Phage display cDNA cloning and expression analysis of hydrophobins from the entomopathogenic fungus *Beauveria (Cordyceps) bassiana*

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Hydrophobins are small amphipathic proteins that function in a broad range of growth and developmental processes in fungi. They are involved in the formation of aerial structures, the attachment of fungal cells to surfaces, and act in signalling in response to surface cues and pathogenesis. *Beauveria bassiana* is an important entomopathogenic fungus used as an arthropod biological control agent. To examine the feasibility of using phage display technology to clone cDNAs encoding hydrophobins, biopanning experiments were performed using a variety of affinity resins, including *N,N'*-diacetylchitobiose-, fucose-, lactose-, maltose- and melibiose-coupled agarose beads. After five rounds of iterative biopanning, cDNAs corresponding to two *B. bassiana* (class I) hydrophobins were selectively enriched using melibiose- or lactose-coupled agarose beads. Expression analysis revealed that the *hyd1* gene was expressed in all samples tested, including aerial conidia, *in vitro* blastospores, submerged conidia, and cells sporulating on chitin and insect cuticle, with *hyd1* expression peaking in growing mycelia. In contrast, the *hyd2* gene was not appreciably expressed in any of the single-cell types (aerial conidia, blastospores and submerged conidia), but was constitutively expressed in growing mycelia and when cells were sporulating on chitin and insect cuticle. MS fingerprinting of an ~10 kDa protein found in boiling SDS-insoluble, trifluoroacetic acid-soluble extracts from aerial conidia identified the major component of the *B. bassiana* rodlet layer to be the *hyd2* gene product. These results reveal the differential regulation of the isolated hydrophobins and indicate that phage display represents a novel approach to cDNA cloning of hydrophobins.

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INTRODUCTION

Fungal hydrophobins represent a class of proteins defined by several amino acid sequence and structural features (Linder *et al.*, 2005; Wessels, 1997; Wosten & de Vocht, 2000). Members of this protein family contain eight cysteine residues with conserved spacing and display characteristic hydropathy patterns. Hydrophobins are often major protein components of the fungal conidial cell envelope, polymerizing to form bundle or rodlet-like structures, or they can be secreted into the medium, where

they self-assemble at the water–air interface, lowering the water surface tension, thus enabling the fungi to breach the interphase to form aerial structures (Kwan *et al.*, 2006; Talbot, 1999; Wosten *et al.*, 1993). Although the role of hydrophobins in some cell processes remains unclear, they have been implicated in a variety of developmental processes, including pathogenesis, fruit body formation, and sporulation, possibly by contributing to key structural features required for differentiation (Beckerman & Ebbole, 1996; Bell-Pedersen *et al.*, 1992; Ebbole, 1997; Girardin *et al.*, 1999; Kazmierczak *et al.*, 2005; Kershaw & Talbot, 1998; Lugones *et al.*, 1996; Nishizawa *et al.*, 2002). Due to their unique biophysical properties, hydrophobins have also been suggested for use in a wide variety of medical and technical applications, such as tissue engineering (by increasing the biocompatibility of medical implants and devices), drug delivery (using hydrophobin-stabilized vesicles), and nanotechnology-based surface patterning and alteration (Corvis *et al.*, 2005; Scholtmeijer *et al.*, 2002, 2004).

Abbreviations: EST, expressed sequence tag; 5'-RACE, 5' rapid amplification of cDNA ends; TFA, trifluoroacetic acid.

The GenBank/EMBL/DDBJ accession numbers for the *hyd1* and *hyd2* sequences of *B. bassiana* are EF452344 and EF520285, respectively.

Figures showing the sequences of hydrophobin genes used in phylogenetic analysis, atomic force micrographs and SDS-PAGE results obtained during rodlet extraction, MS results for a peptide fragment resulting from in-gel tryptic digestion, and BLAST matches of peptide fragment data are available with the online version of this paper.

Beauveria bassiana is an important entomopathogenic fungus that displays a broad host range, and is able to target a diverse range of arthropod species (Clarkson & Charnley, 1996; Leathers *et al.*, 1993; Maurer *et al.*, 1997). Strains of *B. bassiana* have been selected for the control of insects and other arthropods that act as disease vectors, including mosquitoes and ticks, crop pests such as whiteflies and borers, and even ecologically hazardous, invading pests such as fire ants and termites (Blanford *et al.*, 2005; Brownbridge *et al.*, 2001; Cruz *et al.*, 2006; Kirkland *et al.*, 2004; Klinger *et al.*, 2006; Marti *et al.*, 2005; McCoy, 1990).

Phage display technology is based upon the expression of protein or peptide sequences on the surface of phage particles by genetic fusion with phage capsid proteins. Applications include biopanning for antibody-, protein-, peptide-, DNA-, RNA- and oligonucleotide-binding phage-fusion particles (particularly using peptide libraries), as well as cDNA cloning of proteins that act as allergens (Danner & Belasco, 2001; Mullen *et al.*, 2006; Paschke, 2006; Rhyner *et al.*, 2004; Sergeeva *et al.*, 2006). Phage display has also been used to clone a protein with carbohydrate affinity, namely galectin-3 from a HeLa cell display library by mucin affinity selection (Yamamoto *et al.*, 1999).

In this report, we describe the successful use of phage display for the isolation of hydrophobins. A phage display cDNA library was constructed using RNA from *B. bassiana* grown in the presence of insect cuticle. Biopanning experiments were performed using a number of carbohydrate affinity resins, including *N,N'*-diacetylchitobiose-, fucose-, lactose-, maltose- and melibiose-agarose beads. Hydrophobins appeared to be selectively enriched by melibiose-agarose beads, which yielded two different hydrophobin proteins highly represented in the final library enrichment, and to a lesser extent by lactose-agarose beads, which yielded one of the hydrophobins. Expression studies were performed using RNA isolated from a variety of fungal developmental stages, including: (i) single-cell types such as aerial conidia, *in vitro* blastospores and submerged conidia; (ii) during fungal mycelial growth; and (iii) during sporulation on chitin and insect cuticle. Additionally, extraction and mass spectrometric analysis of the major protein constituent of the hydrophobic aerial conidia rodlet layer revealed it to be composed of the *hyd2* gene product.

METHODS

Construction of phage display and 5' rapid amplification of cDNA ends (5'-RACE) cDNA libraries. A phage display cDNA library was constructed in the bacteriophage T7Select10-3 system (Novagen). *B. bassiana* (ATCC 90517) was routinely grown on Sabouraud dextrose (Difco) + 0.5% yeast extract or potato dextrose (PD; Difco) media, either on agar plates or in liquid broth. Plates were incubated at 26 °C for 10–12 days and conidia were harvested by flooding the plate with sterile distilled H₂O (dH₂O) containing 0.01%

Tween 20. Conidial suspensions were filtered through glass wool, and final spore concentrations were determined by direct counting using a haemocytometer. For total RNA preparation, a Sabouraud liquid broth culture (250 ml) supplemented with 0.1% (w/v) sterilized dissected mole cricket (*Scapteriscus abbreviatus*) cuticle was inoculated (1:50, v/v) with conidia harvested from plates to a final concentration of 1×10^6 conidia ml⁻¹. The culture was grown for 3–4 days at 26 °C with aeration. Fungal samples, including mycelia, were harvested by centrifugation, washed with sterile dH₂O, and incubated with RNeasy lysis buffer (RNA stabilizing solution, Ambion) for 10 min at room temperature, before being frozen in liquid nitrogen and lyophilized overnight. Lyophilized samples were ground in liquid nitrogen, resuspended in RNeasy lysis buffer (Ambion), and homogenized using a Polytron homogenizer, before continuing the extraction according to the manufacturer's protocols. Poly-A⁺ RNA was selected using the Poly(A)Purist MAG system (Ambion). A directional cDNA library was constructed using the OrientExpress Oligo(dT) library construction system and cloned into the T7Select10-3 vector, all according to the manufacturer's recommendations (Novagen). A 5'-RACE library was constructed using the SMART RACE cDNA system (Clontech).

Biopanning protocol. *N,N'*-Diacetylchitobiose-, fucose-, lactose-, maltose- and melibiose-coupled agarose beads were obtained from Seigakagu (Tokyo). Beads were washed three times with PBS (50 mM sodium phosphate, pH 7.4, 0.15 M NaCl) before being resuspended in PBS + 1% BSA before use. The phage stock (10 ml of 10^{10} p.f.u. ml⁻¹) was precipitated using PEG-8000 (4% final concentration) and NaCl (3% final concentration) and resuspended in 1 ml PBS + 1% BSA. Beads (200 µl) were mixed with 0.5 ml precipitated phage and incubated at 4 °C for 2 h with gentle shaking, before being washed five times with 1 ml PBS containing 0.5 M NaCl. Bound phage was eluted (twice) with a 0.2 M solution (300 µl total volume, incubated at room temperature for 30 min) of the carbohydrate moiety bound to each respective agarose-coupled bead. The titre of the eluted phage was amplified by passage through an *Escherichia coli* host, and the resultant phage stock was used for the next round of biopanning. Five rounds of the biopanning procedure were performed successively with each affinity resin.

Molecular manipulations. cDNA inserts of phage plaques were analysed by PCR amplification and sequencing (ICBR Sequencing Facility, University of Florida) using vector primers (T7Up and T7Down). Genomic DNA was extracted from fungal mycelium using hexamethyltrimethyl ammonium bromide (CTAB), as described by Talbot (2001). DNA hybridization probes were labelled using the BioPrime chemiluminescent DNA labelling system (Invitrogen). Plasmid preparation, restriction enzyme digestion, agarose gel electrophoresis and Southern blotting were performed using standard procedures (Ausubel *et al.*, 1996).

RNA extraction. Total RNA was extracted from *B. bassiana* cells using either RNeasy lysis buffer or TRI Reagent (Ambion) according to the manufacturer's recommendations, including the high-salt precipitation step for removal of proteoglycans and polysaccharides. Culture conditions for RNA extraction were as follows: *B. bassiana* was grown on PD agar (PDA) or on Sabouraud dextrose + 1% yeast extract either on agar plates (SDAY) or in liquid broth (SDY). Plates were incubated at 26 °C for 10–12 days and aerial conidia were harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through Miracloth and final spore concentrations were determined by direct counting using a haemocytometer. Liquid broth cultures were inoculated (1:50, v/v) with conidia harvested from plates to a final concentration of $0.5\text{--}5 \times 10^5$ conidia ml⁻¹. Cultures were grown for 3–4 days at 26 °C with aeration. Cultures were filtered through glass wool or Mira cloth to remove mycelia, and the concentration of blastospores was determined by direct counting.

Submerged conidia were isolated from TKI broth (per litre: 50.0 g fructose, 10.0 g KNO₃, 5.0 g KH₂PO₄, 2.0 g MgSO₄·7H₂O, 50.0 mg CaCl₂, 50.0 mg yeast extract), as previously described (Cho *et al.*, 2006b). A time course of growing mycelia was prepared by growing *B. bassiana* on PDA. At the desired time points (3, 5, 10, 18 and 28 days), conidia were removed by washing the plates by flooding two to three times with dH₂O, and the mycelium was obtained by lightly scraping off the fungal biomass from the resultant agar plates. Mycelia were examined by light microscopy for the presence of conidia, and samples containing less than 1% conidia were used for further experimentation. Chitin and insect cuticle liquid broth cultures (50–100 ml), consisting of 1:4 diluted Sabouraud dextrose broth supplemented with 1% (w/v) chitin, 1% powdered, sterilized *Manduca sexta* cuticle (kind gift of D. Boucias, Department of Entomology and Nematology, University of Florida), or 1% chitin + 1% *M. sexta* cuticle, were inoculated with conidia harvested from plates to a final concentration of $0.5\text{--}5 \times 10^5$ conidia ml⁻¹ and were grown for 3 days at 26 °C with aeration, after which the total fungal cell culture was harvested by centrifugation (10 000 g, 10 min), washed twice with dH₂O, flash-frozen in liquid nitrogen, and stored at -70 °C.

Semi-quantitative RT-PCR analysis. Total RNA isolated as described above was precipitated once with LiCl before being DNase-treated using the DNA-free reagent (Ambion). RNA samples were then treated with SUPERase-In (RNase inhibitor, Ambion) and stored at -70 °C until use. Total RNA concentration was quantified for each sample preparation using the Ribogreen RNA quantification kit (Molecular Probes). cDNA for each sample was synthesized using 1.0 µg of total RNA plus Superscript III reverse transcriptase with oligo dT₁₈ priming, following the manufacturer's protocol (Invitrogen). PCR reactions were as follows: 1.0 µl of a twofold dilution of the cDNA sample was PCR-amplified using 6.0 µl DNA polymerase MasterMix (Eppendorf), 0.3 µl of 0.01 mM of each primer, and dH₂O to a final volume of 17.0 µl. PCR amplification was performed at 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 35 s. Samples (10 µl) were taken and analysed by agarose gel electrophoresis after 15, 20, 25 and 30 PCR cycles. Amplifications of portions of the *B. bassiana* actin and β -tubulin genes (see Table 2) were performed as controls during all reactions, and were used as internal standards to normalize the expression levels of the *hyd1* and *hyd2* genes in the various RNA samples. The relative intensity of the bands was determined after densitometric scanning using Adobe Photoshop. Primer sets used for the amplification of *hyd1*, *hyd2*, actin and β -tubulin are presented in Table 2. In all experiments, controls containing no template or no enzyme were performed. Each PCR reaction was performed in duplicate with duplicate biological samples and duplicate cDNA preparations for each sample.

Rodlet layer extraction, MS fingerprinting, and N-terminal amino acid sequence determination. The rodlet layer proteins were removed from the surface of *B. bassiana* aerial conidia as previously described (Holder & Keyhani, 2005). Briefly, aerial conidia were resuspended in water, and sonicated at 140 W (3 mm diameter microtip, 50% duty cycle) twice for 10 min using a Sonifier cell disrupter B-30 (Branson Ultrasonics). Unlysed cells and cell debris were removed by low-speed centrifugation (10 000 g, 10 min), and the supernatant was centrifuged for 30 min at 50 000 g. The resultant pellet was boiled in SDS-PAGE sample buffer (2% SDS, 5% β -mercaptoethanol, 10% glycerol, v/v, in 62 mM Tris/HCl, pH 6.8), and washed twice with sample buffer and three times with distilled water. The final pellet was lyophilized, then treated with 100% trifluoroacetic acid (TFA) for 10 min at room temperature. The acid was removed under a stream of nitrogen, and dried extracts were stored at room temperature under dry air and resuspended in water prior to analysis. Aliquots of protein sample were mixed with 4 × lithium dodecyl sulfate (LDS) sample buffer plus DTT and run on a

10% Bistris NuPAGE gel with MES-SDS gel running buffer together with standards (Invitrogen). Protein bands were visualized using either Sypro Ruby Red (Biorad) or Coomassie Blue (Biorad). For MS fingerprinting, proteins were separated by SDS-PAGE, and a protein with an apparent molecular mass of 10 kDa was subjected to in-gel tryptic digestion. Capillary reversed-phase HPLC separation of protein digests was performed on a 10 cm × 75 µm internal diameter PepMap C18 column (LC Packings) in combination with a home-built capillary HPLC system operated at a flow rate of 200 nl min⁻¹. Inline mass spectrometric analysis of the column eluate was accomplished by a quadrupole ion trap instrument (LCQ, ThermoFinnigan) equipped with a nanoelectrospray source. Fragment ion data generated by data-dependent acquisition via the LCQ were searched against a local sequence database using the SEQUEST (ThermoFinnigan) database search engine. The score for SEQUEST protein identification was considered significant when dCn was equal to 0.08 or greater and the cross-correlation score (Xcorr) was greater than 2.2.

Rodlet layer extracts were electrophoresed on SDS-PAGE and electroblotted onto Immobilon-P PVDF membranes. After transfer, the blot was stained with Coomassie Blue, an ~10 kDa protein band was cut out, and the N-terminal sequence determined using an Applied Biosystems Procise Sequencer (Protein CORE Facility, University of Florida).

Phylogenetic analysis. A phylogenetic analysis was performed using nearest-neighbour joining analysis of 56 hydrophobin sequences found in GenBank, along with the *B. bassiana* sequences using the CLUSTALW (EMBL-EBI, 1000 bootstrap trails, 500 random number generator) multiple sequence alignment program. The resultant tree was visualized using the online program Phylodendron phylogenetic tree printer (<http://iubio.bio.indiana.edu/treeapp/treeprint-form.html>).

RESULTS

cDNA and genomic cloning of two *B. bassiana* hydrophobin genes

In order to isolate cell-adhesion and fungal-binding proteins, a *B. bassiana* phage display cDNA library was constructed using the T7Select10-3 vector. In this system, proteins up to 1200 aa in size are expressed as C-terminal fusions to the T7 10B capsid protein, with five to 10 copies of the target displayed per phage. Since hydrophobins are small proteins that mediate binding to hydrophobic surfaces, initial attempts to isolate these proteins from the phage display library were performed using phenyl-Sepharose affinity chromatography. These efforts were unsuccessful, due to non-specific adsorption of the phage on the resin at high salt concentrations (2 M NaCl, data not shown). Instead, five different types of carbohydrate-bound agarose bead: *N,N'*-diacetylchitobiose-, fucose-, lactose-, maltose- and melibiose-coupled agarose, were used in biopanning protocols, as described in Methods. In order to maximize the specificity of the enrichment, 0.2 M of the cognate carbohydrate attached to the agarose beads was used to elute bound phage in each biopanning round.

After five rounds of enrichment, inserts from 20 plaques of each library selected by biopanning were amplified by PCR and examined by agarose gel electrophoresis, revealing that

Table 1. Summary of hydrophobin properties

Gene	5'-UTR*	Promoter (TATA)†	Length (nt) of:			3'-UTR‡	Poly-A signal§
			ORF	Exons	Introns		
<i>hyd1</i>	157	−46	411	274, 75, 62	53, 56	172	50 (AATTAA)
<i>hyd2</i>	160	−59	351	306, 45	64	112	61 (AATAAT)
Protein	No. of amino acids	Molecular mass (kDa)	Signal peptide position	Isoelectric point	Grand average of hydropathicity		
Hyd1	136	13.81	12	6.98	0.305		
Hyd2	116	11.98	10	6.23	0.251		

*Length (nt) of 5' untranslated region (before start codon) derived from consensus cDNA sequence.

†Position relative to ATG start codon.

‡Length (nt) of 3' untranslated region (after stop codon) derived from consensus cDNA sequence.

§Position (nt) of putative polyadenylation signal after stop codon (derived from genomic sequence).

||Position in no. of amino acids of putative signal peptide sequence from N-terminal starting Met.

>95 % of the plaques tested had inserts ranging in size from 0.4 to 1.5 kb. Using heat-denatured phage lysate from each cycle of the biopanning procedures, cDNA inserts were amplified by PCR, and enrichment of certain phages was observed, as judged by the increased band intensity through the rounds of selections (data not shown). Sixteen plaques from each library were randomly selected and their insert nucleotide sequences determined. Sequencing reads averaged ~450 bp per read, and their translated amino acid sequences were analysed by BLAST searches of the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/blast/>). Sequences derived from enrichment using two of the five affinity resins, lactose–agarose and melibiose–agarose, corresponded to cDNAs encoding two different *B. bassiana* hydrophobins. Four cDNA clones contained almost identical sequence inserts, which were used to construct a full-length unambiguous cDNA sequence termed *hyd1*. Three additional phage-display-enriched cDNA sequences that shared no homology to *hyd1* clustered to yield *hyd2*.

In order to obtain the positions of possible introns within the coding sequences of the *B. bassiana* genes, primer

oligonucleotides covering the start and stop codons of each hydrophobin gene were used in PCR reactions performed on genomic DNA. A summary of the gene and deduced protein characteristics is listed in Table 1. Based upon the genomic sequence, *hyd1* contained two small (53 and 56 nt) introns, whereas *hyd2* contained a single intron (64 nt). Putative promoters and polyadenylation signals were also detected in the isolated gene sequences. The ORFs corresponding to *hyd1* and *hyd2* coded for proteins of 136 and 116 aa, respectively, with both proteins containing putative export signal peptides. Southern blot analysis using the cloned genes as probes indicated that only one allele of each gene was present in the genome (data not shown).

Sequence analyses

Analysis of the translated protein products of the *B. bassiana* genes revealed them to possess the hallmarks of class I hydrophobins: (i) low molecular mass (10–15 kDa); (ii) a secretion signal; and (iii) eight cysteine residues with characteristic conserved spacing. Phylogenetic analysis of the protein products revealed that Hyd1 was most closely

Table 2. Primer sequences and product sizes for semi-quantitative RT-PCR

F, forward; R, reverse.

Gene product	Primer sequence (5'→3')	Location on gene	Product size
Hyd1	F: CACCATGGTGGAAAGGATCTGCAC	217–240	188
	R: CCGAGAAGGTGGGAAAGAAGACCA	392–415	
Hyd2	F: TGTC AAGACTGGCGACATTGCG	237–259	180
	R: TCGATGGGGACAAGCTGGTTGA	396–417	
β-Tubulin	F: TCCTTCGTACGGTGACCTGA	309–328	103
	R: CGAGCTTGCGAAGATCAGAG	412–393	
Actin	F: TTGGTGCGAAACTTCAGCGTCTAGTC	67–92	322
	R: TCCAGCAAATGTGGATCTCCAAGCAG	364–389	

related to *Giberella moniliformis* (anamorph *Fusarium verticillioides*) H1 and H2 class I hydrophobins (Fuchs *et al.*, 2004), whereas Hyd2 was most closely related to the *Magnaporthe grisea* mpg1 and *Metarhizium anisopliae* ssgA gene products (also class I hydrophobins) (St Leger *et al.*, 1992; Talbot *et al.*, 1993) (Fig. 1). The phylogenetic analysis also revealed the ancient ancestry of the different hydrophobin genes, suggesting that the two genes are not derived from a recent duplication event.

Hydropathy analysis of the proteins revealed that after a hydrophobic signal peptide, Hyd1 and Hyd2 had two small hydrophilic domains spaced by a neutral-to-hydrophilic

region, a hydrophobic central core followed by another small hydrophilic region, and a hydrophobic C terminus (Fig. 2). These data are consistent with that reported for other hydrophobins, and confirm the amphipathic nature of the proteins.

Gene expression analysis of the *B. bassiana* hyd1 and hyd2 genes

The expression pattern of the *B. bassiana* hyd1 and hyd2 genes was analysed by semi-quantitative RT-PCR. Two internal controls, namely β -tubulin and actin, were used to

Fig. 1. Phylogenetic analysis of the *B. bassiana* and other fungal hydrophobins based on sequence analysis of the translated protein products. The *B. bassiana* H1 (Hyd1) and H2 (Hyd2) proteins are highlighted.

Fig. 2. Hydropathy plots of the deduced amino acid sequences corresponding to Hyd1 (a) and Hyd2 (b). The *x* axis shows the amino acid position relative to the N terminal of the proteins. The hydropathy values were calculated using a window size of 5 amino acids (Kyte & Doolittle, 1982).

normalize the expression data, and two internal standards were used to assess variation between samples: total RNA (quantified as described in Methods) and actin/ β -tubulin. Data in which (i) the variation between samples of the total RNA/actin or total RNA/ β -tubulin and (ii) the variation between biological samples was less than twofold were used. In addition, a standard curve of actin concentration determined by PCR against the amount of RNA quantified by Ribogreen was constructed. Only data that fell within the linear portion of the standard curve were considered valid.

The relative expression level of the two genes was assessed in RNA pools derived from aerial conidia, blastospores, submerged conidia, growing mycelia, and from cells grown on insect cuticle, chitin (the main carbohydrate constituent

of insect cuticles), and insect cuticle + chitin (Fig. 3). The data presented were determined to be within the linear portion of the PCR analysis (25 cycles), with the actin concentration closely correlating to the amount of RNA quantified for each sample under test. Hydrophobin expression was normalized to the quantification of the actin band. *B. bassiana hyd1* expression was detected in all developmental stages and media conditions tested. *Hyd1* levels appeared to increase during mycelial growth, with the highest level of expression observed after 28 days of growth on agar plates, although caution should be taken in any interpretation of these results, as they derived from semi-quantitative measurements. On agar plates, cells began sporulating (i.e. producing aerial conidia) after ~14 days; however, conidia were washed from the plates of all the mycelial samples before RNA isolation. This was

Fig. 3. Expression analysis of *hyd1* and *hyd2*. Semi-quantitative RT-PCR was used to determine the expression of the *hyd1* (a) and *hyd2* (b) genes in various *B. bassiana* cell types and during specific developmental conditions, as described in Methods. Only data within the linear portion of PCR amplification were considered valid, and the data shown are derived from 25 cycles of PCR amplification. Actin expression was used as an internal control for normalization of the data (similar results were obtained using β -tubulin). Expression of the hydrophobin and actin genes was examined during mycelial growth on PDA plates from which conidia were removed (3, 5, 10, 18 and 28 days), and from liquid cultures of blastospores (B), aerial conidia (C), submerged conidia (SC), and after 3 days of growth in the presence of chitin (1%), insect cuticle (1%), or chitin + insect cuticle (Cut + Chit; 1% each).

confirmed by microscopic visualization of the mycelial samples, in which <1% of contaminating aerial conidia were visible in the mycelial preparations. Thus, the observed *hyd1* expression appears to be derived from the mycelia. Expression in aerial conidia was similar to that observed in either blastospores or submerged conidia, and was equivalent to that observed in 18 day mycelia. Cells growing in the presence of 1% chitin, 1% cuticle, and 1% chitin + 1% cuticle also showed robust expression of *hyd1*, similar to levels seen in blastospores and submerged conidia, and 5–10 day mycelia. In contrast to *hyd1*, *hyd2* appeared to be constitutively expressed and at about the same level as actin throughout the growing mycelia stages (3–28 days). Little or no *hyd2* was observed in either blastospores or aerial conidia. Some *hyd2* transcript was detected in submerged conidia, which corresponded to about 5–10% of the levels seen in the mycelial samples. Intriguingly, *hyd2* was expressed (at approximately the same levels as actin) in fungal cells growing on 1% chitin or 1% cuticle, but almost no transcript could be detected when the cells were grown on 1% chitin + 1% cuticle.

Identification of Hyd2 as the conidial cell wall hydrophobin from *B. bassiana*

We have previously reported that aerial conidia contain a boiling-SDS-insoluble, TFA-soluble cell wall component that is not found in either blastospores or submerged

conidia under the conditions tested (Holder & Keyhani, 2005). A protein with an apparent molecular mass of 10 kDa was identified by SDS-PAGE and used for *in situ* tryptic digestion followed by MS peptide fingerprinting (SDS-PAGE and MS data are available as supplementary data with the online journal). Two peptide fragments, TGDICNGNTMHCCNDESVTNK and LTGPSVLSDLRL, were obtained. In addition, N-terminal amino acid sequencing of the ~10 kDa band resulted in the sequence (P/H)-GPSHGXPVKTGDI, where (P/H) represents poorly identified residues and X an unresolved residue. The amino acid fingerprints were consistent with the translated ORF of the *hyd2* sequence cloned by phage display, and the N-terminal amino acid sequence matched the sequence of the putative mature Hyd2 sequence: (APH)GPSHG(S)VKTGDI. These data indicate that the *hyd2* gene product constitutes the rodlet layer hydrophobin that surrounds *B. bassiana* conidia.

DISCUSSION

Hydrophobins are unique fungal proteins that function in a diverse array of physiological processes. Since hydrophobins are often highly expressed, the isolation of genes encoding these proteins has largely been accomplished from genomic [e.g. expressed sequence tag (EST)] analyses or, in some cases, by purification of the protein product

and stepwise cloning of genes using nucleotide primers based upon the determined amino acid sequences (Wessels, 1997, 1999; Wosten & de Vocht, 2000). These approaches are limited by expression levels and the success of protein purification protocols. Lectin-like activity has been reported for several hydrophobins. Both monomeric and assembled forms of the SC3 hydrophobin from the basidiomycete *Schizophyllum commune* agglutinate rat blood cells, whereas the SC4 hydrophobin from the same organism agglutinates horse red blood cells (van Wetter *et al.*, 2000). The class II hydrophobin of the plant pathogen *Cryphonectria parasitica*, named cryparin, agglutinates rabbit, and to a lesser extent rat and chicken red blood cells, but not those of ox, horse, human blood group A or guinea pig (Kazmierczak *et al.*, 1996; McCabe & Van Alfen, 1999). This behaviour is considered typical of lectins; however, although crude cell extracts were able to inhibit agglutination, no sugar tested appeared to inhibit agglutination. A lectin has been reported from *B. bassiana* that recognizes the Thomsen–Friedenrich antigen and related structures, although neither the primary amino acid nor the nucleotide sequence of the gene encoding the protein has been reported (Kossowska *et al.*, 1999). Based upon a comparison of the amino acid content of the described *B. bassiana* lectin, it does not appear to be one of the hydrophobins characterized in this report.

Phage display technologies represent a method for isolating proteins and/or peptides that bind molecules of interest. (Danner & Belasco, 2001; Mullen *et al.*, 2006; Paschke, 2006; Rhyner *et al.*, 2004; Sergeeva *et al.*, 2006). Phage display has been used to isolate human galectin-3, a galactose/lactose-specific lectin and member of the S-type lectin family, using blood group glycoproteins during a biopanning selection (Yamamoto *et al.*, 1999). In this report, we demonstrate the feasibility of phage display to isolate cDNAs coding for hydrophobins. Initial attempts using hydrophobic substrata for biopanning were unsuccessful, due to non-specific adsorption of the phage. However, it may be possible to alter the conditions to minimize this background. Using various carbohydrate-derivatized beads, cDNAs encoding two hydrophobins were enriched from a *B. bassiana* phage display library constructed using mRNA isolated from fungal cells grown *in vitro* in the presence of insect cuticle. The interaction between the hydrophobins and the carbohydrate-derivatized beads appeared to be specific, since (1) not all carbohydrate-derivatized beads tested enriched for the *B. bassiana* hydrophobins, and (2) the elution step during each round of enrichment was competitive, using 0.2 M of the cognate carbohydrate. Further experiments, using the purified hydrophobin preparations, are however needed to conclusively demonstrate lectin-like activity for the isolated proteins.

The translated amino acid sequences of *hyd1* and *hyd2* did not match the 16 aa N-terminal sequence of a putative hydrophobin isolated from *B. bassiana* reported elsewhere, although the N terminus of mature *hyd2* did show some

homology (~50 %) to the reported protein (Bidochka *et al.*, 1995b). Similarly, although not an exact match, the N terminus of mature Hyd1 showed homology (~40 %) to the N terminus (as determined by amino acid sequencing) of what was termed an inner cell wall protein (cwp1) of *B. bassiana* (Bidochka *et al.*, 1995a). Although our results indicate that Hyd2 is a major constituent of the spore rodlet layer, additional experiments are needed to determine the cellular localization of Hyd1. Expression analysis indicated that *hyd1* was highly expressed under almost all conditions. These results are consistent with our previous EST analyses of *B. bassiana* grown under different developmental conditions (Cho *et al.*, 2006a, b). Notably, *hyd1* transcript was abundant in all the single-cell spore types (aerial conidia, *in vitro* blastospores and submerged conidia), whereas *hyd2* transcripts were essentially absent from these cells. Thus, even though the spore coat of aerial conidia is composed of the Hyd2 protein, no *hyd2* transcript was detected in these cells. This is perhaps not too surprising, since it is the conidiogenic cells that need to make *hyd2* and its protein product (in order to make the aerial conidia), and therefore one would not expect to see *hyd2* transcript in the final product of this process, namely the aerial conidia themselves. Transcripts corresponding to *hyd2* were also detected in cells grown in 1 % chitin or 1 % insect cuticle, but were not detected in cultures containing chitin + cuticle, each at 1 % concentration. This result was observed in two separate biological samples in which actin was used as a control. Although it is unclear why *hyd2* transcript was not detected under the latter growth conditions, it could be due to the relative nutrient levels in the cultures. Cells growing under 1 % chitin or 1 % cuticle conditions may be nutrient-limited, which would result in sporulation (i.e. conidiogenesis and hence *hyd2* transcript production), whereas cultures containing both nutrient sources may grow in a vegetative state for a longer period of time. Further experiments delineating the expression of *hyd1* and *hyd2*, especially during growth on insect hosts, i.e. during pathogenesis, as well as gene knockouts, are likely to give important insights into the role of these hydrophobins in fungal development and virulence.

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