to quantify and measure the rate of uptake of the fungal cells by the tick cell lines, *M. anisopliae*-specific antibodies were used to discriminate between external and internalized fungal cells. Using an m.o.i. of 3:1, the percentage of tick cells with internalized conidia rapidly

Fig. 4. TEM images of internalization of *M. anisopliae* conidia by the AAE2 tick cell line at various times post-incubation of fungal conidia with the tick cells. (A) 10 min; the arrow indicates a cross-section of a fungal conidium. (B) 30 min; the small arrow indicates a fungal cell with no clear surrounding membrane. (C) 2 h; the small arrow indicates a cell with no clear surrounding membrane; the large arrows indicate fungal cells in large vacuoles. (D) 16 h; the arrow indicates a cross-section of a germinated conidium inside a tick cell.

increased, reaching ~70 % within 8 h of co-culturing (Fig. 6). Uptake was temperature dependent and cells incubated at 4 °C showed only 20 % internalization after 8 h. Treatment of the tick cells with the actin polymerization inhibitor cytochalasin D decreased uptake to a similar extent as incubation at 4 °C. In order to determine whether conidia that were internalized remained viable, tick cells harbouring fungal cells were gently lysed using Triton X-100. The recovered fungal cells were spread onto agar plates and the percentage germination determined as described in Methods (Fig. 7). These data indicated that most of the fungal cells remained viable when internalized by the tick cells.

Internalized conidia were able to grow within the tick cells and SEM images of tick cells harbouring fungal conidia 12–24 h post-inoculation revealed fungal hyphae emerging from the tick cells (Fig. 8). In several instances, during the

early stages of this growth, tick cell membrane could be seen covering the fungal growth, i.e. extending over the growing hyphae (Fig. 8A, B). Hyphal growth out from cells was clearly apparent (Fig. 8C, D) and in several instances a clear distinction between the tick cell membrane and the growing (protruding) hyphae was visible (Fig. 8E, F).

Dual-label time-lapse photography was used to gather dynamic information concerning the process of internalization and growth of the fungal conidia within the tick cells. Three video files spanning the whole time frame of ~18 h are available as supplementary data with the online version of this paper; images from the videos are presented in Fig. 9. Lysosomal compartments of the tick cells were labelled with the fluorescent acidotropic probe LysoTracker Red as described in Methods, and these cells were subsequently incubated with GFP-expressing *M. anisopliae* conidia. These data revealed a dynamic interaction between

Fig. 5. Germination and hyphal growth of *M. anisopliae* within the AAE2 and IDE12 tick cell lines. Host cells were incubated with *M. anisopliae* conidia for 3 h and external conidia removed as described in Methods. Tick cells (harbouring fungal conidia) were placed back in complete L15B medium and incubated for 20–22 h. (A–D) AAE2 cell line; (E, F) IDE12 cell line (F is a fluorescent image of E). The arrows in (E) and (F) indicate hyphae emerging from cells. Bars, 10 µm.

the fungal conidia and the host cell. Fungal conidia could be observed entering tick cells, with multiple conidia often ‘infecting’ single cells. Intriguingly, no apparent fusion of lysosomes with the presumed conidial-phagocytic endosome was observed, although our observations were qualitative. In several instances, LysoTracker-labelled vesicles in the tick cells could be observed to be ‘pushed’ aside as the conidium moved inside the cell (Fig. 9A–D, and supplementary videos). The conidia were observed to germinate and grow within the tick cells, and in some instances several growing fungal hyphae were clearly visible inside (and eventually protruding from) a single tick cell (Fig. 9E–H). Although this was a qualitative experiment, within the time span examined (18 h) no clear instances of tick cell lysis were observed. Tick cells harbouring growing

Fig. 6. Rate of uptake of *M. anisopliae* conidia by the AAE2 tick cell line. The mean percentage ($\pm$ SD) of tick cells containing one or more conidia was determined over the indicated time-course as described in Methods. ●, AAE2 cells + *M. anisopliae*, 30 °C; □, AAE2 cells + *M. anisopliae*, 4 °C; ◇, AAE2 cells + *M. anisopliae* + 10 µM cytochalasin D, 30 °C.

hyphae remained motile (see supplementary videos) and appeared to contain intact nuclei as determined by DAPI staining of fixed cells (data not shown).

DISCUSSION

The entomopathogenic fungus *M. anisopliae* is pathogenic towards a wide range of insect and tick species. Current models of fungal virulence do not include a possible intracellular state or stage. In this study, we used wild-type and GFP-transformed strains of *M. anisopliae* to investigate internalization, survival and growth of conidia within cultured tick cell lines derived from *I. scapularis* and *A. americanum*. Uptake was inhibited at low temperatures and by cytochalasin D, indicating that host phagocytic mechanisms mediated uptake of the *M. anisopliae* cells.

Fig. 7. Rate of survival of internalized fungal conidia within tick cell lines. The mean percentage ($\pm$ SD) of fungal conidia germinating after internalization by host tick cells, AAE2 (●) and IDE12 (■), over the indicated time-course was determined as described in Methods.

Fig. 8. SEM images of growth and emergence of *M. anisopliae* hyphae from AAE2 tick cells. Tick cells were infected with *M. anisopliae* conidia at an m.o.i. of 5 : 1 (conidia : tick cells). After 3 h, external conidia were removed by aspiration and host cells incubated with fresh L15Bd medium and incubated at 30 °C for an additional 10 h (A, B; arrows point to apparently growing hyphae within the tick cells still surrounded by tick cell membrane) or 14 h (C–F; large arrows point to emergent hyphae; the small arrows in F indicate the apparent limit of the tick cell membrane). Bars, 2.5 μm.

Electron microscopy revealed that most conidia appeared to be in host phagosomes, and it is interesting to note that acidification of phagosomes is not likely to pose a serious challenge to *M. anisopliae*, since this fungus (like many fungi) can grow at about pH 3–4. Internalization of conidia by tick cell lines was monitored in real time by time-lapse photography using a dual-labelled system that revealed a dynamic and robust interaction between the fungal conidia and the tick cells. Based upon the time-lapse photography of the internalization process, in which infected ticks cells appeared to remain intact and motile, the tick cells remained apparently viable even as the fungus was growing within and out from the host cells, although further experiments are needed to confirm this observation.

A number of cases of intracellular parasitism by fungi have been examined to date. Perhaps the best-known examples of intracellular parasitism of arthropod cells are the microsporidia. Microsporidia are unicellular eukaryotes that are obligate parasites of a variety of animals. Although they are classically not considered part of the fungal Kingdom, recent, though still controversial, phylogenetic analyses derived from genome datasets place microsporidia as a sister to a combined ascomycete + basidiomycete clade (Gill & Fast, 2006; Keeling *et al.*, 2005). Other unique fungal–host relationships in which intracellular residence

of fungal cells has been noted include certain types of mycorrhiza and dark septate endophytes (DSE) (Harley, 1992; Jumpponen, 2001; Smith & Read, 1997). Although these relationships have not been extensively studied, and the benefit and/or significance of the intracellular state is unknown, the associations appear to be limited to specific root cells of the host plant. Similarly, since our data are derived from *in vitro* cell cultures, at this stage it is unclear what is the physiological and/or environmental role that intracellular survival may play in the life cycle of the fungus; however, future experiments looking for such a stage, possibly in the respiratory (tracheal) or digestive tissues of insects and/or ticks, are warranted.

Interestingly, a number of fungi that are considered as human pathogens are able to survive within host cells (mainly within professional phagocytes or macrophages). *Histoplasma capsulatum* is a dimorphic fungal pathogen, whose essentially non-pathogenic conidia (spores), when inhaled, convert into a yeast form inside the body (terminal bronchioles and alveoli of the lung) (Newman *et al.*, 2006). The yeast forms are phagocytosed by alveolar macrophages, and are able to survive and multiply within the macrophages. The encapsulated fungus *Cryptococcus neoformans* is also able to survive and multiply within macrophages (Chang *et al.*, 2006). Although not an obligate intracellular

Fig. 9. Time-lapse photography of double-label samples examining uptake and growth of *M. anisopliae* within AAE2 tick cells. Host-cell lysosomal compartments were labelled with LysoTracker Red and incubated with GFP-expressing *M. anisopliae* as described in Methods. Images taken at approximately 10 min (A), 17 min (B), 21 min (C), 24 min (D), 5.5 h (E), 8 h (F, G) and 16.5 h (H) post-incubation of host cells with fungal conidia. Three video files spanning the whole time frame of ~18 h are available as supplementary data with the online version of this paper. The arrow in A–D tracks a single conidium entering a tick cell. Note the two small (red) labelled tick vesicles that appear to be pushed out of the way as the fungal cell proceeds through the tick cell. The arrows in E–G indicate germinating conidia.

pathogen, it has the ability to reside in acidic phagolysosomes of human macrophages. *Aspergillus fumigatus* is an opportunistic pathogen responsible for a number of respiratory diseases in normal hosts and severe invasive infections in immunocompromised patients (Latgé & Calderone, 2002). Although in this instance alveolar macrophages and neutrophils are known to phagocytose and kill *Aspergillus* conidia, a small percentage may be able to survive within these cells; moreover, it has been reported that fungal conidia may attempt to escape from professional phagocytes by invading epithelial and endothelial cells (Ibrahim-Granet *et al.*, 2003; Wasylnka & Moore, 2003; Wasylnka *et al.*, 2005). Furthermore, *Aspergillus* infections can cause significant cell injury during invasion, producing tissue damage that can induce host cell defences, with escape into normally non-phagocytic host cells by induction of fungal cell uptake serving as a mechanism to avoid such defences (Lopes Bezerra & Filler, 2004; Filler & Sheppard, 2006).

Unlike that of insects, the tick digestive system consists of highly phagocytic cells that import nutrients from the blood meal (Sonenshine, 1993). Although the exact nature

of the tick cell lines we used is unknown, these cells are phagocytic, and *M. anisopliae* conidia appear to be able to subvert the phagocytic process to gain entry into the cells. The benefits that intracellular colonization would confer to these fungi are several. It should be noted that almost all studies examining the process of fungal-mediated pathogenesis towards arthropods have used inocula containing high numbers of fungal spores (10^6 – 10^9 fungal cells ml^{-1}) sprayed onto or directly applied to the insects. Aimed towards biological control and other applied aspects of these fungi, these conditions are unlikely to reflect the natural (and hence under evolutionary pressures) life cycle of these organisms. In the environment, fungal concentrations are never very high (Quesada-Moraga *et al.*, 2007). How then, can a small inoculum thwart insect defences including behavioural responses such as grooming and heat seeking? Although further research is needed to make such a conclusion, one mechanism could be intracellular colonization. This lifestyle or adaptation would ensure access to nutrients, allow for some level of protection from host responses, and ensure persistence within the host. Intracellular colonization may also allow for a type of

latency in which the parasite can assess the health of the host. In such instances, stress, nutrient depletion, or other factors may then signal rapid fungal growth, leading to the death and classic desiccation and mummification of the host observed with these fungi. It should be noted that although we have examined the uptake of conidial cells, *Metarhizium* conidia infect via the cuticle and would not normally be present in the haemolymph. Future studies examining intracellular invasion by different developmental stages adapted to the haemolymph, such as blastospores and hyphal bodies, are therefore warranted. Furthermore, an examination of survival within host cells using available *M. anisopliae* microarrays should yield important information concerning the genes and their protein products that may mediate these processes. Finally, since pathogenicity to invertebrates is represented by primitive fungi and is postulated to have arisen at least within the same ancestral time frame of both saprophytism and pathogenicity to plants (Berbee & Taylor, 2001), our data may have important implications regarding the evolution of intracellular invasion by fungi.

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