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EST analysis of genes expressed by the zygomycete pathogen *Conidiobolus coronatus* during growth on insect cuticle

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Conidiobolus coronatus (Zygomycota) is a facultative saprobe that is a pathogen of many insect species. Almost 2000 expressed sequence tag (EST) cDNA clones were sequenced to analyse gene expression during growth on insect cuticle. Sixty percent of the ESTs that could be clustered into functional groups ($E \leqslant 10^{-5}$) had their best BLAST hits among fungal sequences. These included chitinases and multiple subtilisins, trypsin, metalloprotease and aspartyl protease activities with the potential to degrade host tissues and disable anti-microbial peptides. Otherwise, compared to the ascomycete entomopathogen *Metarhizium anisopliae*, *Con. coronatus* produced many fewer types of hydrolases (e.g. no phospholipases), antimicrobial agents, toxic secondary metabolites and no ESTs with putative roles in the generation of antibiotics. Instead, *Con. coronatus* produced a much higher proportion of ESTs encoding ribosomal proteins and enzymes of intermediate metabolism that facilitate its rapid growth. These results are consistent with *Con. coronatus* having adapted a modification of the saprophytic ruderal-selected strategy, using rapid growth to overwhelm the host and exploit the cadaver before competitors overrun it. This strategy does not preclude specialization to pathogenicity, as *Con. coronatus* produces the greatest complexity of proteases on insect cuticle, indicating an ability to respond to conditions in the cuticle.

INTRODUCTION

The Zygomycota and the Chytridiomycota represent the earliest divergences within the so-called 'lower' fungi (Berbee & Taylor, 2001). Reflective of their ancient origins and probable polyphyly, the Zygomycota comprise 10 diverse orders (Voigt & Woestermeyer, 2001). However, the ubiquitous saprobes, the Mucorales, are a sister group to the almost equally ubiquitous insect pathogens, the Entomophthorales (Jensen *et al.*, 1998). Members of the Entomophthorales are amongst the most common and potent pathogens of insects, frequently decimating insect populations in spectacular epizootics that have led to their being employed in biocontrol (Hajek, 1997).

Conidiobolus coronatus is the most basal and least specialized branch of the extant entomophthoralean fungi (Jensen *et al.*, 1998) and probably resembles the forerunners of the more evolved genera and species (Evans, 1989). These are often highly host-adapted and show little or no growth on standard mycological media (Humber, 1984). In contrast, there is no evidence of host specificity in *Con. coronatus*; it has been isolated from various homopteran species, but also from Coleoptera, Lepidoptera or Diptera (see http://www.ppru.cornell.edu/Mycology/ARSEF_Culture_Collection.htm). *Con. coronatus* can probably attack any stressed insect (Papierok, 1986) and it is also an opportunistic pathogen of vertebrates (Benny *et al.*, 2001). However, even stressed insects may deploy a range of physical, chemical and cellular defences that will need to be neutralized or evaded and currently there is no information for *Con. coronatus* as to the plasticity of its physiological responses to host behaviour or whether it has any requirement for host-related stimuli, such as insect cuticle, to produce virulence determinants. Indeed it is still not clear how *Con. coronatus* normally infects and colonizes host tissues.

A recent study on the ascomycetes *Metarhizium anisopliae* sf. *anisopliae* 2575 (wide host range) and *Met. anisopliae* sf. *acridum* 324 (specific to grasshoppers) demonstrated that utilizing expressed sequence tags (ESTs) multiple virulence factors and pathways can be viewed simultaneously, allowing the different lifestyles that exist in insect–fungus interactions to be understood from a broader perspective

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Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; EOM, enzyme overlay membrane; EST, expressed sequence tag; IEF, isoelectric focusing; NA, nitroanilide.

(Freimoser *et al.*, 2003). Another advantage of using cDNA sequence data is that it represents a rapid and relatively efficient method for quickly discovering large numbers of genes in organisms which have little or no genetic research history, which include almost all orders within the Chytridiomycota and Zygomycota.

We have therefore adopted an EST strategy for gene discovery in *C. conidiobolus*. However, in the absence of biochemical and molecular data on the factors influencing the pathogenic habit of *Con. coronatus*, we used levels of protease production by this fungus as a marker to test culture conditions for maximum production of pathogenicity-related molecules, our rationale being that as most animal pathogens produce multiple proteases to degrade the proteinaceous outer integuments of their hosts, proteases may represent a 'niche-specific trait', that is, a trait shared by pathogens that occupy the same niche, irrespective of their phylogenetic position. We report here that *Con. coronatus* produces the greatest complexity of proteases on insect cuticle. Consequently, we targeted the EST project at genes expressed during growth on cuticle to identify potential candidate genes conditioning pathogenicity through a comparative analysis with sequences derived from other organisms, particularly *Met. anisopliae*.

METHODS

Strains and culture conditions. Isolates of fungi and their sources were *Metarhizium anisopliae* isolate ARSEF 2575 (*Curculio caryae*; Coleoptera), *Basidiobolus ranarum* isolate ARSEF 1139 (*Dendrolimus spectabilis*; Lepidoptera), *Basidiobolus haptosporus* isolate ARSEF 264 (soil), *Mortierella hyalina* isolate ARSEF 2061 (egg mass, *Lymantria dispar*; Lepidoptera) and *Sporodiniella umbellata* isolate ARSEF 5222 (Homoptera: Jassidae). These were obtained from the USDA-ARS culture collection, Ithaca, New York, USA, as was the major focus of this study, a Malaysian isolate of *Con. coronatus* (isolate ARSEF 512) recovered from *Nilaparvata lugens* (Homoptera: Delphacidae). Additional isolates of *Mucor mucedo* (isolate ATCC 38694), *Mucor rouxii* (isolate ATCC 24905) and *Allomyces macrogynus* (ATCC 38327) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Isolates were routinely grown at 25 °C on solid (SDA) Sabouraud-glucose medium supplemented with 0·5 % yeast extract.

Preparation and analysis of culture filtrate. Cultures were inoculated with spores taken from 5–12-d-old agar plates or with standardized mycelial inocula (0·5 g wet wt per 10 ml) from 20 h Sabouraud-glucose cultures. The conical flasks were incubated with shaking (100 r.p.m) at 25 °C for up to 2 d in basal medium (BM) containing (l^{-1}) 1 g KH_2PO_4, 0·5 g $MgSO_4$, 0·7 mg $Na_2B_4O_7$, 0·5 mg $(NH_4)Mo_7O_{24}$, 10 mg $Fe_2(SO_4)$ and 0·3 mg Zn_2SO_4, adjusted to pH 6·0 with NaOH, supplemented with 0·2 % yeast extract (YE) and additional nitrogenous nutrients. To compare protease production by diverse fungi, conidia (1×10^6) were inoculated into 50 ml BM plus yeast extract (0·2 %) supplemented with (1) insoluble protein (collagen at 1 %), (2) peptone (2 %) or (3) collagen (1 %) and peptone (2 %) (Table 1). The influence of nutrients on protease production by *Con. coronatus* was determined in BM plus yeast extract (0·2 %) supplemented with 1 % bovine serum albumin, 1 % peptone + 2 % glucose, 1 % cellulose, 1 % collagen, 1 % chitin, 1 % chitosan, 1 % *Manduca sexta* cuticle or 1 % cockroach (*Blaberus giganteus*) cuticle. *Manduca sexta* and *Blaberus giganteus* are sources for unsclerotized and sclerotized cuticles, respectively. Cuticles were isolated and prepared as described previously (St. Leger *et al.*, 1994).

Cultures were harvested by filtering through Whatman No. 1 filter paper and then through a 0·2 μm pore Millipore filter unit before being used for enzyme assays. Total protease activity was measured with hide

Table 1. Protease activities of extracellular preparations from different species of fungi grown on nitrogenous substrates for 3 days

Cultures (50 ml) were inoculated with 1×10^6 spores. Filtrates were assayed with either hide protein azure (total protease) or Suc-Ala-Ala-Pro-Phe-pNA (subtilisin activity).

Species	Growth substrate					
	Collagen (1 %) + Peptone (0·1 %)		Peptone (2 %)		Collagen (1 %) + Peptone (2 %)	
	Total protease	Subtilisin	Total protease	Subtilisin	Total protease	Subtilisin
Zygomycota						
Conidiobolus coronatus	2·4 ± 0·01	52 ± 5	18·3 ± 0·7	414 ± 21	25·4 ± 0·8	580 ± 28
*Basidiobolus haptosporus**	0·4 ± 0·03	12 ± 2	1·5 ± 0·2	37 ± 3	1·8 ± 0·3	63 ± 4
*Basidiobolus ranarum**	0·2 ± 0·02	6 ± 1	0·8 ± 0·1	24 ± 4	0·9 ± 0·1	30 ± 3
Mortierella hyalina	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
Spordiniella umbellata	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
Mucor mucedo	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
Mucor rouxii	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
Chytridiomycota						
Allomyces macrogynus	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1	< 0·1
Ascomycota						
Metarhizium anisopliae	10·9 ± 0·6	228 ± 11	0·9 ± 0·1	17 ± 2	0·9 ± 0·1	15 ± 1

*Some phylogenies place *Basidiobolus* spp. among the Chytridiomycota.

protein azure; activities are expressed as μg trypsin equivalents ml^{-1} min^{-1} (St. Leger *et al.*, 1986). Subtilisin-like protease activity (versus Suc-Ala-Ala-Pro-Phe-NA) was determined as previously described for *Met. anisopliae* and other entomopathogens; activities are expressed as nmol nitroanilide (NA) ml^{-1} min^{-1} (St. Leger *et al.*, 1987). To determine dry weight of fungal biomass, mycelia harvested on filter paper were dried to constant weight under vacuum at 80 °C.

Analytical isoelectric focusing (IEF). Samples were desalted and concentrated 50-fold using Centricon-10 ultrafiltration units. Two-microlitre aliquots of the concentrate were run on ultrathin polyacrylamide gels by using 1 % ampholytes (Bio-Lyte 3/10; Bio-Rad) (St. Leger *et al.*, 1994). Immediately following IEF, gels were overlaid with X-ray film (gelatin overlay) or enzyme overlay membranes (EOM) to detect protease activity (St. Leger *et al.*, 1994). The EOM was impregnated with Suc-Ala-Ala-Pro-Phe-AFC (AFC, 7-amino-4-trifluoromethyl coumarin).

Construction of cDNA library. For cDNA library construction, *Con. coronatus* was first grown for 20 h in liquid SDB broth. The fungal biomass was then harvested on filter paper, washed with sterile distilled water and transferred for 18 h to basal medium supplemented with 1 % (w/v) *Manduca sexta* cuticle/0·2 % (w/v) peptone (insect cuticle was prepared as described previously; St. Leger *et al.*, 1994). Total RNA was extracted from frozen fungus using TRI Reagent as described by Joshi *et al.* (1999) and a cDNA library was constructed in the unidirectional λ ZAP II vector (Stratagene), exploiting the *Eco*RI and *Xho*I restriction sites, according to the manufacturer's instructions.

Plasmid isolation and DNA sequencing. Plasmid constructs were transformed into *Escherichia coli* TOP10 (Invitrogen). Individual transformants were picked, grown overnight in LB medium and plasmid DNA was isolated and purified using QIAprep Spin Miniprep Kits (Qiagen) following the manufacturer's protocols. cDNA inserts were sequenced from the 5′ end by employing the M13 primer and ABI chemicals on ABI 377 DNA sequencers (DNA Sequencing Facility, Dupont, DE, USA).

Sequence analysis. Vector sequences were removed by hand. Overlapping sequences were assembled into consensus sequences (contigs) by using the program CAP3 (Huang & Madan, 1999). The program BLASTX (Altschul *et al.*, 1997) was used to search all ESTs against the non-redundant amino acid reference library (NCBI 'nr' database). *Con. coronatus* ESTs with significant BLAST matches ($E \leqslant 10^{-5}$) were sorted into functional groups as outlined in Fig. 3 (see also Table of supplementary data available at http://mic.sgmjournals.org which contains a complete list of all best BLASTX matches for *Con. coronatus* sequences).

RESULTS AND DISCUSSION

Synthesis of proteases in culture

Characteristics needed by fungi to successfully establish disease in insects must be different in some ways from those needed to live as saprobes. These differences, when clearly delineated, will indicate probable key virulence characters for the pathogens. The ability of *Con. coronatus* to produce proteases was thus compared with other fungi (Table 1). All fungi grew in each medium, raising the pH from an initial pH of 6 to above 7·5, consistent with breakdown of nitrogenous nutrients to ammonia (St. Leger *et al.*, 1999). Also consistent with previous studies (St. Leger *et al.*, 1988), production of proteases by *Met. anisopliae* was repressed when low-molecular-mass nitrogenous nutrients (1 % peptone) were added to media containing insoluble protein (collagen) showing that repression overrides the enhancing effect of polymeric substrates. In contrast, protease production by the entomophthoralean fungi (*Basidiobolus ranarum*, *Basidiobolus haptosporus* and *Con. coronatus*) was enhanced by peptone. Mucoralean fungi are reported to produce endocellular proteases and aspartyl (acid) proteases (rennin) (Gray *et al.*, 1986). However, the mucoralean fungi (*Mortierella hyalina*, *Sporodiniella umbellata*, *Mucor mucedo* and *Mucor rouxii*) and the chytrid *Allomyces macrogynus* apparently do not secrete alkaline-active serine proteases, e.g. subtilisins, chymotrypsins and trypsins.

To investigate the link between fungal biomass and protease production, conidia of *Con. coronatus* and *Met. anisopliae* were inoculated into media containing from 0 to 4 % peptone (Fig. 1). At levels up to 1 % peptone, nutrients were a rate-limiting growth factor for *Con. coronatus* (Fig. 1a). Coincident with this, protease production peaked at 1 % peptone (Fig. 1a), implying that their primary role is in nutrient acquisition. Above 1 % peptone, growth rates

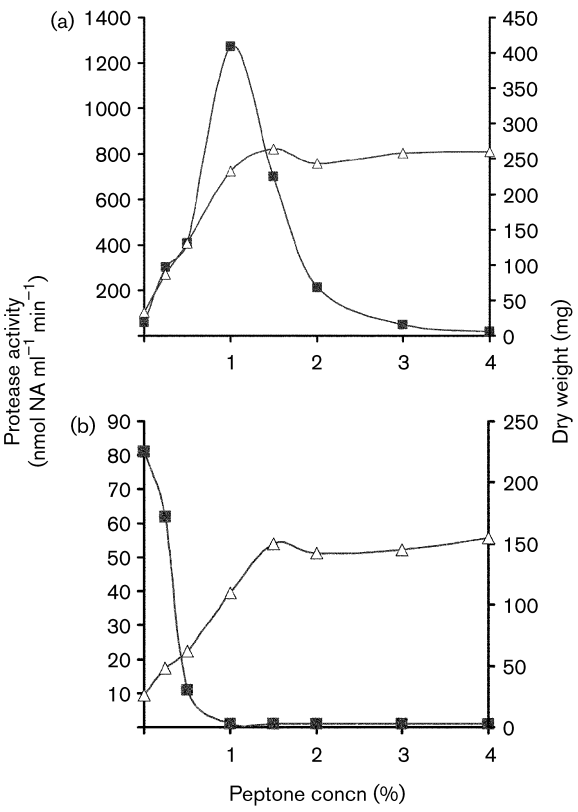

Fig. 1. Growth (dry wt, triangles) and protease production (squares) versus Suc-Ala-Ala-Pro-Phe-NA concentration in *Con. coronatus* ARSEF 512 (a) and *Met. anisopliae* ARSEF 2575 (b) grown for 24 h in basal medium containing from 0 to 4 % peptone. Results are the mean of three replicates. Variation was less than 7 % in each case. The experiment was repeated twice with very similar results.

levelled off and protease production declined, demonstrating catabolite repression at nutrient levels above that required for maximum growth rates (Fig. 1a). Production of proteases by *Met. anisopliae* was repressed at lower concentrations of peptone as compared to *Con. coronatus* (Fig. 1b), consistent with its lower growth rate.

Establishment of growth conditions that maximize secretion of proteases

IEF gels were used to compare proteinase patterns in cultures of *Con. coronatus* grown on different carbon and nitrogen sources (Fig. 2). Basic bands (pI ~ 10) had the highest gelatinase activity, being visible in most cultures within 5 min of applying a gelatin overlay to the IEF gel (Fig. 2a). EOMs containing Suc-Ala-Ala-Pro-Phe-AFC confirmed that these bands hydrolysed a subtilisin substrate. The putative subtilisins were produced both during growth on multiple proteins and in the presence of solubilized readily accessible low-molecular-mass nutrients (peptone and glucose) (Fig. 2). The greatest complexity of proteinase bands was produced during growth on both unsclerotized (*Manduca sexta*) and sclerotized (cockroach) cuticles, with fewer isoforms being produced during growth on other proteins (bovine serum albumin or collagen) or chitin (a component of insect cuticle) (Fig. 2). With the caveat that different proteases may have the same isoelectric points, no new proteases appear to be produced during growth on non-cuticular substrates as compared to cuticle. The large number of proteinases produced in cuticle-containing media suggests an ability of *Con. coronatus* to respond to the environmental conditions in an insect host.

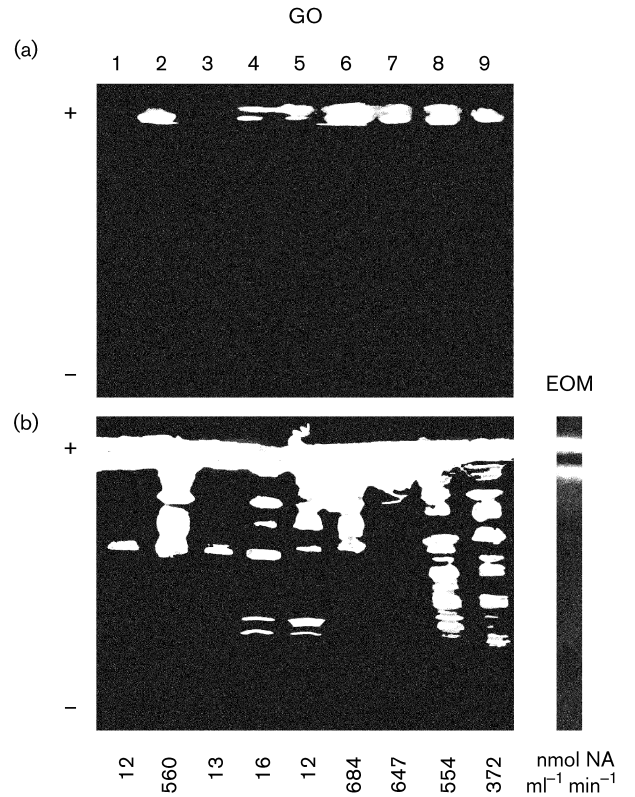

Fig. 2. Analytical (polyacrylamide) IEF (pH 3–10) of proteinases produced by *Con. coronatus* (ARSEF 512) incubated for 24 h in: 1, H_2O; 2, 1 % bovine serum albumin; 3, 1 % peptone + 2 % glucose; 4, 1 % cellulose; 5, 1 % collagen; 6, 1 % chitin; 7, 1 % chitosan; 8, 1 % *Manduca sexta* cuticle; and 9, 1 % cockroach (*Blaberus giganteus*) cuticle. The total proteinase pattern was detected by gelatin zymography using gelatin-coated X-ray films (GO). The gel/gelatin sandwiches were incubated at 37 °C for 5 min (a) or 30 min (b). The zymogram was prepared with an EOM containing Suc-Ala-Ala-Pro-Phe-AFC and was incubated on the gel for 30 min at 37 °C. The numbers at the bottom show protease production versus Suc-Ala-Ala-Pro-Phe-NA.

cDNA library analysis and sequence evaluation

We harvested fungal tissue for library construction 18 h after transfer to cuticle-containing media that maximizes the complexity of protease production by *Con. coronatus*. Around 2000 randomly selected cDNA clones [mean insert size = 1·6 kb (0·6–3·2 kb)] from the *Con. coronatus* cDNA library were selected for single read sequencing. A total of 1831 cDNA sequences were obtained with a mean length after editing of about 450 bp. Comparisons of EST pools with themselves indicated that 53 % of the ESTs were represented by two or more independent clones, the remainder being represented by one clone. BLAST searches of all EST sequences against the NCBI protein database detected significant matches ($E \leqslant 10^{-5}$) for 58 % of the clones of which only 60 % had a fungal sequence as the best match (90 % from ascomycetes, 2 % from chytridiomycetes and 4 % each from zygomycetes and basidiomycetes). The large proportion (40 %) of the ESTs that were most similar to sequences from bacteria, protists, plants or animals probably reflects the very limited amounts of sequence data available from related zygomycete fungi.

Function of expressed *Con. coronatus* genes

The sorting of *Met. anisopliae* (Freimoser *et al.*, 2003) and *Con. coronatus* genes expressed in medium that contains cuticle into the eight general-function categories in Fig. 3 (see also Table of supplementary data available at http://mic.sgmjournals.org which contains a complete list of all best BLASTX matches for *Con. coronatus* sequences) facilitated comparisons between the ascomycete and zygomycete pathogens. Thus Fig. 3 shows that unlike *Met. anisopliae*, metabolism-related genes were collectively the most prevalent among the identified cDNA clones from *Con. coronatus*. The high proportion of ESTs that encode enzymes of intermediary (energy) metabolism indicates that the rapid growth that is characteristic of *Con. coronatus* puts a high demand on central metabolic processes to furnish simple precursors and ATP. Complementary DNA clones encoding various ribosomal proteins were also

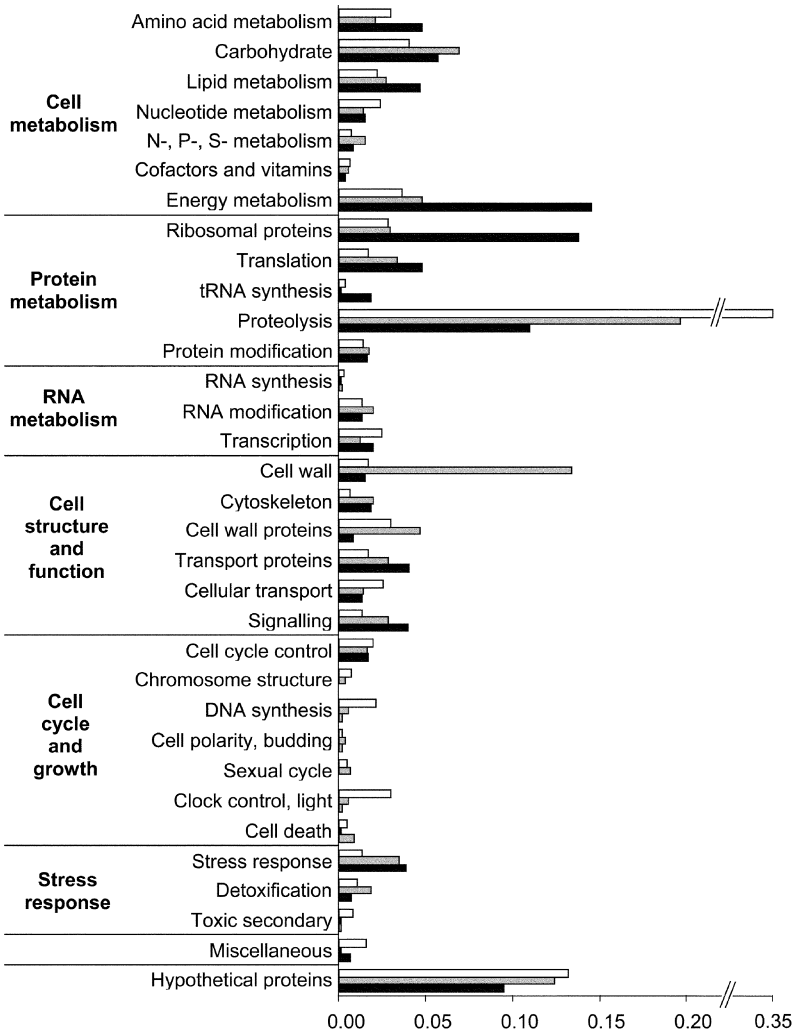

Fig. 3. Proportions of ESTs with significant BLAST hits ($E<10^{-5}$) from *Con. coronatus* (black bars), *Met. anisopliae* sf. *anisopliae* (white bars) and *Met. anisopliae* sf. *acridum* (grey bars) assigned to the listed functional categories.

abundant and contributed to the protein-synthesis-related gene category being the second most prevalent (Fig. 3). The oxidative deamination of amino acids also seems to be an important route in the degradation and assimilation of amino acid skeletons since at least three different dehydrogenase transcripts were represented by four ESTs. These enzymes play a key role in nitrogen metabolism in many organisms and free ammonia is released as a product of catalysis (Garnier *et al.*, 1997). Ammonia alters ambient pH in fungal cultures and regulates the expression of virulence genes in *Met. anisopliae* (St. Leger *et al.*, 1999). *Con. coronatus* also expresses a gene (BQ622518) that is similar ($E=2\times10^{-35}$) to a carbonic anhydrase from *Schizosaccharomyces pombe* that plays a key role in bicarbonate transport and regulation of pH (Sterling *et al.*, 2001).

The *Con. coronatus* EST collection revealed that it produced considerable amounts of proteins generally referred to as stress-related proteins. The most conspicuous species were homologues to molecular chaperones that facilitate protein folding, e.g. the 70 kDa heat-shock protein. Although *hsp70* genes fluctuate in response to cell stress they also function in normal cellular physiology and are developmentally regulated in *Blastocladiella emersonii* (Stefani & Gomes, 1995). *Met. anisopliae* also expresses high levels of protein chaperones during growth on insect cuticle, but the principal component is calnexin (Freimoser *et al.*, 2003). The *Met. anisopliae* EST collection contained a variety of expressed genes encoding antioxidant proteins, including superoxide dismutase, catalase and peroxidase. These proteins are involved in the pathogenicity of animals and plants by fungi, in particular providing defence against active oxygen species produced by the host (Iwanaga & Kawabata, 1998; Wu *et al.*, 1997). *Con. coronatus* has a different antioxidant profile, having homologues to glutathione-dependent antioxidant enzymes (γ-glutamylcysteine synthetase, glutathione reductase) and lacking ESTs for catalase, superoxide dismutase and peroxidase. Recently, it was shown that synthesis of γ-glutamylcysteine is crucial for the survival of the malaria parasite in *Plasmodium*-infected blood cells (Meierjohann *et al.*, 2002).

There is no evidence that *Con. coronatus* responds to light and, unlike in *Met. anisopliae*, we did not find ESTs for clock genes. However, BQ622207 shows similarity ($E=8\times10^{-22}$)

with VIVID, a *Neurospora crassa* protein that is light-induced, clock-regulated and represses light-regulated processes (Heintzen *et al.*, 2001). This implies that components of the *Neurospora* circadian system, to date only found in ascomycetes, have antecedents in the lower fungi.

The products of some pathogenicity genes will be involved in the exchange of signals between the pathogen and its environment, and activation of pathogenic mechanisms. Some of the *Con. coronatus* genes encoding sensors have homologues across a range of phyla. For example, BQ621736 shows similarity ($E=3\times10^{-30}$) to an osmosensor in *Neurospora crassa* that is also similar to osmosensors in bacteria (Schumacher *et al.*, 1997). Likewise, BQ621855 has similarity ($E=7\times10^{-32}$) to notchless (*Drosophila*) and other proteins with WD40 repeats that are involved in protein interactions (Royet *et al.*, 1998). BQ622041 is similar ($E=7\times10^{-22}$) to protein kinase C, involved in the developmental process in the human pathogenic ascomycete *Sporothrix schenckii* (Aquino-Pinero & Rodriguez del Valle, 1997). Production by *Con. coronatus* of a kinase (BQ622118) that is similar ($E=1\times10^{-25}$) to SNF1 is of special interest as SNF1 plays a key role in regulating expression of secreted cell-wall-degrading enzymes in the plant-pathogenic ascomycete *Cochliobolus carbonum* (Tonukari *et al.*, 2000). This implies similarities between the regulatory circuitry of these unrelated pathogens with very different hosts.

Genes with putative roles in host invasion

The ESTs offered insight into components that may aid the fungus in the processes of physical ingress and nutrient solubilization, and thus may constitute quantitative factors that contribute to the overall virulence of the pathogen. Matches to secreted hydrolytic enzymes with substrates in insect hosts included proteases and chitinases. A subtilisin (BQ622771) that is highly similar ($E=7\times10^{-65}$) to the aqualysin 1 precursor from *Thermus aquaticus* comprised 5 % of all the sequences and is thus the most highly expressed gene in *Con. coronatus* grown on cuticle. The EST library revealed two additional subtilisins, a trypsin (BQ622656) similar ($E=4\times10^{-8}$) to Alp1 from *Cochliobolus carbonum*, a metalloprotease (BQ622216) similar ($E=3\times10^{-9}$) to MepB from *Aspergillus fumigatus* and an aspartyl protease (BQ622675) similar ($E=5\times10^{-30}$) to pepsinogen from *Aspergillus niger*.

Subtilisins have been intensively studied in insect–fungal interactions (St. Leger & Screen, 2001). Aspartyl and metalloproteases have been implicated in the infection processes of the plant pathogen *Glomerella cingulata* (Clark *et al.*, 1997) and the human pathogen *Aspergillus fumigatus* (Sirakova *et al.*, 1994), respectively. Trypsins are the most abundant proteases secreted by many plant-pathogenic ascomycetes (St. Leger *et al.*, 1997). Likewise, a trypsin is the most abundant transcript among *Met. anisopliae* sf. *anisopliae* ESTs (Freimoser *et al.*, 2003). The *Con. coronatus* trypsin EST was used as a probe to obtain a full-length cDNA (AF426410) that retained similarities with Alp1 ($E=8\times10^{-32}$) and demonstrated complete conservation of the active site components (unpublished data). It is the first trypsin isolated from a fungus that is not a pathogenic pyrenomycetous ascomycete. Trypsins are lacking in completed genomes from several ascomycetes, including yeast, *Neurospora crassa* and *Magnaporthe grisea* (http://www.genome.wi.mit.edu/annotation/fungi). This suggests that loss of trypsin genes has occurred independently in multiple lineages, indicating that gene loss may be an important factor in fungal evolution. Insertion elements could be a factor promoting genetic instability by increasing the frequency with which DNA sequence is gained or lost. However, in contrast to the *Met. anisopliae* EST project, no active transposable elements were detected in the *Con. coronatus* library.

The patchy distribution of trypsins is consistent with the theme of niche-specific traits, irrespective of their phylogenetic position. From an insect–microbe perspective such traits might include the ability to colonize insect surfaces and tissues, to render host tissues suitable for consumption and overcome cell- and peptide-mediated components of the insect immune system. Broadly dispersed, anti-insect virulence mechanisms might, therefore, include toxins and hydrolytic enzymes capable of degrading host tissues and disabling anti-microbial peptides. The proteases and chitinases produced by *Con. coronatus* could play this role in the same way as similar enzymes do for *Met. anisopliae* (St. Leger & Screen, 2000). Hits to endo- and exo-acting chitinases (BQ622770, BQ622772, BQ622421, BQ622341) indicate that like *Met. anisopliae*, *Con. coronatus* produces a chitinolytic complex to degrade cuticular components.

Conidiobolus spp. are reported to secrete toxins (Roberts & Humber, 1981). However, these were not characterized and the described symptoms (haemorrhaging, blackening of the blood) are also consistent with the action of fungal subtilisins on the prophenoloxidase system of the insect (St. Leger *et al.*, 1996). In contrast to the *Met. anisopliae* strain 2575 EST project that identified many toxin-encoding genes (Freimoser *et al.*, 2003), *Con. coronatus* appears to produce few if any toxic enzymes (e.g. no phopholipases) or secondary metabolites (although it is possible that novel toxin-encoding genes may be included in the hypothetical category). However, BQ622484 is similar ($E=8\times10^{-11}$) to L-ornithine N5-oxygenase involved in pyoverdin biosynthesis by *Pseudomonas aeruginosa*. Pyoverdin plays a predominant role in mobilizing iron from mammalian hosts (Leoni *et al.*, 1996). *Con. coronatus* also expressed an EST (BQ622285) with similarity ($E=4\times10^{-32}$) to a ferritin subunit (iron storage protein) from the insect *Galleria mellonella* (Aisen *et al.*, 2001). It is probable that iron is often a rate-limiting growth factor for *Con. coronatus* as is the case for many other pathogens (Weinberg, 1999).

Many pathogens produce a broad array of antimicrobials to defend against opportunistic colonizers of insect cadavers (Pirozynski & Hawksworth, 1988), e.g. *Met. anisopliae* strain

2575 has many ESTs encoding products with similarity to known antimicrobial agents, including lyzosyme and thaumatin. *Con. coronatus* expressed no such ESTs. Instead it produced sequences such as BQ622839, similar ($E=3\times10^{-24}$) to cephalosporin C acetylhydrolase from *Acremonium chrysogenum* which detoxifies bacterial products, and BQ622726 similar ($E=2\times10^{-18}$) to a xenobiotic metabolizing cytochrome p450 (monooxygenase) enzyme in tobacco. Zygomycetes apparently lack antibiotics and in consequence early death of the host can lead to the fungus being displaced from the cadaver by competing opportunistic micro-organisms (Evans, 1989).

Like *Con. coronatus*, the specific acridid pathogen *Met. anisopliae* strain 324 also expressed very few toxins and antimicrobials (Freimoser *et al.*, 2003). This relates to lifestyles. Strain 2575 kills hosts quickly via toxins and grows saprophytically in the cadaver. In contrast, like many specialists, 324 causes a systemic infection of host tissues before the host dies. Unlike *Met. anisopliae* strain 324, *Con. coronatus* is an opportunistic pathogen of stressed insects. Assuming that the paucity of antimicrobials in the EST collection of *Con. coronatus* is a reflection of its survival strategies, this points to adaptation to environments with a low degree of combatative competition from other microbes. This is consistent with an R-selected strategy and exploitation of ephemeral habitats (Cooke & Rayner, 1984). *Con. coronatus* resembles the Mucorales (classic R-strategists) in being coenocytic with rapid spore germination and high mycelial extension rates, characteristics that permit Mucorales to utilize available soluble carbohydrates in advance of more slowly developing fungi. As would be expected from this lifestyle, *Con. coronatus* had a large number of ESTs for glucosidases and carriers, such as BQ622767 similar ($E=5\times10^{-16}$) to the monosaccharide transporter (Mst-1) of *Amantia muscaria* and BQ622015 similar ($E=4\times10^{-23}$) to the sucrose carrier (Sca1) of *Pneumocystis carinii*. These would permit uptake of the soluble carbohydrates that would be available to initial colonizers of recently living plant tissues. However, *Con. coronatus* also possesses amino acid transporters and secretes proteases that would permit it to solubilize recently living animal tissues and thus quickly access a longer lasting resource than simple sugars. This may guarantee a more prolonged existence and is a strategy presumably not available to the mucoralean fungi assayed in this study. This extra enzymic competence would allow niche differentiation between the Mucorales and the Entomophthorales that may have pre-adapted the latter to entomopathogenicity or may itself have derived from adaptation to entomopathogenicity. The ephemeral nature of Mucorales in a habitat is determined by their lack of combative ability as well as their inability to utilize carbon sources other than mono- or disaccharides (Cooke & Rayner, 1984). In the absence of an ability to combat other microbes, *Con. coronatus* is likewise not adapted to exploit an insect cadaver over a long period in the presence of competitors. However, production of high levels of proteases against a background of soluble nutrients, which would repress protease production by *Met. anisopliae*, will facilitate *Con. coronatus* to colonize wounds containing soluble nutrients, and hydrolyse and utilize other nutrient-rich insect resources such as the haemolymph in such a way that they are quickly exhausted.

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