The autophagy gene \textit{BbATG5}, involved in the formation of the autophagosome, contributes to cell differentiation and growth but is dispensable for pathogenesis in the entomopathogenic fungus \textit{Beauveria bassiana}

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Autophagy is a highly conserved process, representing the major eukaryotic degradative pathway of cellular components. Autophagy-mediated recycling of cellular materials contributes to cell differentiation, tissue remodelling and proper development. In fungi, autophagy is required for normal growth and cell differentiation. The entomopathogenic fungus \textit{Beauveria bassiana} and its invertebrate targets represent a unique model system with which to examine host–pathogen interactions. The \textit{ATG5} gene is one of 17 involved in autophagosome formation, and the \textit{B. bassiana} homologue (\textit{BbATG5}) was identified. The role of autophagy in \textit{B. bassiana} growth and virulence was investigated via construction of a targeted gene knockout of \textit{BbATG5}. The mutant strain displayed increased sensitivity to nutrient limitation, with decreased germination and growth as compared with the wild-type parent. Conidiation was severely compromised and conidia derived from the \textit{DBbATG5} strain were altered in morphology. Cell differentiation into blastospores was also greatly reduced. Despite the significant growth and developmental defects, insect bioassays using the oriental leafworm moth, \textit{Spodoptera litura}, indicated a modest (~40\%) decrease in virulence in the \textit{DBbATG5} strain. The phenotypic defects of the \textit{DBbATG5} strain could be restored by introduction of an intact copy of \textit{BbATG5}. These data suggest that unlike several plant and animal pathogenic fungi, where \textit{ATG5} is required for infection, in \textit{B. bassiana} it is dispensable for pathogenesis.

\textbf{INTRODUCTION}

Autophagy is a ubiquitous, non-selective degradation process in eukaryotic cells that is conserved from yeast to man (Pollack \textit{et al.}, 2009). The autophagy pathway proceeds via formation of double-membrane vesicles termed autophagosomes, which deliver excess or damaged cell components to vacuoles or lysosomes for proteolytic degradation and recycling. Autophagy-based cellular remodelling allows organisms to tolerate extensive phases of nutrient starvation and other stress conditions by elimination or turnover of superfluous and/or dysfunctional cell organelles and constituents. In higher eukaryotes, autophagy has been implicated in physiological processes that span development, proliferation, remodelling, ageing, tumour suppression, neurodegeneration, antigen presentation, innate immunity, regulation of organismal lifespan, and cell death (Levine & Klionsky, 2004; Reumann \textit{et al.}, 2010). Approximately 34 \textit{ATG} (autophagy-related) genes have been identified, of which at least 18, including \textit{ATG5}, represent the ‘core’ machinery involved in all autophagy-related pathways and are considered indispensable for formation of the autophagosome (Suzuki & Ohsumi, 2007; Reumann \textit{et al.}, 2010).

In filamentous fungi, autophagy appears to play a significant role not only in endogenous nutrient recycling in response to starvation, but also in normal fungal development,
In various filamentous fungi, including *Magnaporthe grisea*, *Aspergillus fumigatus*, *Aspergillus oryzae* and *Podospora anserina*, targeted gene knockout mutants of autophagy genes (amongst the 17 ‘required’ genes or others) result in a loss of cellular differentiation, which includes reduction of hyphal density, decreased production of conidia, aerial hyphae, and loss of secondary metabolites and sexual structures (perithecia and proetoperithecia) (Pinan-Lucarré et al., 2005; Pollack et al., 2009; Bartoszewska et al., 2011; Kikuma & Kitamoto, 2011).

For a number of fungal pathogens, the autophagy process has been shown to contribute to virulence. In the rice blast fungus *Magnaporthe oryzae*, deletion mutants of *Mgatg1*, *Mgatg4*, *Mgatg5*, *Mgatg8* and *Mgatg9* result in an inability to form the infection structure and loss of the ability of the fungus to infect its plant host (Veneault-Fourrey et al., 2006; Liu et al., 2010, 2007; Dong et al., 2009; Lu et al., 2009). In the basidiomycete plant-pathogenic fungus *Ustilago maydis*, deletion of *agt1* or *agt8* also blocks autophagy and affects morphogenesis, but the *Delta*1 strain is only slightly less pathogenic than the wild-type (WT), whereas the *Delta*8 strain is significantly reduced in fungal pathogenicity (Nadal & Gold, 2010). The double mutant strain *Delta*1*Delta*8 displays even greater morphogenic and developmental defects than the single mutants, and is completely suppressed in plant gall induction. In the animal pathogen *Candida glabrata*, mobilization of intracellular resources via autophagy has also been shown to participate in virulence, supporting the viability of the fungus within the phagosomal compartment of infected innate immune cells (Roetzer et al., 2010).

*Beauveria bassiana* is an insect pathogen that is currently actively being studied as an alternative to chemical pesticides for insect control, including as a means for reducing disease transmission of human vector-borne agents (Feng et al., 1994; Fang et al., 2011; Fan et al., 2012). Fungal infection is percutaneous, with conidia attaching and germinating on the host cuticle (Holder & Keyhani, 2005). The growing hyphae penetrate the cuticle via expression of a host of cuticle-degrading enzymes, ultimately reaching the insect haemocoel (Fang et al., 2009; Zhang et al., 2012). Once inside the haemocoel, the fungus undergoes a dimorphic transition, elaborating in vivo cells termed hyphal bodies that are able to evade the host immune system and consume the nutrients available in the haemocoel (Wanchoo et al., 2009; Lewis et al., 2009). The fungus then works its way back out of the insect body, ultimately sporulating on the insect cadaver. To date, nothing is known concerning the potential role of autophagy in insect-pathogenic fungi. Given the oligotrophic conditions present on the host, the contribution of autophagy to the development and virulence of *B. bassiana* was investigated.

The autophagy-related gene *ATG5* is part of a ubiquitin-like protein system in which an ATG12–ATG5 covalent conjugate interacts with ATG16, forming a multimeric complex involved in assembly of pre-autophagosomal structures, playing an essential role in autophagosome formation (Kuma et al., 2002). In this study, the *B. bassiana* *ATG5* homologue, *BbATG5*, was isolated. A targeted gene knockout mutant of *BbATG5* was constructed and its phenotype characterized. The *DeltaBbATG5* strain was sensitive to nutrient limitation, displaying slower germination and growth rates as compared with the WT and complemented strains. The *BbATG5* mutant strain produced abnormal conidia, and cell differentiation into blastospores was also greatly reduced. Insect bioassays, however, revealed only a small to moderate (~40%) decrease in virulence in the *DeltaBbATG5* strain. These data suggest that unlike several plant and animal pathogenic fungi where *ATG5* is required for infection, in *B. bassiana*, *ATG5* contributes to, but is dispensable for, pathogenesis.

### METHODS

#### Fungal strains and growth conditions

The fungal strain *B. bassiana* 2860 (ARSEF 2860; RW Holley Center for Agriculture and Health) was preserved as dry conidia mixed with sterile sand at ~76°C. *Escherichia coli* DH5α (Invitrogen) was used for propagation of the plasmids and was cultured in Luria–Bertani medium supplemented with 100 μg ampicillin ml⁻¹ or 50 μg kanamycin ml⁻¹, depending upon the plasmid resistance marker used. Fungal strains were cultured either on Sabouraud dextrose medium (4% glucose, 1% peptone, 1% yeast extract (SDB), and supplemented with 1.5% agar to make SDJ or on Czapék–Dox (Cz) medium. Czapék–Dox agar (CzA) was used to culture and screen the transformants, and CzA-N plates or Cz-N broth medium lacking a nitrogen source was used as the nitrogen starvation medium. Oatmeal agar (OMA; 3.0% oatmeal, 1.5% agar) was used as a nutrient-poor medium.

#### Cloning and analysis of genomic and cDNA sequence of *BbATG5*

Total RNA was extracted from mycelia using TRIzol Reagent (Invitrogen) from 3-day colonies grown on CzA as previously described (Ying & Feng, 2011), and fungal genomic DNA was extracted using a phenol/chloroform method as described by Raeder & Broda (1985). The predicted conserved region (PRFSYLA) of known *ATG5* genes from filamentous fungi (fungal species, accession no.: *Neurospora crassa*, XP_959205; *Cordyceps militaris*, EGX96255; *Metarhizium acridum*, EFY90145; *Metarhizium anisopliae*, EFZ01682; *Gloeomycellula graminicola*, EFQ32306; *Verticillium dahlia*, EY13478; *Verticillium albo-atrum*, XP_003008867) was used as a template for the design of degenerate primers (ATG5F and oligo-dT 17) for amplification of the partial genomic sequence for *BbATG5*. Total genomic DNA from 3-day old colonies was used as a template for amplification with the partial *B. bassiana* cDNA sequence (Table 1). The PCR product (and all subsequent PCR products) was cloned into pGEM-T Easy (Promega), and the nucleotide sequence of the insert was determined using a commercial facility (Invitrogen). The *B. bassiana* partial *ATG5* cDNA sequence was then used to design forward and reverse primers (ATG5F and ATG5R) for isolating a partial genomic sequence for *BbATG5*. The resultant PCR product was used to design four primers for PCR-cloning of 5’ and 3’ sequences of *BbATG5* in order to obtain the full-length genomic DNA sequence of the gene (DNA walking, primers listed in Table 1). The resultant fragments and the full-length gene sequences were assembled by two rounds of DNA walking using the SpeedUp Premix kit II (Segene).
Table 1. PCR primers used for gene cloning, and generation and identification of gene replacement mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>ATG5F</td>
<td>CCCCCGTCTTTCTTAYTNGCC</td>
<td>Gene cloning</td>
</tr>
<tr>
<td>Oligo(dT&lt;sub&gt;17&lt;/sub&gt;)</td>
<td>GACTCGAGTCGACATCGA(T)&lt;sub&gt;17&lt;/sub&gt;</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>ATG5R</td>
<td>CAAAACAAAGGCAACACCAT</td>
<td>Gene cloning</td>
</tr>
<tr>
<td>ATG5UR1</td>
<td>GTTCGACCGAGTCGCCAGGG</td>
<td>Upstream DNA walking</td>
</tr>
<tr>
<td>ATG5UR2</td>
<td>TTCTATCGATGCTGTCGCC</td>
<td>Downstream DNA walking</td>
</tr>
<tr>
<td>ATG5DF1</td>
<td>AATGCCCCCGCGAGGCTGC</td>
<td>Cloning 5′ fragment for disruption vector</td>
</tr>
<tr>
<td>ATG5DF2</td>
<td>GCTGTGAGCTGACCCACC</td>
<td>Cloning 3′ fragment for disruption vector</td>
</tr>
<tr>
<td>ATG5R</td>
<td>CCGAGAATTTCCGGTGCATTGGGG</td>
<td>Confirming knockout and complemented mutant</td>
</tr>
<tr>
<td>A5F</td>
<td>GCCGTGCCACATGCTGACACC</td>
<td>Probe for Southern blotting</td>
</tr>
<tr>
<td>A5R</td>
<td>GGCGGACTAGCGAAGCACTCTT</td>
<td>Cloning the full gene for complementation</td>
</tr>
<tr>
<td>A5CF</td>
<td>GGGGACCTCATATTCCGGTGAAGGTTGGATTGATACGCCTGCCC</td>
<td></td>
</tr>
<tr>
<td>A5CR</td>
<td>GGGGACCTATTCCGGTGAAGGTTGGATTGATACGCCTGCCC</td>
<td></td>
</tr>
<tr>
<td>A5pF</td>
<td>ATGGGAAGCGAGGAGGAGGCC</td>
<td></td>
</tr>
<tr>
<td>A5pR</td>
<td>ATGGGAAGCGAGGAGGAGGCC</td>
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</tr>
<tr>
<td>A5tF</td>
<td>TGGATGCGAATGAGGAGGCC</td>
<td>RT-PCR</td>
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<tr>
<td>A5tR</td>
<td>CGGGACGAGCAATAGGACCC</td>
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</tr>
<tr>
<td>TubulinF</td>
<td>TACTCTACGATGGGTTGGG</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>TubulinR</td>
<td>TGCTGGAACAGGAGGCTGTCCT</td>
<td>RT-PCR</td>
</tr>
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</table>

Disruption and complementation of BbATG5 in fungi. For targeted gene knockout vector construction, separate upstream and downstream flanking sequences of BbATG5 were amplified from B. bassiana genomic DNA and inserted into the plasmid vector p0380-bar containing the phosphothionin resistance marker gene (Xie et al., 2012). The 5′ (1.55 kb) and 3′ (1.49 kb) flanking sequences were amplified using primers ATG5UF/ATG5UR and ATG5DF/ATG5DR, respectively, and inserted into the EcoRI–BamHI and XhoI–SpeI sites of p0380-bar, respectively, to generate p0380-bar-BbATG5s. The complementation vector was constructed using the entire BbATG5 gene plus 2.17 and 0.5 kb of upstream and downstream sequences, respectively. Primer pair A5CF/A5R (Table 1) was used for PCR amplification using genomic DNA as the template, and the resultant PCR fragments were recombined into p0380-sur-Gateway (Xie et al., 2012) using Gateway BP Clonase II Enzyme Mix (Invitrogen), generating plasmid p0380-sur-BbATG5 containing the sulfonylurea resistance marker for selection of transformants. The disruption and complementation vectors were propagated in E. coli DH5α and transformed into Agrobacterium tumefaciens AGL-1. All B. bassiana transformants were obtained using an Agrobacterium-mediated transformation protocol as described by Michielse et al. (2008). B. bassiana targeted gene knockout mutants were selected on CzA supplemented with 200 μg phosphothionin ml⁻¹, and the complemented mutants were screened on the same medium containing 15 μg chorimuron ethyl ml⁻¹ (Ying & Feng, 2006; Xie et al., 2012).

PCR and Southern blotting were used to confirm correct integration events in the transformants. PCR confirmation was performed using genomic DNA extracted from transformants as the template and primers ATG5F/ATG5R (Table 1). BbATG5 gene expression (transcript production) was examined by RT-PCR using BbATG5 gene-specific primers (A5tF/A5tR), and primers for the amplification of tubulin (TubulinF/TubulinR) as a control (Table 1). Southern blotting was performed using 10 μg genomic DNA digested with BglII/BamHI. The digested DNA was separated in a 1.0 % agarose gel and subsequently transferred to a Biodyne nylon membrane (Gelman Laboratory) using standard protocols. Blots were probed with a 552 bp PCR-amplified product generated using primers ATG5pF and ATG5pR. Probe preparation, membrane hybridization and visualization were performed using a DIG High Prime DNA Labeling and Detection Starter kit II with a chemiluminescence detection method (Roche).

Fluorescence microscopy and cytological analysis. For visualization of autophagosomal structures in mycelium, conidia were inoculated into SDB medium at a concentration of 10<sup>6</sup> conidia ml⁻¹, and cultured at 25 °C for 48 h with constant shaking at 120 r.p.m. The mycelia were collected and rinsed with sterile water to remove any media, after which the mycelia were transferred to Cz-N liquid medium containing 50 mM LiCl as an inducer of autophagy (Park et al., 2011). Cultures were incubated for an additional 3 h with shaking at 120 r.p.m. After induction, the mycelia were rinsed and stained in PBS (pH 7.0) containing 0.1 mM monodansylcadaverine (MDC; Sigma) at 37 °C for 10 min (Niemann et al., 2001). After incubation, the cells were washed three times in PBS, and mounted on glass slides under coverslips. Fluorescence images were obtained by using a DMR microscope (Leica). MDC was excited at 375 nm, and the emission was collected at 455 nm. Bright-field and fluorescence images were taken of the same fields. The ultrastructural features of the autophagosome in B. bassiana were examined using transmission electron microscopy (TEM) as
Assessment of conidial quality, vegetative growth, sporulation capacity and virulence. All phenotypic analyses were performed among the WT, the targeted gene knockout mutant (ΔBbATG5) and the complemented mutant (ΔBbATG5::BbATG5) strains. All experiments were performed in triplicate and repeated using at least two independent batches of cells.

Germination. To assay conidial germination rates under different conditions, 50 μl aliquots of conidial suspensions (from 10-day-old SBA plates, adjusted to 10^6 conidia ml\(^{-1}\)) were spread onto various media, and incubated for 24 h at 25 °C. Percentage germination was determined via microscopic examination. Conidia were considered germinated if the length of the germ tube was at least the same as the width of the conidia. For each sample at least three different microscope fields were examined with at least 100 conidia counted. The various media tested in germination assays included SDA, CzA, CzA-N and 0.02 % Tween-80 agar (TWA).

Vegetative growth. Hyphal and mycelial growth was assessed using SDA, CzA, OMA and CzA-N. Aliquots of 100 μl cell suspensions (10^6 conidia ml\(^{-1}\)) were spread onto sterilized cellophane sheets and placed in Petri dishes. The cell-impregnated sheets were incubated for 3 days at 25 °C, after which 5 mm diameter discs were cut from the cellophane, placed on various agar plates, and incubated for an additional 7 days at 25 °C, after which the radial growth (colony diameter) of vegetative mycelia was measured.

Sporulation (conidia and blastospore) capacity. To examine the effects of growth conditions on conidial production, the conidial yield on SDA and OMA plates was quantified as previously described (Xie et al., 2012). Briefly, aliquots of 100 μl cell suspensions (10^6 conidia ml\(^{-1}\)) were evenly spread on SDA and OMA plates and incubated for 7 days at 25 °C. After incubation, 5 mm diameter plugs were cut from the agar and the conidia were washed into 0.02 % Tween-80. Cell concentrations of the conidial suspensions were determined via counting using a haemocytometer and converted to the number of conidia per square centimetre of colony.

The production of blastospores (yield) was quantified in SDB medium. Conidia were inoculated into the broth medium at a concentration of 10^6 conidia ml\(^{-1}\), and after culturing at 25 °C for 36 h, the concentration of blastospores in the medium was assessed using microscopic counts with a haemocytometer and converted to the number of cells per millilitre of culture medium.

Insect bioassays. Fungal virulence was assessed using second-instar larvae of Spodoptera litura. Batches of 30–35 larvae were placed in a Petri dish and treated with 1 ml conidial suspensions (10^6 conidia ml\(^{-1}\)) atomized using an automatic Potter spray tower (Burrhard Scientific) at a working pressure of 0.7 kg cm\(^{-2}\). Larvae were fed daily via changes of leaf discs, and were maintained at 25 °C using a light cycle of 12 h light/12 h dark for 7 days. The number of dead insects was recorded daily, and the median lethal time (LT\(_{50}\)) was calculated by Kaplan–Meier statistics. All experiments were repeated three times using the conidial suspension solution (0.02 % Tween-80) as the mock infection control.

Data analysis. All measurements, including conidial germination percentage, vegetative growth rate, LT\(_{50}\) and sporulation capacity from triplicate assays, were subjected to one- or two-way ANOVA. Nucleotide and amino acid sequence analyses were performed using the online BLAST portal (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL W.

RESULTS

Molecular characterization of the B. bassiana ATG5 gene and generation of ΔBbATG5 complemented (ΔBbATG5::BbATG5) strains

A partial cDNA sequence for B. bassiana ATG5 was amplified using degenerate primers designed to a conserved region based upon known ATG5 genes identified in other filamentous fungi or otherwise referred to in Methods. To obtain the complete BbATG5 nucleotide sequence, the partial B. bassiana cDNA sequence was used to design primers for genome walking. After two rounds of DNA walking, a total of 4416 bp of sequence was obtained, which included the complete BbATG5 ORF (905 bp) and 2382 and 1129 bp of flanking upstream and downstream sequences, respectively. Only one intron was identified in the BbATG5 genomic sequence, and the coding sequence of BbATG5 was deduced to be 810 bp long, encoding a protein consisting of 269 amino acids.

To examine the role of BbATG5 in B. bassiana and to characterize its phenotype, a targeted gene disruption mutant was constructed in which a 49 bp region of the gene sequence was replaced via homologous recombination by the 942 bp bar gene cassette conferring resistance to phosphinothricin (see Fig. S1a available with the online version of this paper). A complementation vector was constructed using the intact gene fragment with its endogenous promoter (2.6 kb of upstream sequence) and the sulfonyl urea (sur) resistance cassette that was then transformed into the knockout mutant. After transformation and selection (as described in Methods), putative recombinants were randomly selected and subjected to PCR verification for the correct homologous replacement event. As expected, a 193 bp fragment of the partial BbATG5 ORF was PCR-amplified from the WT and ΔBbATG5/ΔBbATG5 but not from ΔBbATG5, while a ∼1100 bp fragment harbouring bar was present in the knockout mutant and complemented mutant but not in the WT strain (Fig. S1b). RT-PCR experiments using primers corresponding to the BbATG5 cDNA sequence confirmed complete loss of the BbATG5 transcript in the knockout strain and recovery of the transcript in complemented strains (Fig. S1c). Further confirmation of the integrity of the targeted gene disruption and

previously documented (Lu et al., 2009). Briefly, the induced mycelia (as above) were washed three times in PBS to remove any medium. After fixation overnight at 4 °C in modified Karnovsky’s fixative containing 2 % paraformaldehyde and 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and post-fixation in 1 % OsO\(_4\), the mycelia were dehydrated in a graded ethanol series, embedded in Spurr resin, and stained with 2 % uranyl acetate and Reynold’s lead solution. The samples were examined under an H-7650 transmission electron microscope (Hitachi).

To examine conidial morphology, conidia were harvested from 10-day-old cultures grown on SDA. Conidial suspensions were adjusted to a concentration of 10^5 conidia ml\(^{-1}\) and flow cytometry (model FC500, Beckman Coulter) was used to analyse cell size using a forward scatter detector (FSc), and cell complexity using a side scatter detector (SSc) (Puttikamonkul et al., 2010).
complemented clones was obtained by Southern blotting (Fig. S1d). The mutant and complemented strains were designated ΔBbATG5 and ΔBbATG5::BbATG5, respectively. In all phenotypic aspects examined below, the complemented strain was indistinguishable from the WT parent.

**Disruption of the ATG5 gene blocks normal autophagy**

Changes of the autophagic process in *B. bassiana* hyphae were examined using MDC, which represents/requires acidified compartments and/or vesicle formation and is used to monitor the induction and vesicle formation steps of autophagy (Klionsky *et al.*, 2007). Fungal cells were stained with MDC and examined by fluorescence microscopy as detailed in Methods. After culturing in nitrogen-limited liquid medium in the presence of 50 mM LiCl for 3 h, abundant autophagic bodies as well as lysosomes were detected in the WT and complemented (ΔBbATG5::BbATG5) strains, whereas few autophagic bodies were observed in cells derived from the ΔBbATG5 strain, with the larger lysosomes still apparent (Fig. 1a). TEM also verified that many autophagic bodies were observed inside vacuoles of the WT strain and complemented strain when autophagy was induced, but few were

![Gene disruption of BbATG5 blocks the autophagic process and conidial development in B. bassiana](http://mic.sgmjournals.org)

**Fig. 1.** Gene disruption of BbATG5 blocks the autophagic process and conidial development in *B. bassiana*. Autophagic bodies in mycelia of the WT (left), ΔBbATG5 (middle) and ΔBbATG5::BbATG5 (right) strains were examined by bright-field microscopy (top panel) and fluorescence microscopy (bottom panel) (a), and confirmed by TEM (b). (c) Morphological variation in conidia size and complexity was examined via flow cytometry and visualized by bright-field microscopy (inset). Analysis of conidia size (FSc) and complexity (SSc) was performed by examining 50 000 events (in triplicate). Bars: 10 μm (a, c); 1 μm (b).
found in the ΔBbATG5 mutant (Fig. 1b). These results confirmed that autophagy occurs in B. bassiana under nitrogen starvation, but was blocked when the autophagy gene BbATG5 was disrupted.

Microscopic examination of the conidia of the ΔBbATG5 mutant indicated some alteration in the morphology of these cells. To examine any such changes more quantitatively, conidial size and complexity were examined by flow cytometry using FSc and SSs detectors, respectively (Fig. 1c). The BbATG5 ORF disrupted strain showed an ~20% increase in the values of both FSc and SSs, compared with the values of the WT strain and complemented strain. These data revealed enlargement and distortion in conoidal morphology in the ΔBbATG5 strain that was also visually apparent (insets in Fig. 1c).

Disruption of BbATG5 results in developmental defects affecting conidial germination, vegetative growth and sporation

Except for germination on rich SDA media, significant differences were seen between the germination rates of conidia derived from the ΔBbATG5 strain as compared with the WT and complemented (ΔBbATG5::BbATG5) strains on a variety of nutrient agar media, including CzA, CzA-N (lacking nitrogen) and Tween-20 agar (minimal, starvation condition) after 24 h incubation (Fig. 2a, *P* < 0.05, two-way ANOVA for the various media except as indicated). In CzA, the WT and complemented strains retained almost 100% germination rates, whereas germination of the ΔBbATG5 mutant was reduced by ~20%. In media lacking nitrogen (Cz-N) and in the minimal Tween-20 agar,

germination of the parent and complemented strains was reduced to ~60 and ~43%, respectively, but only ~20% and less than 5% germination of conidia derived from the ΔBbATG5 mutant was observed under identical conditions.

Vegetative growth was assessed on SDA, OMA, CzA and CzA-N, as described in Methods. No significant differences were noted for growth on SDA and OMA between the WT and ΔBbATG5 strains (Fig. 2b). A slight but significant (*P* < 0.05) decrease in growth was noted for ΔBbATG5 on CzA, with a more dramatic ~50% decrease in growth seen on CzA-N (starvation media) for ΔBbATG5 as compared with the WT and complemented strains. Colony morphology was also affected on CzA-N for the ΔBbATG5 strain, with sparse mycelial growth noted. Despite no significant differences noted for the ΔBbATG5 strain with respect to vegetative growth (as measured by colony diameter) on SDA or OMA, conidial yield was dramatically reduced in both media, with the WT producing 1.80 ± 0.13 and 0.47 ± 0.05 × 10⁷ conidia cm⁻² on SDA and OMA, respectively, whereas conidial yield for the ΔBbATG5 strain was decreased by >80% to 0.29 ± 0.01 and 0.08 ± 0.01 × 10⁷ conidia cm⁻² on SDA and OMA, respectively. Conidial yield for the complemented mutant was essentially identical to the WT (Fig. 2c).

In rich broth culture, B. bassiana produces single yeast-like cells termed blastospores (Wanchoo et al., 2009). Blastospore production was dramatically reduced (>90%, *P* < 0.05) in the ΔBbATG5 strain, with the WT parent producing 9.33 ± 0.65 × 10⁷ blastospores ml⁻¹, ΔBbATG5 0.52 ± 0.09 × 10⁷ blastospores ml⁻¹ and the complemented

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**Fig. 2.** Germination, vegetative growth and sporulation capacity of B. bassiana WT, targeted gene knockout (ΔBbATG5) and complemented (ΔBbATG5::BbATG5) strains. (a) Percentage germination of conidia from the various strains plated on different nutrient agars: SDA, CzA, CzA-N and TWA. (b) Diameter of 7-day-old colonies grown on different media: SDA, CzA, OMA and CzA-N. (c) Amount of conidia produced (yield) per cm² after 7 days growth on SDA and OMA plates. All experiments were performed in triplicate with different batches of conidia. Error bars represent SD of the mean. A different letter above a bar indicates a statistical difference.
strain $\Delta BbATG5:: BbATG5$ 9.03 \pm 0.51 \times 10^7$ blastospores ml$^{-1}$ (Fig. 3).

**Effect of BbATG5 on pathogenicity**

The effect on virulence of $BbATG5$ was determined in insect bioassays using larvae of the oriental leafworm moth, *Spodoptera litura*, as the host. Conidia of the WT, $\Delta BbATG5$ and $\Delta BbATG5:: BbATG5$ strains were adjusted to $10^8$ c.f.u. ml$^{-1}$ and applied topically using a Potter spray tower. Mortality was measured over a 7-day period, and the data showed that percentage survival decreased with time post-inoculation (Fig. 4a). On the basis of the combined results from three independent bioassays of each strain, the LT$_{50}$ values of the WT, $\Delta BbATG5$ and $\Delta BbATG5:: BbATG5$ strains were calculated as 5, 7 and 5 days, respectively, by the Kaplan–Meier method. Statistical significance between curves was calculated by the logrank test. The results revealed that the survival curve of the knockout mutant differed significantly from the WT or complemented strain, although there was no significant difference between the curve for the WT and complemented strain (Fig. 4b). These data indicated a 40% decrease in the effectiveness of the fungus, and appeared to be more pronounced at the later stages of infection.

**DISCUSSION**

*Beauveria* species exhibit various lifestyles and have long been considered as important pathogens of insects. *B. bassiana*, in addition to being an insect pathogen, is able to form intimate endophytic associations with certain plants, and the cycling of nutrients from insects to plants via entomopathogenic fungi has recently been demonstrated (Gurulingappa *et al.*, 2010; Behie *et al.*, 2012). Autophagy is an evolutionarily conserved physiological process in eukaryotic cells that is increasingly being recognized as a mechanism regulating programmed cell fate, tissue and cellular remodelling, and development in many organisms (Pollack *et al.*, 2009). A number of aspects of the machinery involved in autophagy remain to be uncovered, although a core group of about 17 proteins appear to be required for proper autophagosome development, with perhaps up to 24 additional proteins involved in various aspects of the autophagic process. Amongst the core or
essential ATG proteins, ATG5 along with ATG12 and ATG16 (the ATG16-ATG5-ATG12 complex) are involved in the initial nucleation step of autophagosome formation, where ATG5 is localized to the outer face of the expanding ‘isolation’ membrane that will form the autophagosome (Suzuki & Ohsumi, 2007). In addition, the ATG12-ATG5 conjugate possesses an E3-like protein lipidation activity (Hanada et al., 2007).

The ATG5 gene was originally identified in a genetic screen for autophagy-deficient mutants in the yeast Saccharomyces cerevisiae (Tsukada & Ohsumi, 1993). In this study, an orthologue of the autophagy gene, ATG5, was isolated from the entomopathogenic fungus B. bassiana (BbATG5). Similar to other fungi, further genome screening indicated the presence of a single ATG5 gene in the B. bassiana genome (Xiao et al., 2012). To probe the functions of BbATG5 in B. bassiana, a targeted gene knockout mutant strain (ΔBbATG5) was constructed. Similar to what has been reported for other filamentous fungi, including M. grisea and Trichoderma reesei (Liu et al., 2011; Lu et al., 2009), ablation of ATG5 blocked autophagosome biogenesis in B. bassiana, thus limiting autophagy. In addition, a general phenotype of autophagy mutants, namely sensitivity to nutrient-limiting conditions including decreased growth and conidial germination on minimal media, was also seen for the ΔBbATG5 strain. Although growth did not appear impaired, even in rich media, the B. bassiana ΔBbATG5 mutant was defective in conidiation, producing ~75% fewer spores than the WT under identical conditions, a phenotype also reported for M. oryzae and T. reesei ATG5 knockout mutants (Lu et al., 2009; Liu et al., 2011). Furthermore, ΔBbATG5-derived conidia appeared distorted in morphology and their germination rates were decreased as compared with the parental strain on various media with the exception of the rich SDA medium.

In rich liquid broth, B. bassiana produces yeast-like single cells known as blastospores (Holder & Keyhani, 2005). Like the asexual spores or conidia, blastospores are infectious (Kirkland et al., 2004); however, conidia and blastospores display different surface properties, and comparative transcriptome analyses have revealed different sets of genes expressed in the two cell types (Wanchoo et al., 2009; Cho et al., 2006). Little is known concerning the biogenesis of these cells; however, blastospore production was almost completely blocked in the ΔBbATG5 mutant, implying a role for ATG5 and autophagy in blastospore development. Deletion of the autophagy genes atg8 and atg1 in U. maydis alters the budding process in this fungus, although the mechanism behind this phenotype remains obscure (Nadal & Gold, 2010). Our data indicate that although hyphal growth did not appear to be significantly affected, terminal differentiation, whether to conidia or to blastospores, appears to involve aspects of the autophagy process. Further research examining the connections between ATG5/autophagy and conidia/blastospore development is warranted.

Perhaps our most surprising phenotype was the relatively minor effect of disruption of ATG5 on B. bassiana insect virulence. Despite the blockage of blastospore development and the reduced conidial germination particularly on nutrient-limiting media (especially given that insect cuticles are considered a nutrient-poor microenvironment), virulence was reduced only ~40% for the ΔBbATG5 strain as compared with the WT parent. In contrast, deletion of ATG5 or ATG8 in M. grisea results in an avirulent strain, and disruption of atg1 or atg8 (with phenotypes further exacerbated in the atg1atg8 double mutant) in U. maydis reduces maize infection. Regarding M. grisea, infection proceeds via elaboration of an infectious structure known as an appressorium, which combines enzyme degradation with generation of immense appressorium-mediated turgor pressure to penetrate the plant host surface. During this process, the conidium that produces the appressorium undergoes cell death. In contrast, B. bassiana infection does not appear to involve a similar structure (unlike its sister insect fungal pathogen, Metarhizium anisopliae), and potential (poorly defined) appressoria have only been seen in certain B. bassiana strains. Instead, penetration appears more to involve enzyme degradation, although mechanical mechanisms may also play an important role. Furthermore, to date, there have been no reports of conidial death as a requirement during the infection process. It is intriguing to speculate, therefore, that the lack of conidium cell death and appressoria differentiation may account for the relatively minor effect of loss of ATG5 on virulence in B. bassiana. Further work examining additional genes in the autophagy pathway is likely to yield greater insights into the differential roles that autophagy may play in fungi, and in the growth and development of B. bassiana in particular.

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**REFERENCES**


Autophagy in entomopathogenic B. bassiana


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