Growth substrates and caeleosin-mediated functions affect conidial virulence in the insect pathogenic fungus *Beauveria bassiana*

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The entomopathogenic fungus, *Beauveria bassiana*, is a microbial biological control agent capable of infecting a wide range of insect hosts. Conidia (spores) initiate infection via adhesion, growth and penetration of the insect cuticle, whose outermost layer is rich in lipids. Conidial virulence was investigated in *B. bassiana* WT and caeleosin mutants (ΔBbca1), the latter a protein involved in lipid storage and turnover. Topical insect bioassays revealed that conidia of the WT strain showed up to 40-fold differences in LD₅₀ values depending upon the growth substrate. The most virulent conidia were harvested from potato dextrose agar containing oleic acid, and the least potent were those derived from Sabouraud dextrose/yeast extract agar (SDAY). However, with the exception of conidia derived from SDAY and Czapek Dox agar, in which values were reduced, mean lethal times to kill (LT₅₀) were essentially unaffected. In topical bioassays, the ΔBbca1 mutant displayed LD₅₀ values 5–40-fold higher than the WT depending upon the growth substrate, with ΔBbca1 conidia derived from SDAY unable to effectively penetrate the host cuticle. The ΔBbca1 mutant also showed concomitant dramatic increases in LT₅₀ values from a mean of ~4.5 for WT to >8.5 days for the mutant. In contrast, intrahaemocoel injection bioassays that bypass cuticle penetration events revealed only minor effects on virulence for either WT or ΔBbca1 conidia. These data highlight the importance of caeleosin-dependent lipid mobilization and/or signalling in cuticle penetration events but suggest their dispensability for immune evasion and within-host growth.

**INTRODUCTION**

The ascomycete fungus *Beauveria bassiana* is among a group of insect pathogens that have been commercialized as biological control agents, especially within the framework of more environmentally friendly alternatives to chemical pesticides and as part of Integrated Pest Management practices (Glare et al., 2012; Lacey et al., 2015). As a broad host range pathogen, *B. bassiana* conidia (spores) can initiate infection essentially anywhere on the insect surface and do not require any specialized route of entry (Ortiz-Urquiza & Keyhani, 2013, 2016). The insect epicuticle, composed of lipids that include abundant amounts of long-chain hydrocarbons, fatty acids and wax esters, is the first barrier to infection, and the fungus has evolved mechanisms for adhesion, germination on the scant nutrients available and subsequent penetration of the host exoskeleton (Charnley, 2003; Holder & Keyhani, 2005; Jarrold et al., 2007; Zhang et al., 2011). Insects actively resist infection at this cuticular level, beginning with the epicuticle or waxy layer, via production of compounds toxic to microbes, e.g. certain fatty acids, quinones and formic/acetic acid (Golebiowski et al., 2011; Toledó et al., 2011; Tragust et al., 2013). Insect behavioural modifications aimed towards eliminating the pathogen and/or mitigating its infectivity can include heat seeking, burrowing and grooming (de Crecy et al., 2009; Roy et al., 2006; Yanagawa & Shimizu, 2007). Such factors as well as insect chemical defences can lead to an

Abbreviations: DAG, diacylglycerol; LD, lipid droplet; LT₅₀, mean lethal time to kill; TAG, triacylglycerol.
evolutionary arms race between the host and pathogen (Pedrini et al., 2015).

Conidia are the infectious agents most commonly used in pest control formulations, and important knowledge has been gained in our understanding of processes important for conidial viability and application (Faria et al., 2012; Jin et al., 2013; Qin et al., 2014), although other cell types have also been shown to be virulent and of potential commercial use (Holder et al., 2007; Mascarín et al., 2015). As the insect epicuticle is the first contact point for any infectious cell, B. bassiana biochemical pathways that utilize fatty acids, alphatic and methyl-branched alkanes and glycerides as substrates can target insect cuticular lipids, and hence are involved in facilitating successful infection (Crespo et al., 2000; Lecuona et al., 1997; Pedrini et al., 2006, 2007). These systems include hydrocarbon oxidative pathways containing a set of cytochrome P450 enzymes implicated in lipid assimilation (Pedrini et al., 2010, 2013; Zhang et al., 2012). Long-chain alkanes, common constituents of the insect epicuticle, are degraded by B. bassiana to free fatty acids, acylglycerols and phospholipids, although important aspects of this process, including how lipids are transported into cells and the biochemical mechanisms of lipid storage and mobilization, remain poorly understood (Crespo et al., 2008; Pedrini et al., 2013). In addition, it is known that culture conditions can affect virulence and that growth on insect-derived alkanes can increase the virulence of conidia as compared to those harvested from standard glucose containing mycological media (Crespo et al., 2002).

Lipid droplets (LDs) are cellular organelles that act as means for lipid storage, impacting metabolism, energy homeostasis and development (Murphy, 2012; Welte, 2015). LDs consist of a phospholipid monolayer, embedded with various proteins that surround a lipid core chiefly consisting of triacylglycerols (TAGs). Caleosins, first described in plants, are LD-associated proteins containing EF-hand calcium-binding motifs (Naested et al., 2000). Some caleosins are capable of binding haem and have been shown to display peroxygenase activity, implicating these proteins in lipid-mediated signalling, e.g. stress response (Hanano et al., 2006; Partridge & Murphy, 2009). Caleosins are widely distributed in plants, typically found as gene families, and are also found in algae and fungi, but not in animals. While more extensively studied in plants, several reports have examined the functions of caleosins in fungi. In Aspergillus flavus, a caleosin-like gene, designated AfPGX, was shown to exhibit peroxygenase activity and to be critical for normal growth and development, impacting aflatoxin accumulation (Hanano et al., 2015). In As. flavus, deletion of the AfPGX gene resulted in severe phenotypes with greatly reduced growth and little to no conidiation apparent. In contrast, targeted gene knockout of the caleosin gene in B. bassiana (∆Bbcal1) resulted in little to no effects on vegetative growth and only small effects on conidiation (Fan et al., 2015). Impairment of spore dispersal was noted, apparently due to clumping of the conidia, and a moderate effect was seen with respect to the mean lethal time to kill (LT₅₀) larvae of the greater wax moth Galleria mellonella using topical bioassays, although only a single growth substrate was examined i.e. the standard mycological media potato dextrose agar (PDA). Here, we sought to expand upon these results to (1) probe the effect of lipid growth substrates on conidial virulence in terms of both the mean lethal dose (LD₅₀) to kill hosts and the mean lethal time (LT₅₀) values and to (2) determine whether caleosin-dependent reduction in virulence occurred mainly at the pre-penetration/penetration stage and/or further downstream i.e. during haemocoeel proliferation and immune evasion. Our data show that growth substrates have significant effects on WT B. bassiana virulence, particularly in topical assays. In addition, the contribution of the caleosin to virulence was greater during pre-penetration/penetration events, with more moderate effects seen once the insect cuticle was breached. These results reveal a critical role for caleosin-mediated lipid mobilization and/or signalling events during the initial phases of fungal infection.

METHODS

Fungal strains and culture conditions. The WT strain B. bassiana ATCC 90517 and a caleosin targeted gene knockout strain (Fan et al., 2015) were routinely grown on PDA, Sabouraud dextrose agar (SDA), SDA + 1 % yeast extract (SDAY), and/or Czapek Dox agar (CZA) supplemented or modified as indicated. For growth on lipid substrates, PDA was supplemented with 0.25 % oleic acid, 0.5 % glyceride trioleate (tri olein), 0.5 % olive oil or 0.2 % hexadecane (C₁₆) prepared in hexane at a concentration of 10 % and was added to the media immediately prior to pouring of the plates. For conidial production, agar plates were incubated at 26 °C for 21 days and aerial conidia were harvested by flooding the plate with sterile distilled H₂O containing 0.02 % Tween 80. Conidial suspensions were filtered through a single layer of Miracloth and final spore concentrations were determined by direct count using a haemocytometer and adjusted to the indicated spore concentration.

Lipid analyses. Fungal conidia were harvested from various growth conditions including CZA, Sabouraud dextrose/yeast extract agar (SDAY), PDA and PDA supplemented with 0.25 % oleic acid, 0.5 % glyceride trioleate, 0.5 % olive oil or 0.2 % alkane (prepared in hexane at a concentration of 10 %). All the plates were cultured at 26 °C for 30 days before harvesting of conidia. The conidia were harvested in sterilized H₂O and 108–109 conidia were used for lipid profiles analysis. Lipids were extracted using the Folch method (Folch et al., 1957) Briefly, 30 µl of a 10 µg ml⁻¹ solution of dilauryl phosphatidylcholine (internal standard) was added, then 1 ml of 2 : 1 chloroform/methanol containing 100 µg l⁻¹ of butylated hydroxytoluene was added to each sample and mixed. The samples were centrifuged at 10 000 g and the supernatant was transferred to a new tube. Next, 200 µl of 0.9 % NaCl was added to induce phase separation. After mixing and gentle centrifugation (1000 g), the chloroform layer was removed and transferred to a clean tube. The extraction process was repeated once on the pellet and the chloroform layers were combined. The combined mixture was dried under a gentle stream of nitrogen. The dried samples were then reconstituted with 300 µl of isopropanol and 2 µl was injected for LC-HRMS analysis.

LC-HRMS analysis was performed on a Thermo Q Exactive with Dionex 3000 UHPLC and autosampler. The mass spectrometer was operated in positive heated electrospray ionization mode with the following conditions: 3.5 kV, 300 °C probe temperature, 30 arb sheath gas, 5 arb aux gas, 1.0 ion sweep gas, s-lens of 35 and 320 °C heated capillary...
temperature. Spectra were collected from 200–1200 at 35 000 mass resolution, mass accuracy was 5 p.p.m. or better and tandem mass spectra were collected using data-dependent scanning (top 5). Separation was achieved on a Waters BEH C18 50×2.1 mm, 1.7 μm column with mobile phase A as 60:40 acetonitrile/water with 0.1% formic acid and 10 mM ammonium formate and mobile phase B was 90:8:2 isopropanol/acetonitrile/water with 0.1% formic acid and 10 mM ammonium formate under gradient elution conditions as previously described (Ulmer et al., 2015). Data processing was performed with MZmine 2.20 for peak alignment and feature selection. An in-house R built script was used to identify lipids based on tandem mass spectra and reference to known fragmentation pathways.

**Insect bioassays.** Fungal strains were bioassayed using the greater wax moth G. mellonella (Pet Solutions, Beavercreek, OH, USA) as the insect host. The larvae were treated topically by dipping for 15 s in solutions of 10^5, 10^4, 10^3, 5×10^2 and 10^0 conidia ml^-1 harvested in sterile distilled H_2O with 0.02% Tween 80. Excess liquid on the insect bodies was removed by placement on a dry paper towel. Control larvae were treated with sterile dH_2O. Mortality was recorded every 24 h and the LD_{50} and LT_{50} were determined by Probit analysis. Each treatment consisted of three replicates with at least 25 insects each, and the entire experiment was repeated three times with different batches of fungal conidia. Additionally, conidia from both WT and ΔBbcal1 strains were injected into G. mellonella larvae. Each larva was injected with 800 conidia using a 1 ml syringe coupled to a programmable syringe pump (World Precision Instruments). Three replicates with 20 insects each were used for every treatment and the whole experiment was repeated three times with different batches of conidia.

**RESULTS**

**Growth substrates and caleosin functioning affect virulence in topical insect bioassays**

WT and a targeted gene knockout of the single identified caleosin gene in B. bassiana [ΔBbcal1 (Fan et al., 2015)] were grown on a variety of substrates including (1) standard complex mycological media (SDAY and PDA), (2) minimal mycological media containing sucrose as the carbon source (CZA) and (3) PDA supplemented with various lipids including hexadecane (C_{16}), oleic acid, triolein and olive oil. No obvious differences were noted in growth rate on the various media and conidia were harvested after 21 days of growth as detailed in Methods. In order to calculate LD_{50} values, five different conidial concentrations namely 10^5, 10^4, 10^3, 5×10^2 and 10^0 cells ml^-1 were used in insect bioassays. G. mellonella larvae were treated topically with fungal cell suspensions as detailed in Methods. WT cells harvested from the standard mycological media, PDA and CZA, displayed similar LD_{50} (at 9 days) values of −1.5×10^6 conidia ml^-1 (Table 1), with an ~10-fold decrease in virulence (i.e. 10-fold higher LD_{50} value) seen for WT conidia isolated from SDAY (LD_{50}^{WT}=15.4×10^6 conidia ml^-1). The ΔBbcal1 mutant fared worse, with LD_{50} values 25–40-fold higher than WT when derived from PDA and CZA media. ΔBbcal1 conidia isolated from SDAY were the least virulent of the conditions tested, being reduced to 77.1×10^6 conidia ml^-1, fivefold lower than WT cells isolated from SDAY.

For the WT strain, supplementation of PDA media with C_{16} olive oil or oleic acid resulted in a two- to fourfold increase in virulence (i.e. increased virulence) in LD_{50} values as compared to PDA. In contrast, conidia isolated from PDA + triolein displayed an LD_{50}^{WT}=3.62×10^6 conidia ml^-1, represented an approximately twofold increase as compared to PDA. Conidia derived from the ΔBbcal1 mutant, isolated from the same media, i.e. PDA + C_{16} (LD_{50}^{Bbcal1} =23.1×10^6 conidia ml^-1), PDA + triolein (LD_{50}^{Bbcal1} =18.1×10^6), PDA + olive oil (LD_{50}^{Bbcal1}=11.7×10^6) and PDA + oleic acid (15.6×10^6), were (two- to threefold) more virulent than mutant conidia harvested from PDA alone but were still 5–45-fold less effective than WT cells grown under correspondingly identical conditions.

For B. bassiana WT, with the exception of conidia derived from CZA and SDAY in which increases in the LT_{50} were seen, little difference was seen in regard to LT_{50} values between cells grown on PDA and PDA supplemented with C_{16} triolein, olive oil or oleic acid, with values ranging from 4.31 to 4.98 days (Fig. 1, Table 1). As compared to PDA, an increase of ~1 and a more dramatic 2 days (reflecting

Table 1. Calculated LD_{50} and LT_{50} values derived from topical infection of B. bassiana WT and ΔBbcal1 conidia harvested from different substrate media using G. mellonella larval insect bioassays

<table>
<thead>
<tr>
<th>Growth substrate*</th>
<th>LD_{50} (~×10^6 conidia ml^-1)</th>
<th>LT_{50} (days)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>ΔBbcal1</td>
</tr>
<tr>
<td>PDA</td>
<td>1.52±0.20</td>
<td>36.43±4.71</td>
</tr>
<tr>
<td>CZ</td>
<td>1.34±0.22</td>
<td>51.90±3.71</td>
</tr>
<tr>
<td>SDAY</td>
<td>15.41±2.33</td>
<td>77.07±1.30</td>
</tr>
<tr>
<td>PDA + C_{16}</td>
<td>0.83±0.11</td>
<td>23.12±1.80</td>
</tr>
<tr>
<td>PDA + triolein</td>
<td>3.62±0.14</td>
<td>18.08±0.21</td>
</tr>
<tr>
<td>PDA + olive oil</td>
<td>0.49±0.07</td>
<td>11.74±0.79</td>
</tr>
<tr>
<td>PDA + oleic acid</td>
<td>0.35±0.04</td>
<td>15.57±1.57</td>
</tr>
<tr>
<td></td>
<td>4.61±0.13</td>
<td>8.69±0.35</td>
</tr>
<tr>
<td></td>
<td>5.4±0.38</td>
<td>8.75±0.37</td>
</tr>
<tr>
<td></td>
<td>6.59±0.7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4.8±0.20</td>
<td>8.66±0.51</td>
</tr>
<tr>
<td></td>
<td>4.3±0.11</td>
<td>7.9±0.32</td>
</tr>
<tr>
<td></td>
<td>4.48±0.07</td>
<td>6.52±0.23</td>
</tr>
<tr>
<td></td>
<td>4.98±0.15</td>
<td>8.00±0.30</td>
</tr>
</tbody>
</table>

*Conidia were harvested from indicated agar media.
†Calculated using 5×10^7 conidia ml^-1.

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decreased virulence) in the LT$_{50}$ were seen for the WT conidia derived from CZA and SDAY respectively. The ΔBbcal1 mutant displayed severely reduced LT$_{50}$ values overall and with the exceptions of conidia from SDAY and PDA + olive oil, requiring 3–4 days longer to kill 50% of infected hosts as compared to corresponding WT cells. Conidia from PDA + olive oil displayed LT$_{50}$ of 6.52 days, which was 2 days more than its corresponding WT, and ΔBbcal1 conidia isolated from SDAY were almost avirulent and an accurate LT$_{50}$ value could not be calculated for these cells.

**Minor impairment of virulence after intrahaemocoel injection**

Direct injection of fungal spores into the host haemocoel bypasses penetration events, while maintaining the requirement for haemolymph proliferation and immune evasion. For WT *B. bassiana* cells, with the exception of conidia harvested from PDA + oleic acid, the LT$_{50}$ values were essentially unaffected when comparing cells grown on PDA, CZA, SDAY, PDA + C$_{16}$, PDA + triolein and PDA + olive oil using intrahaemocoel injection assays into *G. mellonella* larvae (Table 2 and Fig. 2). Under these conditions, LT$_{50}$ values ranged from 2.42 to 2.93 days. A moderate decrease (~1 day) in the WT LT$_{50}$ was seen for conidia harvested from PDA + oleic acid (to 3.62 days). In general, small (~0.5 days for CZA, PDA + triolein and PDA + oleic acid) to moderate (~1 day for PDA, PDA + C$_{16}$ and PDA + olive oil) increases in LT$_{50}$ values were seen for the Bbcal1 knockout mutant as compared to their corresponding WT conidia. *B. bassiana* ΔBbcal1 conidia harvested from SDAY were more severely affected, showing a 3 days increase in LT$_{50}$ values as compared to WT cells.

Regardless of the mode of infection, i.e. for both topical infection and intrahaemocoel injection assays, visual inspection of the cadavers revealed alterations in the melanization patterns during infection and death of the insect. Infection of *G. mellonella* larvae by the WT strain results in a characteristic gradual darkening (melanization) of the insect during the course of the infection, which by the time the infected insect is near death or has died (~24 h post-mortality) renders the cuticle a brown to dark brown discoloration (Fig. 3). In contrast, at or immediately following death of larvae infected by the ΔBbcal1 strain, only discrete patches of melanization are visible on the insects, and melanization over the entire cuticle as seen for WT infections does not occur. Within 5–7 days post-mortality, a profusion

**Table 2.** Calculated LT$_{50}$ values derived from intrahaemocoel injection of *B. bassiana* WT and ΔBbcal1 conidia into *G. mellonella* larvae

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>WT</th>
<th>ΔBbcal1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA</td>
<td>2.67±0.10</td>
<td>3.32±0.33</td>
</tr>
<tr>
<td>CZ</td>
<td>2.93±0.28</td>
<td>2.97±0.34</td>
</tr>
<tr>
<td>SDAY</td>
<td>2.99±0.10</td>
<td>5.97±0.45</td>
</tr>
<tr>
<td>PDA + C$_{16}$</td>
<td>2.51±0.16</td>
<td>3.46±0.22</td>
</tr>
<tr>
<td>PDA + triolein</td>
<td>2.42±0.11</td>
<td>3.02±0.08</td>
</tr>
<tr>
<td>PDA + olive oil</td>
<td>2.57±0.14</td>
<td>3.47±0.12</td>
</tr>
<tr>
<td>PDA + oleic acid</td>
<td>3.62±0.17</td>
<td>3.67±0.21</td>
</tr>
</tbody>
</table>

*LT$_{50}$ calculated using 800 conidia per larval injection. Values indicate mean±SE.*
of mycelia and conidiation is seen for both the WT and ΔBbcal1 strains on infected cadavers (Fig. 3).

Neutral lipid analysis in B. bassiana

Changes in total neutral lipid contents, i.e. diacylglycerol (DAG) and TAG levels in the WT and ΔBbcal1 strains, were

examined in conidia harvested from different growth substrates. Growth substrates included PDA, CZA, SDAY and PDA supplemented with oleic acid, C₁₆, olive oil and glycerol trioleate. No significant changes in DAG content were seen between the WT and the ΔBbcal1 mutant strain in conidia isolated from the various growth substrates with the exception of growth on olive oil, in which the DAG content in the ΔBbcal1 mutant was significantly higher than the WT (Fig. 4a, P<0.05). TAG content was much higher (20–30×) than DAG content in the cells examined; however, no significant differences were noted between the WT and mutant strains in TAG content, although under a number of conditions, i.e. CZA and PDA + oleic acid, a large variation was seen (Fig. 4b).

DISCUSSION

Media composition, i.e. the growth substrates from which fungal spores are isolated, is known to influence virulence of fungal insect pathogens (Kim et al., 2014; Maldonado-Blanco et al., 2014; Pelizza et al., 2011), with complex relationships between various spore parameters, e.g. stress response, germination rate and cuticle degrading enzyme activities, reported (Mascarin et al., 2013; Rosas-Garcia et al., 2014). Conidia derived from media containing lower carbon/nitrogen ratios, including those derived from insect passage but subsequently grown on different synthetic media, were found to display lower LT₅₀ values (i.e. were more virulence) (Safavi et al., 2007). However, it has also been reported that B. bassiana conidia isolated directly from insect cadavers were less virulent that those harvested from rice or synthetic media and that the method of application can influence bioassay results (Santoro et al., 2007). A comparison of B. bassiana grown on colloidal chitin, insect (Sphenarium purpurascens) cuticle, wheat bran or Sabouraud dextrose agar, revealed similar LT₅₀ values for all conidia against adults of the mealworm beetle (Tenebrio molitor) but differential mortality against T. molitor larvae (Rodriguez-Gomez et al., 2009). Similarly, small effects
were reported for the entomopathogenic fungus *Metarhizium anisopliae* when conidia were isolated from media containing various carbon and nitrogen ratios and only moderate correlations were seen between protease and lipase activities and virulence when tested against larvae of the diamondback moth (*Plutella xylostella*) (Wu et al., 2010). Stress conditions have also been shown to affect *M. anisopliae* virulence, with the highest mortality reported for conidia grown on minimal media containing lactose (Pedrini et al., 2008). *B. bassiana* conidia grown on C16 as the sole carbon source displayed decreased LT50 values (increased virulence) when tested against the bean weevil (*Acanthoscelides obtectus*) as compared to cells isolated from glucose grown agar (Crespo et al., 2002), and inducible pathways for assimilation of long-chain hydrocarbons that are prevalent on the insect epicuticle have been reported (Pedrini et al., 2010; Zhang et al., 2012). For the most part, however, relatively small effects have been reported and only the LT50 parameter has been examined.

Our data show that *B. bassiana* WT, grown on standard PDA and CZA mycological media, produces conidia that have lower LD50 values (15-fold more infective) when tested using topical bioassays, as compared to the carbon/nitrogen-rich medium, SDAY. Conidia isolated from PDA were more efficacious than those derived from either CZA or SDAY, with the latter showing a dramatic ~2 days shift in LT50. These data are in general agreement with previous reports (see above) indicating that production on more minimal media results in more virulent spores. Among the mid-chain to long-chain alkanes, C16 is known to be one of the preferred carbon sources for *B. bassiana*, and oleic acid can be used as an energy source that can feed directly into LD formation pathways (Pedrini et al., 2010, 2013). Olive oil consists of TAGs and/or other compounds, or the combined constituents may act to induce other aspects of fatty acid metabolism. Supplementation of PDA with C16 olive oil or oleic acid increased the infectivity of conidia two- to fourfold but had little effect on the efficacy of the conidia. However, conidia isolated from PDA containing triolein (TAG, glycerol trioleate) were twofold less infective than those isolated on PDA alone, although equally efficacious. These data imply that the components of olive oil, i.e. free fatty acids, mixture of TAGs and/or other compounds, or the combined constituents result in the production of more virulent conidia.

In addition to the effects on virulence, a distinct alteration in host melanization that occurs during the infection process was noted. During WT infections, whether topical or via artificial intrahemocoele injection of the fungal conidia, a gradual darkening of the insect cuticle occurs up to and after mortality of the insect. During the last stages of infection, as the insect is dying, internal fungal hypha penetrate outwards, growing as mycelia and sporulating on the cadaver within 5–6 days postmortality (Ortiz-Urquiza & Keyhani, 2013, 2016). This host melanization is typically considered to be part of the host defence response; however, in the *ΔBbcal1* mutant, which is impaired in virulence, host melanization also appears to be dramatically reduced, with only small, localized melanized patches visible on infected host during the time of death. The darkening of the host cuticle may also be linked to the production to fungal secondary metabolites. This raises an intriguing alternative hypothesis that this darkening/melanization response during the late stages of infection is either actively induced by the fungus (rather than being an insect defends response) or that a lack of production of a critical late-stage fungal metabolite(s) occurs in the caleosin mutant. Although speculative, this phenomenon may help fungal infection in several ways including diverting resources away from other defence responses and/or minimizing potential competition by other microbes as the insect dies.

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**Fig. 4.** DAG (a) and TAG (b) contents in WT and *ΔBbcal1* conidia. DAG and TAG levels were examined in *B. bassiana* WT and *ΔBbcal1* mutant conidia harvested from PDA, CZA, SDAY, PDA + oleic acid, PDA + C16, PDA + olive oil and PDA + glycerol trioleate as detailed in Methods. Error bars±SE.
Our data strongly support the idea of growth substrate ‘priming’ of conidia. This priming may entail several processes that can include (pre-) induction of pathways in conidia via accumulation of (1) gene transcripts and/or proteins (e.g. enzyme, transporters and regulators) as determined by the original growth substrate that would allow for utilization of similar carbon and nitrogen sources more rapidly, (2) metabolites and energy stores that can act as stress response modulators and rapid sources of energy and/or (3) factors that directly affect host interactions, e.g. cuticle degrading enzymes, secondary metabolites and toxins, compounds needed for adhesion, more rapid germination and penetration of insect cuticle. As expected, the neutral lipids seen in the fungal conidia were mainly composed of TAGs (10–30-fold higher as compared to DAGs); however, little difference was seen between either TAG or DAG content between the WT and ΔBbcal1 mutant. This is in contrast to significant changes seen in phospholipid, ceramide and even ergosterol levels in the caleosin mutant as compared to the WT (Fan et al., 2015). The only significant difference between the WT and ΔBbcal1 mutant was seen in total DAG content when grown on PDA containing olive oil; intriguingly, these conditions also result in the formation of copious amounts of LDs in the mutant fungal cells (Fan et al., 2015).

LD formation has been linked to virulence in a number of fungi including via regulation of cellular DAG in the rice blast fungus, Magnaporthe oryzae (Sadat et al., 2014), by the fat storage-inducing transmembrane protein 2 in Candida parapsilosis and through the activity of a glycerol-3-phosphate acetyltransferase that contributes to TAG biosynthesis in Metarhizium robertsii (formerly Me. anisopliae) (Gao et al., 2013), where lipid metabolism has also been linked to autophagy (Duan et al., 2013). In the plant fungal pathogen Colletotrichum orbiculare, LDs appear to accumulate and then disappear during appressorial maturation, the latter specialized fungal infection structures used to penetrate host tissues (Asakura et al., 2012), and a perilipin (Plin1 homologue), a major protein constituent of LDs, has been implicated in LD maintenance, appressorial turgor pressure and virulence in Me. robertsii (Wang & Leger, 2007). These data suggest the importance of lipid mobilization in infection by certain fungi. A B. bassiana caleosin (BbCal1), another protein constituent of LDs, has recently been characterized (Fan et al., 2015). Targeted gene inactivation of Bbcal1 did not significantly affect normal growth and germination or stress response; however, altered cellular phospholipid profiles were noted and changes in intracellular vesicle-like structures, that may represent distorted LDs, vacuoles and/or endoplasmic reticulum elements, were seen. In addition, a decrease in the LT50 was seen in topical insect bioassays from conidia harvested from PDA plates. Here we have extended the analysis of the virulence deficiency of ΔBbcal1 mutants to examine both infectivity and efficacy and, in particular, to determine whether impairment occurred during pre/penetration events or post-penetration, the latter during growth in the insect haemocoel and requiring competent immune evasion. Conidia of the ΔBbcal1 mutant derived from PDA showed a >20-fold decrease in infectivity as compared to WT and a dramatic loss (4 days) of efficacy in topical bioassays. On CZA, infectivity was even lower (~40-fold compared to WT CZA conidia), and efficacy was also 4 days lower than the WT counterpart. ΔBbcal1 conidia harvested from SDAY were the least infectious of all conditions tested, although they were only fivefold lower than SDAY WT conidia. However, the efficacy of the ΔBbcal1SDAY conidia was so low that an accurate LD50 could not be calculated. These data indicate that caleosin functioning is critical for both infectivity and efficacy of B. bassiana infection of G. mellonella larvae. Addition of C16, olive oil or oleic acid to PDA improved the infectivity of the resultant ΔBbcal1 conidia; however, LD50 values were still 20–44-fold higher than their WT counterparts. The only exception in the trends observed was seen using PDA + triolein, in which conidia fared worse (approximately twofold) in terms of infectivity than those isolated from PDA for the WT, whereas they fared better (approximately twofold) for the ΔBbcal1 mutant. However, overall, the ΔBbcal1triolen conidia were fivefold less infective than their WT counterparts. These data imply that loss of caleosin functioning impacts the ameliorating effects of growth on the various lipid substrates in terms of infectivity. The efficacy of the ΔBbcal1 mutants grown in the presence of various lipids was also significantly affected: ~3–4 days increase for PDA + C16 triolein and oleic acid, but only an ~2 days increase for PDA + olive oil.

In conclusion, our data illustrate two key points concerning insect virulence mediated by the entomopathogenic fungus, B. bassiana. The first is the growth substrate dependence on the virulence of resultant conidia, with the major consequences seen with respect to (1) topical infection, with in general, minor effects seen once the cuticle has been breached and (2) infectivity, with only minor effects seen with respect to efficacy. Growth substrates that included lipids commonly found on insect cuticles generally increased (topical) infectivity, and the C/N-rich medium, SDAY, resulted in spores with lower infectivity and efficacy. These data are potentially useful in production strategies for the biological control agent. The inability to properly produce and/or regulate LD formation and turnover via a caleosin-dependent pathway significantly decreased both infectivity and efficacy, with the major effect seen in topical assays. These latter data support a model in which lipid mobilization is critical for pre- and/or penetration events, but less important for subsequent proliferation within the haemocoel and immune evasion. Important questions that remain include determining the molecular contributions and functioning of the caleosin within the context of LDs, interacting proteins and their regulation in fungi.

ACKNOWLEDGEMENTS

This work was supported in part by National Science Foundation grant IOS-1557704 to N. O. K.
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Edited by: V. J. Cid and A. H. Herrera-Estrella