Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*

Florian M. Freimoser,† Gang Hu and Raymond J. St Leger

Department of Entomology, University of Maryland, 4112 Plant Sciences Building, College Park, MD 20742, USA

*Metarhizium anisopliae* infects a broad range of insects by direct penetration of the host cuticle. To explore the molecular basis of this process, its gene expression responses to diverse insect cuticles were surveyed, using cDNA microarrays constructed from an expressed sequence tag (EST) clone collection of 837 genes. During growth in culture containing caterpillar cuticle (*Manduca sexta*), *M. anisopliae* upregulated 273 genes, representing a broad spectrum of biological functions, including cuticle-degradation (e.g. proteases), amino acid/peptide transport and transcription regulation. There were also many genes of unknown function. The 287 down-regulated genes were also distinctive, and included a large set of ribosomal protein genes. The response to nutrient deprivation partially overlapped with the response to *Man. sexta* cuticle, but unique expression patterns in response to cuticles from another caterpillar (*Lymantria dispar*), a cockroach (*Blaberus giganteus*) and a beetle (*Popilla japonica*) indicate that the pathogen can respond in a precise and specialized way to specific conditions. The subtilisins provided an example of a large gene family in which differences in regulation could potentially allow virulence determinants to target different hosts and stages of infection. Comparisons between *M. anisopliae* and published data on *Trichoderma reesei* and *Saccharomyces cerevisiae* identified differences in the regulation of glycolysis-related genes and citric acid cycle/oxidative phosphorylation functions. In particular, *M. anisopliae* has multiple forms of several catabolic enzymes that are differentially regulated in response to sugar levels. These may increase the flexibility of *M. anisopliae* as it responds to nutritional changes in its environment.

INTRODUCTION

Current molecular and genomic methods are being applied to *Metarhizium anisopliae*, the causative agent of green muscardine disease, because of its importance for the biological control of insect pests. It is a very versatile fungus, being able to infect a broad range of insects, 200 species from over 50 insect families (Samuels *et al*., 1989), and is also adapted to life in the root rhizosphere (Hu & St Leger, 2002). Consistent with its promiscuous nature, an array of expressed sequence tags (ESTs) from *M. anisopliae* strain 2575 identified large numbers of genes dedicated to host interaction and countering insect defences, as well as regulators for coordinating their implementation (Freimoser *et al*., 2003). Sequence comparisons and conserved motifs suggest that about 60 % of the ESTs of strain 2575 expressed during growth on cuticle encode secreted enzymes and toxins. Acting collectively, the number and diversity of these effectors may be the key to this pathogen’s ability to infect a wide variety of insects. In contrast, ESTs from the specialized locust pathogen *M. anisopliae* sf. *acridum* strain 324 revealed very few toxins (Freimoser *et al*., 2003). This relates to lifestyles. Strain 2575 kills hosts quickly via toxins, and grows saprophytically in the cadaver. In contrast, strain 324 causes a systemic infection of host tissues before the host dies. This shows that by utilizing ESTs, multiple virulence factors and pathways can be viewed simultaneously, and the different lifestyles that exist in insect–fungus interactions can be understood from a broader perspective.

†Present address: Institute of Plant Sciences, ETH Zurich, Universitätsstr. 2, CH-8092 Zurich, Switzerland.

Abbreviations: BC, beetle (*Popilla japonica*) cuticle; CC, cockroach (*Blaberus giganteus*) cuticle; ESTs, expressed sequence tags; GC, gypsy moth (*Lymantria dispar*) cuticle; HL, haemolymph; MC, *Manduca sexta* cuticle; RT-PCR, reverse-transcription PCR.

The expression ratios for *Metarhizium anisopliae* ESTs in different cuticle-containing media are shown in Supplementary Table S1 with the online version of this paper at http://mic.sgmjournals.org.
In this report, we use cDNA microarrays for high-throughput expression profiling of how *M. anisopliae* strain 2575 responds over a 24 h period to cuticle from tobacco hornworm caterpillars (*Manduca sexta*). As a control, we also define the response of *M. anisopliae* to nutrient deprivation. In addition we obtained snapshots of gene expression at 24 h to compare and contrast the responses of *M. anisopliae* to gypsy moth caterpillar cuticle (*Lymantria dispar*), and hard (sclerotized) cuticles from a beetle (*Popilla japonica*) and a cockroach (*Blaberus giganteus*). Each of these insects is a susceptible host for *M. anisopliae*.

These studies demonstrated that *M. anisopliae* can rapidly adjust its genomic expression patterns to adapt to insect cuticle, and identified specific responses to different cuticles. Genes specifically induced by cuticle included a plethora of cuticle-degrading enzymes, transporters for cuticle degradation products and a subset of transcription factors.

**METHODS**

**Strains and culture conditions.** To measure the variation in the expression of genes during starvation conditions or during adaptation to growth on different insect cuticles, we transferred cultures to minimal medium (MM) or cuticle-containing media after a period of unrestricted growth on a nutrient-rich medium. This is an effective and reproducible procedure for obtaining proteins that require release from catabolite repression and/or specific induction by a cuticular component (St Leger et al., 1994). *M. anisopliae* sf. *anisopliae* (strain ARSEF 2575) was routinely grown at 27 °C, either in liquid (SDB) or on solid (SDA) Sabouraud dextrose medium supplemented with 0.5% yeast extract. For RNA extraction, the fungus was grown for 48 h in 50 ml liquid SDB broth. The cultures were then washed with sterile distilled water and 2 g wet weight of the fungal biomass was transferred for up to 24 h to 10 ml MM containing 0.1% KH$_2$PO$_4$, 0.05% MgSO$_4$, and 50% tap water, supplemented with 1% of the following additives: tobacco hornworm cuticle (*M. sexta* cuticle, MC); cockroach cuticle (*CC, B. giganteus*); beetle cuticle (*BC, P. japonica*); gypsy moth cuticle (*GC, L. dispers*). Cuticles were prepared as described previously (St Leger et al., 1986b). Alternatively, *M. anisopliae* sf. *anisopliae* was transferred to 10 ml of *M. sexta* haemolymph (HL) obtained and treated as described by Grundsohber et al. (1998).

**cDNA microarray experiments.** All unique ESTs with significant BLAST matches (Freimoser et al., 2003) were amplified using T3 and T7 primers and standard PCR protocols. It should be noted that, as with most other bioinformatic studies, gene identities are based on computer-predicted homologies, and in very few cases (e.g. serine proteases and hydrophobins) have the protein products of these genes been demonstrated experimentally. Genes found among the EST sequences of *M. anisopliae* sf. *acidium* (ARSEF 324), such as chitinases and chitosanase (Freimoser et al., 2003), which were absent from the *M. anisopliae* sf. *anisopliae* (ARSEF 2575) EST collection, were amplified from *M. anisopliae* sf. *anisopliae* genomic DNA with specific primers and included on the array. This resulted in 837 clones, which were precipitated and resuspended in 3 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) to give a final DNA concentration between 100 and 300 ng ml$^{-1}$.

Printing, hybridization and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner (see http://www. umbi.umd.edu/~cab/macro/macrostart.htm for detailed protocols) at the University of Maryland Biotechnology Institute's Microarray Core Facility located at the Center for Biosystems Research. PCR products were spotted in triplicate on poly-lysine-coated glass slides, with a mean spot diameter of 100 μm and a spot spacing of 375 μm. Following printing and cross-linking, slides were washed with 1% SDS to remove background, treated with blocking solution (0.2 M succinic anhydride, 0.05 M sodium borate, prepared in 1-methyl-2-pyrrolidinone) and washed with 95 °C water and 95 % ethanol. After drying, slides were kept in the dark at room temperature.

RNA was extracted as previously described for *M. anisopliae* (Joshi & St Leger, 1999). For experiments comparing different media, RNA from a culture transferred to SDB was used as the reference sample. For time-course experiments, mycelium was collected after 1, 2, 4, 8, 12, 18 and 24 h from MM or from medium containing MC. RNA from the 0 h time-point was used as a reference. Hybridizations were done with Cy3- and Cy5-labelled probes derived from 50–80 μg of total RNA. All hybridizations were repeated at least three times with RNA from independent experiments and with switched labelling for the reference and test RNA samples.

**Analysis of microarray data.** The images of the scanned slides were analysed with Scanalyse (available from Eisen Lab: http://rana.lbl.gov/) and the data obtained from each scanned slide were normalized using global normalization, as performed by J-Express (Dysvik & Jonassen, 2001). All data were log-transformed, and for further analysis the mean (Em) and the standard deviation (SD) of the log-transformed expression ratios of the replicates were calculated for all genes. A gene was defined as differently regulated if the expression varied by at least a factor of two (1 < Em < −1). Expression ratios not fulfilling this requirement (−1 < Em < 1) were defined as zero, and the same was done for cases where the interval Em ± 1.96 × SD (95% confidence interval around the mean value for the three replicate spots) included the value 0. Further analysis of the processed data was performed using J-Express (Dysvik & Jonassen, 2001), EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/) and Excel.

**Validation of differentially expressed clones through real-time PCR.** A total of 16 clones predicted to be differentially expressed by microarray analysis were tested by quantitative reverse-transcription PCR (RT-PCR) by using an Applied Biosystems GeneAMP 5700 sequence detection system and an Applied Biosystems TaqMan RT kit. Transcript abundance was calculated by using the comparative ΔCt method relative to the amount of the tubulin alpha chain transcript A1273998 or 18S rRNA in the sample, with primers and conditions as described by Parsley et al. (2002). Differential expression based on RT-PCR measurements was defined as a change in transcript abundance accumulation of twofold or more.

**RESULTS**

**Overall patterns of cuticle-induced gene expression**

The libraries we employed to obtain ESTs were made from fungi growing on *M. sexta* cuticle (MC) for 24 h (Freimoser et al., 2003). The complete list of ESTs classified into functional groups is available at http://mic.sgmjournals.org/cgi/content/full/149/1/239/DC1. These ESTs were hybridized with labelled RNA probes isolated from mycelium harvested up to 24 h after the transfer from a nutrient-rich medium (SDB) to media containing an insect cuticle or HL. As a control for time-course studies with MC, mycelium was challenged by transfer to MM,
revealing the response to nutrient deprivation after growth in SDB. The responses to MC and MM were studied in parallel time-course experiments, each with seven time-points (1–24 h) that, together with the redundant sequence representation in the microarrays, ensured the robustness of the expression profiles. The expression ratios for ESTs in the different media are shown in Supplementary Table S1 with the online version of this paper at http://mic.sgmjournals.org. In addition, the ESTs are characterized in Table S1 with their accession numbers, E values and a description of the best BLAST hit.

An overview of the microarray results is presented in Fig. 1. They illustrate the rapid changes in expression of some genes in response to MC. Overall, these changes increased in magnitude with time. Thus at 4 and 18 h post-inoculation in MC medium, 88 and 154 genes were upregulated, respectively. Similarly, 66 genes were down-regulated at 8 h, and 143 genes were down-regulated at 18 h. During the first hour, there was no overlap between genes upregulated in response to MC and those upregulated in response to starvation conditions (MM). However, by 18 and 24 h, 30% of the genes were concomitantly upregulated in MC and MM, indicating that catabolite repression is involved in regulating at least some cuticle-induced genes. A cluster of 41 genes was rapidly activated (<2 h) by MM, but down-regulated in response to cuticle (Fig. 1). Only eight of these genes had homologues with known biological activity in databases, and these included the subtilisin Pr1G and ribosomal proteins. At least with respect to the regulation of these 41 genes, nutrient deprivation may be perceived as having an effect distinct from and even opposite to that of induction by cuticle.

In contrast, the magnitude of expression of most genes upregulated by nutrient deprivation, including the majority of the secreted proteases, was sharply increased by the presence of cuticle (Supplementary Table S1). In addition, a large subset of diverse genes was upregulated by MC, and not by MM, during the first 2 h, suggesting that these genes are specifically involved in adaptation to growth on cuticle (Fig. 2). A broad view of the nature of the adaptations made by M. anisopliae following transfer from nutrient-rich (SDB) medium to MC was obtained by grouping functionally related genes (Fig. 3). Changes involving upregulation, measured on the microarray for each functional category during the 24 h of growth on cuticle, were either gradual following the first hour, as for secreted proteases, or abrupt during the first hour followed by a slow decline, as for genes for amino acid/peptide uptake. Down-regulated genes included many for protein synthesis machinery, excluding RNA synthesis and processing. Genes encoding ribosomal proteins and translational machinery were coordinately regulated, showing an initial decrease, followed by an increase and a decrease, resulting in an 8- to 13-fold down-regulation. The repression of ribosomal protein genes has been reported in yeasts during multiple stress responses, including glucose deprivation (Warner, 1999), and may therefore be a general feature of fungi transferred to a low-nutrient medium. Overall, housekeeping genes for cell metabolism, including endocellular proteases, showed stable expression.

**Fig. 1.** Gene expression patterns of M. anisopliae in response to starvation conditions (MM), haemolymph (HL) from Man. sexta, or cuticles from a beetle (BC), a cockroach (CC) and caterpillars (Man. sexta, MC, and Lymantria dispar, GC). Mycelia growing on MC or in starvation conditions were assayed in time-course experiments. The 837 cDNA clone set was analysed by hierarchical clustering based on their expression patterns. Genes showing at least twofold regulation, compared with a reference probe from mycelia grown on SDB, are shown in red (upregulated) and green (down-regulated). Colour intensity is directly relative to magnitude of differential expression ratios. Experiments were carried out in triplicate, and representative clusters are shown.
Based on the time-course experiments under starvation conditions or in MM supplemented with MC, we chose the 24 h time-point to obtain a snapshot of gene expression during growth on other insect cuticles. The large-scale features of the expression patterns illustrate shared features between the responses to different cuticles and to starvation conditions, indicative of a stereotyped programme of gene expression. However, no two expression patterns were identical in terms of the genes affected and the magnitude of expression alteration (Fig. 1). Of the 136 genes upregulated on MC at 24 h, 87 (64 %) were similarly regulated on CC, 96 (71 %) were similarly regulated on BC and 95 (70 %) were similarly regulated on GC. Among these commonly regulated genes, 64 were upregulated on all four cuticles at 24 h. The balance of genes demonstrated specific responses to different cuticles, including up- or down-regulation of genes not observed at any time-point on MC (Fig. 4). This implies that the pathogen can precisely respond to different conditions. In some cases, genes coordinately upregulated on a particular cuticle were functionally related. Thus, several sequences upregulated at 24 h on GC, but not MC, CC or BC at this time-point, have homologues in yeast that are involved in integrating nutrient and growth signals with morphogenesis. These include LAS1, a nuclear protein required for cell-surface growth and bud formation (Doseff & Arndt, 1995); SLA2, required for morphogenesis and polarization of the membrane cytoskeleton (Holtzman et al., 1993); Ecm15p, involved in yeast cell-wall biogenesis (Goffeau et al., 1996); and PIG-L, essential in the synthesis of glycosylphosphatidylinositol, used as a membrane anchor by cell-surface proteins (Watanabe et al., 1999).

**Fig. 2.** The cluster of *M. anisopliae* genes upregulated within 2 h in medium containing *Man. sexta* cuticle, and which were not upregulated in minimal medium (starvation conditions). Samples were reordered from Fig. 1 according to the time-scale shown across the top, and genes were hierarchically clustered. Gene names and accession numbers are shown to the right of the figure.
Identification of genes regulated by nutrient deprivation and by insect cuticles

Energy metabolism. While the overall expression of several functional categories, including cell metabolism, was largely unaltered by transfer to cuticle (Fig. 3), individual ESTs among categories were altered in regulation, possibly indicative of pivotal enzymes involved in metabolic reprogramming (Supplementary Table S1). Although pathways are incomplete in the *M. anisopliae* array, inferences can be made from the differential expression of representative genes, as this likely reflects alterations in the pathways in which these genes are involved. We thus compared our results with experiments performed with *Saccharomyces cerevisiae* (DeRisi et al., 1997) and *Trichoderma reesei* (Chambergo et al., 2002), using very similar nutrient-rich and nutrient-poor media. In all three species, the regulation of many genes that participate in key metabolic processes is not affected by being in sugar-rich media, such as SDB (Supplementary Table S1). However, in *T. reesei*, expression of genes encoding tricarboxylic acid (TCA) cycle components and mitochondrial proteins favours the oxidation of pyruvate via the TCA cycle, rather than its reduction to ethanol by fermentation. In contrast, *S. cerevisiae* preferentially ferments glucose, even in the presence of oxygen. Only when glucose is exhausted do yeast cells use the ethanol as a carbon and energy source for aerobic respiration (the ‘diauxic shift’). *M. anisopliae* resembled *T. reesei* in that the abundance of transcripts encoding enzymes of the glycolytic pathway and TCA cycle (e.g. isocitrate dehydrogenase, AJ272972) was mostly unaffected upon transfer from a sugar-rich (SDB) to a sugar-deficient medium (MM). In yeast, these genes are strongly repressed in sugar-rich media. Yeast mitochondrial genes are also subject to strong repression by glucose. However, levels of *M. anisopliae* transcripts encoded by the mitochondrial genome (e.g. NADH ubiquinone dehydrogenase, AJ273010) and nuclear genes encoding mitochondrial proteins (e.g. cytochrome c oxidase chain V, AJ272726) were the same or higher in sugar-rich media than in MM. These results indicate that, like *T. reesei*, but unlike yeast, *M. anisopliae* will respire in the presence of sugar.

However, *M. anisopliae* appears to differ from *T. reesei* in the extent to which aerobic respiration prevails. As in yeast and *T. reesei*, a *M. anisopliae* pyruvate decarboxylase (AJ274332) is upregulated in the presence of sugar.

![Fig. 3. Regulation of functionally related genes. The curves represent the average induction or repression ratios for all the genes in each indicated group. The total number of genes in each group was as follows: cell metabolism, 71; cofactors/vitamins, 6; energy metabolism, 27; ribosomal proteins, 25; translation, 15; rRNA synthesis, 4; secreted protease, 23; intracellular proteases, 12; transport proteins, 14; amino acid/peptide transporters, 6; cell wall structure/formation, 26; stress response, 26; RNA metabolism, 28.](http://mic.sgmjournals.org)
However, in contrast to these fungi, *M. anisopliae* has an additional pyruvate decarboxylase (AJ274298) that is repressed in nutrient-rich medium but upregulated within 1 h on MC (Fig. 2). In *S. cerevisiae*, the acetaldehyde formed from pyruvate decarboxylase is reduced to ethanol by alcohol dehydrogenase, and is not converted to acetate, due to repression of aldehyde dehydrogenase by glucose. Two paralogous genes for aldehyde dehydrogenase have been identified in *T. reesei*, only one of which is repressed by nutrient-rich conditions. In contrast, both the aldehyde dehydrogenases (AJ272833 and AJ273869) in *M. anisopliae* are down-regulated in SDB compared to cuticle-containing media, suggesting that readily utilized nutrients repress acetate production. It is of interest that AJ272833 is upregulated at an earlier time-point on MC than in MM (Fig. 2). We also identified two paralogues of acetyl coenzyme A synthetase: the AJ273955 transcript is upregulated early during growth on cuticle and late during growth in MM (Fig. 2), while regulation of AJ274191 is not affected. If both enzymes have comparable specificity, production of acetyl coenzyme A in glucose-poor media such as cuticle will increase the entry of acetate, produced via the pyruvate bypass route, into the TCA cycle. Interestingly, *M. anisopliae* also has two paralogous genes for alcohol dehydrogenase. AJ273792 is regulated in a similar fashion to pyruvate decarboxylase AJ274298, whereas AJ273547, like pyruvate decarboxylase AJ274298, is repressed in SDB. Thus, *M. anisopliae* has multiple gene families of catabolic enzymes, some of which include isoforms that are differentially regulated by sugars. These alternative forms may give *M. anisopliae* the flexibility to shunt any available pyruvate into fermentation or the TCA cycle, irrespective of sugar levels.

**Fig. 4.** Subclusters of genes specifically upregulated on only one of the cuticles (BC, beetle; CC, cockroach; MC, *Man. sexta*; GC, *Lymantria dispar*). Samples were reordered from Fig. 1 according to the time-scale shown across the top, and genes were hierarchically clustered. Gene names and accession numbers are shown to the right of the figure.
Amino acid, carbohydrate and lipid metabolism. Genes with homologues involved in amino acid catabolism and which were upregulated on cuticle included glutaminase A (AJ273512) and NADH-specific glutamate dehydrogenase (AJ274362). Glutamate is the preferred amino acid substrate for *M. anisopliae* (St Leger et al., 1986a). Otherwise, diverse genes involved in amino acid synthesis were commonly down-regulated in MM and on cuticle, consistent with the reduced availability of raw materials for biosynthesis. Insect cuticle also contains diverse lipids, and seven of 13 genes for lipid metabolism were upregulated on at least one cuticle. Only a cytochrome P450 monooxygenase (AJ274003) was also upregulated during growth in MM. Lipases are the last class of depolymerases to be secreted in insect cuticle (St Leger et al., 1986b), consistent with which, lipase AJ274124 was upregulated in late cuticle-containing cultures (24 h) only. Enzyme consistent with which, lipase AJ274124 was upregulated in late cuticle-containing cultures (24 h) only. Enzyme assays have also detected a secreted DNase activity during growth on cuticle (St Leger et al., 1986b), and in this study DNase (AJ273950) was upregulated in cuticle-containing media.

Protein aside, the major component of insect cuticle is chitin, and predictably therefore chitinases were upregulated on cuticle. Chitinase AJ274366 was expressed within 1 h on MC, but was not expressed in MM (Fig. 2). Chitosanase was only produced on GC (Fig. 4). As this coincides with the GC-specific expression of genes involved in morphogenesis, it is possible that the chitosanase may be involved in modifying cell wall components. However, five additional enzymes involved in metabolizing carbohydrates not known to occur in cuticle were also upregulated in one or more of the cuticle media: formate dehydrogenase (AJ274347), usually involved in detoxification reactions; 1,2-α-D-mannosidase (AJ273630); β-D-galactosidase (AJ273808); L-sorbose dehydrogenase (AJ273834); and β-glucosidase (AJ273623). These could be involved in digesting glycoproteins, but were also more weakly upregulated in starvation conditions, consistent with catabolite repression in SDB. Only one of the genes for carbohydrate metabolism (AJ272928) was upregulated in response to HL, while seven genes were down-regulated (Supplementary Table S1). Seven carbohydrate-metabolizing enzymes were down-regulated on cuticle-containing media, including a transketolase (AJ274194) and fructose-bisphosphate aldolase (AJ273952).

RNA synthesis. Elements required for mRNA synthesis, such as RNA polymerase (AJ272996) and RNA polymerase transcription factor (AJ274125) were upregulated in cuticle-containing media, but not in MM or HL. This presumably adapts the fungus for the rapid synthesis of cuticle-degrading enzymes.

Transport proteins. The ESTs included homologues of two distinct peptide transport systems, one for di-/ tripeptides (PTR transporter AJ273551 and PTR-2 transporter AJ272830) and another for tetra-pentapeptides (OPT transporter AJ273568), as well as diverse amino acid transporters (e.g. the INDA1 homologue AJ272773). These all required induction by cuticle, and most were upregulated 8- to 12-fold within 1 h on MC (Figs 2 and 3). In contrast, the PTR transporter in *T. reesei* is upregulated by glucose exhaustion alone (Chambergo et al., 2002), consistent with the *M. anisopliae* transporters having acquired more specialized functions in pathogenicity. Only the *M. anisopliae* oligopeptide transporter OPT2 (AJ273118) was not upregulated in cuticle-containing media. Regulation of peptide/amino acid transporters was not altered in HL compared to growth on SDB.

Proteolytic enzymes. It had been shown previously that total subtilisin activity is produced in response to nutrient deprivation, but that production is enhanced by the addition of cuticle to media (Paterson et al., 1994). Consistent with this, subtilisins Pr1A and Pr1B were upregulated on MM, and to a greater extent on insect cuticles (Fig. 5). Increased induction by cuticle compared with nutrient deprivation alone suggests that subtilisin production is controlled by multiple regulatory systems evoked under different environmental conditions. In contrast, Pr1C, Pr1D, Pr1E, Pr1F, Pr1H and Pr1J were down-regulated at most time-points in MM. Of these, Pr1C and Pr1D were rapidly upregulated (Pr1C within 1 h of transfer to MC; Fig. 2), while upregulation of Pr1E and Pr1K in MC was delayed by 4 and 8 h, respectively. Pr1J was upregulated on all the cuticles, except BC. Pr1G was sharply down-regulated in CC. Pr1F and Pr1H were upregulated on MC and on GC. Expression of Pr1H was slightly upregulated by transfer to MC and MM.

The exo-acting carboxypeptidase AJ274343 was upregulated after 18 h in MM, but showed earlier and much stronger upregulation in all cuticle-containing media. Most other categories of exopeptidases (e.g. aminopeptidases AJ273806 and AJ274061) and endopeptidases, including trypsin (AJ272743), chymotrypsin (AJ273663), metalloprotease (AJ273481) and aspartyl protease (pepsinogen) (AJ274168) were only upregulated in the presence of cuticle.

Transcription factors and signal transduction. Of the 17 arrayed ESTs encoding homologues of proteins known to be involved in transcription in other organisms, ten (AJ272823, AJ272967, AJ273078, AJ273134, AJ273171, AJ273219, AJ273260, AJ273589, AJ273694 and AJ274235) were upregulated on at least one cuticle. The positive sulfur transcription regulator homologue (AJ273134) was down-regulated in MM and BC, suggestive of particularly low sulfur levels in these media (sulfite reductase, AJ273620, but not sulfite oxidase, AJ272866, was upregulated on cuticle, within 1 h in MC, but not in MM and HL). In contrast, the pH signalling transcription factor PacC (AJ273219) was upregulated on cuticle, but not in MM or HL. AJ272977 was unique in being upregulated in HL. In contrast, AJ273694 was very strongly down-regulated in HL and strongly upregulated on the lepidopteran.
cuticles GC and MC. Among gene products involved in signalling (category 4f), adenylate cyclase (AJ251971) (the enzyme that produces cAMP) and protein kinase A (AF116597) (PKA: the major effector of cAMP responses) were not upregulated on cuticle-containing media, while a downstream activity, MAP kinase kinase 2 (AJ273356) was upregulated in GC- and BC-containing media, and at two time-points in MC.

**Cell wall proteins.** Of 30 genes encoding proteins involved in cell structure and function, 18 were upregulated in at least one cuticle-containing medium. The hydrophobins are differentially regulated. Thus, AJ273847 was upregulated in HL and MM, and down-regulated in cuticle-containing media, while AJ274156 was upregulated in MM and on sclerotized cuticles (CC and BC), unaltered on lepidopteran cuticles (GC and MC) and down-regulated in HL. The other cell wall proteins upregulated on cuticle were AJ273845, a homologue to an antigenic cell wall protein from the human pathogen *Aspergillus fumigatus*, and AJ274019, which is very similar to the antifungal glucan 1,3-β-glucosidase from *Trichoderma atroviride* (Donzelli et al., 2001). Clearly, besides cell wall biosynthesis and structure, these proteins may have additional functions in pathogenicity or in protecting scarce resources from competitors.

**Stress response.** Several arrayed *M. anisopliae* ESTs are similar to peptide synthases, reductases and other enzymes that take part in the synthesis of fungal toxins, such as destruxins, trichothece and enniatin (Freimoser et al., 2003). This is in agreement with the observation that *M. anisopliae* strain 2575 rapidly kills its host after infection through the action of toxins, and subsequently colonizes the insect host by saprobic growth (Samuels et al., 1989). Genes upregulated in at least one cuticle-containing medium included those encoding a peptide synthase (AJ272930, in BC and GC), a protein involved in sterigmatocystin biosynthesis (AJ273515, in GC), versicolorin B synthase (AJ272697, in CC, GC, MC and HL) and a bacteriolytic enzyme (AJ272917, in CC, BC, GC, early in MC and after 12 h in MM).

**Validation of microarray results.** An external quality control check on the lists of differentially expressed clones generated through microarray profiling was provided by analysing a subset of 16 clones by quantitative RT-PCR. Each clone tested by RT-PCR was measured in triplicate for each of two independent RNA isolations. All 16 clones were confirmed by RT-PCR, indicating a very high success rate for predicting differentially expressed clones. However, expression ratios were consistently underestimated at least 10-fold by cDNA microarrays, compared to PCR-based methods.

**DISCUSSION**

The construction of a *M. anisopliae* cDNA microarray provides many advantages over previous labour-intensive techniques to monitor transcriptional responses to host tissues. For this study, it provided a powerful tool with which to examine the influence of culture conditions on the magnitude and spectrum of cuticle-induced gene expression. The analysis presented here has expanded the number of identified *M. anisopliae* genes that respond to cuticle from about 20 (Joshi & St Leger, 1999; Joshi et al., 1997) to more than 200. Expression patterns of known pathogenicity genes, including Pr1A subtilisin, hydrophobin, trypsins, chymotrypsin and carboxypeptidase, matched previously published data (Screen & St Leger, 2000; St Leger et al., 1986b, 1987, 1992, 1996). This provides a high level of confidence that the arrays accurately identify differentially expressed clones. For selected genes, including subtilisins (Pr1A, Pr1H and Pr1K), a trypsin (Try1) and tubulin, the
expression patterns were also verified by quantitative real-time RT-PCR. Normally (e.g. Yuen et al., 2002), expression ratios are greatly underestimated by cDNA microarrays, compared to PCR-based methods. Together with our strict analysis of the replicates within and between different arrays, this suggests that our estimates of the magnitude of changes in expression are conservative.

The demonstration of the differential regulation of genes encoding cuticle-degrading enzymes, cell wall proteins, toxins and toxin-producing enzymes on the different cuticles, and in HL and MM suggests that *M. anisopliae* may have the ability to target the production of these proteins to different hosts. Like other ascomycete pathogens, *M. anisopliae* secretes a great variety of proteases (Hu and St Leger, 2004), some of which have been associated with virulence, because they allow rapid physical ingress, nutrient solubilization and the disabling of antimicrobial peptides (St Leger et al., 1996). The subtilisin cluster provides a good example of *de novo* protein synthesis required for adaptation to growth on cuticle (Figs 1 and 5, Supplementary Table S1), particularly as the differences in regulation of subtilisins imply differences in their function. This supports homology-modelling studies based on sequences that predict differences between the Pr1s in their secondary specificities, adsorption properties to cuticle and alkaline stability (Bagga et al., 2004). It is likely that these differences in regulation and structure–function allow *M. anisopliae* to respond flexibly, producing proteases that are appropriate to the composition of the environment, consistent with its opportunistic lifestyle. Thus the proteases such as Pr1A produced as part of a general response to nutrient deprivation could also function outside of pathogenesis by scavenging for nutrients during saprophytic existence. During early infection processes, they could also function in concert with the exopeptidases to provide host degradation products. These may include specialized signals that allow the fungus to ‘sample’ the cuticle and then respond with the secretion of a plethora of cuticle-induced proteins. This will include the proteases that require cuticle for induction, as they presumably have specialized roles in breaching host barriers. The very early induction of peptide/amino acid transport systems (Figs 2 and 3) would enhance the ability of the fungus to rapidly and precisely monitor host degradation products.

Hydrophobins provide another example besides subtilisins where members of a family are differentially regulated, consistent with different functions. Thus, AJ1273847 was upregulated in HL, while AJ274156 was down-regulated in HL. This suggests that adaptation to HL may include alterations in cell wall composition.

Of key importance to understanding the mechanisms behind adaptation to cuticles is the identification of components of signal transduction that will allow *M. anisopliae* to screen its surroundings to regulate protein synthesis and secretion. PacC-mediated pH signalling is crucial to the pathogenicity of the human pathogen *Candida albicans* and the plant pathogen *Fusarium oxysporum* (Caracuel et al., 2003; Davis et al., 2000). Consistent with a crucial role for PacC in *M. anisopliae*, extracellular pH rises during cuticle degradation and acts as a key signal for the production of alkaline-active enzymes such as subtilisins (St Leger et al., 1998). Significant for their absence of response were adenylate cyclase and protein kinase A, as transcriptional regulation in response to the cAMP signalling pathways seems central to infection-related development in *M. anisopliae* (St Leger, 1993). Constitutive expression may be a feature of some primary initiators of physiological processes, so their importance will not be detected in microarray analyses. A downstream activity, MAP kinase kinase 2 (AJ273356) was upregulated in GC- and BC-containing media. This enzyme, and other transcription factors, may constitute downstream ‘ground-level’ components which are immediately concerned with recognizing and responding to specific host features, and which do not control fungal metabolism as a whole. As such, they may be useful for strain improvement purposes.

Microarray technology has made it possible to decipher the transcriptional programmes of organisms by studying gene expression en masse while assessing individual gene function in a detailed manner (Brown & Botstein, 1999). Thus, knowing when and where a gene is expressed often provides a strong clue as to its function (DeRisi et al., 1997). Almost 50 % of the arrayed ESTs upregulated in cuticle-containing media have undiscovered biological activities (Table 1 and Supplementary Table S1), and 25 % of these are not upregulated in MM or HL. These genes have never been recognized to have a role in pathogenicity, but are now implicated by co-regulation with known virulence factors. They thus provide an additional rich resource for future research.

Evolutionary theory has long held that the process of adaptation is driven by competition for limited resources. Among heterotrophic micro-organisms, the availability of carbon limits the ability of these organisms to multiply. As a result, the machinery of central metabolism is tuned to exploit reduced carbon resources in natural environments, where they vary greatly in both form and abundance (Ferea et al., 1999). Comparisons between *M. anisopliae*, *T. reesei* and *S. cerevisiae* suggest that the three fungi will respond differently to environmental changes, presumably reflecting their adaptation to predictable differences in the composition of these environments. The similarities between *M. anisopliae* and *T. reesei* may reflect their close relationship as clavicipitaceous pyrenomycetes. However, the alternatively regulated forms of catabolic enzymes in *M. anisopliae* and *T. reesei* suggest they will differ in how they coordinate the regulation of key parts of metabolism, such as fermentation at different levels of glucose. This could affect the extent to which aerobic respiration prevails in glucose-rich media.

Evidently, gene-duplication events and altered patterns of regulation could provide mechanisms for evolution to
fine-tune ATP-producing pathways, allowing these organisms to adapt to their different environments and nutritional requirements. It is tempting to speculate that fermentation may play a more pivotal role in the life of *M. anisopliae*, compared to that of *T. reesei*, to enable it to exploit sugars in the anaerobic environment of the dead host. However, complicating interpretation of these results, ATP-producing pathways can be co-opted to other functions. Thus, some fungal acetyl coenzyme A synthetases are involved in the biosynthesis of secondary metabolites such as penicillin, as well as in primary metabolism (Martinez-Blanco et al., 1993). It is axiomatic that as more is learned about the function of each gene, comparative studies on transcriptomes will become an increasingly powerful tool allowing predictive insights into the behavioural plasticity of each saprophyte or pathogen.

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