

## A pilot-scale Expressed Sequence Tag analysis of *Beauveria bassiana* gene expression reveals a tripeptidyl peptidase that is differentially expressed in vivo

Aurélien Tartar & Drion G. Boucias

Department of Entomology & Nematology, University of Florida, Gainesville, Florida 32611-0620, USA

Received 15 December 2003; accepted in revised form 28 May 2004

### Abstract

The entomopathogen *Beauveria bassiana* is a dimorphic fungus that displays an in vivo-specific, yeast-like parasitic phase. In order to study the transcriptome of *B. bassiana* during this unique developmental phase, we developed a method to harvest in vivo *B. bassiana* cells from infected *Manduca sexta* larvae. The infected hemolymph was collected just prior to insect death and subjected to gradient centrifugation, which allowed for separation of the *B. bassiana* in vivo-produced cells from remaining insect hemocytes. Total RNA was extracted from the harvested fungal cells and used to construct a cDNA library that is representative of *B. bassiana* gene expression in vivo. Expressed Sequence Tags (ESTs) were generated and led to the cloning of two protease genes. One of these proteases was identified as a tripeptidyl peptidase (Bb TPP). The Bb TPP protease was shown to be up-regulated during infection, and identification of a signal peptide suggested that the enzyme is secreted in the host hemolymph. Although its activity and role have yet to be characterized, the Bb TPP protease appears as a likely candidate for being involved in *B. bassiana* pathogenesis. The identification of this novel, up-regulated protease also suggests that random sequencing from our in vivo cDNA library may be a valuable step towards identifying biologically active metabolites produced in vivo by *B. bassiana*.

**Key words:** *Beauveria bassiana*, entomopathogen, expressed sequence tags, in vivo, protease, tripeptidyl peptidase

### Introduction

The fungus *Beauveria bassiana* is one of the most common mycopathogens infecting insects [1]. It has long been recognized as a potential biocontrol agent and is actively being developed to control various pest insects [2]. Like other fungal pathogens [3], *B. bassiana* exhibits a dimorphic life cycle. In artificial media and soil *B. bassiana* grows as a filamentous form and produces conidia. Under proper conditions, conidia bind to the insect cuticle and germinate to form germ-tubes that digest and penetrate the insect integument and reach the hemocoel. Once in the insect hemolymph, the fungus may develop through a yeast-like hyphal body phase, which is an obligatory parasitic phase

and is not normally observed outside the insect host. *In vivo*-produced hyphal body cells are free-floating in the hemolymph and develop rapidly by budding. At time of insect death, fungal cells in the hemolymph switch back to the filamentous form and colonize the remaining tissues. The mycelial phase eventually exits the cuticle and produces diagnostic hyaline conidiophores over the external surface of the host (see review on *B. bassiana* dimorphic life cycle in [1]).

Although a yeast-like phenotype has recently been obtained in vitro [4], the in vivo growth of *B. bassiana* remains poorly understood. Pendland et al. [5] demonstrated that in vivo-produced hyphal bodies, unlike in vitro cells, lack a well defined cell wall. The modified cell wall lacks

antigenically important constituents, and has been associated with the ability of fungal cells to evade host defense mechanisms. Additionally, the in vivo yeast phase has also been shown to secrete toxic metabolites in the insect hemolymph [6]. These toxic metabolites differ from the low molecular weight cyclic peptides isolated from in vitro *B. bassiana* cultures (such as beauvericin and bassianolide, see [1] for review). In vivo-secreted toxins have been described as high-molecular weight proteins (>10 kDa) but have never been fully purified or characterized [6, 7].

In an effort to characterize the pathogenesis-associated events better, we developed a rapid method to isolate in vivo-produced yeast cells, extracted mRNA, and initiated an Expressed Sequence Tag (ESTs) analysis of *B. bassiana* gene expression in vivo. Herein, we report the identification of a high-molecular weight protease, a tripeptidyl peptidase, that is up-regulated during *B. bassiana* infection.

## Materials and methods

### *Maintenance of fungal culture*

*B. bassiana* strain UFL 5477, originally isolated from adult mole crickets, *Scapteriscus vicinus*, was maintained on Sabouraud maltose agar plus 2% yeast extract (SMY) at 25 °C. Conidia produced on these plates were inoculated into flasks containing Sabouraud dextrose broth plus 2% yeast extract (SDY) and shaken at 23 °C on a gyratory shaker for 3–4 days. Blastospores produced in broth cultures were separated from mycelial fragments by filtering cultures through a layer of Miracloth. Blastospores were then collected by low speed centrifugation and resuspended in a 0.85% w/v saline (NaCl) solution. The concentration of washed blastospores was estimated with a Brite-line hemacytometer and adjusted to  $2 \times 10^8$  cells per ml. The cells were used immediately.

### *Production of in vivo fungal cells*

The tobacco hornworm, *Manduca sexta*, accessed from laboratory colonies maintained at North Carolina State University, was used as host to propagate the in vivo cell phenotype of *B. bassiana*. Larvae were reared on artificial diet (diet recipe

on <http://www.entm.purdue.edu/Entomology/outreach/recipe/manduca.diet.htm>) at 25 °C until they reached the 5th larval instar. Cohorts of fifth instar larvae were immobilized on ice and challenged by injection of blastospores into the hemocoel, as previously described [8]. A total of  $1 \times 10^6$  cells were injected per *M. sexta* larva. At this concentration, treated larvae were able to undergo limited development for 24 h but succumbed synchronously to mycosis within 48–60 h post-inoculation. In vivo-produced fungal cells were harvested from *M. sexta* larvae just before death (*ca.* 48 h post-challenge), when the concentration of fungal cells in the blood is maximal [8]. Before being bled, individual larvae were injected with 0.5 ml citrate EDTA buffer (0.14 M NaCl, 0.1 M glucose, 26 mM citric acid, 30 mM trisodium citrate, 10 mM EDTA, pH 4.6), which both inhibited the phenoloxidase cascade and served as an anticoagulant.

Hemolymph from infected insects was harvested on ice and immediately applied on top of a step gradient of 25 and 50% Centricoll (where 100% Centricoll is defined by 9 volumes of pure Centricoll and 1 volume of 2.5 M sucrose, in accordance with the manufacturer's instructions). Centrifugation was performed in 2 ml tubes, at 10,000 g for 10 min in a cold room (4 °C). In vivo fungal cells separated from the insect hemocytes and were collected as a pellet at the bottom of the centrifuge tube. Pellets were washed free of gradient material in 1 ml of ice-cold sterile water (10,000 g, 2 min, 4 °C), immediately frozen in liquid nitrogen, and stored at -70 °C until processed for RNA extraction.

### *Library preparation and DNA sequencing*

Frozen in vivo cell pellets from individual larvae were pooled under liquid nitrogen and ground into a fine powder using mortar and pestle. Total RNA was isolated using TriReagent, resuspended in formamide, and its concentration was estimated using a spectrophotometer. The cDNA library was prepared using 0.6 µg of total RNA and the PCR-based method of Clontech's SMART cDNA library construction kit. Following the manufacturer's protocol, the cDNAs were ligated directionally into  $\lambda$ TriplEx2 vectors, the library was titered and amplified, and mass excision was performed to convert the phage into the pTriplEx2 plasmid. Recombinant bacterial colonies were

randomly transferred to 96-well plates (192 colonies, corresponding to two 96-well plates) and processed by the University of Florida Interdisciplinary Core for Biotechnological Research (ICBR) for high-throughput sequencing. ESTs were obtained by single-pass sequencing of the 5' end of the cDNA, using the 5'  $\lambda$ TriplEx2 sequencing primer (5'-TCCGAGATCTGGACGAGC-3').

#### Sequence analysis

The sequencing reads were imported into the ICBR software package "Finch-Suite" (Geospiza Inc., Seattle, WA) where various third-party algorithms are used to estimate the quality of the read (Phred), trim down the vector sequences (Crossmatch) and assemble contigs (Phrap). ESTs were stored in a database and compared with the non-redundant NCBI database by performing sequence similarity searches using BlastX [9]. The predicted amino acid sequences that showed very significant similarity ( $E$ -value  $< 10^{-15}$ ) to known proteins were categorized based on broad cellular functions [10]. Screening of the EST database for signal peptides at the N-terminus of putative proteins was performed using the SignalP (version 2.0) server online ([www.cbs.dtu.dk/services/SignalP-2.0/](http://www.cbs.dtu.dk/services/SignalP-2.0/), [11]). Additional analyses of protein sequences, such as molecular weight estimation and motifs recognition, were done using the ExpASY molecular server (<http://us.expasy.org/>, [12]). The Bb TPP protein sequence was aligned with its closest homologues (according to BlastX analysis) using ClustalX, and Unweighted Pair Group Method using arithmetic Averages (UPGMA) analysis was run in PAUP\* with default parameters.

RACE-PCR: The 5' ends of selected ESTs were amplified using the SMART RACE cDNA amplification kit (Clontech). The obtained PCR

fragments were cloned into pGEM-T vectors (Promega) and sent to the University of Florida ICBR for sequencing.

#### *In vitro* RNA extraction and RT-PCR

Mycelia and blastospores from *B. bassiana* SDY culture were collected by filtration, frozen in liquid nitrogen and ground for RNA extraction using TriReagent as described above. The *in vitro* RNA concentration was estimated using a spectrophotometer, and an equal quantity of *in vitro* and *in vivo* total RNA was used for cDNA synthesis. First strand cDNA syntheses and RT-PCR reactions were performed using the ThermoScript RT-PCR system from Life Technologies, following the manufacturer's directions. The primer names and sequences used for amplification of *B. bassiana*  $\beta$ -tubulin, DPP and TPP transcripts are listed in Table 1. The thermal cycling conditions were 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min performed for 20, 25, 30 or 35 cycles. The samples were subjected to 1% agarose gel electrophoresis (in TBE buffer) and stained in ethidium bromide. Transcript abundance variations between the *in vitro* and *in vivo* RNA pools were compared using the semi-quantitative RT-PCR method previously described by Zhang et al. [13].

## Results and discussion

#### *Purification of in vivo cells*

The gradient centrifugation method, similar to the one used by Mead et al. [14], proved to be very efficient in separating fungal cells from the remaining insect hemocytes. After centrifugation, virtually no insect cells were present in the pellet (Figure 1C). About 1 ml of blood, containing

Table 1. Primers used to amplify  $\beta$ -tubulin, Bb DPP and Bb TPP transcripts in RT-PCR

Genes	Primer names and sequences
Beta tubulin	TubF: 5'-TGGGCYAARGGYCACTACACYGA-3' TubR: 5'-TCAGTGAACTCCATCTCRTCCAT-3'
Bb DPP	DPPF: 5'-CCAAGACCCTCAGGCTTTGAATAC-3' DPPR: 5'-CAATGGTTCTCGTCGGGAAACACTA-3'
Bb TPP:	aorF: 5'-CGGGCAGCTAGTCAAGGGAAACAAG-3' aorR: 5'-CGTTGGCATGGTGGTATTGGTGTTTC-3'

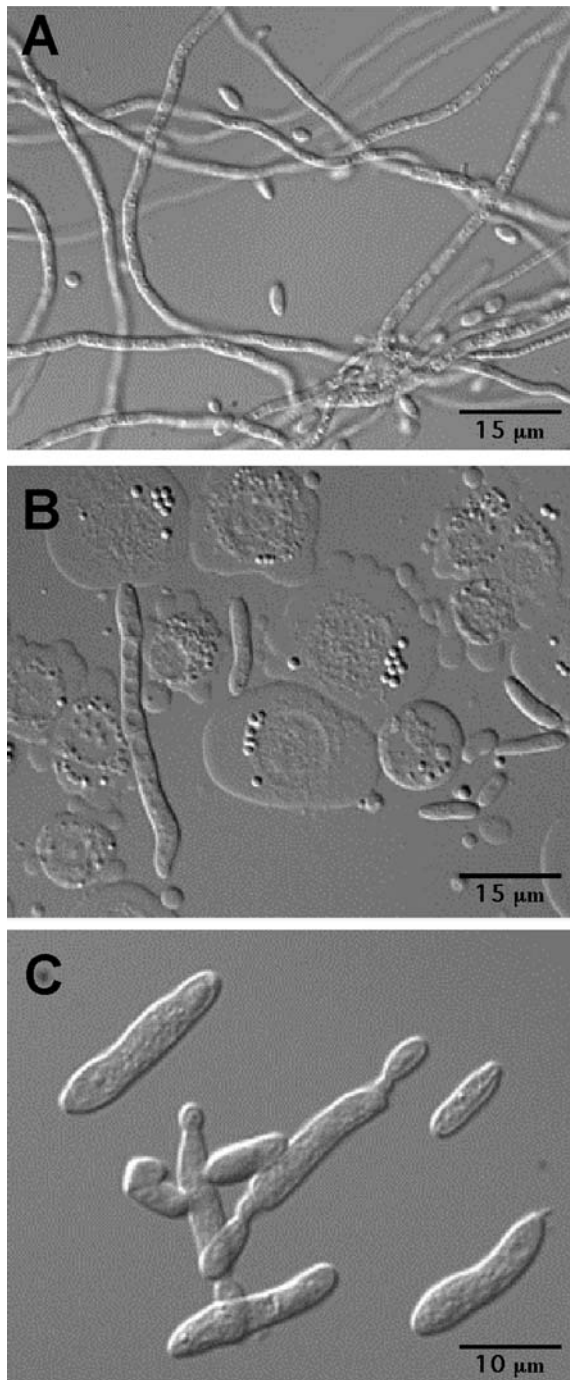


Figure 1. Light micrographs illustrating *B. bassiana* dimorphism. (A) In SDY, *B. bassiana* grows as mycelium and produces blastospores, (B) In vivo, *B. bassiana* grows as hyphal bodies, which are observed floating in the hemolymph, and (C) A sample obtained after gradient centrifugation: the *B. bassiana* hyphal bodies were separated from insect cells and used for RNA extraction.

ca.  $10^7$  in vivo-produced fungal cells, was obtained per *M. sexta* larva, which is a concentration ( $10^7$  fungal cells per ml of hemolymph) consistent with previously published results for *Spodoptera exigua* [8].

The cells were purified in less than 1 h, which prevented the in vivo-produced yeast cells from switching back to the walled in vitro phenotype. This was confirmed by probing collected cells with FITC-labeled peanut lectin. After each purification and prior to freezing, aliquots of in vivo cells were subjected to the fluorescent studies described by Pendland et al. [5]. These studies demonstrated that the peanut lectin did not label the purified in vivo cells, but did label the in vitro blastospores (data not shown). Therefore, we are confident that we purified the thin-walled, in vivo phenotype of *B. bassiana* cells, and that the cDNA library produced from these cells is representative of fungal gene expression in vivo.

To test our cDNA library, two 96-well plates containing transformant bacterial colonies were produced and processed for sequencing. After elimination of the non-readable sequencing reactions, and vector-only reads, a total of 163 successful EST, with an average length of 780 bp, were analyzed. Following contig assembly, 98 unisequences were identified and 57 of these sequences were found to have very significant similarities (BlastX *E*-value  $< 10^{-15}$ ) with sequences present in public databases. These 57 putatively identified genes are listed in Table 2. All the sequences but two are similar to genes sequenced from other fungi (Table 2). These results confirm that the separation of insect and fungal cells was very efficient. No ESTs were found to be similar to insect-related sequences.

#### Cloning of two in vivo-produced protease genes

*B. bassiana* is thought to kill its host by nutrient depletion, dehydration and/or toxin production [1]. Cheung and Grula [15] showed that *B. bassiana*-infected larvae exhibited a general decrease in hemolymph proteins but did not show any significant alteration in hemolymph carbohydrates. Accordingly, *B. bassiana* has been shown to secrete several proteases in the host hemolymph [16, 17]. Among our 57 unigenes, we identified two proteases: a dipeptidyl peptidase (Bb DPP), and a tripeptidyl peptidase (Bb TPP). The two clones

Table 2. List of *B. bassiana* unigenes displaying significant amino acid sequence similarity to the non-redundant GenBank protein database. The two ESTs found to be homologous to non-fungal sequences are marked by an asterisk (\*)

Accession number	Putative protein	BlastX E-value	Species with best match
<i>Metabolism</i>			
CF350241	Alpha mannosidase	9e-48	<i>Emericella nidulans</i>
CF350242	Lumazine synthase	5e-38	<i>Magnaporthe grisea</i>
<i>Energy</i>			
CF350243	ATP synthase protein 9 (lipid binding protein)	2e-37	<i>N. crassa</i>
CF350244	Dihydrolipoamide succinyltransferase	5e-35	<i>Podospora anserina</i>
CF350245	Hexokinase	3e-57	<i>A. oryzae</i>
CF350246	Transaldolase	2e-89	<i>N. crassa</i>
<i>Cell division/cycle</i>			
CF350247	Guanine nucleotide-binding protein	4e-99	<i>N. crassa</i>
CF350248	Histone H3	5e-69	<i>N. crassa</i>
<i>Protein synthesis</i>			
CF350249	40S ribosomal protein S11	2e-57	<i>N. crassa</i>
CF350250	40S ribosomal protein S12	8e-33	<i>Paracoccidioides brasiliensis</i>
CF350251	40S ribosomal protein S18	8e-56	<i>N. crassa</i>
CF350252	40S ribosomal protein S21	1e-34	<i>N. crassa</i>
CF350253	40S ribosomal protein S22	4e-58	<i>N. crassa</i>
CF350254	40S ribosomal protein S25	3e-37	<i>N. crassa</i>
CF350255	40S ribosomal protein S26	4e-37	<i>N. crassa</i>
CF350256	40S ribosomal protein S28	2e-75	<i>N. crassa</i>
CF350257	40S ribosomal protein S29	1e-23	<i>N. crassa</i>
CF350258	40S ribosomal protein S30	4e-15	<i>N. crassa</i>
CF350259	40S ribosomal protein S5	2e-63	<i>N. crassa</i>
CF350260	60S ribosomal protein L10	5e-81	<i>N. crassa</i>
CF350261	60S ribosomal protein L11	2e-67	<i>Saccharomyces cerevisiae</i>
CF350262	60S ribosomal protein L12	4e-78	<i>N. crassa</i>
CF350263	60S ribosomal protein L23a	8e-41	<i>N. crassa</i>
CF350264	60S ribosomal protein L28	7e-37	<i>N. crassa</i>
CF350265	60S ribosomal protein L29	4e-28	<i>N. crassa</i>
CF350266	60S ribosomal protein L30	1e-43	<i>Ophiostoma novo-ulmi</i>
CF350267	60S ribosomal protein L31	2e-35	<i>N. crassa</i>
CF350268	60S ribosomal protein L36	2e-38	<i>Trichoderma hamatum</i>
CF350269	60S ribosomal protein L37	1e-24	<i>E. nidulans</i>
CF350270	60S ribosomal protein L37a	4e-19	<i>Branchiostoma belcheri</i> (*)
CF350271	60S ribosomal protein L38	2e-19	<i>N. crassa</i>
CF350272	60S ribosomal protein L42	5e-29	<i>S. cerevisiae</i>
CF350273	Elongation factor 1 alpha	6e-28	<i>Rhizomucor racemosus</i>
CF350274	Elongation factor 3	e-127	<i>N. crassa</i>
CF350275	Elongation factor EF1 alpha chain	8e-50	<i>Candida albicans</i>
CF350276	Eukaryotic translation initiation factor 5A	2e-52	<i>C. albicans</i>
<i>Protein destination</i>			
CF350277	Polyubiquitin	9e-58	<i>N. crassa</i>
AY380550	Protease: dipeptidyl peptidase	0	<i>N. crassa</i>
AY380551	Protease: tripeptidyl peptidase	9e-89	<i>N. crassa</i>
CF350278	Proteasome subunit beta type 2	5e-95	<i>N. crassa</i>
CF350279	Ubiquitin-carboxy extension protein fusion	1e-39	<i>Filobasidiella neoformans</i>
<i>Intracellular transport</i>			
CF350280	ADP/ATP carrier protein	e-122	<i>N. crassa</i>
CF350281	DigA protein	7e-44	<i>E. nidulans</i>
<i>Cell structure</i>			
CF350282	GTPase rho1	8e-95	<i>Blumeria graminis</i>
CF350283	Peroxisomal protein POX18	2e-42	<i>N. crassa</i>

Table 2. Continued

Accession number	Putative protein	BlastX <i>E</i> -value	Species with best match
<i>Signal transduction</i>			
CF350284	Calmodulin	2e-77	<i>N. crassa</i>
<i>Cell defense</i>			
CF350285	Cu/Zn superoxide dismutase	2e-17	<i>Cordyceps militaris</i>
<i>Unknown function</i>			
CF350286	67 kDa streptococcal antigen	3e-59	<i>Bradyrhizobium japonicum</i> (*)
CF350287	Hypothetical protein	1e-68	<i>N. crassa</i>
CF350288	Hypothetical protein	1e-45	<i>N. crassa</i>
CF350289	Hypothetical protein	2e-45	<i>N. crassa</i>
CF350290	Hypothetical protein	3e-34	<i>N. crassa</i>
CF350291	Hypothetical protein	1e-29	<i>N. crassa</i>
CF350292	Hypothetical protein	6e-24	<i>N. crassa</i>
CF350293	Hypothetical protein	1e-17	<i>N. crassa</i>
CF350294	Hypothetical protein	4e-16	<i>N. crassa</i>
CF350295	PEP5-related protein	7e-66	<i>N. crassa</i>

corresponding to the Bb DPP and Bb TPP–ESTs were sequenced completely and, since the 5' end was truncated for both clones, 5' RACE PCR was performed for both genes in order to sequence the entire Open Reading Frame (ORF). The two complete sequences have been deposited in GenBank, and accession numbers are AY380550 and AY380551 for Bb DPP and Bb TPP, respectively. Following the complete sequencing of both genes, gene-specific primers were designed for RT–PCR (Table 1). Both Bb DPP and Bb TPP gene-specific primers were tested on *B. bassiana* genomic DNA, and produced single bands of the expected size (data not shown). Furthermore, these PCR products were sequenced, confirming the fungal nature of both enzymes and ruling out the possibility of insect gene contamination in the cDNA library.

#### Sequence analyses

The Bb DPP–ORF is 2061 bp – long and encodes a predicted 687 aa protein, with an estimated molecular weight of *ca.* 76 kDa. The amino acid sequence is similar to an enzyme previously identified from the human pathogen *Aspergillus fumigatus*, DPP V [18]. Interestingly, this enzyme has been shown to correspond to one of the two major antigens produced *in vivo* by *A. fumigatus*. Its function remains unknown, but, as stated by Beauvais et al. [18], the DPP V protease is likely to play a nutritional role and produce a source of

amino acids for fungal growth during infection. Whereas the DPP V enzyme has been shown to be secreted by *A. fumigatus*, computational analysis of the Bb DPP *N*-terminal sequence did not identify a signal peptide, suggesting that the enzyme is not secreted by *B. bassiana*.

The Bb TPP–ORF is 1731 bp-long, encoding a predicted 577 aa protein. The programs SignalP and Prosite identified a signal peptide (aa 1–19) and a putative active site (aa 488–498), respectively. Molecular weight estimation indicated that the enzyme is *ca.* 63 kDa when the signal peptide is included, and *ca.* 61 kDa when the signal peptide is removed. Detection of a signal peptide at the *N*-terminal of the Bb TPP protein suggests that this protease is actively secreted by *B. bassiana* in the insect hemolymph. The protein sequence is similar to a sequence identified as a tripeptidyl peptidase A (tppA) from *Aspergillus oryzae* (GenBank accession number BAC56232), as well as two hypothetical proteins from *Neurospora crassa*. These Bb TPP homologues (as identified by BlastX analysis) have been aligned and compared using the UPGMA method. The UPGMA analysis is not meant to represent phylogenetic relationships but rather allows for classification of protein sequences according to the level of similarity. The UPGMA tree (Figure 2) identified Bb TPP as part of a group of fungal tripeptidyl peptidases that are similar to a clade of mammalian tripeptidyl peptidases I. Alignment of the putative active sites (Figure 3)

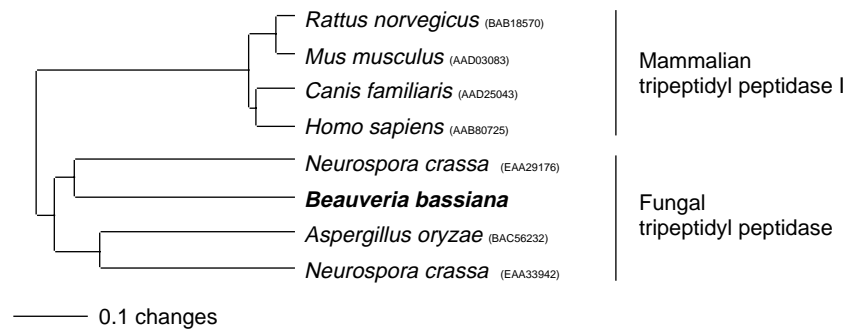


Figure 2. Unweighted pair group method using arithmetic average (UPGMA) dendrogram inferred from an alignment of the Bb TPP protein sequence and its closest homologues (as identified by BlastX analysis). The tree shows that Bb TPP belongs to a clade of fungal proteases that includes the tppA.

confirmed that the four fungal sequences encode for a similar enzyme belonging to the subtilase family and possessing a serine protease-type activity (as recognized by the ProSite algorithm). Lastly, in addition of being similar to mammalian and fungal tripeptidyl peptidases, the Bb TPP sequence also exhibits similarity with aorsin, a novel serine proteinase isolated from *A. oryzae* [19].

#### Gene expression and role of Bb TPP and Bb DPP during *B. bassiana* infection

Semi-quantitative RT-PCR reactions (Figure 4) showed that the expression of both Bb DPP and Bb TPP is not in vivo-specific, and that both transcripts are present in the RNA pool extracted from SDY-grown *B. bassiana*. Since the reaction conditions were the same for all cDNA amplifications, the variation in amplification between in vivo and in vitro samples reflects the initial difference in mRNA abundance in each growth condition. The  $\beta$ -tubulin gene was used as a control. As is the case for  $\beta$ -tubulin, the expression of DPP seems independent of growth conditions.

Consensus	GTSASAPxxAA
<i>Neurospora crassa</i> (EAA29176)	GTSASTPVVAA
<i>Beauveria bassiana</i> (AY380551)	GTSASAPVFAA
<i>Aspergillus oryzae</i> (BAC56232)	GTSASAPAFSA
<i>Neurospora crassa</i> (EAA33942)	GTSASAPMFAG

Figure 3. Alignment of the amino acid sequence corresponding to the active site of the fungal tripeptidyl peptidases. The active site sequence is highly conserved, and the consensus sequence is identified as a serine protease (subtilase family) by the Prosite algorithm.

Contrastingly, the TPP gene appeared to be up-regulated in vivo (Figure 4).

The differential expression of Bb TPP during infection suggests that this protease may play a role in *B. bassiana* pathogenesis. Our primary hypothesis is that both Bb TPP and Bb DPP are involved in protein digestion and amino acid uptake from the host. However, due in part to the fact that Bb TPP and Bb DPP belong to groups of fungal proteases with little prior characterization, their exact enzymatic activities and roles during the infection have yet to be clarified. Because of the two preliminary characteristics highlighted in this study (differential expression in vivo and signal peptide), we are currently focusing on Bb TPP, and have initiated studies to determine the role of this protease, either by purification and subsequent injection of pure enzymes in insects, or by disruption of the Bb TPP gene expression.

#### Potential of the *B. bassiana* in vivo cDNA library

The differential expression of the Bb TPP gene reveals that the in vivo transcriptome of *B. bassiana* may be specific to the growth conditions provided by the host hemolymph. These growth conditions are very unique and, to date, irreproducible in vitro. Our hypothesis is that the majority of the genes involved in pathogenesis are up-regulated and/or expressed specifically when the fungal cells recognize the peculiar growth conditions of the hemolymph. Although the number of ESTs is limited, this pilot-scale analysis of *B. bassiana* gene expression shows that producing sequences from our cDNA library may reveal metabolic pathways that are used by the fungus in vivo, and identify

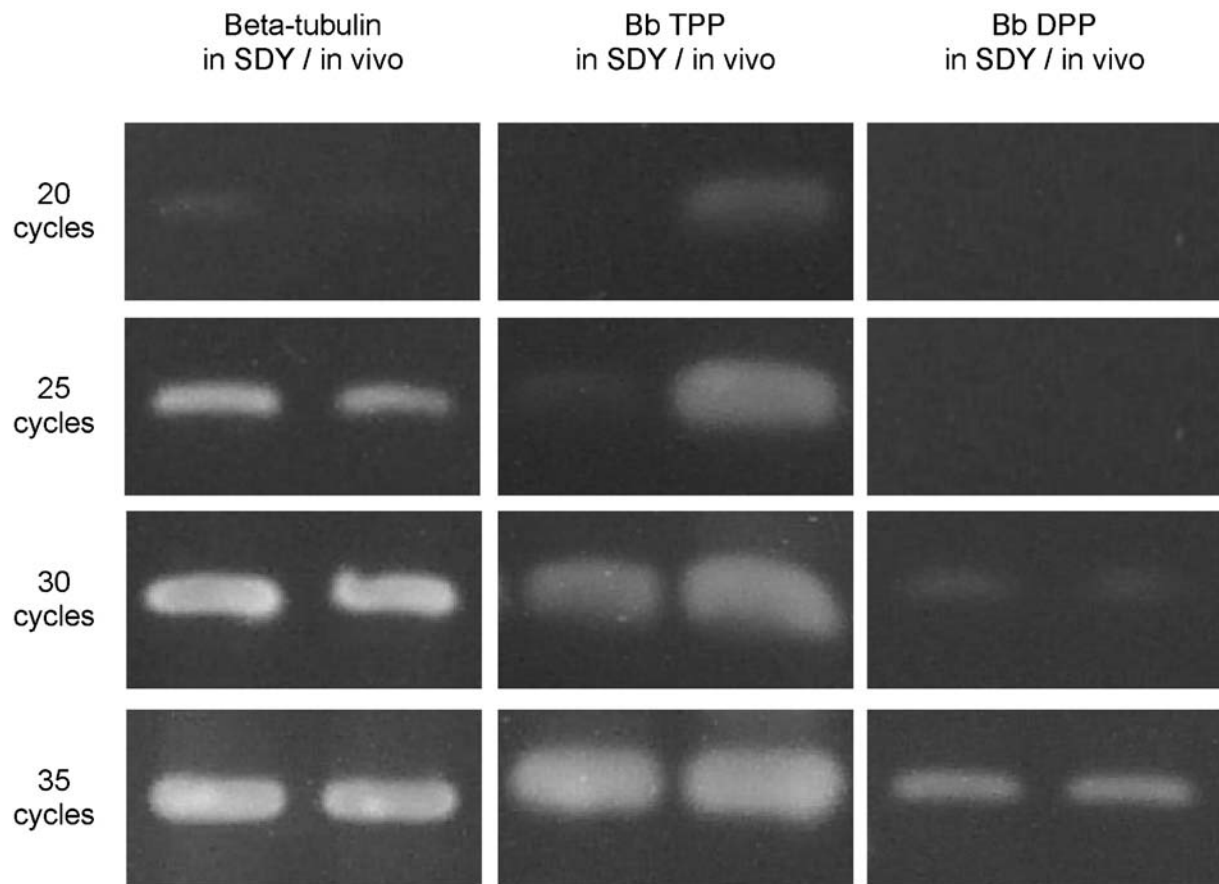


Figure 4. RT-PCR quantitative estimation of  $\beta$ -tubulin, Bb DPP and Bb TPP transcripts under in vitro and in vivo conditions. As opposed to  $\beta$ -tubulin and Bb DPP, the expression of Bb TPP appears to be up-regulated when *B. bassiana* grows in the insect hemolymph.

genes that are involved during pathogenesis. As noted above, *B. bassiana* has been shown to produce several active metabolites in vivo. A recent study by Fuguet and Vey [20] confirmed previous reports [6, 7, 21] that *B. bassiana* insecticidal toxins are proteinaceous. In addition, in vivo-secreted fungal proteases have also been reported to inhibit the cellular component of the host defense system [17]. Potentially, the genes encoding for these active proteins have been cloned and are present in our in vivo cDNA library. We are confident that a larger-scale sequencing effort of this library will lead to the identification of several of these genes.

#### Acknowledgements

The authors thank Bill Farmerie and Regina Shaw from the Interdisciplinary Core for Biotechnology

Research (ICBR) at the University of Florida for their assistance in sequencing and managing the EST database. We also thank J.E. Maruniak, V.-U. Bläske and B. Magalhaes for early revision of the manuscript. Florida Experiment Station Journal Series no. R-09761.

#### References

1. Boucias DG, Pendland JC. Principles of insect pathology. Kluwer Academic Publishers Boston, 1998.
2. Butt TM, Jackson CW, Magan N. Fungi as biocontrol agents. Progress, problems and potential, CABI Publications, 2001.
3. Sanchez-Martinez C, Perez-Martin J. Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis* – similar inputs, different outputs. Curr Opin Microbiol 2001; 4: 214–221.
4. Alves SB, Rossi LS, Lopes RB, Tamai MA, Pereira RM. *Beauveria bassiana* yeast phase on agar medium and its pathogenicity against *Diatraea saccharalis* (Lepidoptera:

- Crambidae) and *Tetranychus urticae* (Acari: Tetranychidae). *J Invertebr Pathol* 2002; 81: 70–77.
5. Pendland JC, Hung S-Y, Boucias DG. Evasion of host defense by in vivo-produced protoplast-like cells of the insect mycopathogen *Beauveria bassiana*. *J Bacteriol* 1993; 175: 5962–5969.
  6. Mazet I, Hung S-Y, Boucias DG. Detection of toxic metabolites in the hemolymph of *Beauveria bassiana* infected *Spodoptera exigua* larvae. *Experientia* 1994; 50: 142–147.
  7. Boucias DG, Mazet I, Pendland JC, Hung S-Y. Comparative analysis of the in vivo and in vitro metabolites produced by the entomopathogen *Beauveria bassiana*. *Can J Bot* 1995; 73: S1092–S1099.
  8. Hung S-Y, Boucias DG. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm *Spodoptera exigua*. *J Invertebr Pathol* 1992; 60: 152–158.
  9. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215: 403–410.
  10. Bevan M, Bancroft I, Bent E, Love K, Goodman H, Dean C, Bergkamp R, Dirkse W, Van Staveren M, Stiekema W, Drost L, Ridley P, Hudson SA, Patel K, Murphy G, Piffanelli P, Wedler H, Wedler E, Wambutt R, Weitzenegger T, Pohl TM, Terryn N, Gielen J, Villarroel R, Chalwatzis N. Analysis of 1.9 Mb of contiguous sequence from chromosome four of *Arabidopsis thaliana*. *Nature* 1998; 391: 485–488.
  11. Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997; 10: 1–6.
  12. Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res* 2003; 31: 3784–3788.
  13. Zhang Z, Hall A, Perfect E, Gurr SJ. Differential expression of two *Blumeria graminis* chitin synthase genes. *Mol Plant Pathol* 2000; 1: 125–138.
  14. Mead GP, Ratcliffe NA, Renwranz LR. The separation of haemocyte types on Percoll gradients: methodology and problems. *J Insect Physiol* 1986; 32: 167–177.
  15. Cheung PYK, Grula EA. In vivo events associated with entomopathology of *Beauveria bassiana* for the corn earworm (*Heliothis zea*). *J Invertebr Pathol* 1982; 39: 303–313.
  16. Shimizu S, Tsuchitani Y, Matsumoto T. Production of an extracellular protease by *Beauveria bassiana* in the haemolymph of the silkworm, *Bombyx mori*. *Lett Appl Microbiol*. 1993; 16: 291–294.
  17. Griesch J, Vilcinskis A. Proteases released by entomopathogenic fungi impair phagocytic activity, attachment and spreading of plasmatocytes isolated from haemolymph of the greater wax moth *Galleria mellonella*. *Biocontrol Sci Technol* 1998; 8: 517–531.
  18. Beauvais A, Monod M, Debeaupuis JP, Diaquin M, Kobayashi H, Latgé JP. Biochemical and antigenic characterization of a new dipeptidyl-peptidase isolated from *Aspergillus fumigatus*. *J Biol Chem* 1997; 272: 6238–6244.
  19. Lee BR, Furukawa M, Yamashita K, Kanasugi Y, Kawabata C, Hiranos K, Andos K, Ichishima E. Aorsin, a novel serine proteinase with trypsin-like specificity at acidic pH. *Biochem J* 2003; 371: 541–548.
  20. Fuguet R, Vey A. Comparative analysis of the production of insecticidal and melanizing macromolecules by strains of *Beauveria* spp.: in vivo studies. *J Invertebr Pathol* 2004; 85: 152–167.
  21. Kucera M, Samsinakova A. Toxins of the entomophagous fungus *Beauveria bassiana*. *J Invertebr Pathol* 1968; 12: 316–320.
- Address for correspondence:* Drion G. Boucias,  
Department of Entomology & Nematology, University of Florida, Gainesville,  
Florida 32611-0620, USA  
Phone: +1 352 392 1901 ext. 202; Fax: +1 352 392 0190  
E-mail: [dgb@mail.ifas.ufl.edu](mailto:dgb@mail.ifas.ufl.edu)