EST analysis of cDNA libraries from the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana*. I. Evidence for stage-specific gene expression in aerial conidia, *in vitro* blastospores and submerged conidia

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The entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana* holds much promise as a pest biological control agent. *B. bassiana* produces at least three *in vitro* single cell infectious propagules, including aerial conidia, vegetative cells termed blastospores and submerged conidia, that display different morphological, biochemical and virulence properties. Populations of aerial conidia, blastospores and submerged conidia were produced on agar plates, rich liquid broth cultures and under conditions of nutrient limitation in submerged cultures, respectively. cDNA libraries were generated from mRNA isolated from each *B. bassiana* cell type and ~2500 5' end sequences were determined from each library. Sequences derived from aerial conidia clustered into 284 contigs and 963 singlets, with those derived from blastospores and submerged conidia forming 327 contigs with 788 singlets, and 303 contigs and 1079 contigs, respectively. Almost half (40–45 %) of the sequences in each library displayed either no significant similarity (e value > 10⁻⁴) or similarity to hypothetical proteins found in the NCBI database. The expressed sequence tag dataset also included sequences representing a significant portion of proteins in cellular metabolism, information storage and processing, transport and cell processes, including cell division and posttranslational modifications. Transcripts encoding a diverse array of pathogenicity-related genes, including proteases, lipases, esterases, phosphatases and enzymes producing toxic secondary metabolites, were also identified. Comparative analysis between the libraries identified 2416 unique sequences, of which 20–30 % were unique to each library, and only ~6 % of the sequences were shared between all three libraries. The unique and divergent representation of the *B. bassiana* transcriptome in the cDNA libraries from each cell type suggests robust differential gene expression profiles in response to environmental conditions.

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INTRODUCTION

Insect fungal pathogens are dispersed within the major fungal taxonomic groups, and represent unique lifestyle adaptations that have evolved numerous times (Goettel *et al.*, 2000; Khachatourians, 1996). *Beauveria bassiana* belongs to the order *Hypocrealean* within the *Ascomycota*, and is one of the most widely studied members of the *Cordycipitaceae* clade. *B. bassiana* has been environmental protection agency approved for commercial use against a

variety of agricultural pests, including whiteflies, beetles, grasshoppers and psyllids. *B. bassiana* has also been used as a model system to study fungal-mediated tick (*Acari: Ixodidae*) biological control, and recent studies have highlighted the potential for entomopathogenic fungi, including *B. bassiana*, as agents in combating the spread of malaria by controlling mosquito populations (Blanford *et al.*, 2005; Kirkland *et al.*, 2004; Scholte *et al.*, 2005). The *Beauveria* lifestyle is further unique in that it is a facultative saprophyte and can exist as a plant endophyte and/or form intimate interactions with plant roots (Bing & Lewis, 1992; Elliot *et al.*, 2000; Lewis *et al.*, 2001; Wagner & Lewis, 2000; White *et al.*, 2002).

B. bassiana has evolved sophisticated mechanisms for penetrating the formidable barrier that constitutes the

Abbreviations: EST, expressed sequence tag; GO, gene ontology.

The GenBank/EMBL/DDBJ accession numbers for the EST sequences reported in this paper are shown in the Supplementary Tables.

A figure and tables with further details about the EST data are available as supplementary data with the online version of this paper.

insect/arthropod exoskeleton or cuticle. (Binnington & Retnakaran, 1991; Boman, 1981; Clarkson & Charnley, 1996; Ferron, 1981; Neville, 1975; St Leger, 1991). Interspersed within the cuticle barrier are biochemical components, such as toxic lipids and phenols, enzyme inhibitors, proteins and other defensive compounds that entomopathogens must overcome for successful virulence (Anderson *et al.*, 1995; Hackman, 1984; Renobales *et al.*, 1991). Pathogens must cope with hydrophobic barriers, electrostatic charges, low relative humidity, low or sequestered nutrient levels, endogenous microbial flora, and cross-linked proteins that contribute to a stiff and desiccated cuticle (St Leger, 1991). Successful pathogenic fungi must also thwart infection-induced responses such as melanization and haemocyte activation (Boman, 1981; Pendland & Boucias, 1985, 1986; Pendland *et al.*, 1993; Riley, 1997).

The overall process of arthropod infection by pathogenic fungi involves many steps (Boucias & Pendland, 1991; Carruthers & Soper, 1987; Charnley, 1990; Charnley & St Leger, 1991) most of which remain unelucidated, particularly at the molecular level. Entomopathogenic fungi possess complex systems for (1) finding the appropriate insect host(s), (2) adhering to the exoskeletal substrata, (3) evading host defences, (4) penetrating and degrading the cuticle, (5) transporting to the cytoplasm and catabolizing necessary nutrients (carbon/nitrogen, external products of the degradation), and (6) dispersing from the catabolized host(s). The proteins and enzymes that contribute to this process include those that are secreted, and those that are found in the cell envelope, vesicles and vacuoles, as well as the cytoplasm and nucleus. *B. bassiana* does not require an opening or ingestion for attachment or germination, and is presumably able to penetrate anywhere on the cuticle surface (although preferential sites of infection have been described for some insects).

B. bassiana produces at least three single cell infectious propagules, known as aerial conidia, *in vitro* blastospores and submerged conidia, which display different morphological and biochemical properties (Bidochka *et al.*, 1987; Jeffs *et al.*, 1999; Thomas *et al.*, 1987). Aerial conidia are relatively resistant to varying environmental conditions and represent the most commonly used cell form in biological control applications. Aerial conidia contain a rodlet layer that presumably contributes to the hydrophobic nature of these cells (Holder & Keyhani, 2005). In contrast, blastospores are hydrophilic, do not appear to contain a rodlet layer and bind poorly to hydrophobic surfaces, but germinate and grow at much higher rates than aerial conidia. Submerged conidia are also hydrophilic, but appear to be able to bind to both hydrophobic and hydrophilic surfaces, contain a rough surface morphology, and may be an important developmental adaptation for growth under nutrient limiting conditions.

B. bassiana contains a haploid nuclear genome of between 34 and 44 Mb, consisting of 7–8 chromosomes, which by comparison to equivalent fungal genomes would contain

10 000–15 000 genes (Pfeifer & Khachatourians, 1993; Viaud *et al.*, 1996). We have employed an expressed sequence tag (EST) analysis of aerial conidia, *in vitro* blastospores and submerged conidia in order to characterize the *B. bassiana* transcriptome. Analysis of the libraries revealed a robust diversity in expressed transcripts, with the majority of sequenced ESTs unique to each library. These data will provide a basis for investigating the gene expression profile of distinct developmental stages as well as the overall genomic potential of *B. bassiana*. It also will facilitate gene identification, ORF calling and mRNA processing site identification for *B. bassiana* genome sequencing efforts.

METHODS

Cultivation of fungi. *B. bassiana* (ATCC 90517) was routinely grown on potato dextrose agar (PDA). Plates were incubated at 26 °C for 10–14 days and aerial conidia were harvested by flooding the plate with sterile dH₂O. Conidial suspensions were filtered through a single layer of Miracloth and final spore concentrations were determined by direct count using a haemocytometer. Blastospores were produced in Sabouraud dextrose/0.5–1 % yeast extract (SDY) liquid broth cultures inoculated with conidia ($0.5\text{--}5 \times 10^5$ conidia ml⁻¹ final concentration). Cultures were grown for 3–4 days at 26 °C with aeration (shaking incubator, 150 r.p.m.). Cultures were filtered (twice) through glass wool to remove mycelia, and the concentration of blastospores determined by direct count. Submerged conidia were produced in TKI broth using fructose as the carbon source (Thomas *et al.*, 1987). For all cell types, Miracloth or glass wool filtered cell suspensions were harvested by centrifugation (10 000 g, 15 min, 4 °C), washed twice with sterile dH₂O and resuspended at $10^7\text{--}10^8$ cells ml⁻¹.

Construction of cDNA libraries. Fungal cells were lysed by grinding in liquid nitrogen and total RNA was extracted using RNeasy (Ambion). Partially purified mRNA was recovered from the total RNA pool using the Poly(A)Purist mRNA purification system (Ambion), both according to the manufacturer's recommendations. cDNA libraries were constructed using the unidirectional pBluescript II XR system (Stratagene). Colonies picked for sequencing (~2500 per library) were derived from primary libraries containing $0.5\text{--}1.0 \times 10^6$ c.f.u. without amplification.

Plasmid isolation and DNA sequencing. Plasmid constructs were transformed into XL10-Gold ultracompetent cells (Stratagene) and the presence of inserts selected for using the blue/white screening on X-Gal/LB agar plates. Randomly selected bacterial cDNA clones were grown in 96-well microplates overnight in LB/8 % glycerol. A small portion of each bacterial culture was used for sequence template production and the remainder of the culture retained in a frozen archive. All sequencing templates were prepared using Amersham Biosciences TempliPhi rolling circle DNA amplification kits as specified by the supplier, with minor protocol modification. For DNA sequencing reactions Amersham Biosciences ET Terminator dye terminator cycle-sequencing chemistry was used. The reactions were precipitated with ethanol to remove unincorporated terminator, resuspended in loading solution and analysed by capillary gel electrophoresis using Amersham MegaBACE 1000 DNA sequencing units. All DNA sequence electropherogram files were processed by a Geospiza Finch Suite DNA sequencing management system (version 2.10.1) for base calling by Phred (version 0.020425.c) and archival storage of sequence electropherograms.

Sequence analysis. EST-sequence clustering and assembly was performed using TranscriptAssembler (PTA) version 2.7.0 (Paracel).

The SCYLLA component of PTA masked vector sequences, filtered *Escherichia coli* chromosomal DNA contamination, filtered rRNA components, trimmed low quality bases from the ends of EST sequences and annotated intrinsic repeats such as simple sequence repeats and SINE elements prior to clustering and assembly. EST sequences with fewer than 100 high quality nucleotides were excluded from the clustering and assembly steps. After filtering, EST sequences were passed to the PTA clustering module for pairwise comparison. EST sequences with a minimum Smith–Waterman score of 50 were placed together in individual clusters. Each cluster of related EST sequences was assembled using the CAP4-based PTA assembly module. All BLAST searches were conducted using a local compute cluster using Paracel BLAST (version 1.5.6). The results were parsed and stored in a local SQL database that facilitated search and summary of BLAST results on the basis of user-defined criteria through a web browser-based interface.

Gene ontology (GO) assignments. For functional classification according to GO assignments, all contig and singlet sequences were queried against the NCBI non-redundant (NR) protein database using BLASTX with the *e* value threshold set to $\leq 10^{-4}$. The top 100 BLAST hits were selected for further analysis. GO terms associated with the best scoring BLAST hits were assigned back to the original query nucleotide sequence. Based on these GO term assignments, the complete UniGene EST set for the cDNA library was organized around GO hierarchies divided into biological processes, cellular components and molecular functions (Harris *et al.*, 2004). BLAST results were parsed and stored in BlastQuest, a SQL database that facilitates BLAST results management and GO term browsing (Farmerie *et al.*, 2005).

RESULTS AND DISCUSSION

Characterization of the EST database

EST libraries were constructed from mRNA derived from *B. bassiana* aerial conidia, *in vitro* blastospores and submerged conidia. Cell preparations were deemed greater than 95 % homogeneous as determined by microscopic analysis of samples used for RNA extraction. Approximately 2500 clones were picked at random from each library and subjected to single pass 5' sequencing. The mean insert size of the cDNA libraries ranged from 0.8 to 1.2 kb with an overall range of 0.4–2.8 kb. After editing the mean length of the single read sequences ranged from 0.4 to 0.5 kb. An analysis of the total number of runs, the number of valid ESTs, assembly of the ESTs into groups of cDNAs that share

sequence identity and are considered to represent transcripts of the same gene (contigs), the number of singlets, and the diversity index for each library is given in Table 1. The representative nature of the libraries was confirmed by the high degree of transcript diversity (diversity index ranging from 57 to 66 %). The distribution of sequences per contig is presented in Fig. 1. No EST obviously representing rRNA or prokaryotic contamination was found in any of the sequences (prior to filtering).

Functional analysis of the EST libraries

The clustered and singlet sequences constituting the unigene set of each library were used for BLASTX searches. Significant hits were defined based upon *e* value scores $\leq 10^{-4}$, which identified contigs with amino acid sequence similarity to previously characterized proteins deposited in the GenBank NR and/or Swissprot databases (as of September 2005). Search results were also used to extract gene annotation terms for grouping sequences into functional classifications. The complete search results, with GenBank accession numbers, for each EST are available as supplementary data with the online journal (Supplementary Table S1).

The number of contigs that displayed matches to proteins in the GenBank/Swissprot databases (September 2005) were 581 out of 1247 ESTs (47 %), 508/1115 (46 %) and 725/1382 (52 %), for the aerial conidia, blastospore and submerged conidia libraries, respectively. Of the ESTs with significant matches, 75–90 % could then be assigned GO terms. Most (70–80 %) of the ESTs displayed a fungal sequence as the best match, with the greatest number of hits among ascomycete sequences and only 1–4 % among the basidiomycetes. ESTs displaying similarity to sequences from animals or prokaryotes represented 8–10 % of the total, and less than 5 % showed similarity to plant, archaeal or protist sequences.

GO representations of the unigene EST set for each library were constructed around the GO organizing hierarchies of (i) biological processes (Figs 2a, 3a, 4a), (ii) cellular components (Figs 2b, 3b, 4b) and (iii) molecular functions (Figs 2c, 3c, 4c) (see Supplementary Tables S2 and S3 available with the online journal). These tables and figures

Table 1. Summary of *B. bassiana* EST sequencing data

	Aerial conidia	Blastospore	Submerged conidia
Total no. of runs	2380	2329	2540
Total no. of valid ESTs	1952	1947	2092
Total no. of contigs	284	327	303
No. of EST in contigs	989	1159	1012
Total no. of singlets	963	788	1079
Total no. of unique sequences	1247	1115	1382
Diversity index* (%)	64	57	66

*Diversity index = total no. of unique sequences/total no. of valid ESTs.

Fig. 1. EST redundancy in contig assembly: (a) aerial conidia, (b) blastospores and (c) submerged conidia.

provide a representation by major GO categories including (i) metabolism, nutrient uptake and cell communication; (ii) cytoplasmic, nuclear, membrane and extracellular localization; (iii) ligand binding; (iv) catalytic, transport and signal transduction activities.

Comparative analysis between the aerial conidia, *in vitro* blastospore and submerged conidia libraries

Functional categorization of the ESTs isolated from aerial conidia, *in vitro* blastospores and submerged conidia revealed similar representations of most metabolism categories including protein, phosphorus, nucleic acid,

carbohydrate, and amino acid catabolism and biosynthesis. Likewise, the distribution of sequences within ribosomal, cytoskeletal, mitochondrial, nuclear and membrane intracellular subcategories, as well as between enzymic functional categories, were similar between the three libraries (see Supplementary Fig. S1 available with the online journal). Although this global analysis revealed similar distributions within these functional categories, few of these represented overlapping genes (i.e. instead we observed different genes that were categorized within the same functional group).

An overall analysis of unique and shared sequences between the libraries was performed (Fig. 5). In all, 2193 different transcripts were identified between the three libraries. The library generated from submerged conidia contained the largest number of unique sequences (739 corresponding to 30 % of the total clustered unigene set), with unique aerial conidial sequences representing 26 % of the total unique gene set and blastospores accounting for 21 % of the total.

An analysis of the 10 most abundant ESTs found in each dataset is presented in Table 2 (for the top 25 most abundant transcripts see Supplementary Table S4 available with the online journal). For comparative purposes these tables also list the number of times each sequence was found in the other libraries. Overall, the top 25 most highly represented transcripts accounted for between 15–17 % of the total ESTs in the datasets for each library.

Aerial conidia. Aerial conidia are produced sympodially, in acropetal succession on elongating conidiogenous cells from hyphae and mycelia growing on solid surfaces (Hegedus *et al.*, 1992; MacLeod, 1954). Due to their spore-like qualities and relative greater stability and resistance to environmental conditions, these cells are often used as the infectious agents for biological control. Thus, much effort has gone into creating stable formulations and optimizing large scale production and delivery protocols (Desgranges *et al.*, 1993; Inglis *et al.*, 1995; Lord, 2001; Wraight *et al.*, 2001). A list of the clone IDs, BLAST scores and top search result of the ESTs described in the following paragraphs have been compiled in Supplementary Table S5 available with the online journal. A hydrophobin was the most highly represented transcript in both the aerial conidia and submerged conidia datasets (and second in representation in the blastospore EST library). Hydrophobins are unique small molecular mass fungal proteins, and different members of this protein family play roles in a diverse range of physiological processes, including adhesion, development (formation of aerial structures) and pathogenesis (Linder *et al.*, 2005; Wosten, 2001). The aerial conidia library contained a number of unique ESTs similar to depolymerizing enzymes, including (i) proteases and peptidases, e.g. subtilisin protease, tripeptidyl-peptidase and an EST similar to a thermophilic serine protease from *Bacillus* Ak.1 (MacIver *et al.*, 1994), (ii) glycosidases, e.g. *N,O*-diacetylmuramidase and chitinase, and (iii) lipases, and an EST similar to

(a) Biological processes

(b) Cellular components

(c) Molecular functions

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Fig. 2. Percentage representation of GO mappings for *B. bassiana* ESTs derived from the aerial conidia cDNA library.

the platelet-activating factor acetylhydrolase. This secreted phospholipase (A2) is unusual in that it is calcium dependent and contains amino acid motifs characteristic of neutral lipases and serine esterases (Tjoelker *et al.*, 1995). These enzymes play important roles in fungal virulence towards arthropods (El-Sayed *et al.*, 1993; Freimoser *et al.*, 2003; Charnley & St Leger, 1991; Gupta *et al.*, 1994) and our data suggest that conidia (the dispersive spores) are pre-loaded with some of the enzymes (or with transcripts at least) that are required for cuticle degradation and establishment of the fungal infection on target hosts. The aerial conidia library contained transcripts potentially involved in spore cell wall formation including an EST similar to the sporulation specific 1,3- β -glucan synthase from *Schizosaccharomyces pombe* (Martin *et al.*, 2000) and a class V chitin synthase, as well as a septin homologue (Homann *et al.*, 1996) possibly involved in cell surface organization. A diverse number of cytochrome p450 ESTs were also found, including a sterol α -14-demethylase, an alkane hydroxylase, a trichodiene oxygenase, a sterol 26-hydroxylase, an enzyme similar to the plant (*Arabidopsis thaliana*) 89A2 p450 and a bifunctional p450/NADPH-p450 reductase, indicating the availability of a robust detoxification and cell defence response. In terms of secondary metabolism, transcripts similar to the

Gibberella fujikuroi farnesylpyrophosphate synthase, a key enzyme in isoprenoid biosynthesis leading to the biosynthesis of several classes of essential (terpenoid) metabolites, including sterols, quinones, carotenoids and gibberellins (Homann *et al.*, 1996), was identified, along with an enterobacterial aerobactin siderophore biosynthesis protein (Martinez *et al.*, 1994). A number of ESTs related to drug resistance and remediation were identified. These included a transcriptional activator containing a Zn₂C₆-type zinc finger motif required for the induction of fluconazole resistance (Talibi & Raymond, 1999), a mitomycin oxidase (August *et al.*, 1994), a multidrug ABC transporter and a salicylate hydroxylase, which catalyses the formation of catechol from salicylate (You *et al.*, 1991), a key intermediate in naphthalene catabolism.

In vitro blastospores. *In vitro* blastospores represent a single celled vegetative state produced in rich nutrient broth. Depending upon nutrient conditions, these cells are released laterally as well as terminally from growing hyphae (Bidochka *et al.*, 1987). *In vitro* blastospores are cylindrical, somewhat irregular in size, smooth walled (i.e. no visible rodlet layer) infectious propagules that can germinate to form new hyphae or under certain nutrient conditions can produce microcycle conidia. Unlike aerial

(a) Biological processes

(b) Cellular components

(c) Molecular functions

Fig. 3. Percentage representation of GO mappings for *B. bassiana* ESTs derived from the blastospore cDNA library.

conidia, the blastospore EST collection contained few hydrolases, although a number of unique enzymes were noted. These included (i) peptidases, represented by two different aminopeptidases, one an EST similar to the secreted acid protease from the yeast *Saccharomycopsis fibuligera* (Hirata *et al.*, 1988) and one an EST displaying similarity to the avian aminopeptidase H/bleomycin hydrolase (Adachi *et al.*, 1997), (ii) cellulytic glycosidases, one similar to the *Trichoderma reesei* cellulase and another EST similar to the *Aspergillus aculeatus* cellobiase, and (iii) an EST similar to the *Penicillium notatum* phospholipase enzyme that displays intrinsic lysophospholipase and phospholipase B activities (Masuda *et al.*, 1991). Since blastospores are infectious agents, the lack of hydrolases may imply either a rapid induction of these enzymes when in contact with hosts or that some of these enzymes are present (translated) even though their transcripts were not found in our dataset. The blastospore library contained a number of ESTs encoding transporters. These included ESTs displaying similarity to a high affinity methionine permease, the *Pseudomonas putida* phthalate transporter, a mammalian chloride channel, a putative yeast copper transporter and the *Aspergillus nidulans* mirB triacetylfusarinine C siderophore transporter. Vegetative incompatibility regulates the fusion of hyphae, which is

generally followed by the exchange of cytoplasm and nuclei. ESTs displaying similarity to the *Podospira anserina* vegetative incompatibility protein HET-E-1 were identified (Saupe *et al.*, 1995). Finally, an EST with similarity to a novel antifungal peptide presumed to be an important element of the innate immunity system from the insect *Acrocis longimanus* (Coleoptera) was identified (Barbault *et al.*, 2003).

Submerged conidia. The production of submerged conidia by *B. bassiana* represents an adaptation to nutrient-stress conditions. These cells are smaller and more uniform in size than blastospores, spherical in shape, refractile or hyaline, and have a rough surface morphology with no rodlet bundles (Bidochka *et al.*, 1987; Holder & Keyhani, 2005). Two different modes of submerged conidia production have been noted, (a) sporogenous cells occurring on mycelium or on short branches of hyphae and (b) germinating blastospores that give rise to (submerged) conidia at the tip of short germ tubes (Bidochka *et al.*, 1987). These cells are infectious and germinate slightly faster than aerial conidia. With respect to development, cell wall remodelling and nutrient uptake, the submerged conidia library contained unique ESTs similar to (a) the catalase peroxidase (catalase 2) of

(a) Biological processes

(b) Cellular components

(c) Molecular functions

Fig. 4. Percentage representation of GO mappings for *B. bassiana* ESTs derived from the submerged conidia cDNA library.

Neurospora crassa implicated in cell differentiation and fungal morphogenic transitions (Peraza & Hansberg, 2002), (b) the 1,3- β -glucan synthase component, Bgs4, of

Fig. 5. Comparative analysis of sequenced ESTs derived from the *B. bassiana* aerial conidia, blastospore and submerged conidia cDNA libraries: the Venn diagram shows unique and overlapping sets of transcripts between the libraries.

Schizosaccharomyces pombe and the chitin synthase B from *A. nidulans* (Yanai *et al.*, 1994), and (c) the *Candida albicans* peptide transporter, PTR2 (Basrai *et al.*, 1995) and mammalian pepsinogen/gastricsin. Two ESTs within the submerged conidia library corresponded to parts of the 25 gene sterigmatocystin cluster of *A. nidulans* (Brown *et al.*, 1996), including a moderately represented EST cluster (four sequences) similar to the *A. nidulans* *verA* gene product that converts the aflatoxin precursor versicolorin A to sterigmatocystin, and a sterigmatocystin biosynthesis monooxygenase. Intriguingly, another EST was similar to the mammalian aflatoxin (B1) aldehyde reductase, a putative detoxifying enzyme (Knight *et al.*, 1999), suggesting that *B. bassiana* possess mechanisms for both production and protection from toxins. Submerged conidia also expressed a number of efflux pump genes including ESTs similar to the *Cochliobolus carbonum* cyclic peptide efflux pump, ToxA, to a *Schizosaccharomyces pombe* multidrug efflux transporter similar to mammalian *p*-glycoproteins, implicated in mediating leptomycin B resistance, and to the Pur8 protein from *Streptomyces alboniger* that confers puromycin resistance (Tercero *et al.*, 1993). Finally, the submerged conidia library contained an EST similar to mammalian soluble epoxide hydrolase, an enzyme involved

Table 2. The most abundantly represented transcripts in the *B. bassiana* aerial conidia, *in vitro* blastospore and submerged conidia libraries

EST	No. of ESTs	Putative function	Related taxon	Accession no.	<i>e</i> value	No. of ESTs in other libraries		
						Blastospore	Submerged conidia	Aerial conidia
Aerial conidia								
conidia.014.c1	47	Rodlet protein precursor (rodA)	<i>Aspergillus nidulans</i>	P28346	1.91e−11	23	93	
conidia.009.c1	43	—	—	—	—	0	1	
conidia.021.c1	40	—	—	—	—	0	6	
conidia.059.c1	18	—	—	—	—	0	5	
conidia.020.c1	17	—	—	—	—	0	0	
conidia.078.c1	17	Elongation factor 1- α (TEF)	<i>Podospora anserina</i>	Q01520	0	12	25	
conidia.024.c1	15	ADP/ATP carrier protein (acp)	<i>Neurospora crassa</i>	P02723	2.16e−42	13	9	
conidia.028.c1	14	—	—	—	—	0	0	
conidia.131.c1	11	Hypothetical UPF0057 protein T23F2.5 in chromosome X	<i>Caenorhabditis elegans</i>	Q22702	1.94e−14	10	4	
conidia.034.c1	10	—	—	—	—	0	12	
conidia.062.c1	10	—	—	—	—	0	10	
In vitro blastospore								
blastospores.030.c1	25	—	—	—	—		4	0
blastospores.081.c2	23	Rodlet protein precursor (rodA)	<i>Aspergillus nidulans</i>	P28346	1.85e−11		93	47
blastospores.005.c1	22	Glucose-repressible gene protein (grg-1)	<i>Neurospora crassa</i>	P22151	1.38e−18		3	8
blastospores.099.c3	21	—	—	—	—		11	4
blastospores.048.c1	16	Hypothetical 34.1 kDa protein in WHI3-CHS1 intergenic region	<i>Saccharomyces cerevisiae</i>	P40169	2.86e−13		6	1
blastospores.024.c1	14	Clock-controlled protein 6 (ccg-6)	<i>Neurospora crassa</i>	Q01302	6.55e−04		13	3
blastospores.014.c1	13	Histone H3 (hh3)	<i>Neurospora crassa</i>	P07041	0		8	3
blastospores.028.c1	13	ADP/ATP carrier protein (acp)	<i>Neurospora crassa</i>	P02723	0		9	15
blastospores.050.c1	13	—	—	—	—		5	4
blastospores.003.c1	12	—	—	—	—		0	0
blastospores.116.c1	12	Nucleoside diphosphate kinase (swoH)	<i>Aspergillus nidulans</i>	Q8TFN0	0		2	3
blastospores.123.c1	12	Elongation factor 1- α (TEF)	<i>Podospora anserina</i>	Q01520	0		25	17
Submerged conidia								
TKL001.c1	93	Rodlet protein precursor (rodA)	<i>Aspergillus nidulans</i>	P28346	2.01e−11	23		47
TKL024.c1	25	Elongation factor 1- α (TEF)	<i>Podospora anserina</i>	Q01520	0	12		17
TKL039.c1	13	Clock-controlled protein 6 (ccg-6)	<i>Neurospora crassa</i>	Q01302	5.23e−04	14		3
TKL017.c1	12	—	—	—	—	0		10
TKL048.c1	11	—	—	—	—	6		2
TKL080.c1	11	—	—	—	—	21		4
TKL015.c1	10	—	—	—	—	7		5
TKL053.c1	10	Aquaporin-like protein (AQY1)	<i>Saccharomyces cerevisiae</i>	P53386	4.43e−43	4		1
TKL098.c1	10	Alcohol dehydrogenase I (adh-1)	<i>Neurospora crassa</i>	Q9P6C8	0	7		3
TKL145.c1	10	—	—	—	—	0		10

—, No significant search results for query (*e* value $> 10^{-3}$).

in detoxification of environmental mutagens and carcinogens playing an important role in xenobiotic metabolism by degrading potentially toxic epoxides.

Shared transcripts. The ESTs shared between only the aerial conidia and blastospores accounted for 4.9 % (119/2416) of the total unigene set. This common set included a number of ABC-type multidrug transporters, an EST similar to the *Schizosaccharomyces pombe* multidrug resistance transporter conferring brefeldin A resistance (Nagao *et al.*, 1995), and an EST similar to the *Saccharomyces cerevisiae* PDR5 protein that confers resistance to cycloheximide, sporidesmin and other mycotoxins.

Blastospore and submerged conidia libraries uniquely shared 130 transcripts corresponding to 5.4 % of the total unigene set. A cell wall β -glucan synthase component was shared, as was a transcript with similarity to the fruit fly, *Drosophila melanogaster*, salivary glue protein Sgs3. The latter protein is a member of a family of glycosylated proteins that are part of the salivary glue secreted by larvae as a means of fixing themselves to an external substrate, and this transcript may encode for a novel type of fungal surface adhesion protein. Both cell types expressed two different free radical detoxifying enzymes, a Mn-superoxide dismutase and Cu-Zn-superoxide dismutase, a phenol detoxifying enzyme (phenol 2-monooxygenase) and an EST with similarity to enzymes (gibberellin 2- β -dioxygenases) that modify or inactivate the gibberellin family of plant signalling compounds. Blastospore and submerged conidia shared transcripts also included a vegetative incompatibility gene and a moderately to highly represented EST (transcript found 5 and 8 times in the blastospore and submerged conidia libraries, respectively) similar to a dehydroquinase synthase family enzyme (2-*epi*-5-valiolone synthase), possibly involved in the biosynthesis of amylostatin/aminoglycoside-like antibiotics.

Aerial conidia and submerged conidia shared 146 transcripts (6 % of the total), although a number of these transcripts corresponded to housekeeping genes such as ribosomal components, transcription factors and metabolic enzymes. Common transcripts between the aerial conidia and submerged conidia included a putative cuticle-degrading (serine) protease, and a number of ESTs responsible for nutrient uptake, including an amino acid permease similar to a transporter induced by the fungal biocontrol agent *Trichoderma harzianum* during mycoparasitism, a high-affinity glucose transporter and a galactose-proton symporter. In addition, an EST similar to 14-3-3 proteins, a family of conserved small acidic proteins that have been implicated in playing major roles in a wide variety of signalling cascades and that are essential for normal hyphal and cell development, was identified.

ESTs found in all three libraries accounted for 6.5 % of the total unique set. Many of these transcripts coded for ribosomal proteins, histones, protein-turnover enzymes, and basic metabolic and ATP generating enzymes. As noted

before, one of the most abundant transcripts found in all three libraries was a hydrophobin similar to the *Aspergillus* rodlet-layer protein. Hydrophobins have been best characterized as being the major protein constituent of the rodlet layer (outer cell envelope layer) found on aerial conidia; however, they have also been implicated in hyphal development and virulence (Kazmierczak *et al.*, 2005; Linder *et al.*, 2005). Hydrophobins can also be secreted into the media where they self-assemble into 2D arrays at water-air interfaces that act to decrease the surface tension of water, enabling hyphae and other structures to grow into the air (Talbot, 1999; Wosten *et al.*, 1999).

An EST similar to the *Malassezia furfur* major allergen Malf1 (Schmidt *et al.*, 1997) was also found in all three libraries and may correspond to one of the *B. bassiana* antigens previously identified in Western blots of fungal extracts probed with human sera (Westwood *et al.*, 2005). A covalently linked cell wall protein belonging to the PIR (proteins with internal repeats) family was moderately represented in the submerged conidia library (six times), and also found in the aerial conidia and blastospore libraries (twice and once, respectively). A number of depolymerases likely to function in general processes were also represented in all three libraries. These included a vacuolar aspartate protease similar to saccharopepsin or protease A, implicated in the posttranslational regulation of *S. cerevisiae* vacuolar proteinases, a transcript similar to the *S. cerevisiae* cytoplasm to vacuole targeting lipase, and an EST similar to a *S. cerevisiae* cell wall glycoprotein that displays lectin-like binding to 1,3- β -glucan and chitin, and possesses 1,3- β -exoglucanase activity with laminarin as substrate (Klebl & Tanner, 1989; Teter *et al.*, 2001; Woolford *et al.*, 1986).

Conclusions

A small number of transcriptome analyses have been reported for filamentous fungi, particularly with respect to plant and arthropod pathogens. Transcript analyses performed on distinct developmental stages (germinating teliospores, diploid filamentous growth and microsclerotia development) or during pathogenic growth of the plant pathogens *Ustilago maydis* and *Verticillium dahliae* have provided bases for genome annotation and further exploration of these fungi (Neumann & Dobinson, 2003; Nugent *et al.*, 2004; Sacadura & Saville, 2003). EST analyses of two subspecies of the entomopathogenic fungus *Metarhizium anisopliae* revealed distinct patterns of expression of secreted proteases and pathogenicity factors that have led to the ability to examine gene expression patterns during infection of various insect hosts (Freimoser *et al.*, 2003, 2005). Our analyses revealed a robust and dynamic gene expression repertoire available to the entomopathogenic fungus *B. bassiana*. Aerial conidia, *in vitro* blastospores and submerged conidia are morphologically and biochemically different, a distinction borne out through the analysis of their transcripts. The EST collection offers an insight into components that may aid the fungus in the process of insect virulence. A large number of depolymerases including

proteases, glycosidases and lipases were identified, as were numerous carbohydrate and amino acid transporters. Similarity based searches identified transcripts corresponding to the production of toxic secondary metabolites, including a putative sterigmatocystin gene cluster. The EST collection also contained a large variety of expressed genes encoding free radical scavenging, antioxidant proteins, including superoxide dismutases, catalases and peroxidases. These proteins have been implicated in providing protection against host oxidative defence systems and are important components mediating fungal virulence. Furthermore, a large number of ESTs similar to efflux or multidrug transport systems as well as numerous cytochrome p450s similar to xenobiotic and antimicrobial-detoxifying enzymes were noted, and may help account for the remarkable antibiotic resistance of *Beauveria* species. *B. bassiana* ESTs were also identified encoding a number of potential antimicrobial compounds, including lysozyme and an antimicrobial peptide that may defend against endogenous microflora or secondary colonizers. It should be noted that the room for novel gene discovery remains quite vast since the annotated portion of the EST collection represents only ~50 % of the total. From approximately 7500 clones, 2400 unique sequences were identified, although the absolute number might be lower (10–15 %) due to failure of non-overlapping sequences and splice variants to cluster, but still represents a significant portion of the genome. Further sequencing of the libraries should yield additional new transcripts.

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