



Fungal dimorphism in the entomopathogenic fungus *Metarhizium rileyi*: Detection of an *in vivo* quorum-sensing system



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ABSTRACT

This investigation documents the expression of the *in vivo* dimorphic program exhibited by the insect mycopathogen *Metarhizium rileyi*. This insect mycopathogen represents the key mortality factor regulating various caterpillar populations in legumes, including subtropical and tropical soybeans. Using two hosts and *M. rileyi* isolates, we have measured *M. rileyi* growth rates under *in vivo* and *in vitro* conditions and have assessed the pathogen's impact on host fitness. Significantly, the hyphal bodies-to-mycelia transition that occurs at the late infection stage is regulated by a quorum-sensing molecule(s) (QSM) that triggers hyphal bodies (Hb) to synchronously switch to the tissue-invasive mycelia. Within hours of this transition, the host insect succumbs to mycosis. The production of the QS chemical(s) occurs when a quorum of Hb is produced in the hemolymph (late-stage infection). Furthermore, the QS activity detected in late-stage infected sera is unique and is unrelated to any known fungal QSM. The lack of similar QS activity from conditioned media of *M. rileyi* suggests that the chemical signal(s) that mediates the dimorphic switch is produced by host tissues in response to a quorum of hyphal bodies produced in the host hemolymph. The serum-based QS activity is retained after lyophilization, mild heat treatment, and proteinase digestion. However, attempts to extract/identify the QSM have been unsuccessful. Results suggest that the observed hyphal body-to-mycelia transition is a multi-step process involving more than one chemical signal.

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1. Introduction

Fungal pathogens are recognized as important disease-causing agents of insects. Virtually all insects host a complex of fungal diseases; representative insect mycopathogens can be found in all the major taxa of mycota (Boucias and Pendland, 1998). Many insect mycopathogens have homologues to species that infect plant and animal hosts. Unlike other insect pathogens (viruses, bacteria, microsporidia, algae, protists) that are typically transmitted *per os*, the majority of insect mycopathogens penetrate the cuticle barriers of healthy hosts to gain ingress into the hemocoel. In light of their unique attributes, extensive research has examined these pathogens both as microbial controls of medical and agricultural insect pests and as potential sources of biologically active metabolites (Roy et al., 2010; Khan et al., 2012).

Similar to zoopathogenic fungi (Szaniszlo, 1985), many insect-pathogenic hyphomycetes, including species within the genera

Metarhizium, *Beauveria*, and *Isaria*, exhibit a defined *in vivo* dimorphic developmental program (Boucias and Pendland, 1998). This program involves switching between apical and budding growth, which provides mycopathogens with both tissue-invasive and vegetative growth capabilities. The budding, yeast-like vegetative cells absorb nutrients in the hemocoel without apparent damage to tissues, allowing the insect to continue to feed and develop. The trigger(s) responsible for the switch from the budding to the apical growth program for these insect mycopathogens is unknown. However, the ability to switch cell phenotypes is crucial for successful *in vivo* development. It should be noted that continued passage of some species under *in vitro* conditions selects for strains that lose the capability to switch; these attenuated strains exhibit lowered pathogenicity to their homologous insect hosts (Morrow et al., 1989). *Metarhizium (Nomuraea) rileyi*, a mycopathogen that exhibits a dimorphic life style under both *in vitro* and *in vivo* conditions (Pendland and Boucias, 1997) was selected for detailed studies on the yeast-to-mycelial transition.

In nature, the fungus *M. rileyi*, previously known as *Nomuraea rileyi* (Kepler et al., 2014), is a pathogen of lepidopteran larvae

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and, under proper environmental conditions, represents the key mortality factor regulating caterpillar populations in various crops, including subtropical and tropical soybeans (Kish and Allen, 1978). *M. rileyi* exhibits a defined developmental program that involves the sequential production of cellular phenotypes designed to perform spatially and temporally unique functions. The initial stage of the infection involves passively dispersed hydrophobic conidiospores that are encased in rodlet bundles comprised of hydrophobins that bind to hydrophobic surfaces such as that found on the insect epicuticle (Boucias et al., 1988). Impregnated within the conidial surface coat are hydrolytic enzymes that, under moist conditions, will actively degrade the epicuticle, producing substances that serve as germination triggers. The second stage of development involves the production of apical-growing penetrant hyphae or germ tubes, which release a cocktail of cuticle-degrading enzymes that mediate the ingress of the germ tube through the multilaminar insect cuticle (St Leger et al., 1986, 1987). The ability of these fungi to breach the cuticle barrier is the key pathogenic determinant that dictates their ability to infect and replicate in host insects.

Upon reaching the nutrient-rich hemolymph, the penetrant germ tubes switch from an apical to a budding growth program (Boucias and Pendland, 1982) leading to the formation of freely circulating hyphal bodies (Hb). These cells lack pathogen-associated molecular patterns and produce cell wall surface epitopes that are not recognized as non-self by either insect opsonins or circulating phagocytic cells (Boucias and Pendland, 1998; Pendland and Boucias, 1991). The yeast-like hyphal bodies grow exponentially in the nutrient-rich hemolymph, reaching densities that far outnumber circulating hemocytes (Lopez-Lastra and Boucias, 1994). When a critical threshold density is achieved, these hemolymph-borne cells revert synchronously to an apical growth program, forming the tissue-invasive mycelial (Myc) cell phenotype. The hyphal tips of these cells are characterized by being coated with a layer of laminin-binding material that attaches each converting cell to the basement membrane that encases insect tissues (Pendland et al., 1994). The ensuing mycelial (tissue-invasive) phase produces and secretes a suite of metabolites that modulates host development, rapidly kills the host, and efficiently digests insect tissue, leading to the mummification of infected larvae. For example, under *in vitro* conditions the mycelial phase of *M. rileyi* produces biologically active levels of ecdysteroid-22-oxidase (Kiuchi et al., 2003). This enzyme inactivates the insect-molting hormone ecdysone and prevents metamorphosis, a process that is detrimental to *M. rileyi* development. Within hours of the transition of Hb to mycelia, the host insect is killed and mummified by the extensive mycelial development of the pathogen. The mycelial phase, like the penetrant germ tube, releases a complex of hydrolases that lyse and digest insect tissues, providing nutrients to the rapidly developing mycelia. In addition, the mycelial phase produces large quantities of exocellular β 1-3 glucan moieties believed to function in the preservation of the mycosed insect (Latgé et al., 1988), as well as other metabolites such as ergosterol peroxide (Prompiboon et al., 2008). This latter compound has been reported to inhibit melanization (Keishi et al., 2001); products of this cascade (quinones) are detrimental to conidiophore development (Cerenius and Söderhäll, 2004; Yassine et al., 2012; Kim et al., 2013). Depending on the microclimate, mycelial cells will then either egress through the epicuticle and differentiate into specialized conidiophore cells that produce numerous infectious conidiospores (Boucias and Pendland, 1982) or produce thick-walled resting structures designed for long-term survival (Pendland, 1982).

In other fungal systems, multiple inputs, including changes in nutrition, aeration, pH, and temperature, affect morphogenesis and define the cell phenotype (Klein and Tebbets, 2007; Nadal

et al., 2008). In a few cases, the switch in cell phenotype has been correlated with both cell population density (quorum density) and the production of autoinducers that mediate switches in the developmental programs that modulate either cell behavior or phenotype (Albuquerque and Casadevall, 2012; Han et al., 2011; Nickerson et al., 2006, 2012). To date, several quorum-sensing molecules (QSMs) that mediate fungal development have been purified and identified from conditioned media. These include the isoprenoid farnesol and farnesoic acid, compounds that block the yeast-to-mycelia transition in various strains of the polymorphic fungus *Candida albicans* (Hornby et al., 2001; Oh et al., 2001). Farnesol also plays a role in dispersing cells from established biofilm (Alem et al., 2006). Farnesol added to *C. albicans* cells may inhibit initial biofilm formation or, if added to mature biofilms, may stimulate the production of progeny yeast (planktonic) cells that invade new microhabitats (Cao et al., 2005; Mosel et al., 2005). It should be noted that quorum-sensing (QS) behavior may be host-/strain-specific. For example, the different isomers of farnesol applied at different concentrations trigger variable responses and may display fungistatic and fungitoxic effects (Albuquerque and Casadevall, 2012). A second identified fungal QSM is tyrosol, a phenolic antioxidant detected in conditioned medium, a compound that acts as an accelerator by shortening the lag time required by *C. albicans* to enter the exponential growth phase and by stimulating the production of filamentous cells comprising *C. albicans* biofilms (Chen et al., 2004). It should be noted that other QS activities have been reported in other fungal systems but the key QSMs have yet to be identified.

In the following investigation, we detail the hyphal body-to-mycelia transition of *M. rileyi* in insect hosts and provide evidence for quorum-sensing using an *in vitro* assay to monitor the dimorphic switch. Furthermore, we provide indirect evidence that the QS activity detected in late-stage infected sera is unique and is unrelated to any known fungal QSM molecule. The lack of similar QSM activity from conditioned media of *M. rileyi* suggests that the chemical signal(s) that mediates the dimorphic switch is produced by host tissues in response to a quorum of hyphal bodies produced in the host hemolymph.

2. Materials and methods

2.1. *In vitro* growth studies

The *M. rileyi* strains F174, F178-6, Nr26b, and Nr5772 used in these assays were isolated from mycosed *Anticarsia gemmatalis* larvae collected over the past four decades in soybeans (Gadsden Co. and Alachua Co., FL). For the duration of this study, these fungi were maintained on Sabouraud maltose supplemented with yeast extract (2%)(SMY) agar and on 3% fish-soluble extract, 1% maltose, 1% yeast extract, and 1.2% agar plates (FMAY, Prompiboon et al., 2008). In addition to solid media, fungi also were cultured in a series of liquid media, including Vogels N broth, McCoy's broth (10 g glucose, 5 g sucrose, 5 g yeast extract, 2.5 g peptone and 12.5 g agar per liter), SMY broth supplemented with yeast extract (2%)(SMY), SF-900 II SFM (Gibco), and Serum-Free Insect Medium (Orbigen).

2.2. Insect bioassays

In vitro hyphal bodies (Hb) of the Nr5772 strain were produced by inoculating SMY broth and shaking for 3–4 days incubation at 26 °C at 200 rpm. Hb were collected from broth cultures by filtration through Miracloth™ (Calbiochem®), pelleted and suspended in sterile phosphate-buffered saline (pH 7.2, PBS), and counted with a hemacytometer. A total of 5×10^3 Hb in 2.5 μ l were injected into the hemocoel of lab-reared, weighed, early-to-late fifth instar

Spodoptera exigua (Hübner) larvae (Fig. S1). For comparative purposes, additional cohorts of late-instar cabbage looper, *Trichoplusia ni* (Hübner), also were injected with Hb preparations. Control larvae were injected with sterile PBS. Treated larvae were placed on a pinto bean artificial insect diet (Guy et al., 1985) and incubated at 26 °C. Groups of 20–25 larvae were injected either with PBS or the Hb suspension in replicate assays.

At various times post-injection (pi), cohorts of treated and control larvae were weighed and selected for examination. A posterior proleg of individual larvae was amputated and hemolymph collected onto a sheet of parafilm placed on crushed ice. A total 75–150 µl hemolymph were withdrawn from each larva; the volume depended on larval size and time of sampling. Twenty µl of each hemolymph bleed was added to an equal volume of anticoagulant buffer and used for subsequent microscope observation. Aliquots of hemolymph were centrifuged immediately for 5 min at 5000 g at 4 °C and resulting supernatants collected and frozen at –80 °C for subsequent biochemical analysis. Additional hemolymph samples were collected from larvae at late-stage infection (72–90 h) and pooled into tubes containing several crystals of the antioxidant phenylthiourea. These samples were centrifuged and supernatants frozen at –80 °C for subsequent assay and fractionation.

An additional set of insects was injected either with buffer or hyphal bodies, then re-injected subsequently at 1, 2, and 3 days post-challenge with an aliquot containing 1×10^5 Fluoresbrite carboxylate polystyrene microspheres (diameter 1.75 µm) (Polysciences Inc., Warrington, PA). Within one hour post-injection, hemolymph was withdrawn and examined using epifluorescent optics and imaged with Insight digital camera fitted with SPOT software (Diagnostics Instruments Inc., MI).

2.3. Microscopy

Hemolymph samples diluted in anticoagulant buffer were loaded onto a Bright-Line hemocytometer to determine the relative densities of hemocytes and fungal cells. Late-stage samples, due to amplification of the fungus, required additional dilution in anticoagulant buffer prior to quantitation. In addition to hemacytometer counts, samples were examined under DIC optics and imaged with Insight digital camera.

2.4. Bioassay of collected sera samples

The effects of insect hemolymph on fungal development were assessed under *in vitro* conditions (Fig. S1). Both individual samples and pools of hemolymph were collected from fungus-challenged (5×10^3 Hb/larva) and control larvae (buffer-injected) at different times (24–72 h pi, see above). Over the course of this study, a minimum of 10 pooled samples/time interval were collected and processed from healthy and infected larvae. Hemolymph samples, collected in microcentrifuge tubes containing several crystals of phenylthiourea, were subjected to centrifugation at 5000 g for 5 min at 4 °C to pellet fungal cells and/or hemocytes. The supernatants were filter-sterilized by centrifugation in Ultrafree[®] MC Millipore microcentrifuge tubes with a low protein-binding Durapore[®] membrane, 0.45-µm filter unit (Millipore, Billerica, MA) at 16,000 g for 10 min at 4 °C and filtrates frozen at –70 °C until assayed.

Cell culture assays were conducted in sterile 96-well microtiter plates that contained 5×10^3 Hb/well of the four *M. rileyi* strains suspended in either SF-900 II SFM or Serum-Free Insect Medium (Orbigen). Both of these cell culture media supported Hb replication in stationary culture. Each filtered hemolymph serum sample was added to the first well at a 5% concentration then diluted two-fold through a series of 6–8 wells. Controls included both wells inoculated with filtered serum collected from PBS-injected larvae

and wells inoculated with an aliquot of PBS. Plates, incubated at 26 °C, were examined after 24 and 48 h with Hoffman Modulation optics to assess cell phenotypes. The degree of conversion was ranked from 1 to 4; with (1) being 100% Hb, (2) containing >75% Hb, (3) having <50% Hb, and (4) containing >90% transitioned mycelia.

2.5. *In vitro* assay of potential quorum-sensing chemistries

In light of published reports, the ability of reported QSMs (mixed isomer and trans, trans-farnesol and tyrosol (2-(4-hydroxyphenyl) ethanol), Sigma–Aldrich) to suppress or induce the Hb → mycelia transition were assayed under *in vitro* conditions (see above). Stock solutions (25 mM) prepared in 10% DMSO were added to wells containing Hb suspended in serum-free medium or in Vogels N and diluted 2-fold, producing a range of test concentrations. A second series of assays involved adding test materials to Hb in serum-free media amended with 5% concentration late-stage infected sera determined previously to elicit *in vitro* the Hb-to-mycelia transition. In addition to compounds reported to possess quorum-sensing activities, other test chemistries, including insect hormones (JH I, II, III, methoprene, methyl farnesoate, and ecdysone, stocks at 500 µM Sigma–Aldrich), *M. rileyi* metabolites β-1,3 glucan at 10 mg/ml (Latgé et al., 1988), and ergosterol and ergosterol peroxide at 500 µM (Prompiboon et al., 2008) were screened for QSM activity for *M. rileyi*.

2.6. Protein analysis

Total protein levels in sera samples, diluted in 50 mM phosphate buffer (pH 7.6), were estimated using Pierce[™] BCA protein assay kit (Thermo Scientific, Rockford, IL). The relative protein levels were estimated from standard curves generated with a BSA standard. Additional aliquots of sera were assessed on commercial 6–15% gradient SDS-PAGE gels following the protocol outlined for Criterion[®] TGE gels (BioRad Corp, Hercules, CA). Resulting peptide bands were visualized with the Ruby Red[®] stain (Life Technologies Invitrogen[™], Grand Island, NY) and digital images captured on GelDoc XR system (BioRad). Precision[®] molecular weight reference standards (BioRad) were included to estimate relative size of hemolymph peptides.

2.7. Treatment and fractionation of hemolymph sera

Pools of late-stage infected sera, assayed for their ability to induce the Hb → mycelial transition under *in vitro* conditions, were selected for additional fractionation. These pools initially were subjected to freeze drying and re-assayed to determine activity levels. Aliquots of the late-stage infected sera were incubated with proteinase K (0.1 µg to 1.0 µg/µl hemolymph, Sigma–Aldrich) at 37 °C for 2 h and 24 h intervals. A second series of sera samples was subjected to either 56 °C or 95 °C heat treatment for 30 min. Treated sera were centrifuged at 12,000 g for 15 min at 4 °C and supernatants and control proteinase K solutions assayed for Hb → mycelia transition activity. The second phase involved attempts to use either solvents (ice-cold acetone, methanol) or (NH₄)₂SO₄ to selectively partition the transition fraction from sera. Four volumes of either ice-cold methanol or acetone were slowly added to 200 µl of late-infected sera, incubated at –20 °C for 2 h, then centrifuged at 12,000 g for 15 min at 4 °C. Supernates were collected and solvents removed with nitrogen. The pellets were dried at RT and resuspended in 200 µl PB. Additional sera samples were sequentially precipitated with 30% and 70% (NH₄)₂SO₄. Pellets from 0% to 30% and 30% to 70% were resuspended in 200 µl PB. The third phase involved partitioning either whole sera or proteinase K-treated sera fractions on various chromatographic resins.

Mini-columns containing 1 ml of Phenyl Sepharose CL4B resin were used to partition sera fractions containing 1 M $(\text{NH}_4)_2\text{SO}_4$. A combination of Pierce strong anion- and cation-exchange filters (Thermo Scientific), following manufacturer's protocol, were used to fractionate transition activity from sera samples. All of the different treatment samples and corresponding buffer controls were assayed for relative transition activity using the previously described microtiter assay (see above). Selected fractions were analyzed on SDS-PAGE as described above.

3. Results

The development of all four *M. rileyi* strains under *in vitro* conditions was impacted by specific growth conditions. Inoculated onto McCoy's agar, conidiospores germinated within 24 h at 27 °C and produced small mucoid colonies that contained budding hyphal bodies. Within 48 h, these cultures transitioned to white colonies characterized by apical-growing hyphae. The production of conidiophores on this media after 5–7 days was restricted to small zones in the periphery of the colonies. Alternatively, both SMY and FMAY agar plates supported extensive hyphal body growth; these budding cells formed mucoid colonies that merged over the plate surface. After 5–6 days at 28 °C, the budding Hb transitioned into mycelia. Unlike the McCoys agar, both the SMY and FMAY agar plates supported conidiophore formation and extensive sporulation. Similarly, the McCoys broth stimulated extensive mycelial growth, whereas both FMAY and SMY stimulated Hb production. Inoculating Hb preparations from SMY broth into the defined, nutrient-limited Vogels N broth stimulated a synchronous production of the mycelial phenotype (Fig. 1). Alternatively, serum-free insect medium inoculated with Hb produced the budding hyphal body stage in stationary broth or shake cultures and reached peak concentrations of $\sim 2.5 \times 10^5$ cells/ μl within 72–96 h post-inoculation. The stationary cultures, produced in serum-free insect media, failed to transition into mycelia but formed heavily vesiculated, thick-walled hyphal bodies (Fig. S2).

Observations of the *in vivo* replication of *M. rileyi* in both *S. exigua* and *T. ni* larvae revealed a tightly orchestrated development program. For example, in *S. exigua*, after an initial 16–30 h lag phase, the Hb of strain Nr5772 underwent an exponential growth phase (30–62 h) with cell numbers doubling every 3–4 h in the hemolymph (Fig. 2). During this period, the fungal cells replicated by a combination of apical and lateral budding from parent cells, with Hb densities reaching $\sim 3 \times 10^5$ cells/ μl of hemolymph. At this peak density, budding was replaced with apical growth; Hb elongated and produced septae between adjacent cells. During this transition, cell replication was reduced with cell-doubling time increasing to an estimated 7–8 h. At the late (host death) stage of infection, these elongate cells transitioned synchronously into the tissue-invasive or mycelial phenotype (Fig. 2E). These cells

attached to the basement membrane of insect tissues, resulting in the clearance of free-floating fungal cells from the hemocoel as observed at 88 and 96 h post-challenge (Fig. 2).

The total hemocyte counts (THC) in the PBS control *S. exigua* larvae remained somewhat constant, fluctuating between $\sim 16,000$ and 19,000 cells per μl hemolymph over the three days preceding the onset of the non-feeding wandering stage before pupal molt. In comparison, the THC in *M. rileyi*-infected larvae were significantly reduced; at 24, 48, and 72 h pi, infected larvae contained 44%, 37%, and 47% fewer hemocytes than their control counterparts (Table 1). The *in vitro*-produced Hb delivered into the hemocoel elicited no detectable non-self recognition by the circulating phagocytic hemocytes (Fig. 3). The injected cells and the progeny budding hyphal bodies remained free-living in the hemolymph throughout the vegetative development of the hyphal bodies. The lack of recognition was not due to Hb cells inhibiting the phagocytic response; hemocytes throughout the three-day, post-challenge period were competent in their phagocytosis of Fluoresbrite microspheres. Although the phagocytes remained functioning, there was a marked alteration in coagulation; heated, healthy hemolymph produced a major clot (2/3 of pellet post-centrifugation), whereas the pellet from heat-treated, mid-stage infected hemolymph (~ 48 h pi) produced a small pellet comprised of hemocytes and Hb.

The replication of the Hb in the hemocoel induced no significant changes in serum protein levels during the initial 48 h pi; both control and infected *S. exigua* larvae contained approximately 25–30 $\mu\text{g}/\mu\text{l}$ of sera. At 72 h pi, protein levels (51 $\mu\text{g}/\mu\text{l}$) in the infected larvae increased significantly to those observed in the uninfected larvae (39 $\mu\text{g}/\mu\text{l}$). SDS-PAGE of sera demonstrated that even in the presence of $>10^5$ Hb/ μl hemolymph, infected insects maintained a full complement of hemolymph peptides; the gel profiles of infected larvae were nearly identical to profiles of control hemolymph (Fig. 4). In the single-dimension SDS-gradient gels, only three minor bands having M_r s of 12, 18, and ~ 42 kDa were observed in the late-infection samples that were not observed in control samples.

Larval development, estimated by weight gain, demonstrated that during the initial 48 h, infected *S. exigua* developed at rates that corresponded to controls (Fig. 5). The leveling off of control weight gain at 64 h was due to transition to the non-feeding, wandering stage that preceded the pupal molt. The leveling of the weight of infected larvae corresponded to the massive increase in fungal biomass and subsequent decrease in hemocyte levels. At 72 h post-challenge, uninfected larvae spun loose silken cocoons at the top of the rearing cells. At this time, infected larvae undergoing Hb \rightarrow mycelia transition did not enter the wandering stage but instead ceased to feed and remained associated with the diet surface until death. Neither infected *T. ni* nor *S. exigua* larvae produced prepupae or pupae, whereas all control (PBS-injected) larvae by 96 h pi formed silkened pupal cells and pupated (Fig. S3).

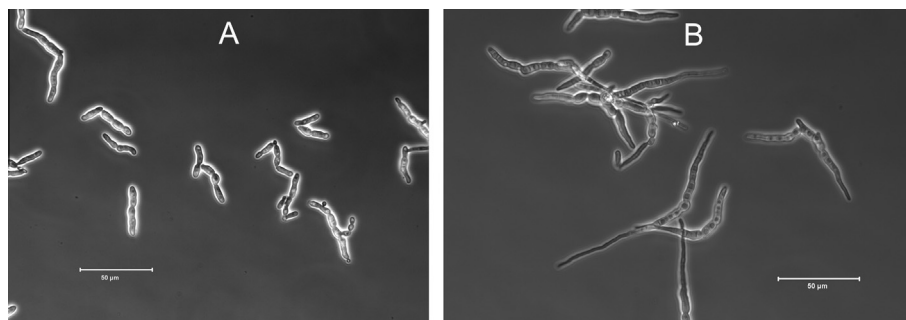


Fig. 1. Light micrographs of *Metarhizium rileyi* inoculated as hyphal bodies (Hb) into insect tissue culture broth (panel A) and Vogels media (panel B). Note that the transfer into nutrient-poor media (panel B) induced the Hb to convert synchronously to an apical growth program leading to mycelial phenotype.

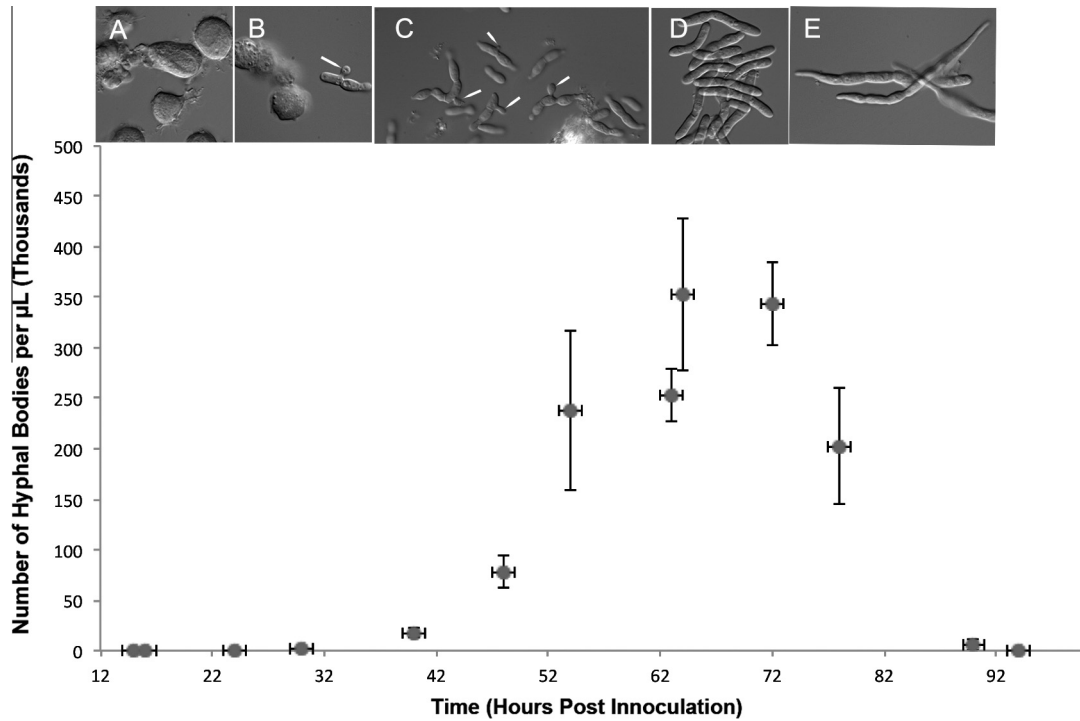


Fig. 2. A composite of the growth curve of *Metarhizium rileyi* in host *Spodoptera exigua* larvae depicting the average number of hyphal bodies (Hb) per microliter of hemolymph and associated cell phenotype. During the exponential phase (24–62 h post-injection) the hyphal bodies multiply by budding (A–C). After reaching threshold ($\sim 3 \times 10^5$ cells/ μ L), lateral budding of the Hb (see arrows) ceases, and elongate hyphal bodies are formed (D, 62–72 h post-injection) that transition synchronously into apical growing hyphae (E, 72–80 h post-injection). The differential contrast images A–E were taken at 1000 \times magnification.

Table 1
The effect of *Nomuraea rileyi* replication on the total hemocyte count of *Spodoptera exigua* larvae.

Time post injection	Mean number hemocytes/ μ L haemolymph (SE)		P-value
	Control	Infected	
24 h	1.9×10^4 (1.9×10^3) n = 5	1.1×10^4 (1.0×10^3) n = 18	0.0008 ^a
48 h	1.6×10^4 (1.2×10^3) n = 8	1.0×10^4 (0.8×10^3) n = 18	0.0005 ^a
72 h	1.7×10^4 (1.0×10^3) n = 7	0.9×10^4 (1.9×10^3) n = 16	0.0237 ^a
P-value	0.7706	0.3938	

^a Denotes significant differences between infected and control larvae at the different sampling intervals.

The cell-free sera samples harvested from both control and infected *S. exigua* and *T. ni* larvae were assayed for their ability to initiate the Hb \rightarrow mycelia transition under *in vitro* conditions (Fig. 6). All control sera samples and PBS controls, regardless of concentration and sampling interval, failed to induce the Hb from any of the four *M. rileyi* strains to transition to an apical growth program. All wells at 48 h incubation produced a contiguous monolayer of Hb. Similar to these controls, sera sampled from larvae containing budding Hb (12–36 h pi) did not alter the Hb replication pattern under *in vitro* conditions. However, the addition of cell-free sera extracted at the late stage of infection (hyphal body elongation to mycelial transition) induced Hb to synchronously switch to an apical growth program. It should be noted that samples of infected sera, collected at the Hb \rightarrow mycelia transition at

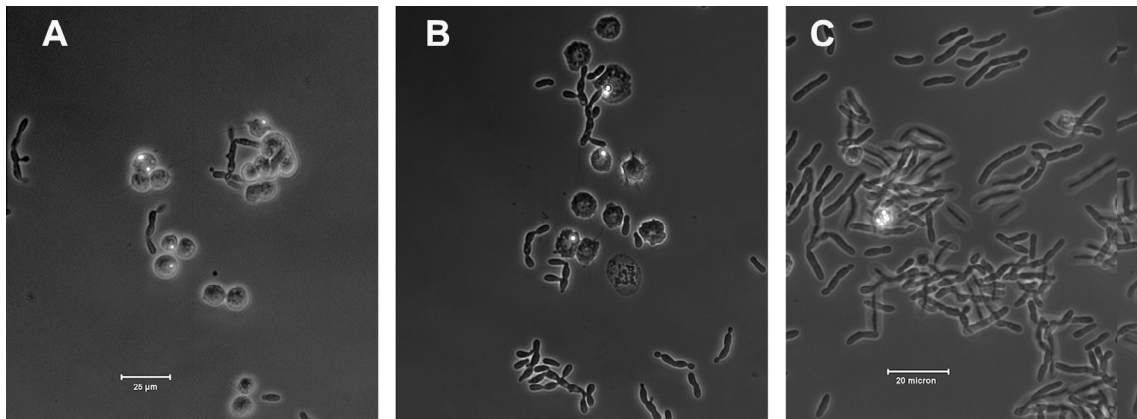


Fig. 3. A combination of epifluorescent and phase-contrast optics that depict the association of hyphal body (Hb) and hemocytes in hemolymph sampled at 20, 40, and 60 h pi. Note that the phagocytic hemocytes capable of recognizing and ingesting the fluorescent microbeads (see arrows) did not recognize the Hb as nonself.

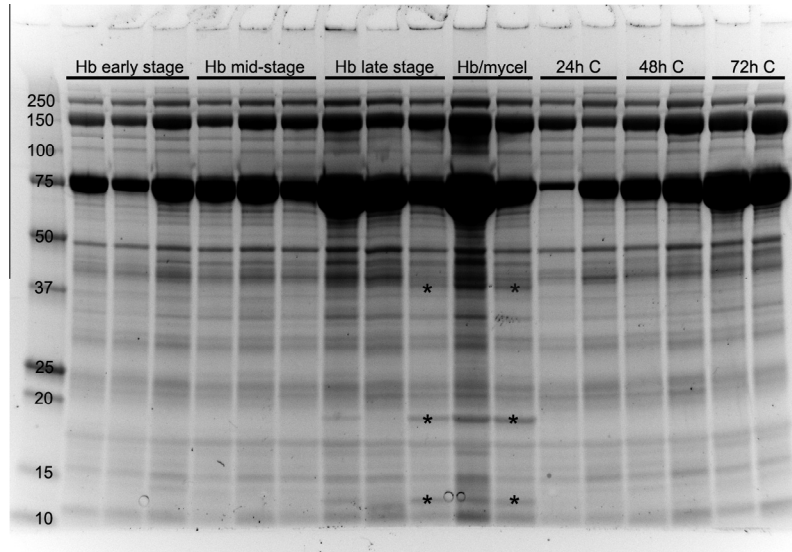


Fig. 4. SDS-PAGE of infected and healthy (C) hemolymph sera sampled at different times post-injection. The first lane contains Precision plus protein standards (BioRad Corp) with M_r denoted by numbers. The asterisks represent peptides having M_r of ~40, 18, and 12 kDa that were consistently observed in the late-infection stages (60–72 h pi).

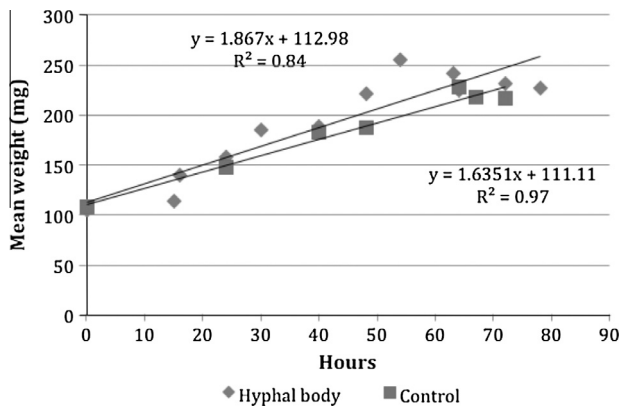


Fig. 5. Average weight of early fifth-instar *S. exigua* larvae that were injected with hyphal bodies or with PBS (control) sampled over 3–4 days post-injection (pi). It should be noted that by 72 h pi, the control larvae had formed either prepupae or pupae, whereas by 72–80 h, infected larvae were mummified.

concentrations $\geq 0.16\%$ in serum-free media, induced the phenotypic switch in *M. rileyi*. The timing of the Hb \rightarrow mycelia switch in these assays was concentration-dependent; at the higher concentrations, the switch could be observed as early as 12 h post-incubation, whereas at the more dilute sera concentrations, the Hb formed elongate cells at 24 h and fully formed mycelia after 48 h incubation at 27 °C.

Tyrosol, the insect hormones, and the *M. rileyi* metabolites (β -1,3 glucan, ergosterol and ergosterol peroxide) assayed at varying concentrations, did not stimulate or inhibit the Hb \rightarrow mycelia transition under *in vitro* conditions. The Hb cells inoculated into serum-free media containing these test compounds or their respective carriers continued to develop as budding cells throughout the 48 h test interval. The Hb \rightarrow mycelia transition induced by the addition of late-stage sera to serum-free media was not suppressed by the addition of any of the above test compounds. Furthermore, Hb cells incubated in Czapeks Dox broth with or without the amendments converted within 24–48 h to the mycelial phenotype. However, assays involving farnesol, depending on the test concentration,

Hours post-inoculation	Rating of HB to mycelia transition			
	Well 1	Well 2	Well 3	Well 4
<i>M. rileyi</i> 15	1	1	1	1
<i>M. rileyi</i> 24	1	1	1	1
<i>M. rileyi</i> 40	1	1	1	1
<i>M. rileyi</i> 48	3	1	1	1
<i>M. rileyi</i> 54	4	3	2	1
<i>M. rileyi</i> 63	4	4	4	4
<i>M. rileyi</i> 72	4	4	4	4
PBS injected 24	1	1	1	1
PBS injected 48	1	1	1	1
PBS injected 60	1	1	1	1
PBS control	1	1	1	1

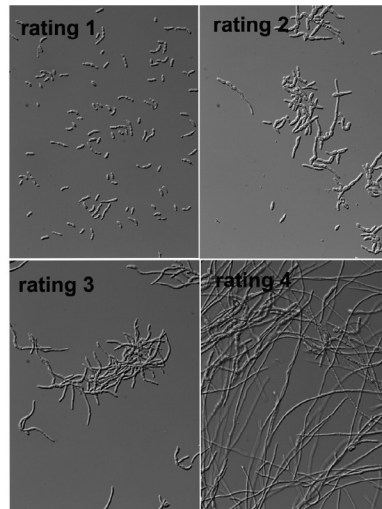


Fig. 6. An example of the activity of sera fractions extracted from infected *S. exigua* larvae assayed in microtiter wells containing *M. rileyi* hyphal bodies in insect sera-free cell culture media. The different wells (1–4) contain 2-fold dilutions ranging from 5% to 0.625% sera concentrations.

impacted Hb development. At concentrations >50 μM , the farnesol isomer complex inhibited Hb growth but did not impact the transition at nontoxic concentrations. The tetra, tetra-farnesol at high concentrations (>2 mM) inhibited fungal growth but at concentrations of 250 μM – 2 mM, this compound stimulated the Hb \rightarrow mycelia transition; below this concentration, this isomer had no impact on fungal development.

The late-stage infected insect sera heat-treated at 55 °C for 30 min retained the transition activity detected in unheated sera, whereas treatment at 95 °C caused a massive precipitation of sera and removed ~75% of the transition activity detected in the untreated sera. The insect sera from either *S. exigua* and *T. ni* displaying conversion activity (Hb \rightarrow mycelia) retained 100% activity after being lyophilized and reconstituted in water. Subsequent attempts to partition the active component(s) from sera preparations met with partial success. Initial fractionation of sera with solvents such as methanol or acetone/TCA resulted in the either a partial or total loss of transition activity in the pellet and supernatant fractions, respectively. However, fractionation of the reconstituted lyophilized late-stage infected sera with $(\text{NH}_4)_2\text{SO}_4$ resulted in activity being localized in the 30–70% fraction. Control preparations containing equivalent concentrations of $(\text{NH}_4)_2\text{SO}_4$ did not stimulate Hb \rightarrow mycelia transition. The 30–70% $(\text{NH}_4)_2\text{SO}_4$ pellet, resuspended in 1 M $(\text{NH}_4)_2\text{SO}_4$, did not bind to the phenyl Sepharose® CL4B resin but remained in the 1 M $(\text{NH}_4)_2\text{SO}_4$ eluant. The active 1 M $(\text{NH}_4)_2\text{SO}_4$ cut subjected to either dialysis and fractionation on a PD10 gel permeation column resulted in >75% loss of the transition activity. The chemistry responsible for transition activity failed to effectively bind to either the strong cation-(S) or anion-(Q) exchange filters. With both resins, <50% of the transition activity in the original fractions were recovered in the initial eluants. Fractions from elution with either low pH (4.0) or high pH (9.0) buffers, or with buffers amended with 2 M NaCl, contained no detectable transition activity.

Proteinase K treatment added at 0.1 and 1.0 $\mu\text{g}/\mu\text{l}$ of late-stage infected *T. ni* sera after two h and 24 h incubation at 37 °C retained >75% of the transition activity detected in the untreated insect sera samples. Proteinase K added at equivalent concentrations to control wells did not alter Hb development. SDS-PAGE analysis of these samples showed that the degree of digestion corresponded with the concentration and duration of proteinase K treatment (Fig. 7). After 2 h at 37 °C, proteinase K (1.0 $\mu\text{g}/\mu\text{l}$) digested the major large-molecular-weight peptides in *T. ni* and *S. exigua* sera, whereas very small amounts of proteinase-resistant peptides remained in the enzyme-treated fractions (Fig. 7, lanes 1–4). Several of the faint bands detected in the digested infected and control sera aligned with bands detected in the proteinase K control (lane 5). Assays of the proteinase K digest of the late-stage infected *T. ni* sera subjected to a subsequent methanol precipitation step suggested that the transition activity may due to multiple chemistries. The resuspended pellet from the solvent precipitation of late-stage proteinase K digested infected *T. ni* sera induced the production of elongate Hb (see Fig. 2D), but after 48 h, these fractions did not induce mycelial formation. However, the supernatant fraction, after solvent evaporation, had no effect on Hb development. Reconstitution of the pellet and supernatant fractions resulted in 75% of the initial transition (elongation) and the production of the mycelial phenotype activity detected in the parental late-stage infected sera.

4. Discussion

The ability of pathogenic yeasts' fungi to exhibit dimorphic lifestyles (or phenotypic duality, yeast versus hyphae) has been known for decades; this trait allows these microbes to adjust to changing microhabitats. In many cases, the switch is associated

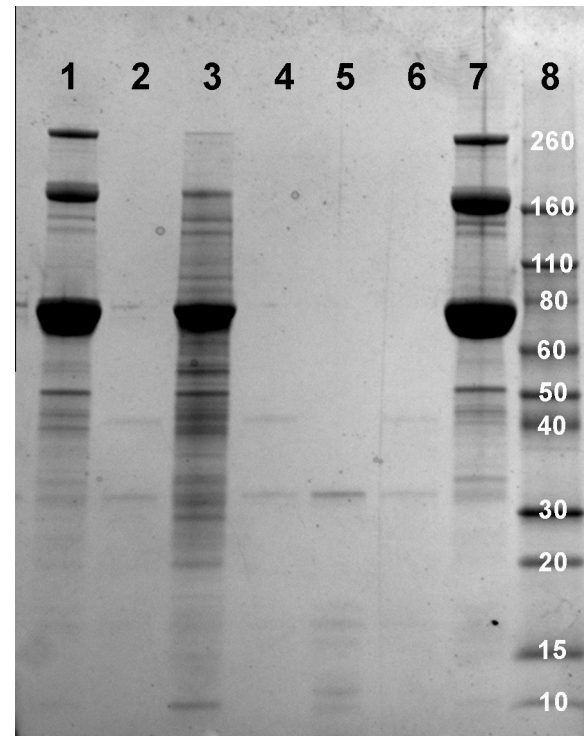


Fig. 7. SDS-PAGE of the lanes loaded with the following: lane 1, late-stage infected *S. exigua* sera (0.5 μl); lane 2, late-stage infected *S. exigua* sera (0.5 μl) digested 2 h with proteinase K; lane 3, loaded late-stage infected *T. ni* sera (0.5 μl); lane 4, late-stage infected *T. ni* sera (0.5 μl) digested 2 h with proteinase K; lane 5, proteinase K control (5 μg); lane 6, late-stage control *S. exigua* sera digested 2 h with proteinase K; lane 7, late-stage control *S. exigua* sera; and lane 8, the MW ladder (Novex Sharp Pre-stained standards, Invitrogen).

with a change from the saprophytic to parasitic (pathogenic) phase. Ascomycetes such as *Histoplasma capsulatum*, *Coccidioides immitis*, and *Paracoccidioides brasiliensis* produce the yeast phase *in vivo* and the hyphal phase under saprophytic conditions (Klein and Tebbets, 2007). Alternatively, the opportunistic yeast *C. albicans* exhibits a different program. Under *in vitro* conditions, it typically forms a budding yeast, whereas as a pathogen, it produces parasitic hyphal phase. It has been suggested that various insect-pathogenic fungi exhibit a dimorphic lifestyle during their *in vivo* development (Boucias and Pendland, 1998). Upon breaching the cuticle barrier of host insects, penetrant germ tubes of these pathogens contact the nutrient-rich hemolymph that is believed to induce conversion to budding yeast-like Hb. These cells divide exponentially and reach a critical density (quorum) that triggers a synchronous conversion to an apical growing tissue-invasive phenotype. The time required to reach the quorum depends on both host (age, species) and pathogen (strain, inoculum density); ambient temperature drives the rate of Hb replication in their respective poikilothermic hosts (Boucias et al., 1984).

M. rileyi has the ability to undergo selective exponential growth in the hemocoel of infected larvae. One of the remarkable features of this *M. rileyi*/host interaction is the ability of the host to tolerate extremely high levels of the hemolymph-borne hyphal bodies. Centrifugation of late-stage infected hemolymph at the threshold density ($\sim 3 \times 10^5$ cells/ μl hemolymph) produces an Hb pellet that comprises ~40% of the mass of the hemolymph (Fig. S2b). *M. rileyi*-infected larvae continue to feed, develop, and behave as healthy insects until the Hb transition to mycelia (Pendland et al., 1994). Replication of this pathogen, although not disrupting larval growth and molting, does inhibit the larval-to-pupal transition. The observed disruption of metamorphosis may reflect the production of ecdysteroid-22-oxidase by this pathogen (Kiuchi et al., 2003).

In prior research, we have proposed that the co-existence between the host system and circulating hyphal bodies is due in part to the lack of surface epitopes that activate the insect cell defense system (Pendland et al., 1988). The Hb phenotype neither elicits a phagocytic response nor possesses surface epitopes recognized by insect pathogen recognition receptors (PRRs). In addition, these budding cells assimilate amino acids and sugars from the hemolymph and do not secrete the metabolites (toxins, enzymes, etc.) that characterize the mycelial phenotype. At the point of Hb → mycelia conversion, the outnumbered, remaining phagocytic plasmatocytes and granulocytes are incapable of mounting an effective defense against the tissue-invasive mycelia. The concept of a pathogen reaching a critical density *in vivo*, producing a signal that controls virulence determinants to overwhelm the host defense response, is not novel and has been reported for various plant and vertebrate bacterial infections (Williams et al., 2000). It should be noted that throughout the Hb replication phase, phagocytes of infected larvae recognized and internalized nonself. Infection inhibited the heat-induced hemolymph coagulation reaction, but both sera and hemolymph sampled at 48 h pi contained phenoloxidase levels similar to those detected in healthy larvae (data not shown). These observations differ from those reported previously for *Beauveria bassiana* replicating in *S. exigua* larvae (Pendland et al., 1993; Mazet et al., 1994; Hung and Boucias, 1996). *In vivo* replication of *B. bassiana* reduced the numbers of circulating hemocytes and suppressed both the phagocytic capabilities and hemolymph phenoloxidase levels.

In vitro, the budding hyphal body phenotype can be maintained readily and indefinitely by frequent transfer (two-day intervals) in nutrient-rich media such as the serum-free insect cell media. However, by switching the nutrient inputs, it is possible to manipulate the *M. rileyi* cell phenotype (Pendland and Boucias, 1997). Hyphal body cultures grown in nutrient-rich SMY broth (or serum-free insect cell media) transferred to a defined Vogels (Pendland and Boucias, 1997) or Czapeks Dox broth (this study) converted to mycelia within 12–24 h post-transfer. Furthermore, if the mycelia culture is transferred back into a nutrient-rich media, the mycelia, over time, will revert to the budding Hb phenotype.

Based on these *in vitro* observations, we believed initially that the observed *in vivo* Hb switch to the tissue-invasive mycelial phenotype in the hemolymph was due to the depletion of key nutrients required to maintain the Hb phenotype. However, the results of *in vitro* assays conducted herein suggest that this was not the case; amending nutrient-rich, serum-free insect cell media with minute amounts of sera derived from late-stage infected larvae induced a synchronous switch of Hb cultures to an apical growth pattern. The elicitor(s) responsible for this switch was localized only in sera sampled from late-stage infected larvae at the time of Hb → mycelia transition. Buffer controls (void of nutrients) and sera sampled from either healthy larvae or at early-stage infection (0–48 h pi, Hb stage) failed to elicit the Hb → mycelia transition. These observations implicated the presence of a soluble component that was produced when Hb reach a threshold density or quorum.

It is important to note that we do not know if the elicitor(s) is produced by the fungus and/or by the infected insect. In other fungal systems, QSM activities have been detected in conditioned culture supernatants and not from infected host material. For example, the two well-characterized QSMs farnesol and tyrosol, that either modulate the yeast-to-mycelium conversion or maintain the biofilm in *C. albicans*, were both detected from conditioned media (Hornby et al., 2001; Alem et al., 2006). In other fungi, QSM activity (unidentified) has been associated with conditioned media from other yeast and filamentous fungi (Hornby et al., 2004; Chen and Fink, 2006; Kugler et al., 2000). Unlike these prior reports, QSM activity has not been detected from conditioned media of *M. rileyi* cultures that have been induced to switch from Hb to mycelia by

altering nutrient inputs (Pendland and Boucias, 1991). Significantly, the transition is not observed when hyphal bodies are incubated in SF-900 II SFM; without the amendment of late-stage infected sera, the Hb cultures incubated at 28 °C within 72–96 h attain a density (2×10^5 cells/ μ l), comparable to maximum densities observed in the hemolymph. The QSM activity reported herein has been observed only with hemolymph sera extracted from late-stage infected host insects.

Based on recent reports that conditioned media of *M. rileyi* sampled in its mycelial stage contained ecdysteroid-22-oxidase, we speculated that a potential trigger of the *in vivo* Hb to mycelia switch may involve host hormone titers. However, our assays demonstrated that neither ecdysone nor sesquiterpene epoxide (JH) amendments stimulated Hb → mycelia conversion or prevented the infected sera-induced Hb → mycelia transition. Similarly, various fungal metabolites produced by *M. rileyi* failed impact fungal development. Tyrosol, a reported QSM that stimulated mycelial formation and biofilm production in *C. albicans* (Chen et al., 2004), had no impact on *M. rileyi* development. Unexpectedly, the tetra, tetra-farnesol, a QSM reported to inhibit filament hyphae in *C. albicans* (30), at high concentrations induced the *M. rileyi* Hb → mycelia transition under *in vitro* conditions but was unable to block the insect serum-induced Hb → mycelia at lower test concentrations.

This later finding stimulated solvent (methanol, acetone) fractionation of late-stage infected sera samples; small, nonpolar compounds such as farnesol should be retained in the solvent phase. However, neither the supernatant or pellet fractions from the solvent partitioning of the infected sera induced Hb to transition to mycelia. However, the reconstituted pellet fraction, at high concentrations, did elicit the Hb to produce elongate cells similar to those detected *in vivo* at ~60 h pi. The chemical(s) responsible for the transition activity appeared to be uncharged and did not bind to the tested HIC or ion-exchange resins. Many of the serum proteins present in the 30–70% $(\text{NH}_4)_2\text{SO}_4$ cut bound to these resins, whereas the transition activity was only detected in the sample eluant fractions. The chemistry responsible for transition appears to be less than 10 KDa, as implicated by the activity loss after dialysis. The proteinase K digestion of sera effectively degraded the bulk of hemolymph peptides but did not destroy the Hb → mycelia transition activity. Solvent-partitioning the proteinase K-digested sera provided evidence that the observed QSM activity may be due to multiple chemistries. The pellet fraction was able to induce Hb elongation, whereas this fraction supplemented with the supernatant was able to induce Hb → mycelia transition. Indirect support for this theory derives from observations of the Hb → mycelia transition event; the initial phase is the cessation of budding and a concomitant elongation of Hb cells ending with the production of apically growing mycelia.

In summary, this work has documented the expression of the dimorphic program exhibited by *M. rileyi*. Using two hosts and various *M. rileyi* isolates, we have validated this program, measured *M. rileyi* growth rates under *in vivo* and *in vitro* conditions, and assessed the pathogen's impact on host fitness. Significantly, we provide evidence that the Hb-to-mycelia transition is regulated by a QSM(s). The production of this chemical(s) occurs when a quorum of Hb is produced in the hemolymph (late-stage infection). The serum-based QS activity is retained after lyophilization, mild heat treatment, and proteinase digestion. However, our attempts to extract the QSM have failed. Results suggests that the observed HB → mycelia transition is a multi-step process involving more than one chemical signal.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jip.2016.03.013>.

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