

# Mitogen-Activated Protein Kinase *hog1* in the Entomopathogenic Fungus *Beauveria bassiana* Regulates Environmental Stress Responses and Virulence to Insects<sup>∇†</sup>

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*Beauveria bassiana* is an economically important insect-pathogenic fungus which is widely used as a biocontrol agent to control a variety of insect pests. However, its insecticide efficacy in the field is often influenced by adverse environmental factors. Thus, understanding the genetic regulatory processes involved in the response to environmental stress would facilitate engineering and production of a more efficient biocontrol agent. Here, a mitogen-activated protein kinase (MAPK)-encoding gene, *Bbhog1*, was isolated from *B. bassiana* and shown to encode a functional homolog of yeast *HIGH-OSMOLARITY GLYCEROL 1* (*HOG1*). A *Bbhog1* null mutation was generated in *B. bassiana* by targeted gene replacement, and the resulting mutants were more sensitive to hyperosmotic stress, high temperature, and oxidative stress than the wild-type controls. These results demonstrate the conserved function of HOG1 MAPKs in the regulation of abiotic stress responses. Interestingly,  $\Delta Bbhog1$  mutants exhibited greatly reduced pathogenicity, most likely due to a decrease in spore viability, a reduced ability to attach to insect cuticle, and a reduction in appressorium formation. The transcript levels of two hydrophobin-encoding genes, *hyd1* and *hyd2*, were dramatically decreased in a  $\Delta Bbhog1$  mutant, suggesting that *Bbhog1* may regulate the expression of the gene associated with hydrophobicity or adherence.

Mycoinsecticides are important insect pest control agents (10, 19, 41). Unlike entomopathogenic bacteria and viruses that invade insects through their digestive tracks, fungal pathogens penetrate the host integument and are considered the only group of microbial biocontrol agents active against sucking-type insect pests (21, 49). However, low killing speed and sensitivity to adverse environment factors such as desiccation, high temperature, and UV radiation limit the widespread use of entomopathogenic fungi (6, 22, 40). Thus, an understanding of the regulatory processes involved in response to environment stress is essential for commercial development and improvement of these biocontrol fungi.

Mitogen-activated protein kinases (MAPKs), a family of serine-threonine protein kinases, are widespread in eukaryotic cells and play crucial roles in transduction of a variety of extracellular signals and regulation of various development and differentiation processes (37, 45). MAPKs are usually activated by MAPK kinases, which are in turn activated by MAPK kinase kinases. These MAPK kinase kinase-MAPK kinase-MAPK cascades are conserved in eukaryotic cells and have been studied extensively in many organisms (45). In *Saccharomyces cerevisiae*, at least five MAPK pathways have been identified. These pathways are designated the FUS3, KSS1,

HOG1, SLT2, and SMK1 MAPK cascades, and they are involved in mating, filamentous growth, the high-osmolarity response, cell integrity, and ascospore formation, respectively (20). Recent studies showed that homologs of HOG1 are involved in responses to osmotic stress (12, 29, 44, 58), oxidative stress (28, 47), heat shock (28), and tolerance to a phenylpyrrole fungicide (12, 29, 58) in several other fungi. For instance, deletion of HOG1-encoding genes in *Candida albicans* (44), *Neurospora crassa* (58), *Magnaporthe grisea* (12), and *Colletotrichum lagenarium* (29) causes a defect in adaptation to high-osmolarity conditions. A *hog1*-silenced strain of *Trichoderma harzianum* was highly sensitive to osmotic stress and showed intermediate levels of sensitivity to oxidative stress (11). HOG1 MAP kinase is activated by high osmolarity, oxidative stress, and UV stress in *Debaryomyces hansenii* (47). In *Aspergillus nidulans*, a SakA protein kinase related to HOG1 has been implicated in stress signal transduction, sexual development, and spore survival (28). Inactivation of HOG1 orthologs in *M. grisea*, *N. crassa*, and *C. lagenarium* results in increased resistance to the phenylpyrrole fungicide fludioxonil (12, 29, 58). A *T. harzianum* strain overexpressing the *hog1*(*F315S*) allele was highly resistant to the calcineurin inhibitor cyclosporine A, which suggests that there are links between the two pathways (11). In addition, HOG1 orthologs participating in fungal pathogen-host interactions have been demonstrated to be present in the plant pathogen *Mycosphaerella graminicola* and the mycoparasitic fungus *T. harzianum*. Deletion of the HOG1 kinase gene in *M. graminicola* results in mutants that are non-pathogenic to the host plant (35). In *T. harzianum*, either hyperactivation [with the *hog1*(*F315S*) allele] or silencing (using RNA interference) of a HOG1 protein leads to strongly reduced antagonistic activity against some plant fungal patho-

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gens (11). These results suggested that HOG1 MAPKs might provide a clue for understanding the regulatory mechanism that responds to environmental stresses in fungi and fungal pathogenesis. However, whether HOG1-type MAPKs in entomopathogenic fungi are involved in the response to environmental stress and pathogenesis is far from clear.

Here, we characterized a HOG1-related MAPK gene, designated *Bbhog1*, in *Beauveria bassiana*, an important entomopathogenic fungus which targets a variety of insect pests, including agricultural pests and vectors of human pathogens (5, 8, 32). Our data showed that *Bbhog1* regulates responses to stress stimuli. Interestingly, disruption of *Bbhog1* resulted in a significant reduction in virulence and impairment of characteristics associated with pathogenicity.

## MATERIALS AND METHODS

**Fungal and bacterial strains.** *B. bassiana* single-spore isolate Bb0062 has been described previously (17). Cultures were grown on Sabouraud's dextrose agar (Difco Laboratories, Detroit, MI) supplemented with 1% (wt/vol) yeast extract for 14 days at 26°C with a cycle consisting of 15 h of light and 9 h of darkness. *Escherichia coli* DH5 $\alpha$  was employed for DNA manipulations and transformations. *Agrobacterium tumefaciens* AGL-1 was used for fungal transformations.

**Identification of the HOG1 MAPK-encoding gene.** Fungal genomic DNA was prepared as described previously (39), and total RNA was isolated from *B. bassiana* using the method of Wan and Wilkins (53). RQ1 RNase-free DNase (Promega) was used to remove DNA contamination in RNA samples. To PCR amplify a fragment of the HOG1 gene from *B. bassiana*, degenerate primers Bh-F and Bh-R (Table 1) were designed based on alignments of *C. lagenarium* OSC1 (29), *N. crassa* OS-2 (58), *M. grisea* OSM1 (12), and *S. cerevisiae* HOG1 (7). PCR was performed using ExTaq DNA polymerase (TaKaRa). The resulting PCR product was shown to be a fragment of a HOG1-related MAPK gene. The complete gene was subsequently cloned by PCR walking using the Y-shaped adaptor-dependent extension (YADE) method as described previously (14). The primers used for PCR walking are shown in Table 1. All PCR products were cloned into the A-T cloning vector pGEM-T Easy (Promega) according to manufacturer's instructions and subsequently sequenced. Designated *Bbhog1*, the complete gene was resolved using the PCR and YADE products based on overlapping regions. Primers (Table 1) were designed to clone the cDNA sequence of this gene using the 3' full random amplification of cDNA ends (RACE) core set (TaKaRa).

**Disruption of *Bbhog1*.** Plasmid pK2-BarGFP containing the herbicide resistance gene *bar* and the enhanced green fluorescent protein gene *egfp* was generated by inserting a *Bar::GFP* fusion gene (27) into EcoRI and HindIII sites of the binary vector pPK2 (34) in which an *hph* cassette was deleted. The 3' end of *Bbhog1* (3' *hog1*) was cloned by performing PCR with primers R1 and R2 (Table 1) using ExTaq DNA polymerase (TaKaRa). The resulting PCR product was digested with SpeI and SmaI and inserted into HindIII and XbaI sites of pK2-BarGFP which was blunt ended using T4 DNA polymerase at a HindIII site, to form pK2-BarGFP:3'*hog1*. The 5' end of *Bbhog1* (5' *hog1*) was amplified with primers L1 and L2 with ExTaq DNA polymerase (TaKaRa). The PCR product was cloned into pGEM-T Easy (Promega) to form pGEM-5'*hog1*. Then the 5' end of *Bbhog1* was excised from pGEM-5'*hog1* with EcoRI and inserted into the unique EcoRI site of pK2-BarGFP:3'*hog1*. The resulting gene replacement vector was confirmed by performing PCR with primers L1 and B2 (5' end sequence of *Bar*) (Table 1) to be a 3.5-kb fragment and designated pBG $\Delta$ *hog1*, and then it was mobilized into *A. tumefaciens* AGL-1 for fungal transformation (17).

*Bbhog1* disruption mutants were initially selected on the basis of herbicide (glufosinate ammonium) resistance and expression of green fluorescent protein. PCR performed with the primer pairs G1/R2 and G1/R2' (Table 1), Southern blotting, reverse transcription-PCR (RT-PCR), and Western blotting were used to confirm disruption of *Bbhog1* in *B. bassiana* transformants.

**Blotting analysis.** Southern gel blot analysis was performed with about 25  $\mu$ g DNA digested with appropriate restriction enzymes for each sample. The DNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a labeling kit (Amersham).

Fungal proteins were extracted as described previously (28). For Western blot analysis, 20  $\mu$ g proteins was transferred from a sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis gel to a polyvinylidene difluoride membrane (Roche), and immunoblotting and blot development were carried out according to the instructions provided with an Opti-4CN Western blot kit (Bio-Rad).

Proteins related to HOG1 were detected using anti-Hog1p antibody y-215 (Santa Cruz Biotech, United States).

**Quantification of spore yield.** Twenty-milliliter portions of 1:4-diluted Sabouraud's dextrose agar supplemented with 1% (wt/vol) yeast extract or Czapek agar containing 0.5% (wt/vol) peptone (CZP agar) were tempered to 45°C, mixed with 50  $\mu$ l of conidial suspensions at a concentration of  $1 \times 10^7$  conidia ml<sup>-1</sup>, and poured into 90-mm-diameter petri dishes. After incubation at 26°C using a cycle consisting of 15 h of light and 9 h of darkness for a total of 15 days, conidia were collected in 0.05% (vol/vol) Tween 80 and filtered through four layers of lens-cleaning tissue (Hangzhou Special Paper Industry Co., Ltd., People's Republic of China) to remove mycelial debris. The number of conidia produced was determined by counting the conidia with a hemocytometer using a Motic microscope (E220G). The experiment was repeated four times with three replicates for each strain.

**Identification of compatible solutes for *B. bassiana*.** Two grams (fresh weight) of mycelia harvested from 48-h liquid cultures was inoculated into 100 ml Sabouraud's dextrose broth containing 1% (wt/vol) yeast extract (pH 7.0) (SDY broth) supplemented with 0 or 0.4 M NaCl and incubated at 26°C on a rotary shaker (180 rpm) for 24 h. Mycelia were harvested, frozen in liquid nitrogen, and freeze-dried under a vacuum for 3 to 5 days for carbohydrate extraction using a method described previously (12). A 0.5-g (dry weight) portion of mycelia was used to extract carbohydrates after addition of 20  $\mu$ g ribitol as the internal standard by a previously described method (12). The mannitol, trehalose, erythritol, arabinol, and glycerol contents of mycelia were determined using a gas-liquid chromatography method described previously (25). The experiment was repeated three times.

**Bioassay.** Apterous adults of *Myzus persicae* ( $\leq 2$  days after the last ecdysis) reared on cabbage in the greenhouse were prepared by using a previously described method (51) and used for a bioassay. A fungal conidial suspension was diluted to obtain suspensions containing  $5 \times 10^7$  and  $1 \times 10^7$  conidia ml<sup>-1</sup> using 0.05% (vol/vol) Tween 80. For inoculation, the aphids on each cabbage leaf (replicates were used for each treatment) were exposed to a spray consisting of 1 ml of a conidial suspension using a Potter precision laboratory spray tower (Burkard Manufacturing Co., Ltd., United Kingdom) and then transferred to an HPG-280H artificial climate cell (Harbin Donglian Electronic & Technology Development Co. Ltd. of China) kept at 22 to 24°C. Control aphids were treated with water plus 0.05% (vol/vol) Tween 80. For each treatment there were three replicates, each with 30 to 40 aphids, and the experiments were repeated three times. Aphid mortality was examined daily and corrected using Abbott's formula:  $P_T = [(P_O - P_C)/(100 - P_C)] \times 100$ , where  $P_O$  is the observed mortality and  $P_C$  is the control mortality (1).

**Determination of conidial germination, adherence, appressorium formation, and hyphal body differentiation.** One hundred microliters of a conidial suspension containing  $1 \times 10^7$  spores ml<sup>-1</sup> was spread onto CZP agar plates and incubated at 26°C. After 8 h, the percentage of germinated conidia was assessed hourly. Three hundred conidia were examined for each time, and all experiments were repeated on three different dates. A conidium was considered to have germinated if the length of its germ tube was greater than its width.

Conidial adherence to cicada hind wings was investigated by using a previously described method (55). Appressoria were induced on cicada hind wings using a method described previously (54), and the frequency of appressorium formation was determined by counting the number of appressoria that developed from 300 germinated conidia after 22 h. All experiments were repeated three times.

Hyphal body differentiation was investigated by injecting conidia into insects. Third instars of *Pieris brassicae* larvae were inoculated with 2  $\mu$ l of a conidial suspension containing  $5 \times 10^7$  spores ml<sup>-1</sup> and bled at 12-h intervals to observe hyphal bodies with a microscope.

**RT-PCR.** Two micrograms of total RNA was reverse transcribed using an oligo(dT)-primed cDNA synthesis kit (MBI Fermentas), and the first-strand cDNA was amplified with the *Bbhog1*, *Bgpd*, and  $\beta$ -*tubulin* primers listed in Table 1. The expression of these three genes was investigated during growth in CZP broth supplemented with 0.4 M NaCl for 0, 10, and 20 min.

**Real-time RT-PCR analysis.** First-strand cDNA was synthesized using oligo(dT) primers according to the manufacturer's instructions for a cDNA synthesis kit (MBI Fermentas). Five micrograms of total RNA was used for cDNA synthesis, and the cDNA was subsequently diluted with nuclease-free water to obtain a concentration of 10 ng  $\mu$ l<sup>-1</sup>. One microliter of diluted cDNA was used as a template for subsequent PCR amplification using a quantitative real-time PCR kit (Bio-Rad). The transcript levels of two hydrophobin-encoding genes, *hyd1* and *hyd2* (9), the Pr1 protease gene *cdepl* (18), and the chitinase gene *Bbchit1* (14) were analyzed using real-time RT-PCR. The stability of expression of four potential reference genes, the *18S rRNA*,  $\beta$ -*tubulin*, *actin*, and *Bgpd* genes, was evaluated using the wild type and *Bbhog1* disruption mutants grown in insect

TABLE 1. Oligonucleotides used in this study

Category and oligonucleotide	Sequence (5'–3') <sup>a</sup>	Remarks
PCR-based cloning of a fragment of <i>Bbhog1</i>		
BH-F	TCGGCATGGGNGCNTTYGGN	
BH-R	TCGTCCGTNGGRTTCRTGRTA	
YADE-based PCR walking		
Bh3X	AGATATAGCGATCGATCTGA	Used to clone the 3' fragment obtained with BH-F and BH-R
Bh3Z	ATCGATCTGATGGAGCGCAT	
Bh5X	GTCGGAGATGCTTGAGCAAT	Used to clone the 5' fragment obtained with BH-F and BH-R
Bh5X	AGGGCTTCATGATCTTCTTG	
Cloning cDNA of <i>Bbhog1</i> using 3' RACE		
BH-3	TTGCCCTCACAATGGCCGA	
<i>Bbhog1</i> disruption vector construction		
L1	GAATTCATTCACCCTTTGCGT	
L2	AAAGTCGCAAATCTTGAGAT	
R1	<u>GGACTAGTCCCTTGAGTCATAGGT</u>	
R2	<u>TCCCCGGGGGAAGTAATACCCTTTCAGAGCA</u>	
Disruption vector and disruptant confirmation		
B2	TTAGATCTCGGTGACGGGCA	3' end sequence of <i>Bar</i>
G1	ATGAGTAAAGGAGAAGAACT	5' end sequence of <i>GFP</i>
R2'	AGATATTGGAGCAGATTGGT	5' flanking sequence of R2
RT-PCR		
Hog1 RT-1	TACTCTACGATTCGTCAAGT	Used for RT-PCR analysis of <i>Bbhog1</i>
Hog1 RT-2	TGCTGGAACAGAGCCGTCTT	
Hyd1-1	ATCTACTGCTGCAACGAGAA	Used for real-time RT-PCR analysis of <i>hyd1</i>
Hyd1-2	TACTGGATAAGACTGCCAAT	
Hyd2-1	AGTGTCAAGACTGGCGACAT	Used for real-time RT-PCR analysis of <i>hyd2</i>
Hyd2-2	ATCCGAGGACGGTGATGGGA	
Cdep1-1	TGCACCGTCGGAGCTACCGA	Used for real-time RT-PCR analysis of <i>cdep1</i>
Cdep1-2	AGTTGACGGTGCTGAAGGA	
Bbchit1-1	TACCTGGACAATGGTCGTCT	Used for real-time RT-PCR analysis of <i>Bbchit1</i>
Bbchit1-2	TGGAGCCGTCCCAGTTCAGA	
18S rRNA-1	CGGGTAACGGAGGGTTAGG	Used for real-time RT-PCR analysis of <i>18S rRNA</i>
18S rRNA-2	AGTACACGCGGTGAGGCCG	
$\beta$ -tubulin 1	TACTCTACGATTCGTCAAGT	Used for RT-PCR and real-time RT-PCR analysis of $\beta$ -tubulin
$\beta$ -tubulin 2	TGCTGGAACAGAGCCGTCTT	
Bgpd-1	TCGACCTGACTGCTCGTCTT	Used for RT-PCR and real-time RT-PCR analysis of <i>Bgpd</i>
Bgpd-2	TAGGAGATAAGGTCCAGGA	
Actin-1	TTGGTGCAGAACTTCAGCGTCTAGTC	Used for real-time RT-PCR analysis of <i>actin</i>
Actin-2	TCCAGCAAATGTGGATCTCCAAGCAG	

<sup>a</sup> N = A, G, C, or T; Y = C or T; R = A or G. Underlining in the R1 and R2 sequences indicates introduced SpeI and SmaI sites, respectively.

cuticle or chitin medium (9) and a method described previously (52). Three of the four reference genes with the most stable gene expression were selected to normalize levels of gene transcription using geNORM (52). Primer sequences for the eight genes used are shown in Table 1.

The transcript levels of *hyd1* and *hyd2* were ascertained using insect cuticle media as described previously (9). Conidia harvested from plates were inoculated into 1:4-diluted Sabouraud's dextrose broth containing 1% (wt/vol) cicada cuticle at a final concentration of  $5 \times 10^5$  conidia ml<sup>-1</sup> and were grown for 3 days at 26°C with aeration, after which the total RNA was extracted and subjected to real-time RT-PCR analysis.

The levels of expression of *cdep1* and *Bbchit1* in insect cuticle broth (basal salt

medium supplemented with 0.5% [wt/vol] cicada cuticle as the sole carbon and nitrogen source) were also assessed. Two grams of washed mycelia was transferred from SDY medium into cicada cuticle broth and grown for an additional 12, 24, 36, 48, 60, and 72 h. Total RNA was extracted and subjected to real-time RT-PCR analysis.

**Data analysis.** Variation among the treatments in a given experiment was differentiated by using one-way analysis of variance, and the significance between treatments was tested using the least significant difference or the Dunnett T3 method. All statistical analyses were carried out with the SPSS 8.0 program.

**Nucleotide sequence accession numbers.** Data reported here have been deposited in the GenBank database under the following accession numbers:



AY572854 for *Bbhog1* genomic DNA, EF452344 for *hyd1* mRNA, EF520285 for *hyd2* mRNA, AY040532 for *cdep1* genomic DNA, AY145440 for *Bbchit1* genomic DNA, and AY679162 for *Bgpd* genomic DNA.

## RESULTS

**Cloning of the *Bbhog1* gene.** A HOG1-like MAPK gene, designated *Bbhog1*, was cloned from *B. bassiana* by performing PCR with degenerate primers (Table 1) and YADE (14). Based on the putative coding sequence identified using BLASTX, additional primers (described in Materials and Methods) were designed to clone the cDNA using 3' RACE. The cDNA fragment contained a 1,077-bp open reading frame which encodes a 358-amino-acid protein with a predicted molecular mass of 49.9 kDa and a deduced pI of 8.7. Eight introns were identified in the DNA sequence by comparison to the cDNA sequence. The predicted *Bbhog1* contained the conserved TGY activation loop at positions 171 to 173 found in the stress-activated protein kinase subgroup of MAPKs (42). The amino acid sequence of *Bbhog1* showed 96%, 94%, 94%, and 80% identity to *C. lagenarium* OSC1 (29), *M. grisea* OSM1 (12), *N. crassa* OS-2 (58), and *S. cerevisiae* HOG1 (7), respectively. Southern analysis indicated that a single copy of *Bbhog1* is present in *B. bassiana* (data not shown).

**Disruption of *Bbhog1* leads to sensitivity to high osmolarity, oxidative stress, and high temperature and resistance to the fungicide fludioxonil.** To investigate the roles of *Bbhog1*, a gene disruption strategy was used. The gene replacement vector pBG $\Delta$ hog1 was constructed to delete 677 bp of the *Bbhog1* coding region and to replace it with a 4.2-kb *Bar::GFP* fusion gene cassette (see Fig. S1A in the supplemental material). The disruption of *Bbhog1* was first screened by PCR with both primer pair G1/R2 and primer pair G1/R2' (Table 1) as a 2.8-kb fragment (data not shown) and then confirmed by Southern blotting (see Fig. S1B in the supplemental material), RT-PCR (see Fig. S1C in the supplemental material), and Western blotting (see Fig. S1D in the supplemental material). Transformants were selected randomly for further study.

On standard medium (CZP medium), no obvious variation in the growth rate (Fig. 1A) or hyphal morphology (Fig. 1B) was observed in  $\Delta$ *Bbhog1* mutants. However, the mutants did not form obvious daylight rings resulting in rhythmic sporulation like the wild-type strain (Fig. 1A), which was confirmed by a significant reduction in conidiation on agar plates. The numbers of conidia produced by  $\Delta$ *Bbhog1* mutants on 1:4-diluted Sabouraud's dextrose agar supplemented with 1% (wt/vol) yeast extract ( $3.3 \times 10^5 \pm 0.6 \times 10^5$  conidia  $\text{mm}^{-2}$ ) and CZP agar ( $1.0 \times 10^5 \pm 0.1 \times 10^5$  conidia  $\text{mm}^{-2}$ ) plates were 75.6% ( $df = 6$ ;  $t = 11.33$ ;  $P < 0.001$ ) and 90.5% ( $df = 6$ ;  $t = 22.69$ ;  $P < 0.001$ ) less than the numbers of conidia produced by the wild-type strain, respectively.

Under hyperosmotic stress conditions, the growth of the mutants was dramatically reduced compared to that of the wild-type strain (Fig. 1C). Hyperosmotic stress also led to a distinct morphological change in the mutant hyphae, which became swollen and vacuolated (Fig. 1D). The reduction in radial growth of the mutants was also found to be consistent with the biomass formation during osmotic stress in shake flasks (see Fig. S2 in the supplemental material). Similar reductions in growth were observed when  $\Delta$ *Bbhog1* mutants were

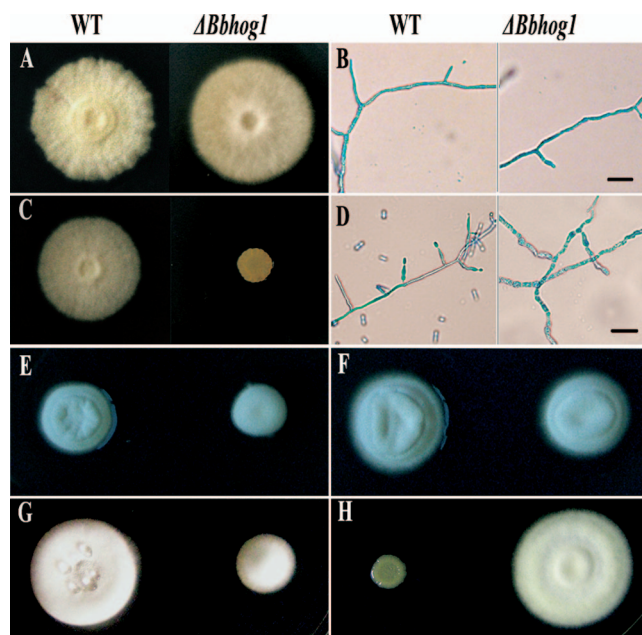


FIG. 1. Disruption of *Bbhog1* influences the growth of *B. bassiana*. Wild-type strain Bb0062 (WT) and the *Bbhog1* disruption mutant ( $\Delta$ *Bbhog1*) were incubated on CZP agar plates at 26°C for 10 days (A) or in CZP broth at 26°C on a rotary shaker (180 rpm) for 12 h (B) (control). The test strains were incubated on CZP agar plates supplemented with 0.4 M NaCl at 26°C for 10 days (C), in CZP broth containing 0.4 M NaCl at 26°C on a rotary shaker (180 rpm) for 12 h (D), on CZP agar plates supplemented (E) or not supplemented (F) with 0.4 M NaCl at 32°C for 10 days, or on CZP agar plates containing 20 mM  $\text{H}_2\text{O}_2$  (G) or  $2.5 \mu\text{g ml}^{-1}$  fludioxonil (H) at 26°C for 10 days. Scale bars in panels B and D, 20  $\mu\text{m}$ .

subjected to chronic hyperosmotic stress by exposure to concentrations of glycerol or KCl generating identical osmotic potentials (data not shown). The sensitivity to osmotic stress of  $\Delta$ *Bbhog1* mutants was alleviated at a higher temperature (32°C) (Fig. 1E), like that of *S. cerevisiae* (48). Furthermore, disruption of *Bbhog1* increased the sensitivities to high temperature and oxidative stress. At 32°C or in medium containing 20 mM  $\text{H}_2\text{O}_2$ ,  $\Delta$ *Bbhog1* mutants showed a significant reduction in growth compared to the wild-type strain (Fig. 1F and G). In addition,  $\Delta$ *Bbhog1* mutants exhibited remarkable resistance to the fungicide fludioxonil (Fig. 1H), demonstrating that *Bbhog1* has the same function in regulating sensitivity to fludioxonil as HOG1 MAPKs in other fungal species.

**Disruption of *Bbhog1* alters erythritol and arabitol accumulation.** To determine the effect of the  $\Delta$ *Bbhog1* mutation on the cellular response to hyperosmotic stress, we prepared mycelial extracts from wild-type and  $\Delta$ *Bbhog1* mutant cultures under isosmotic and hyperosmotic conditions and measured mannitol, trehalose, erythritol, arabitol, and glycerol concentrations using the gas-liquid chromatography method (25).

Disruption of *Bbhog1* in *B. bassiana* did not interfere with the accumulation of mannitol and glycerol in mycelium. Under normal conditions, *B. bassiana* accumulated mannitol as a major carbohydrate in the mycelium ( $400.11 \pm 6.71 \mu\text{mol g}^{-1}$  [dry weight] mycelium). During hyperosmotic stress, which was imposed by incubating fungal mycelium in 0.4 M NaCl, the level of mannitol increased by 30.0% (Fig. 2A). Compared to the

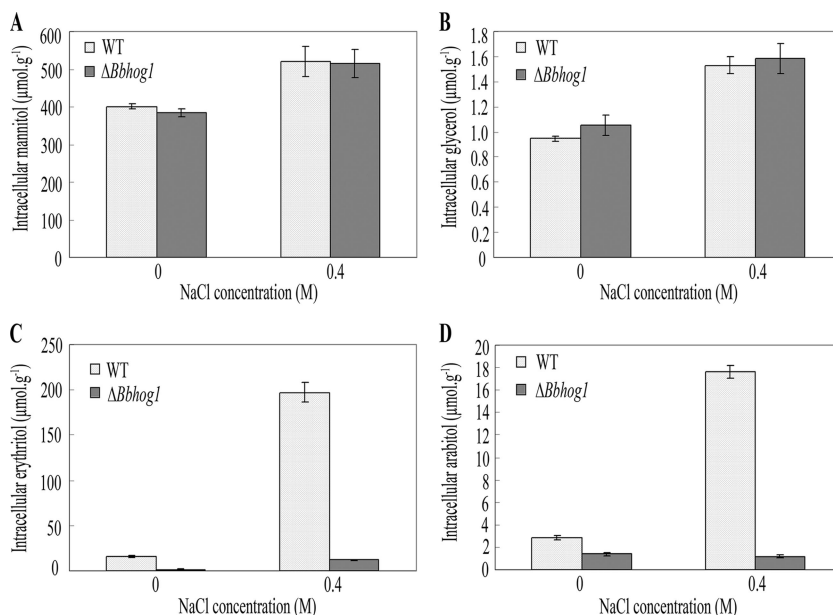


FIG. 2. Compatible solute production in mycelia of the wild-type strain and the  $\Delta Bbhog1$  mutant. Mycelia were grown for 48 h in SDY broth before they were transferred to SDY broth or SDY broth containing 0.4 M NaCl and incubated for a further 24 h. Carbohydrates were extracted and quantified by gas-liquid chromatography (25). (A) Mannitol; (B) glycerol; (C) erythritol; (D) arabinol. The error bars indicate standard deviations from three repeats of the experiment. WT, wild type.

mannitol contents of the wild-type strain, no significant changes in the mannitol contents of the  $\Delta Bbhog1$  mutant were observed under normal conditions or under hyperosmotic stress conditions (Fig. 2A). Smaller amounts of glycerol were present both in the wild-type strain and in the  $\Delta Bbhog1$  mutant, and the amounts increased by 50.5% and 61.8% during osmotic stress, respectively (Fig. 2B). The trehalose accumulation patterns were similar in the wild-type and  $\Delta Bbhog1$  strains, although the levels were decreased during hyperosmotic stress (data not shown).

Significant changes in erythritol and arabinol accumulation in the  $\Delta Bbhog1$  mutant were found when the mycelium was exposed to the osmotic stress. Erythritol is a major solute that accumulates in *B. bassiana* under osmotic stress conditions (23). When the organisms were stressed with 0.4 M NaCl, the concentration of erythritol increased rapidly in wild-type cells, reaching levels that were 11.7-fold higher than the levels under normal conditions (Fig. 2C). In cells of the  $\Delta Bbhog1$  mutant, erythritol accumulated at very low levels under normal conditions, reaching only 7.8% ( $F_{3,11} = 38.27$ ; least significant difference for the post hoc test,  $P < 0.001$ ) of the level in the wild-type strain. Although osmotic stress led to an erythritol level in the  $\Delta Bbhog1$  mutant mycelium that was 8.9-fold-higher than the level under normal conditions, the level was still much lower than that in the wild-type strain (Fig. 2C). Arabinol, which was present at only low levels ( $2.82 \pm 0.20 \mu\text{mol g}^{-1}$  [dry weight] mycelium) during growth of *B. bassiana* under isosmotic conditions, accumulated dramatically in response to hyperosmotic stress, and the level was 17.7-fold greater than the level under normal conditions (Fig. 2D). In contrast, the levels of arabinol in cells of the  $\Delta Bbhog1$  mutant during growth under normal conditions were significantly lower than those in cells of the wild-type strain ( $F_{3,11} = 1935.45$ ; Dunnett T3 for the

post hoc test,  $P < 0.001$ ) and did not increase like the levels in the wild-type strain in response to hyperosmotic stress (Fig. 2D). These results suggested that accumulation of erythritol and accumulation of arabinol were regulated by *Bbhog1* in *B. bassiana*.

**Disruption of *Bbhog1* leads to a significant decrease in virulence.** The bioassay results showed that disruption of *Bbhog1* in *B. bassiana* significantly reduced the virulence against *M. persicae*. Significantly more aphids survived after inoculation of the  $\Delta Bbhog1$  mutant at concentrations of  $1 \times 10^7$  and  $5 \times 10^7$  conidia  $\text{ml}^{-1}$  than after inoculation of the wild-type strain. No distinct difference in survival rates was observed for aphids inoculated with the ectopic integration transformant T270 and aphids inoculated with the wild type (Fig. 3). For instance, at day 6 after inoculation, the survival rates for aphids inoculated using concentrations of  $5 \times 10^7$  and  $1 \times 10^7$  conidia  $\text{ml}^{-1}$  in assays with the  $\Delta Bbhog1$  mutant were in the range from 75.2 to 81.7% (df = 2 and 8), compared to 20.3 to 53.3% (df = 2 and 8) for assays with the wild-type strain (Fig. 3). Meanwhile, 6 days after inoculation, the corrected mortality rates for *M. persicae* treated with the  $\Delta Bbhog1$  mutant using concentrations of  $1 \times 10^7$  and  $5 \times 10^7$  conidia  $\text{ml}^{-1}$  were reduced by 66.5% and 72.5%, respectively, compared to the mortality rates for *M. persicae* inoculated with the same concentrations of the wild-type strain. The results suggested that *Bbhog1* is an important virulence factor of *B. bassiana*.

**Disruption of *Bbhog1* decreases spore viability and adherence and impairs appressorium formation.** To investigate the effect of *Bbhog1* disruption on virulence-related factors, we examined the conidial viability, adherence, appressorium formation, and hyphal body differentiation of  $\Delta Bbhog1$  mutants. The conidial germination rates of the  $\Delta Bbhog1$  mutant on agar plates during the 8 to 14 h after inoculation were significantly

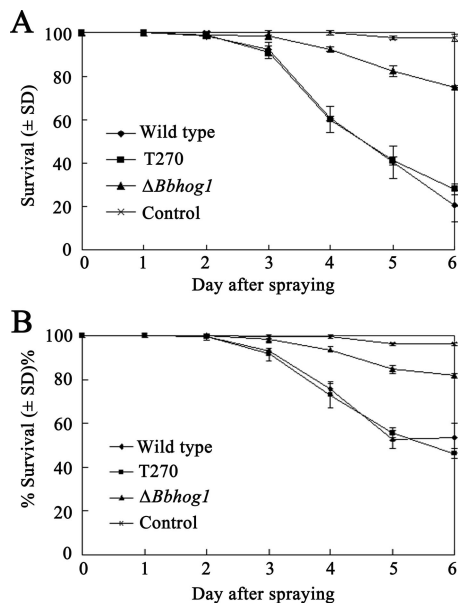


FIG. 3. Trends for mean survival of *M. persicae* after spraying. *M. persicae* peach aphids were sprayed with 1-ml portions of conidial suspensions containing  $5 \times 10^7$  conidia  $\text{ml}^{-1}$  (A) and  $1 \times 10^7$  conidia  $\text{ml}^{-1}$  (B), and the survival was recorded every day after inoculation. Control insects were sprayed with a conidial suspension prepared with the ectopic integration transformant T270 and sterilized water. For each treatment there were three replicates with 30 to 40 aphids each, and the experiments were repeated three times.

lower ( $P < 0.01$ ) than those of the wild-type strain (Fig. 4A). The median germination time of the mutant was ca. 1.50 h longer than that of the wild-type strain. The results suggested that disruption of *Bbhog1* in *B. bassiana* caused a reduction in spore viability.

Conidial adherence was assayed using cicada hind wings and a method described previously (55). At 8 h after inoculation, only  $37.7\% \pm 9.3\%$  of the mutant conidia remained on the wings and could not be washed off by 0.05% (vol/vol) Tween 20, compared to  $81.1\% \pm 10.5\%$  of the wild-type strain conidia ( $df = 4$ ;  $t = 6.17$ ;  $P < 0.01$ ) (Fig. 4B), indicating that conidial adherence to the insect cuticle was severely impaired by the gene disruption.

Appressoria were induced on cicada hind wings using a method described previously (54). The results showed that although the appressorium structure of the  $\Delta Bbhog1$  mutant was morphologically similar to that of the wild type (Fig. 5A), the frequency of appressorium formation for the  $\Delta Bbhog1$  mutant was significantly lower than that for the wild-type strain. Twenty-two hours following inoculation, only  $18.2\% \pm 6.4\%$  of germinated conidia of the mutant differentiated into appressoria, a 77.9% decrease compared to the results for the wild-type strain ( $82.5\% \pm 7.5\%$ ) ( $df = 4$ ;  $t = 27.09$ ;  $P < 0.001$ ) (Fig. 4C).

In addition, we also investigated the hyphal body differentiation of  $\Delta Bbhog1$  mutants inside the insect body. At day 3 after injection, the  $\Delta Bbhog1$  mutant produced the same amount of hyphal bodies in the hemolymph of the third-instar larvae of *P. brassicae* as the wild-type strain (Fig. 5B),

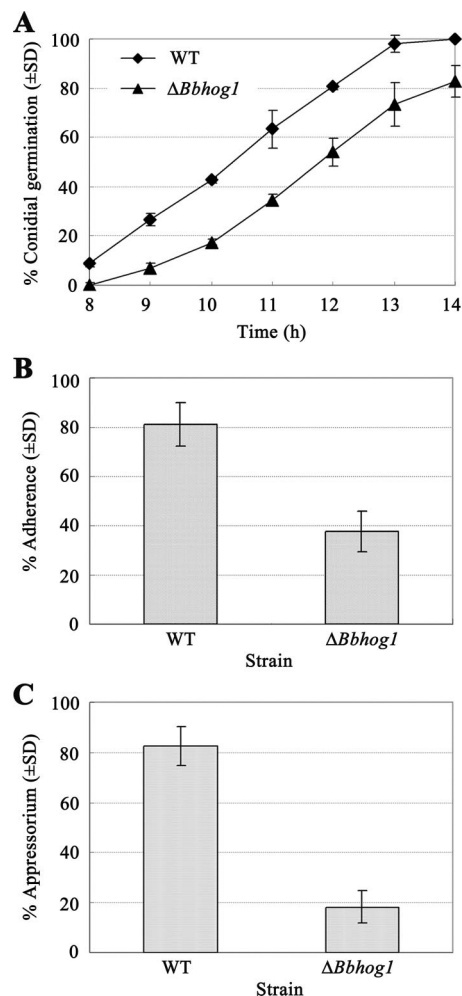


FIG. 4. Determination of conidial germination, adherence, and appressorium formation. (A) Conidial germination. Conidia were inoculated onto CZP agar plates, and germinated conidia were counted hourly beginning 8 h after inoculation. (B) Conidial adherence on cicada hind wings. The assay was performed by using a previously described method (48). (C) Frequency of appressorium formation on cicada hind wings after 22 h of incubation. All experiments were repeated three times with three replicates for each repeat. WT, wild type.

suggesting that inactivation of *Bbhog1* may not influence the postpenetration development of *B. bassiana*.

***Bbhog1* disruption suppresses expression of the hydrophobin-encoding genes *hyd1* and *hyd2*.** The ranking of the four internal reference genes after determination of gene stability was as follows (from least stable to most stable): *actin*, *18S rRNA*,  $\beta$ -*tubulin*, *Bgpd*. Thus, we selected the *Bgpd*,  $\beta$ -*tubulin*, and *18S rRNA* genes to normalize the levels of gene transcription using geNORM (52). The transcript levels of two hydrophobin-encoding genes, *hyd1* and *hyd2*, were determined by real-time RT-PCR when the fungal cells were grown in the presence of insect cuticle for 3 days. The results showed that the transcript levels of *hyd1* and *hyd2* in the  $\Delta Bbhog1$  mutant were 46.4-fold and 41.5-fold lower, respectively, than those in the wild-type strain (Fig. 6). Similar reductions in the levels of



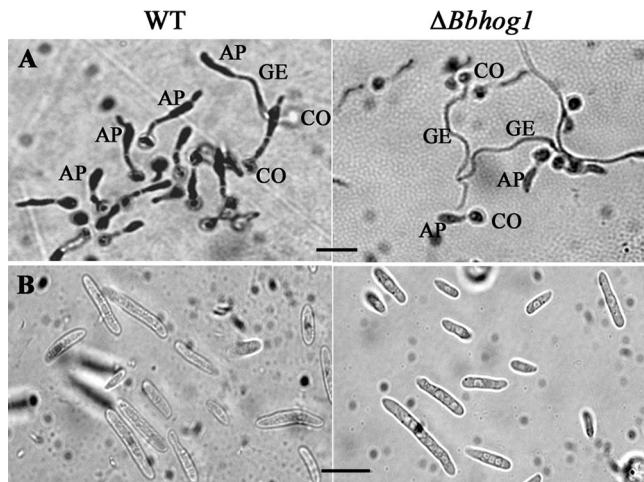


FIG. 5. (A) Appressorium morphology. Appressorium formation was induced on cicada hind wings using a method described previously (54). AP, appressorium; GE, germ tube; CO, conidium. Bar = 20  $\mu$ m. (B) Hyphal body differentiation at 3 days after injection of conidia into *P. brassicae* larva. Bar = 20  $\mu$ m. WT, wild type.

expression of *hyd1* and *hyd2* were observed when  $\Delta$ *Bbhog1* mutants were grown in chitin broth (data not shown).

We also analyzed the transcript levels of the genes encoding two cuticle-degrading enzymes, the Pr1 protease CDEP1 and the chitinase Bbchit1, in  $\Delta$ *Bbhog1* mutants and the wild type by real-time RT-PCR using RNA from mycelia induced by using the cicada cuticle. The transcript patterns of these two genes were similar in the wild-type and  $\Delta$ *Bbhog1* strains (data not shown), suggesting that inactivation of *Bbhog1* in *B. bassiana* does not affect the expression of genes encoding cuticle-degrading enzymes.

## DISCUSSION

*Bbhog1* disruption mutants showed sensitivity to hyperosmotic stress, high temperature, and oxidative stress and exhibited a remarkable resistance to the fungicide fludioxonil. These results demonstrate that there is a conserved function of HOG1 MAPKs in regulation of abiotic stress responses. Similar to the situation in *S. cerevisiae* (48, 57), the sensitivity to osmotic stress of  $\Delta$ *Bbhog1* mutants could be alleviated by a higher temperature (32°C) (Fig. 1E), suggesting that a similar mode of response to hyperosmotic conditions and an elevated temperature may be present in *B. bassiana*. In addition, two strong bands were detected using HOG1 antibody in mycelial extracts of the *B. bassiana* wild-type strain, but no signal was observed in *Bbhog1* disruption mutants (see Fig. 1SD in the supplemental material), demonstrating that both of the bands are related to HOG1 MAPK. This phenomenon has also been observed for *Botrytis cinerea* (29). The relationship of these two bands is unclear.

Under osmotic stress conditions, fungal cells often accumulate high levels of a compatible solute to maintain cellular turgor (33). The HOG1 pathway is required for regulation of the accumulation of some carbohydrates to increase osmolarity in several fungal species. In *S. cerevisiae*, hyperosmotic conditions induce an increase in the level of intracellular glycerol

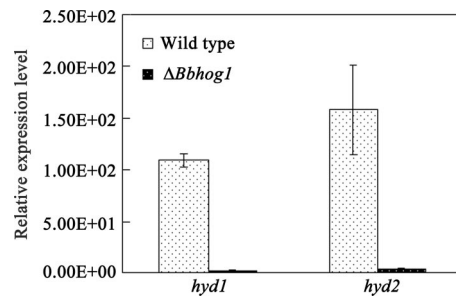


FIG. 6. Expression of the hydrophobin-encoding genes *hyd1* and *hyd2* in the  $\Delta$ *Bbhog1* mutant and the wild-type strain. Real-time RT-PCR was used to determine the relative levels of expression of *hyd1* and *hyd2* using *Bgpd*,  $\beta$ -*tubulin*, and *18S rRNA* as loading controls to normalize samples by a method described previously (52) when the fungal cells were grown in the presence of cicada cuticle for 3 days. The error bars indicate standard deviations.

(36). In this process, HOG1 MAPK is involved in glycerol synthesis by regulating expression of the glycerol synthesis genes *GPD1* and *HOR2* (20). In *C. albicans*, glycerol accumulation under hyperosmotic stress conditions is also partially controlled by this pathway (44). However, in the phytopathogenic fungus *M. grisea*, it is arabinol and not glycerol that is the major compatible solute for adaptation to acute and chronic hyperosmotic stress. The accumulation of glycerol and turgor generation in appressoria are not altered by disruption of the HOG1 MAPK gene *osm1* (12). Disruption of *Bbhog1* in *B. bassiana* resulted in a significant decrease in erythritol and arabinol accumulation under both normal and hyperosmotic stress conditions (Fig. 2). Thus, HOG1-mediated carbohydrate accumulation varies among fungal species.

Although inactivation of *Bbhog1* did not result in a measurable defect in the growth of *B. bassiana* under normal conditions, a significant reduction in conidiation in agar plates was observed for *Bbhog1* disruption mutants. This phenomenon was also observed for the rice blast fungus *M. grisea*, in which disruption of the *osm1* gene (a homologue of *HOG1*) leads to a dramatic reduction in conidial production in standard medium (12). Furthermore, a different MAPK in the corn leaf pathogen *Cochliobolus heterostrophus* (CHK1, an ortholog of FUS3/KSS1) was required for conidiation (31). However, the mechanism of conidiation regulated by MAPKs is poorly understood. In several filamentous fungi, the heterotrimeric G protein signaling pathway is involved in conidiation, as well as morphogenesis and pathogenesis (3, 16, 30). The regulatory G protein signaling gene *Bbrgs1* is also required for conidiation in *B. bassiana* (15). Although G proteins are generally upstream of MAPK pathways, it is not yet known whether conidiation of *B. bassiana* mediated by *Bbrgs1* occurs through activation of MAPKs. Further experiments are needed to elucidate the cross talk between MAPKs and G protein signal pathways for regulation of conidiation.

Accumulating data have shown that HOG1 kinases are involved mainly in stress responses. However, there is little evidence correlating HOG1 kinases with the virulence or pathogenicity of pathogens. In the phytopathogenic fungus *M. grisea*, disruption of the *osm1* gene homologous to the HOG1 gene does not alter the virulence, and  $\Delta$ *osm1* mutants are fully pathogenic (12). In contrast, *MgHog1* disruption mutants of

the plant pathogen *M. graminicola* do not infect wheat leaves due to impaired initiation of infectious germ tubes (35). Either hyperactivation or silence of a HOG1 protein, ThHog1, in *T. harzianum* results in strongly reduced antagonistic activity against the plant pathogens *Phoma betae* and *Colletotrichum acutatum* (11). Here we found that Bbhog1 was an important virulence determinant of *B. bassiana* and influenced at least three aspects of infection, spore viability, adherence to the insect cuticle, and appressorium formation.

The rate of conidial germination is an important indicator of virulence in entomopathogenic fungi and is to some degree correlated with virulence (2, 26, 43, 46). It was demonstrated previously that physical manipulation of growth conditions can significantly modify the endogenous compounds synthesized and channeled into the propagules of fungi (33, 38). Optimization of erythritol and glycerol contents in conidia of entomopathogenic fungi, such as *B. bassiana*, *Metarhizium anisopliae*, and *Paecilomyces farinosus*, has increased the germination rate and improved the pathogenicity at a low relative humidity (22, 23, 33). A reduction in the spore viability of  $\Delta$ Bbhog1 mutants may be due to lower levels of some compatible solutes in mature conidia compared to the levels in the wild-type strain (Fig. 2).

Cuticle attachment has been considered a reliable determinant of pathogenicity (2, 24, 55). Fungal cell attachment to the cuticle may involve specific receptor-ligand and/or nonspecific hydrophobic and electrostatic mechanisms (4, 5, 13). Hydrophobins are generally thought to be ubiquitous proteins of fungal walls. These proteins form an outer layer of hyphae and conidia and play a role in a broad range of processes in the growth and development of filamentous fungi. They are involved in the formation of aerial structures and in the attachment of hyphae to hydrophobic surfaces (9, 56). For instance, the hydrophobin MPG1 of *M. grisea* directs formation of a rodlet layer on conidia, which contributes to the hydrophobicity of the surface. A  $\Delta$ mpg1 mutant showed reduced infectivity and an inability to form appressoria (50). Recently, two hydrophobins, Hyd1 and Hyd2, were also isolated from *B. bassiana*, and Hyd2 has been identified as the major component of the rodlet layer in the aerial conidium surface (9). Here we found that transcript levels of *hyd1* and *hyd2* were markedly reduced in a  $\Delta$ Bbhog1 mutant when the fungal cells were grown in the presence of insect cuticle (Fig. 6), suggesting that Bbhog1 may influence the expression of some genes associated with hydrophobicity or adherence. In addition, *hyd2* is expressed more strongly than *hyd1* both in wild-type strain Bb0062 and in a  $\Delta$ Bbhog1 mutant, which is not consistent with the data described previously (9). The difference may be due to genetic diversity of different isolates.

Like the conidia of many plant-pathogenic fungi, conidia of entomopathogenic fungi, such as *M. anisopliae* and *B. bassiana*, often differentiate to form appressoria on the host surface. The formation of appressoria is pivotal for penetrating the host cuticle and establishing a pathogenic relationship with the host (10). Consequently, impairment of appressorium formation reduces the virulence of the fungal pathogen to host. It has been demonstrated that attachment is required to trigger the appropriate stimuli necessary to initiate appressorium development (50). Therefore, we may reasonably speculate that impairment of appressorium formation in  $\Delta$ Bbhog1 mutants is

partially due to the reduction in attachment which is to a certain extent mediated by hydrophobins.

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#### REFERENCES

- Abbott, W. S. 1925. A method for computing the effectiveness of an insecticide. *J. Econ. Entomol.* **18**:265–267.
- Altre, J. A., J. D. Vandenberg, and F. A. Cantone. 1999. Pathogenicity of *Paecilomyces fumosoroseus* isolates to diamondback moth, *Plutella xylostella*: correlation with spore size, germination speed, and attachment to cuticle. *J. Invertebr. Pathol.* **73**:332–338.
- Bölker, M. 1998. Sex and crime: heterotrimeric G proteins in fungal mating and pathogenesis. *Fungal Genet. Biol.* **25**:143–156.
- Boucias, D. G., and J. C. Pendland. 1991. Attachment of mycopathogens to cuticle, p. 101–127. In G. T. Cole and H. C. Hoch (ed.), *The fungal spore and disease initiation in plants and animals*. Plenum Press, New York, NY.
- Boucias, D. G., J. C. Pendland, and J. P. Latge. 1988. Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Appl. Environ. Microbiol.* **54**:1795–1805.
- Braga, G. U. L., S. D. Flint, C. L. Messias, A. J. Anderson, and D. W. Roberts. 2001. Effect of UV-B on conidia and germlings of the entomopathogenic hyphomycete *Metarhizium anisopliae*. *Mycol. Res.* **105**:874–882.
- Brewster, J. L., T. de Valoir, N. D. Dwyer, E. Winter, and M. C. Gustin. 1993. An osmosensing signal transduction pathway in yeast. *Science* **259**:1760–1763.
- Butt, T. M., C. Jackson, and N. Magan. 2001. Fungi as biocontrol agents: progress, problems and potential, p. 1–8. In T. M. Butt, C. Jackson, and N. Magan (ed.), *Fungi as biocontrol agents: progress, problems and potential*. CAB International, Oxford, United Kingdom.
- Cho, E. M., B. H. Kirkland, D. J. Holder, and N. O. Keyhani. 2007. Phage display cDNA cloning and expression analysis of hydrophobins from the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*. *Microbiology* **153**:3438–3447.
- Clarkson, J. M., and A. K. Charnley. 1996. New insights into the mechanisms of fungal pathogenesis in insect. *Trends Microbiol.* **4**:197–203.
- Delgado-Jarana, J., S. Sousa, F. González, M. Rey, and A. Llobell. 2006. ThHog1 controls the hyperosmotic stress response in *Trichoderma harzianum*. *Microbiology* **152**:1687–1700.
- Dixon, K. P., J. R. Xu, N. Smirnov, and N. J. Talbot. 1999. Independent signaling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. *Plant Cell* **11**:2045–2058.
- Doss, R. P., S. W. Potter, G. A. Chastagner, and J. K. Christian. 1993. Adhesion of nongerminated *Botrytis cinerea* conidia to several substrata. *Appl. Environ. Microbiol.* **59**:1786–1791.
- Fang, W., B. Leng, Y. Xiao, K. Jin, J. Ma, Y. Fan, J. Feng, X. Yang, Y. Zhang, and Y. Pei. 2005. Cloning of *Beauveria bassiana* chitinase gene *Bbchit1* and its application to improve fungal strain virulence. *Appl. Environ. Microbiol.* **71**:363–370.
- Fang, W., L. R. Scully, L. Zhang, Y. Pei, and M. J. Bidochka. 2008. Implication of a regulator of G protein signaling (BbRGS1) in conidiation and conidial thermotolerance of the insect pathogenic fungus *Beauveria bassiana*. *FEMS Microbiol. Lett.* **279**:146–156.
- Fang, W., Y. Pei, and M. J. Bidochka. 2007. A regulator of a G protein signalling (RGS) gene, *cag8*, from the insect-pathogenic fungus *Metarhizium anisopliae* is involved in conidiation, virulence and hydrophobin synthesis. *Microbiology* **153**:1017–1025.
- Fang, W., Y. Zhang, X. Yang, X. Zheng, H. Duan, Y. Li, and Y. Pei. 2004. *Agrobacterium tumefaciens*-mediated transformation of *Beauveria bassiana* using an herbicide resistance gene as a selection marker. *J. Invertebr. Pathol.* **85**:18–24.
- Fang, W., Y. Zhang, X. Yang, Z. Wang, and Y. Pei. 2002. Cloning and characterization of cuticle degrading enzyme CDEP-1 from *Beauveria bassiana*. *Acta Genet. Sin.* **29**:278–282.
- Feng, M. G., T. J. Poprawski, and G. G. Khachatourians. 1994. Production, formulation and application of the entomopathogenic fungus *Beauveria bassiana* for insect control: current status. *Biocontrol Sci. Technol.* **4**:3–34.
- Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport. 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **62**:1264–1300.
- Hajek, A. E., and R. J. St. Leger. 1994. Interactions between fungal pathogenesis and insect hosts. *Annu. Rev. Entomol.* **39**:293–322.
- Hallsworth, J. E., and N. Magan. 1994. Effects of KCl concentration on



- accumulation of acyclic sugar alcohols and trehalose in conidia of three entomopathogenic fungi. *Lett. Appl. Microbiol.* **18**:8–11.
23. Hallsworth, J. E., and N. Magan. 1995. Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability. *Microbiology* **141**:1109–1115.
  24. Holder, D. J., and N. O. Keyhani. 2005. Adhesion of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana* to substrata. *Appl. Environ. Microbiol.* **71**:5260–5266.
  25. Hu, L., B. Guo, H. Lu, X. Zou, Y. Li, and X. Jiang. 2004. Gas chromatographic analysis of sugar and sugar alcohol derivatives through 1-methylimidazole catalyzed acetylation. *Chin. Bull. Bot.* **21**:689–699.
  26. James, R. R. 2001. Effects of exogenous nutrients on conidial germination and virulence against the silverleaf whitefly for two hyphomycetes. *J. Invertebr. Pathol.* **77**:99–107.
  27. Jin, K., Y. Zhang, Z. Luo, Y. Xiao, Y. Fan, D. Wu, and Y. Pei. 2008. An improved method for *Beauveria bassiana* transformation using phosphothricin acetyltransferase and green fluorescent protein fusion gene as a selectable and visible marker. *Biotechnol. Lett.* **30**:1379–1383.
  28. Kawasaki, L., O. Sánchez, K. Shiozaki, and J. Aguirre. 2002. SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. *Mol. Microbiol.* **45**:1153–1163.
  29. Kojima, K., Y. Takano, A. Yoshimi, C. Tanaka, T. Kikuchi, and T. Okuno. 2004. Fungicide activity through activation of a fungal signalling pathway. *Mol. Microbiol.* **53**:1785–1796.
  30. Lengeler, K. B., R. C. Davidson, C. D'Souza, T. Harashima, W. Shen, P. Wang, X. Pan, M. Waugh, and J. Heitman. 2000. Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64**:746–785.
  31. Lev, S., A. Sharon, R. Hadar, H. Ma, and B. A. Horwitz. 1999. A mitogen-activated protein kinase of the corn leaf pathogen *Cochliobolus heterostrophus* is involved in conidiation, appressorium formation, and pathogenicity: diverse roles for mitogenactivated protein kinase homologs in foliar pathogens. *Proc. Natl. Acad. Sci. USA* **96**:13542–13547.
  32. Luz, C., I. G. Silva, C. M. T. Cordeiro, and M. S. Tigano. 1998. *Beauveria bassiana* (Hyphomycetes) as a possible agent for biological control of Chagas disease vectors. *J. Med. Entomol.* **35**:977–979.
  33. Magan, N. 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents, p. 239–251. *In* T. M. Butt, C. Jackson, and N. Magan (ed.), *Fungi as biocontrol agents: progress, problems and potential*. CAB International, Oxford, United Kingdom.
  34. McCluskey, K. 2003. The Fungal Genetics Stock Center: from molds to molecules. *Adv. Appl. Microbiol.* **52**:245–262.
  35. Mehrabi, R., L. H. Zwieters, M. A. de Waard, and G. H. J. Kema. 2006. *MgHog1* regulates dimorphism and pathogenicity in the fungal wheat pathogen *Mycosphaerella graminicola*. *Mol. Plant-Microbe Interact.* **19**:1262–1269.
  36. Nevoigt, E., and U. Stahl. 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **21**:231–241.
  37. Nishida, E., and Y. Gotoh. 1993. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* **18**:128–131.
  38. Pascual, S., P. Melgarejo, and N. Magan. 2003. Water availability affects the growth, accumulation of compatible solutes and the viability of the biocontrol agent *Epicoccum nigrum*. *Mycopathologia* **156**:93–100.
  39. Raeder, U., and P. Broda. 1985. Rapid preparation of DNA from filamentous fungi. *Lett. Appl. Microbiol.* **1**:17–20.
  40. Rangel, D. E. N., G. U. L. Braga, S. D. Flint, A. J. Anderson, and D. W. Roberts. 2004. Variations in UV-B tolerance and germination speed of *Metarhizium anisopliae* conidia produced on insects and artificial substrates. *J. Invertebr. Pathol.* **87**:77–83.
  41. Roberts, D. W., and R. J. St. Leger. 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Adv. Appl. Microbiol.* **54**:1–70.
  42. Robinson, M. J., and M. H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **9**:180–186.
  43. Safavi, S. A., F. A. Shah, A. K. Pakdel, G. R. Rasouljan, A. R. Bandani, and T. M. Butt. 2007. Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. *FEMS Microbiol. Lett.* **270**:116–123.
  44. San José, C., R. Alonso Monge, R. Pérez-Díaz, J. Pla, and C. Nombela. 1996. The mitogen-activated protein kinase homologue *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J. Bacteriol.* **178**:5850–5852.
  45. Schaeffer, H. J., and M. J. Webber. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**:2435–2444.
  46. Shah, F. A., C. S. Wang, and T. M. Butt. 2005. Nutrition influences growth and virulence of the insect-pathogenic fungus *Metarhizium anisopliae*. *FEMS Microbiol. Lett.* **251**:259–266.
  47. Sharma, P., N. Meena, M. Aggarwal, and A. K. Mondal. 2005. *Debaryomyces hansenii*, a highly osmo-tolerant and halo-tolerant yeast, maintains activated Dhog1p in the cytoplasm during its growth under severe osmotic stress. *Curr. Genet.* **48**:162–170.
  48. Siderius, M., E. Rots, and W. H. Mager. 1997. High-osmolarity signalling in *Saccharomyces cerevisiae* is modulated in a carbon-source-dependent fashion. *Microbiology* **143**:3241–3250.
  49. St. Leger, R. J., L. Joshi, M. J. Bidochka, and D. W. Roberts. 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc. Natl. Acad. Sci. USA* **93**:6349–6354.
  50. Talbot, N. J., M. J. Kershaw, G. E. Wakley, O. M. H. De Vries, J. G. H. Wessels, and J. E. Hamer. 1996. *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*. *Plant Cell* **8**:985–999.
  51. Vandenberg, J. D. 1996. Standardized bioassay and screening of *Beauveria bassiana* and *Paecilomyces fumosoroseus* against the Russian wheat aphid (Homoptera: Aphididae). *J. Econ. Entomol.* **89**:1418–1423.
  52. Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paep, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**:RESEARCH0034.1–RESEARCH0034.11.
  53. Wan, C. H., and T. A. Wilkins. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* **223**:7–12.
  54. Wang, C. S., and R. J. St. Leger. 2005. Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acidum*. *Eukaryot. Cell* **4**:937–947.
  55. Wang, C. S., and R. J. St. Leger. 2007. The MAD1 adhesion of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesion enables attachment to plants. *Eukaryot. Cell* **6**:808–816.
  56. Whiteford, J. R., and P. D. Spanu. 2002. Hydrophobins and the interactions between fungi and plants. *Mol. Plant Pathol.* **3**:391–400.
  57. Wojda, I., R. Alonso-Monge, J. Bebelman, W. H. Mager, and M. Siderius. 2003. Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways. *Microbiology* **149**:1193–1204.
  58. Zhang, Y., R. Lamm, C. Pillonel, S. Lam, and J.-R. Xu. 2002. Osmoregulation and fungicide resistance: the *Neurospora crassa os-2* gene encodes a *HOG1* mitogen-activated protein kinase homologue. *Appl. Environ. Microbiol.* **68**:532–538.