Major impacts on the primary metabolism of the plant pathogen *Cryphonectria parasitica* by the virulence-attenuating virus CHV1-EP713

Angus L. Dawe,^{1,2} Wayne A. Van Voorhies,² Tannia A. Lau,¹ Alexander V. Ulanov³ and Zhong Li³

Correspondence
Angus L. Dawe
dawe@nmsu.edu

¹Department of Biology, New Mexico State University, Las Cruces, NM 88003, USA

²Molecular Biology Program, New Mexico State University, Las Cruces, NM 88003, USA

³Roy J. Carver Biotechnology Center, University of Illinois, Urbana, IL 61801, USA

Cryphonectria parasitica, the chestnut blight fungus, can be infected by virulence-attenuating mycoviruses of the family *Hypoviridae*. Previous studies have led to the hypothesis that the hypovirus-infected phenotype is partly due to metabolic changes induced by the viral infection. To investigate this, we measured the metabolic rate and respiration of *C. parasitica* colonies grown on solid medium. These experiments supported historical observations of other fungal species done in liquid cultures that the metabolic rate steadily declines with age and differentiation of the mycelium. Hypovirus infection increased metabolic rate in the youngest mycelium, but a subsequent decline was also observed as the mycelium aged. By measuring both CO₂ production and O₂ consumption, we also observed that changes occur in carbohydrate metabolism as a result of ageing in both infected and uninfected mycelium. Mycelium on the periphery of the colony exploited fermentation pathways extensively, before transitioning to aerobic carbohydrate metabolism and finally lipid metabolism in the interior regions, despite abundant remaining glucose. However, the hypovirus affected the extent of these changes, with infected mycelium apparently unable to utilize lipid-related metabolic pathways, leading to an increased depletion of glucose. Finally, we used metabolic profiling to determine the changes in accumulation of primary metabolites in wild-type and hypovirus-infected mycelium and found that approximately one-third of the 164 detected metabolites were affected. These results are consistent with those expected from the physiological measurements, with significant alterations noted for compounds related to lipid and carbohydrate metabolism. Additionally, we observed an increase in the accumulation of the polyamine spermidine in the presence of hypovirus. Polyamines have been implicated in antiviral responses of mammalian systems; therefore this may suggest a novel antiviral response mechanism in fungi.

Received 10 March 2009
Revised 30 June 2009
Accepted 7 July 2009

INTRODUCTION

Investigation of basic fungal metabolism under a variety of nutritional conditions formed the foundation of many early examinations of fungal behaviour (reviewed by Foster, 1949; Hawker, 1950). Historically, fungal metabolism studies measured O₂ consumption using variations of the Warburg–Barcroft constant-volume manometer (Dixon, 1952), with CO₂ production inferred by titration after alkali absorption. For such analyses large cultures of liquid-grown submerged mycelium were typically used regardless of the organism of study [such as *Botrytis cinerea* (Gentile, 1954), *Penicillium* sp. (Koffler *et al.*, 1945),

Ustilago zeae (Shu, 1953) and *Fusarium solani* (Cochrane, 1958)]. Large amounts of biomass were required because the methods used for measuring O₂/CO₂ flux at the time were much less sensitive than methods currently available. These studies were sufficient, however, to conclude that the metabolic rate of liquid-grown mycelium declined with age.

Metabolic rate measurements are of significant interest for monitoring the behaviour of micro-organisms in liquid culture. These may relate to production efficiencies of industrial processes and, therefore, most recent studies concerning fungal metabolism rates have been directed towards cultures in batch or chemostat formats. Efficient reporting methods have recently been developed that can examine metabolic rate by spectrophotometric monitoring of compounds responsive to mitochondrial activity (Moss

A supplementary table and figure with details of the primary metabolites produced by hypovirus-infected and uninfected strains are available with the online version of this paper.

et al., 2008), by direct gas analysis (Bideaux *et al.*, 2006) or by measurement of metabolic precursors and products (Gheshlaghi *et al.*, 2007) when cultures are grown under highly controlled conditions.

Fungal mycelium growing on solid medium represents a distribution of ages from the colony centre (oldest) to the periphery (most recent) that cannot be accurately modelled by a liquid environment. In an ageing colony, this spatial differentiation has been related to four zones, termed (i) extending zone, where hyphae are extending into fresh medium; (ii) productive zone, where biomass is increasing and differentiation begins; (iii) fruiting zone, typically where the generation of aerial hyphae and spore-forming structures occurs; and (iv) aged zone, where compartments are highly vacuolated and autolysis may occur (Carlisle *et al.*, 2001). Analysis of an entire colony will, therefore, not take account of variation in the hyphae of different ages. Equally, an experiment that restricts the hyphae used to those produced during a small window of time requires measurement methods that can accurately determine metabolic rates from very small quantities of biomass. In order to investigate the metabolic behaviour of a fungal colony over time, we have applied methods developed for the analysis of small biomass quantities such as small groups of *Caenorhabditis elegans* nematodes (Van Voorhies & Ward, 1999) or individual *Drosophila melanogaster* fruit flies (Melvin *et al.*, 2007; Van Voorhies *et al.*, 2004) to variously aged samples of fungal colonies.

The organism used in this study, the plant pathogen *Cryphonectria parasitica*, is the agent responsible for the near-elimination of the American chestnut, *Castanea dentata*, from its natural range across the eastern United States. Easily manipulated in the laboratory, this fungus can be infected by a mycovirus that induces characteristic alterations in phenotype that include reduced virulence, reduced asexual sporulation and changes in colony growth rate (reviewed by Dawe & Nuss, 2001; Nuss, 2005) and recently quantified changes in colony morphology (Golinski *et al.*, 2008). Although mycoviruses are associated with all major classes of fungi (Buck, 1986), those of the family *Hypoviridae* that infect *C. parasitica* represent the only viral agents for this entire host kingdom for which infectious cDNA clones have been developed (Chen *et al.*, 1994; Choi & Nuss, 1992; Lin *et al.*, 2007). Furthermore, spotted cDNA microarray analysis revealed significant impacts of hypovirus infection on the expression of genes involved with glycolysis and mitochondrial function (Allen & Nuss, 2004). Consistent with these studies, we have employed the methods for metabolic analysis noted above to measure significant physiological variations that occur as a result of ageing and differentiation in both infected and uninfected mycelium, and found that the presence of the hypovirus affects the extent of these changes.

Recently, the application of highly sensitive metabolic profiling methods to model fungal systems has increased understanding of the production of a variety of polar and

non-polar compounds (reviewed by Jewett *et al.*, 2006). To complement the physiological measurements we have also applied a metabolomic approach, which revealed a response profile supporting the physiological measurements and confirming that infection of *C. parasitica* with a hypovirus causes major changes to primary metabolic pathways. In particular, we have observed that major changes occur in pathways related to carbon and lipid metabolism as well as a large change in polyamine accumulation.

The studies presented here represent a unique combination of physiological and analytical approaches that have provided a novel set of tools for examining the metabolic changes following infection with a virulence-attenuating mycovirus and will likely have relevance to other filamentous fungal systems.

METHODS

Fungal strains, growth conditions and harvesting regimen. *C. parasitica* strain EP155 (ATCC 38755) and the isogenic strain EP155/CHV1-EP713 (ATCC 52571) infected with the prototypic hypovirus CHV1-EP713 (Choi & Nuss, 1992) were each grown on solid medium [3.9%, w/v, Difco potato dextrose agar (PDA, Becton Dickinson)] at room temperature (22–25 °C) with a 12 h light/dark cycle at 1300–1600 lx. Inoculations for measurements used a single plug cut from the periphery of an actively growing culture with a 4 mm cork borer and placed in the centre of the PDA growth medium in 150 mm × 15 mm Petri plates. The edge of the expanding colony was marked at 24 h intervals to track colony expansion. Plugs of mycelium were removed from each colony according to the time periods indicated in Fig. 1, using the 4 mm cork borer, corresponding to a mycelial area of 0.126 cm² per plug. Each measured sample consisted of two plugs; thus each value corresponded to the metabolic measurement from 0.25 cm² of mycelium. To avoid bias across different portions of the colony and between colonies, four sample measurements were made at each growth time point at approximately 90° to one another, across four independently grown plates. The measurements reported represent the combined data from these 16 samples.

Fig. 1. Sampling strategy. A colony of *C. parasitica* strain EP155 with the circles denoting the typical sample ranges used in this study. Numbers refer to the age range of the mycelial zone in hours.

Metabolic activity measurements. To determine metabolic rates, pairs of plugs with attached mycelium were placed into 2.2 ml glass measurement chambers that were sealed with a rubber stopper. Chambers were flushed for 15 s at a flow rate of 90 ml min⁻¹ with CO₂-free, water-saturated (100 % RH) room air introduced through a 22-gauge needle with a second needle inserted to vent the chamber, and then left sealed for 1 h in an incubator at 23 °C with white light illumination of 1200 lx. After 1 h, a 1 ml (STPD; standard temperature and pressure, dry) sample of the chamber gas was removed using a SampleLock syringe (Hamilton) and injected directly into a 150 ml min⁻¹ or 200 ml min⁻¹ ($\pm 1\%$) STPD, CO₂-free carrier air stream scrubbed of water with a magnesium perchlorate filter before entering a 6251 CO₂ gas analyser (Li-Cor Biosciences) and, connected in series, an Oxzilla oxygen analyser (Sable Systems). The measurement chamber was flushed again with CO₂-free air; samples were replaced in the incubator and 1 h later a second chamber gas sample was taken. The quantity of CO₂ produced or O₂ consumed by an individual mycelial sample of two 0.126 cm² plugs was calculated from the analytical instrument measurements using Datacan software (Sable Systems), recorded in Excel and graphs were generated by KaleidaGraph (Synergy Software). The accepted SI unit for a rate of metabolic energy use per unit time is W, usually calculated by relating the O₂ consumption to kJ. However, in this instance, because of the observed variation in O₂ consumption (indicating a transition between utilization of different metabolic pathways) the units have been left as measured in $\mu\text{l CO}_2 \text{ h}^{-1} \text{ cm}^{-2}$. Several empty chambers and chambers containing agar plugs from uncolonized regions of the plates were included in each set of metabolic measurements to control for background CO₂, presence of CO₂ in the air used for flushing, gas absorption by the agar plugs and gas leakage in the assay chambers.

Glucose content in growth medium. Using the same borer and harvesting regimen as above, plugs were recovered from PDA-grown mycelium of hypovirus-infected and uninfected cultures. Individual plugs with associated agar column were weighed, suspended in 9 vols sterile water, and incubated for 10 min in a boiling water bath to dissolve. For liquid cultures, mycelium was grown for 4 days in 50 ml Difco potato dextrose broth (PDB, Becton Dickinson) in stationary 250 ml flasks at room temperature, following inoculation by 4–6 plugs of mycelium from an actively growing solid medium culture. The mycelium was then homogenized with a hand-held homogenizer (Polytron 1600E, Kinematica) and an equal volume of fresh medium added followed by an additional 3 days of growth. Aliquots of medium were removed at the end of the growth period and diluted 1 : 10 in sterile water. Glucose content in solid and liquid medium was measured by absorbance at 630 nm using the QuantiChrom Assay kit (BioAssay Systems) according to the manufacturer's recommendations. Control values were obtained from uncolonized PDA plates or PDB incubated under the same conditions.

Sample preparation for metabolic profiling. Liquid-grown fungal tissue, prepared as described above, was harvested by filtration using Miracloth (EMD Biosciences), rinsed with sterile water, then immediately frozen in liquid N₂. The frozen samples, in triplicate, were ground by hand in liquid N₂ using a pestle and mortar. Then 1.5 ml 70 % methanol was added to 100 mg of each ground sample and the mixture was sonicated in a Sonicator 4000 (Misonix) for 5 min. Samples were then extracted for 15 min at 60 °C with 1.5 ml water at room temperature followed by 0.75 ml chloroform at 37 °C. Polar (methanol+water phase) and nonpolar (chloroform phase) extracts were separately evaporated to dryness under vacuum.

Prior to GC/MS analyses, dried polar extracts were converted to TMS (trimethylsilyl) derivatives with 80 μl methoxyamine hydrochloride (20 mg ml⁻¹) for 60 min at 40 °C followed by 80 μl MSTFA [N-methyl-N-(trimethylsilyl)trifluoroacetamide] at 60 °C for 40 min.

Nonpolar metabolites were converted to methyl esters by mixing with 50 μl chloroform, 30 μl (trimethylsilyl)diazomethane (2 M solution in hexane) and 20 μl methanol at room temperature for 30 min.

Metabolic profiling analysis. The Agilent GC/MS system consisted of a 7890A gas chromatograph, a 5975C mass-selective detector and a 7683B autosampler. Gas chromatography of the polar extracts was performed on an Agilent HP-5MS capillary column (60 m $\times$ 0.25 mm i.d., 0.25 μm film thickness). The inlet and MS interface temperatures were 250 °C, and the ion source temperature was adjusted to 230 °C. An aliquot of 1 μl was injected with a split ratio of 5 : 1. The helium carrier gas was kept at a constant flow rate of 1.5 ml min⁻¹. The temperature programme was an initial 5 min isothermal heating at 70 °C, followed by an oven temperature increase of 5 °C min⁻¹ to 310 °C and a final 10 min at 310 °C.

Nonpolar metabolites were analysed with a Phenomenex ZB-WAX capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness). The inlet and MS interface temperatures were set at 260 °C and 280 °C, respectively. The ion source was 230 °C. An aliquot of 1 μl was injected with a split ratio of 2 : 1. The helium carrier gas was set at a constant flow rate of 2 ml min⁻¹. The temperature programme was a 5 min isothermal heating at 140 °C, followed by an oven temperature increase of 10 °C min⁻¹ to 265 °C and a final 25 min at 265 °C.

In both cases mass spectra were recorded in the m/z 25–800 scanning range.

The acquired GC/MS spectra were processed by the AMDIS software (National Institutes of Standards and Technology, NIST) and compared with standard mass spectrum libraries NIST08 (NIST), WILEY8n (Palisade Corporation), and the custom library. To allow comparison between samples, data were normalized to the added internal standard (hentriacontanoic acid at 10 mg ml⁻¹) and fresh weight of each sample.

RESULTS AND DISCUSSION

A complete fungal colony covers a range of conditions that may affect physiology, particularly changing nutrient conditions and waste product accumulation as well as differentiation and development. Since effects of hypovirus infection on the metabolism of *C. parasitica* have been implied by the transcriptional profile of genes related to glycolysis (Allen *et al.*, 2003), we sought to identify metabolic changes occurring in cultures of *C. parasitica* by using a combination of physiological measurements and metabolic profiling.

Metabolic rate and age in *C. parasitica*

Our measurements of CO₂ production show that the greatest metabolic activity is found at the extending edge of the colony, where the most rapid growth is occurring (Fig. 2). We have also clearly demonstrated that we can detect a decline in metabolic rate of the colony over time. Unexpectedly, the metabolic rate of a mycelium stably infected with mycovirus was found to be greater than that of uninfected mycelium at the periphery of the culture. The subsequent decline in metabolic rate in the older portions of both cultures, however, was very similar (Fig. 2). These data are supported by previous observations that respiratory activity of submerged fungal cultures was highest

Fig. 2. Metabolic rate declines with mycelial age. CO₂ production by 0.126 cm² plugs of mycelium was measured using infrared gas analysis. Each measured sample consisted of two plugs (0.25 cm² mycelium total) removed from PDA cultures of strain EP155 (uninfected, shaded bars) and EP155/CHV1-EP713 (hypovirus infected, white bars) according to the harvesting scheme described in Fig. 1. Four samples were harvested at each time point at approximately 90° to one another, across four independently grown plates. The measurements are the combined data from these 16 samples. Error bars represent the standard error of each dataset.

during times of most rapid growth but declined with age of the culture (Gentile, 1954; Koffler *et al.*, 1945). However, those observations were based on manometric methods that, while simple to construct and operate, may not accurately measure metabolic rate (Van Voorhies *et al.*, 2008). The new data are significant in that they represent measurements of differently aged regions of the colony obtained during the same experiment, and required only small amounts of biomass.

Changes in metabolic pathways of an ageing colony

In order to gain greater insight into the metabolic activities of the colonies at different ages in both infected and uninfected mycelium, we also measured O₂ consumption and used these data to calculate respiratory quotient (RQ) values (the ratio of CO₂ production to O₂ consumption) for each age of mycelium (Fig. 3). RQ values are often used as estimates of the metabolic substrates an organism is using during oxidative phosphorylation (Gessaman & Nagy, 1988; Walsberg & Wolf, 1995). A value of 1.0 indicates that carbohydrates are the main substrate being oxidized. Values above or below 1.0 indicate that the cell is utilizing other metabolic processes or substrates: RQ values >1.0 are common during fermentative metabolism; RQ

Fig. 3. Metabolic pathways change with mycelial age. Mycelial plugs were removed from PDA cultures of strain EP155 (uninfected, shaded bars) and EP155/CHV1-EP713 (hypovirus infected, white bars) according to the harvesting scheme described in Fig. 1. RQ values were calculated from the ratio of CO₂ production to O₂ consumption. RQ values >1.0 are common during fermentative metabolism; RQ values <1.0 occur when the cell utilizes energy sources such as lipids (RQ ≈ 0.7) or proteins (RQ ≈ 0.85). Error bars represent the standard error of each dataset.

values <1 occur when the cell utilizes energy sources such as lipids (RQ ≈ 0.7) or proteins (RQ ≈ 0.85) or potentially ethanol (RQ ≈ 0.66; Gessaman & Nagy, 1988; Walsberg & Wolf, 1995).

The RQ values in Fig. 3 indicate that the outermost hyphae exploiting fresh medium are operating aerobic fermentation pathways extensively. This is consistent with the Crabtree effect (Crabtree, 1929), a phenomenon that has been observed extensively in *Saccharomyces* species (De Deken, 1966) but not to our knowledge previously reported in filamentous fungi. From an ecological perspective, it has been proposed that the rapid consumption of carbohydrate by fermentation pathways during fruit degradation allows *Saccharomyces* species to rapidly convert a substrate that may be suitable for many competitors into one (in this case, ethanol) that is not, thereby sequestering the resources of the fruit from competing organisms (Fleet & Heard, 1993; Thomson *et al.*, 2005). It is possible that the high carbohydrate content of the PDA medium used for growth in these studies elicits a similar response from *C. parasitica*.

After the first 24 h of growth, the metabolism shifts to predominantly complete carbohydrate oxidation, as indicated by the reduced RQ value (approaching 1.0) as O₂ consumption rises relative to CO₂ production (Fig. 3). This also correlates with the productive zone of the colony

where biomass is increasing more rapidly, perhaps requiring both the more energetically efficient pathways of oxidative phosphorylation and the anabolic precursors produced by the TCA cycle. As the colony ages still further, an additional decline in RQ to approximately 0.9 was noted (Fig. 3). This indicates that the older mycelium transitions again into metabolic modes likely based on utilizing lipids and/or proteins.

Interestingly, an analysis of glucose concentrations remaining in the medium at these time points demonstrated that significant resources remained available to the centre of the colony (Fig. 4). Glucose concentration was reduced only to about 40 % of the value of uncolonized medium under the oldest hyphae, even though the uncolonized values were somewhat higher than predicted for PDA (2 %, or ~110 mM), presumably due to dehydration of the plate during incubation. Furthermore, the transition away from carbohydrate metabolism can also be observed; the decline in glucose concentration stops in those regions of the colony that were observed to generate RQ values <1.0. These data indicate that the metabolic transitions as well as the structural changes in colony development occur as a result of ageing and are not a response to starvation.

Fig. 4. Differential glucose depletion in the presence of the hypovirus. Mycelial plugs, including the column of medium directly underneath, were removed from PDA cultures of strain EP155 (uninfected, shaded bars) and EP155/CHV1-EP713 (hypovirus infected, white bars) according to the harvesting scheme described in Fig. 1. Triplicate glucose measurements were determined from each of three independent colonies (nine in total); error bars represent the standard error across these samples. Uncolonized medium measurements were derived from eight samples from plates poured from the same batch of medium and incubated for the same time period, but not inoculated.

Altered metabolism of hypovirus-infected mycelium

Interestingly, colonies infected with the hypovirus CHV1-EP713, while still exhibiting the highest RQ values on the periphery of the colony, did not appear to transition into the other metabolic pathways as seen in the wild-type colonies (Fig. 3). All of the RQ values recorded for hypovirus-infected mycelium were between 1.06 and 1.23, compared with 1.45–0.92 for uninfected mycelium. Given that the metabolic changes in the uninfected mycelium occur in the absence of glucose limitation, we also determined the remaining glucose values underneath the hypovirus-carrying strain. As seen in Fig. 4, we observed a decline in glucose concentration in medium underneath infected mycelium of all ages, consistent with the RQ data, which showed continuous use of carbohydrates in all hyphae regardless of age.

The lack of development in a hypovirus-infected colony (as noted by reduced pigmentation and asexual sporulation) has been likened to retaining the mycelium in a juvenile state (McCabe & Van Alfen, 2001). This is supported by our data showing that the presence of the hypovirus partially prevented metabolic changes characteristic of older, uninfected, fungal tissue. It is not clear what is responsible for the failure in this metabolic transition. One possibility is that the failure is due to regulation of specific enzymes that operate in the pathways concerned, as has been implied by microarray studies (Allen *et al.*, 2003). Another possibility is that the hypovirus causes a sequestration of lipids to particular parts of the fungal cell. Mycoviruses are typically associated with membranous structures (Buck, 1986), and hypovirulent isolates of *C. parasitica* containing dsRNA have been shown to feature a large number of pleiomorphic membranous vesicles (Hansen *et al.*, 1985; Newhouse *et al.*, 1983). It is not known if this redistribution of membrane might affect the use of fatty acids for catabolism in older mycelium.

To investigate the phenomenon of altered metabolism further, we also profiled the primary metabolites produced by uninfected and hypovirus-infected strains. Analysis of both polar and nonpolar metabolites detected a total of 165 positively identified compounds (see Supplementary Table S1, available with the online version of this paper, for the full list of compounds and Supplementary Fig. S1 for statistical analysis). Of these, 54 compounds were altered to a statistically significant degree ($P < 0.05$) in the presence of the hypovirus. Interestingly, many more metabolites (41) showed increased accumulation (Table 1), compared to just 12 compounds that were decreased (Table 2). The compounds found to be changed represented broad metabolic categories, including amino acid metabolism, carbohydrate metabolism, lipid metabolism and purine/pyrimidine metabolism.

The alterations in lipid metabolism are most profound in the three compounds reduced by hypovirus infection to undetectable levels [9,12-hexadecadienoic acid, n-pentade-

Table 1. Metabolites increased in accumulation in the presence of hypovirus CHV1-EP713Changes were deemed significant when $P < 0.05$. ND, Not detected.

Metabolite/role	EP155/CHV1-EP713	SE ($n=3$)	EP155	SE ($n=3$)	P (<0.05)	Fold change
Amino acid metabolism						
Cystathionine	3.519	0.066	0.885	0.054	0	3.97
Methionine	0.199	0.015	0.084	0.008	0.035	2.37
Ornithine	5.934	0.446	3.334	0.045	0.034	1.78
Asparagine	1.359	0.107	0.79	0.02	0.043	1.72
Arginine	0.663	0.027	0.388	0.032	0.003	1.71
Threonine	5.516	0.195	3.254	0.102	0.002	1.7
<i>N</i> -Acetyl-L-lysine	1.107	0.095	0.681	0.028	0.047	1.62
Homocysteine	0.047	0.002	0.03	0.002	0.028	1.6
Alanine	6.923	0.086	4.777	0.374	0.037	1.45
Benzoic acid	0.066	0.006	0.051	0.005	0.005	1.3
Isoleucine	4.742	0.031	4.292	0.088	0.017	1.1
Carbohydrate metabolism						
Galactopyranose	1.721	0.051	0.403	0.04	0.002	4.27
Glucose 6-phosphate	0.404	0.036	0.099	0.005	0.016	4.09
Galactitol	0.844	0.055	0.276	0.021	0.005	3.05
Cellobiose	0.901	0.022	0.332	0.006	0.001	2.72
Fructose 6-phosphate	0.088	0.007	0.034	0.003	0.027	2.57
1-Methyl α -D-glucopyranoside	26.5	0.498	11.235	0.642	0	2.36
Inositol 1-phosphate	0.175	0.014	0.079	0.008	0.047	2.23
Melibiose	3.431	0.371	1.543	0.081	0.041	2.22
1-Methyl β -D-galactopyranoside	1.412	0.044	0.678	0.004	0.004	2.08
Threonic acid	0.197	0.01	0.103	0.001	0.012	1.91
Threitol	0.128	0.006	0.068	0.003	0.02	1.87
<i>N</i> -Acetylglutamic acid	0.532	0.013	0.301	0.026	0.004	1.77
Fumaric acid	0.419	0.025	0.251	0.022	0.005	1.67
Glucose	54.101	1.292	44.479	0.956	0.006	1.22
1-Ethylglucopyranoside	0.336	0.022	ND	–	–	–
Fructofuranoside	0.084	0.004	ND	–	–	–
Lipid metabolism						
Nonanoic acid	0.018	0.001	0.009	0	0.007	1.97
Ethanolamine	0.832	0.022	0.423	0.073	0.044	1.97
Eicosanoic acid	0.431	0.013	0.244	0.014	0.005	1.77
7-Hexadecenoic acid	0.051	0.001	0.034	0.001	0	1.53
Octadecanoic acid	14.467	0.708	10.467	0.457	0.035	1.38
9-Octadecenoic acid	3.441	0.076	2.637	0.088	0.003	1.3
1-Dodecanol	0.646	0.016	0.499	0.016	0.038	1.3
Oxalic acid	0.045	0.001	0.036	0.002	0.011	1.28
Nucleotide metabolism						
Hypoxanthine	0.178	0.011	0.065	0.007	0.024	2.73
Uridine	0.173	0.013	0.073	0.006	0.033	2.35
Cytosine	0.009	0.001	0.006	0	0.04	1.47
Polyamine metabolism						
Spermidine	0.607	0.033	0.012	0.001	0.003	51.1
Various						
Pyrophosphate	1.919	0.041	0.092	0.011	0.001	20.75
Phosphonic acid	0.697	0.02	0.224	0.034	0.002	3.11

cane and 3-hydroxy-3-methylglutaric acid (HMG)]. The former is an unsaturated fatty acid product previously identified in other fungi (Murayama *et al.*, 2006; Pedneault *et al.*, 2006) while the saturated n-pentadecane is likely a precursor for fatty acid synthesis. HMG is usually found as part of HMG-CoA, the reduction of which forms a key step

in ergosterol biosynthesis (reviewed by Ferreira *et al.*, 2005). A variety of lipid-related compounds are also elevated by hypovirus infection, including fatty acids and precursors (Table 2) but with much more modest magnitudes (1.5–2-fold). These changes were not the result of altered media depletion by the different strains. Analysis

Table 2. Metabolites decreased in accumulation in the presence of hypovirus CHV1-EP713

Changes were deemed significant when $P < 0.05$. ND, Not detected.

Metabolite/role	EP155/CHV1-EP713	SE ($n=3$)	EP155	SE ($n=3$)	P (<0.05)	Fold change
Amino acid metabolism						
Tyrosine	1.003	0.034	1.736	0.087	0.022	−1.73
Homoserine	0.254	0.019	0.406	0.032	0.020	−1.60
Propanoic acid	0.150	0.016	0.233	0.023	0.042	−1.55
Tryptophan	0.723	0.032	1.020	0.035	0.006	−1.41
Carbohydrate metabolism						
Arabitol	2.282	0.158	4.793	0.412	0.048	−2.10
Maltose	0.041	0.002	0.061	0.003	0.018	−1.49
Lipid metabolism						
9,12-Hexadecadienoic acid	ND	–	0.087	0.006	–	–
3-Hydroxy-3-methylglutaric acid	ND	–	0.052	0.002	–	–
n-Pentadecane	ND	–	0.076	0.005	–	–
Nucleotide metabolism						
Guanine	0.120	0.008	0.207	0.012	0.016	−1.72
Allantoin	0.108	0.017	0.155	0.009	0.030	−1.44
Polyamine metabolism						
Putrescine	0.075	0.033	0.188	0.018	0.039	−2.51

of the glucose concentration in spent medium from both sets of cultures returned values of 63–68 mM compared to 95 mM in uncolonized medium. These values are noticeably higher than those recorded for agar cultures, presumably due to the addition of fresh medium during incubation, and indicate that the observed metabolite changes are not the result of carbohydrate depletion. Taken together, these observations are consistent with a disruption of lipid catabolism and biosynthesis that contributes to the altered metabolic pathways operating in hypovirus-infected mycelium described above.

The inability of hypovirus-infected mycelium to properly transition into oxidative phosphorylation can also be inferred from the metabolite profiles. Fermentation requires that glycolysis is highly active for pyruvate production. Three compounds at the beginning of this pathway (glucose, glucose 6-phosphate and fructose 6-phosphate) are all elevated in hypovirus-infected mycelium, presumably then directing increased carbon flow towards pyruvate production. However, with pyruvate being employed in fermentative pathways, reduced flow of carbon in the tricarboxylic acid cycle would lower the activity of the electron-transport chain, leading to a reduction in ATP synthase activity. This may explain the >20-fold increase in accumulation of pyrophosphate that occurs in the presence of the hypovirus.

Additionally, there was considerable disruption of polyamine metabolism in infected mycelium, with spermidine levels elevated in excess of 50-fold. Other compounds that feed into spermidine biosynthesis are also elevated, including cystathionine, homocysteine and methionine. These data are also consistent with transcriptional profiling results, which showed elevated expression of genes related

to the synthesis of these compounds (Allen *et al.*, 2003). Disruption of polyamine synthesis is potentially of great interest, since spermidine has been shown to be important in the development of *Aspergillus nidulans* (Jin *et al.*, 2002) and *Sclerotinia sclerotiorum* (Pieckenstain *et al.*, 2001). Given the developmental impairment caused by hypovirus infection of *C. parasitica*, this finding warrants further investigation. Furthermore, oxidized polyamines have been shown to have antiviral activity (reviewed by Bachrach, 2007) and it is possible that such a response from *C. parasitica* implies a polyamine-based antiviral response that has not been previously observed in fungal systems.

Concluding remarks

We have applied a novel approach to assess the metabolic state of a fungal colony utilizing highly accurate gas analysis methods. The experiments reported here not only provide the first data on the metabolic rate of *C. parasitica* and the consequences of hypovirus infection, but also, to our knowledge, represent the first study of the age-related metabolic changes that occur in small regions of single filamentous fungal colonies grown on solid medium. We anticipate that besides providing a novel metric for measuring the impact of mycoviruses on fungal growth, this approach can also be easily applied to investigations of metabolism occurring in the mycelium of other fungi. Our metabolomic analysis, the first of its kind on this organism, has illustrated the tremendous changes in cellular physiology that occur in response to the hypovirus and has both supported our physiological observations and highlighted intriguing parallels with previous microarray analyses, while also indicating potential areas for further study.

ACKNOWLEDGEMENTS

The authors wish to thank John Gustafson and Glenn Kuehn for helpful discussions. The work was supported in part by a NIH MBRS-RISE grant (R25GM061222) undergraduate research fellowship (to T.A.L.), National Science Foundation award MCB-0718735 (to A.L.D.) and National Cancer Institute MSI CCP NCI U56 CA96286 (to W.A.V.V.).

REFERENCES

- Allen, T. D. & Nuss, D. L. (2004). Linkage between mitochondrial hypovirulence and viral hypovirulence in the chestnut blight fungus revealed by cDNA microarray analysis. *Eukaryot Cell* **3**, 1227–1232.
- Allen, T. D., Dawe, A. L. & Nuss, D. L. (2003). Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypoviruses. *Eukaryot Cell* **2**, 1253–1265.
- Bachrach, U. (2007). Antiviral activity of oxidized polyamines. *Amino Acids* **33**, 267–272.
- Bideaux, C., Alfenore, S., Cameleyre, X., Molina-Jouve, C., Uribealarea, J. L. & Guillouet, S. E. (2006). Minimization of glycerol production during the high-performance fed-batch ethanolic fermentation process in *Saccharomyces cerevisiae*, using a metabolic model as a prediction tool. *Appl Environ Microbiol* **72**, 2134–2140.
- Buck, K. (1986). *Fungal Virology – an Overview*. Boca Raton, FL: CRC Press.
- Carlisle, M. J., Watkinson, S. C. & Gooday, G. W. (2001). *The Fungi*, 2nd edn. San Diego: Academic Press.
- Chen, B., Choi, G. H. & Nuss, D. L. (1994). Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. *Science* **264**, 1762–1764.
- Choi, G. H. & Nuss, D. L. (1992). Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. *Science* **257**, 800–803.
- Cochrane, V. W. (1958). *Physiology of Fungi*. New York: Wiley.
- Crabtree, H. G. (1929). Observations on the carbohydrate metabolism of tumours. *Biochem J* **23**, 536–545.
- Dawe, A. L. & Nuss, D. L. (2001). Hypoviruses and chestnut blight: exploiting viruses to understand and modulate fungal pathogenesis. *Annu Rev Genet* **35**, 1–29.
- De Deken, R. H. (1966). The Crabtree effect: a regulatory system in yeast. *J Gen Microbiol* **44**, 149–156.
- Dixon, M. (1952). *Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes*, 3rd edn. London: Cambridge University Press.
- Ferreira, M. E., Colombo, A. L., Paulsen, I., Ren, Q., Wortman, J., Huang, J., Goldman, M. H. & Goldman, G. H. (2005). The ergosterol biosynthesis pathway, transporter genes, and azole resistance in *Aspergillus fumigatus*. *Med Mycol* **43** (Suppl 1), S313–S319.
- Fleet, G. H. & Heard, G. M. (1993). Yeast growth during fermentation. In *Wine Microbiology and Biotechnology*, pp. 27–54. Chur, Switzerland: Harwood Academic Publishers.
- Foster, J. W. (1949). *Chemical Activities of Fungi*. New York: Academic Press.
- Gentile, A. C. (1954). Carbohydrate metabolism and oxalic acid synthesis by *Botrytis cinerea*. *Plant Physiol* **29**, 257–261.
- Gessaman, J. A. & Nagy, K. A. (1988). Energy metabolism: errors in gas-exchange conversion factors. *Physiol Zool* **61**, 507–513.
- Gheshlaghi, R., Scharer, J. M., Moo-Young, M. & Douglas, P. L. (2007). Metabolic flux analysis for optimizing the specific growth rate of recombinant *Aspergillus niger*. *Bioprocess Biosyst Eng* **30**, 397–418.
- Golinski, M. R., Boecklen, W. J. & Dawe, A. L. (2008). Two-dimensional fractal growth properties of the filamentous fungus *Cryphonectria parasitica*: the effects of hypovirus infection. *J Basic Microbiol* **48**, 426–429.
- Hansen, D. R., Van Alfen, N. K., Gillies, K. & Powell, W. A. (1985). Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. *J Gen Virol* **66**, 2605–2614.
- Hawker, L. E. (1950). *Physiology of Fungi*. London: Oxford University Press.
- Jewett, M. C., Hofmann, G. & Nielsen, J. (2006). Fungal metabolite analysis in genomics and phenomics. *Curr Opin Biotechnol* **17**, 191–197.
- Jin, Y., Bok, J. W., Guzman-de-Pena, D. & Keller, N. P. (2002). Requirement of spermidine for developmental transitions in *Aspergillus nidulans*. *Mol Microbiol* **46**, 801–812.
- Koffler, H., Emerson, R. L., Perlman, D. & Burris, R. H. (1945). Chemical changes in submerged penicillin fermentations. *J Bacteriol* **50**, 517–548.
- Lin, H., Lan, X., Liao, H., Parsley, T. B., Nuss, D. L. & Chen, B. (2007). Genome sequence, full-length infectious cDNA clone, and mapping of viral double-stranded RNA accumulation determinant of hypovirus CHV1-EP721. *J Virol* **81**, 1813–1820.
- McCabe, P. M. & Van Alfen, N. K. (2001). Molecular basis of symptom expression by the *Cryphonectria* hypovirus. In *dsRNA Genetic Elements: Concepts and Applications in Agriculture, Forestry, and Medicine*, pp. 125–144. Edited by S. M. Tavantzis. Boca Raton, FL: CRC Press.
- Melvin, R. G., Van Voorhies, W. A. & Ballard, J. W. (2007). Working harder to stay alive: metabolic rate increases with age in *Drosophila simulans* but does not correlate with life span. *J Insect Physiol* **53**, 1300–1306.
- Moss, B. J., Kim, Y., Nandakumar, M. P. & Marten, M. R. (2008). Quantifying metabolic activity of filamentous fungi using a colorimetric XTT assay. *Biotechnol Prog* **24**, 780–783.
- Murayama, S. Y., Negishi, Y., Umeyama, T., Kaneko, A., Oura, T., Niimi, M., Ubukata, K. & Kajiwara, S. (2006). Construction and functional analysis of fatty acid desaturase gene disruptants in *Candida albicans*. *Microbiology* **152**, 1551–1558.
- Newhouse, J. R., Hoch, H. C. & MacDonald, W. L. (1983). The ultrastructure of *Endothia parasitica*. Comparison of a virulent with a hypovirulent isolate. *Can J Bot* **61**, 389–399.
- Nuss, D. L. (2005). Hypovirulence: mycoviruses at the fungal–plant interface. *Nat Rev Microbiol* **3**, 632–642.
- Pedneault, K., Angers, P., Gosselin, A. & Tweddell, R. J. (2006). Fatty acid composition of lipids from mushrooms belonging to the family Boletaceae. *Mycol Res* **110**, 1179–1183.
- Pieckenstain, F. L., Garriz, A., Chornomaz, E. M., Sanchez, D. H. & Ruiz, O. A. (2001). The effect of polyamine biosynthesis inhibition on growth and differentiation of the phytopathogenic fungus *Sclerotinia sclerotiorum*. *Antonie Van Leeuwenhoek* **80**, 245–253.
- Shu, P. (1953). Fermentation, oxygen uptake in shake flask fermentations. *J Agric Food Chem* **1**, 1119–1123.
- Thomson, J. M., Gaucher, E. A., Burgan, M. F., De Kee, D. W., Li, T., Aris, J. P. & Benner, S. A. (2005). Resurrecting ancestral alcohol dehydrogenases from yeast. *Nat Genet* **37**, 630–635.
- Van Voorhies, W. A. & Ward, S. (1999). Genetic and environmental conditions that increase longevity in *Caenorhabditis elegans* decrease metabolic rate. *Proc Natl Acad Sci U S A* **96**, 11399–11403.

Van Voorhies, W. A., Khazaeli, A. A. & Curtsinger, J. W. (2004). Testing the “rate of living” model: further evidence that longevity and metabolic rate are not inversely correlated in *Drosophila melanogaster*. *J Appl Physiol* **97**, 1915–1922.

Van Voorhies, W. A., Melvin, R. G., Ballard, J. W. & Williams, J. B. (2008). Validation of manometric microrespirometers for measuring oxygen consumption in small arthropods. *J Insect Physiol* **54**, 1132–1137.

Walsberg, G. & Wolf, B. (1995). Variation in the respiratory quotient of birds and implications for indirect calorimetry using measurements of carbon dioxide production. *J Exp Biol* **198**, 213–219.

Edited by: N. P. Keller