Colony sectorization of *Metarhizium anisopliae* is a sign of ageing

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Spontaneous phenotypic degeneration resulting in sterile sectors is frequently observed when culturing filamentous fungi on artificial medium. Sterile sectors from two different strains of the insect pathogenic fungus *Metarhizium anisopliae* were investigated and found to contain reduced levels of cAMP and destruxins (insecticidal peptides). Microarray analysis using slides printed with 1730 clones showed that compared to wild-type, sterile sectors down-regulated 759 genes and upregulated 27 genes during growth in Sabouraud glucose broth or on insect cuticle. The differentially expressed genes are largely involved in cell metabolism (18.8%), cell structure and function (13.6%) and protein metabolism (8.8%). Strong oxidative stress was demonstrated in sectorial cultures using the nitro blue tetrazolium assay and these cultures show other syndromes associated with ageing, including mitochondrial DNA alterations. However, genes involved in deoxidation and self-protection (e.g. heat-shock proteins, HSPs) were also upregulated. Further evidence of physiological adaptation by the degenerative sectorial cultures included cell-structure reorganization and the employment of additional signalling pathways. In spite of their very similar appearance, microarray analysis identified 181 genes differentially expressed between the two sectors, and the addition of exogenous cAMP only restored conidiation in one of them. Most of the differentially expressed genes were involved in catabolic or anabolic pathways, but the latter included genes for sporulation. Compared to the mammalian ageing process, sectorization in *M. anisopliae* showed many similarities, including similar patterns of cAMP production, oxidative stress responses and the involvement of HSPs. Thus, a common molecular machinery for ageing may exist throughout the eukaryotes.

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INTRODUCTION

The ascomycete *Metarhizium anisopliae* is a widespread, soil-borne pathogen of insects, ticks and mites (Roberts & St Leger, 2004). Strains of this fungus are being developed for the control of pest species (Butt *et al.*, 2001). Like many other fungi (Morrow *et al.*, 1989; Kale & Bennett, 1992; Li *et al.*, 1994), it frequently shows culture degeneration (sterile sectorization): a loss or reduction in sporulation and virulence with a fluffy-mycelium type growth. Fungal culture degeneration is usually irreversible and inheritable, and can result in great commercial losses (Li *et al.*, 1994; Horgen *et al.*, 1996; Ryan *et al.*, 2003).

Abbreviations: COX, cytochrome c oxidase; Dtx, destruxin; HSP, heat-shock protein; NBT, nitro blue tetrazolium; ROS, reactive oxygen species; RT-PCR, real-time PCR; SAM test, significance analysis of microarray test.

Microarray data demonstrating the transcriptional variations of *Metarhizium anisopliae* sectors grown in different media are available in Supplementary Table S1 with the online version of this paper.

Apart from the reports of secondary metabolite decline in degenerative fungal cultures (Wing *et al.*, 1995; Guzman-de-Pena & Ruiz-Herrera, 1997; Ryan *et al.*, 2002; Kale *et al.*, 2003), little information is available to explain fungal culture instability at the molecular level. However, genomic DNA methylation was reported in a sector of *Fusarium oxysporum* after successive subculturing (Kim, 1997). In another case, fungal morphological instability was linked to dsRNA virus infection (Dawe & Nuss, 2001).

In this study, the spontaneous sterile sectors from two genetically distinct *M. anisopliae* strains were subcultured and characterized in comparison with wild-type parents. Microarray analysis using slides printed with 1730 *M. anisopliae* genes (Wang *et al.*, 2005) demonstrated that the degenerative sectors are under strong oxidative stress and show signs of ageing/senescence. Since conidiation in plant-pathogenic fungi correlates with cAMP levels (e.g. Adachi & Hamer, 1998), we also measured intracellular cAMP in the *M. anisopliae* sectors and attempted to rescue wild-type phenotypes using exogenous cAMP.

METHODS

Fungal cultures. The wild-type strains V275 and V245 were isolated from *Carpocapsa pomonella* (Lepidoptera: Tortricidae) and soil in Austria and Finland, respectively. These two strains exhibit chromosome-length polymorphism (Wang *et al.*, 2003) and differ in their destruxin profiles (Wang *et al.*, 2004). During culture maintenance on Sabouraud glucose agar (SDA, Difco), mycelia-type (non-sporulating) sectors from V275 (Fig. 1A) and V245 (Fig. 1B) were observed and transferred onto fresh SDA plates. Both sector cultures demonstrated similar stable white and sterile growth patterns even after successive subculturing on SDA (Fig. 1C), and were referred to as V275Sec and V245Sec, respectively. The sectors and wild-type cultures were used for further analysis after being subcultured five times (every 14 days) with mycelia from the edge of the colony.

Toxin analysis. The production of destruxins (Dtxs) by the sectors and parent cultures was investigated by HPLC, as described by Wang *et al.* (2003, 2004). Briefly, the analysis was conducted at a flow rate of 1 ml min⁻¹ with a HPLC system (Dionex) equipped with a C18 reverse-phase column (particle size, 5 µm; pore diameter, 120 Å; dimensions, 4.6 × 250 mm) and a UVD 340U diode-array detector. The amounts of Dtxs A, B and E were quantified by calibration with corresponding standards. Experiments were repeated twice with three replicates each.

cAMP assay. Intracellular cAMP levels of sectors and parent cultures were determined as described by Fillinger *et al.* (2002). Briefly, mycelia (1 g) harvested from 36 h cultures grown in Sabouraud glucose broth (SDB) were ground thoroughly under liquid nitrogen and suspended in 0.5 ml extraction buffer (50 mM Tris/HCl, 4 mM EDTA, pH 7.5). A 0.1 ml aliquot of suspension was used for the protein assay. The rest was boiled for 5 min, then centrifuged at 13 000 r.p.m. for 5 min. The cAMP concentration in the supernatant was determined on a microplate reader (Multiskan Ascent, Thermo Labsystem, Finland) using the cAMP enzyme immunoassay

kit (Sigma) according to the supplier's instructions. The concentrations were expressed as pmol (mg protein)⁻¹ (Fillinger *et al.*, 2002). The assays were conducted in triplicate from two batch cultures. In parallel experiments, exogenous cAMP (Sigma) was added to SDA plates to a final concentration of 10 mM to examine whether it could induce sporulation in sterile sector cultures.

cDNA microarray analysis. The conidia of the wild-type strains and the mycelia of the sector cultures were grown in SDB for 36 h. The mycelia were harvested and washed with sterile distilled water. The same amount of mycelia (2 g, wet weight) was then transferred for 24 h to: (1) 1% *Manduca sexta* cuticle medium buffered with 1 g KH₂PO₄ l⁻¹, 0.5 g MgSO₄ l⁻¹ and 10 mg FeSO₄ l⁻¹ (Wang & St Leger, 2005); (2) SDB or (3) SDB amended with 10 mM cAMP. Total RNA was extracted using an RNeasy Plant mini kit including a treatment with DNase I (Qiagen). The RNA from the corresponding wild-type culture was used as the reference sample. Hybridizations were conducted using the slides printed with 1730 cDNA clones from *M. anisopliae* var. *anisopliae* ARSEF 2575 and microarray data were analysed as described previously (Wang *et al.*, 2005; Wang & St Leger, 2005). A *t* test (Pan, 2002) was conducted to identify the genes whose expression varied significantly between the V275Sec and V245Sec groups. A *P* value was estimated based on the *t* distribution and the overall α was set at 0.05. Hybridizations were conducted with RNA from three independent experiments.

Real-time PCR (RT-PCR) validation. The extracted RNA (1 µg) from SDB and insect cuticle media was converted into cDNA for RT-PCR analysis using the anchored oligo-dT primer following the manufacturer's protocol (ABgene, Surrey, UK). Gene-specific primers were designed with an anticipated product size of approximately 200 bp to guarantee high amplification efficiency. The examined genes included those for subtilisins PR1A (M73795) and PR1B (U59484), the chymotrypsin CHY1 (AJ242735), the trypsin TRY1 (AJ242736) and the esterase STE1 (AJ251924), as they are involved in fungal virulence (Freimoser *et al.*, 2003). The primers designed

Fig. 1. Different types of culture colonies of *Metarhizium anisopliae* indicating the occurrence of sterile sectorization and the restoration of conidiation. (A) V275 colony with sector; (B) V245 colony with sector; (C) sterile subculture transferred from the sector of either V245 or V275; (D) partial restoration of conidiation of V245sec on 10 mM cAMP agar.

from the small subunit ribosomal gene (AF218207) of *M. anisopliae* were used as an internal control. PCR was conducted using a Bio-Rad iQ SYBR Green Supermix kit in a volume of 20 µl, including 1 µl 10 × diluted cDNA template and 0.25 pmol of each primer. The cycling parameters were programmed on a Bio-Rad iCycler iQ system. The relative expression ratio of each gene was calculated by calibration with the ribosomal gene, and the fold-change between the sector and wild-type was estimated. Each sample had three replicates and the whole experiment was repeated twice.

Confirmation of oxidative stress. To check if the sectors were under oxidative stress, the production of reactive oxygen species (ROS) was compared with the wild-type strains using the nitro blue tetrazolium (NBT) reduction assay (Lara-Ortiz *et al.*, 2003). The mycelia from 36 h SDB medium were vacuum-filtered for 20 min with a 0.3 mM NBT aqueous solution containing 0.3 mM NADPH to increase sensitivity. NBT, on reduction by ROS, formed a blue/purple formazan precipitate. Mycelial discs were mounted in 30 % glycerol (v/v) and examined in a light microscope.

Mitochondrial DNA (mtDNA) digestion analysis. Mitochondria were extracted from sectors and wild-type cultures using step gradients, based on the method of Lambowitz (1979), and resuspended in 450 µl lysis buffer (0.1 M Tris/HCl, pH 8.0, 1.0 % SDS, 2 % Triton X-100, v/v, 10 mM EDTA, 0.1 M NaCl). The samples were incubated at 65 °C for 30 min and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was precipitated with one volume of cold 2-propanol for 30 min and centrifuged at 20 000 g for 10 min. Pellets of mtDNA were washed with 70 % ethanol, air-dried and resuspended in Tris/EDTA buffer. mtDNA was digested with *Hind*III and the methylation-sensitive enzyme *Hpa*II to determine if any mtDNA rearrangement/methylation had occurred in the sterile cultures.

RESULTS

Biological and physiological characteristics of the sector cultures

Both *M. anisopliae* V275 and V245 spontaneously produced sectors on SDA (Fig. 1A, B). The stable sectors V275Sec and V245Sec failed to produce aerial conidia on SDA plates (Fig. 1C), 1 % insect-cuticle agar or SDA amended with 1 % insect cuticle, and secreted significantly ($P < 0.01$) lower levels of Dtx A, B and E than the parent cultures (Table 1). Immunoassays of intracellular cAMP levels revealed that V275Sec ($P = 2.04 \times 10^{-8}$) and V245Sec ($P = 0.0047$) also had significantly reduced levels of cAMP compared with the wild-type parent. Incorporation of cAMP into SDA restored conidia production in sterile culture of V245Sec (Fig. 1D),

but not V275Sec. However, V245Sec still produced significantly fewer conidia per unit area than the wild-type parent ($P = 0.0085$) 20 days post-inoculation.

Large-scale gene down-regulation in the sector cultures

Microarray analysis revealed that extensive down-regulation of genes occurred during sectorization (Fig. 2A). From 1730 printed clones, the significance analysis of microarray (SAM) test identified 786 differentially expressed genes, of which 759 were down-regulated compared to wild-type cultures. Down-regulated genes were present in all the major functional categories previously classified (Wang *et al.*, 2005), but proportionally the greatest number were in cell metabolism (18.8 %), cell structure and function (13.6 %), and protein metabolism (8.8 %). Examples include regulatory modulators, transcription factors, translation factors and virulence factors, such as subtilisins (Fig. 2A). However, 38.7 % of genes of unknown function were also down-regulated (Fig. 2B). Consistent with the results of the intracellular cAMP assay, the transcription of adenylate cyclase (*Acy*, AJ251971) is down-regulated in both sectors when grown in SDB (7.5-fold in V275Sec and 3.3-fold in V245Sec) (Supplementary Table S1).

Stress-response and respiratory genes

Of the 27 genes differentially upregulated in both sectors, nine encode HSPs (Table 2), indicating that the sectors experienced strong stress responses. Two HSP90 cognates of *Metarhizium* AJ274186 and CN809267 are highly similar to *Podospora anserina* and *Aspergillus nidulans* heterokaryon incompatibility regulatory component Mod-E, respectively (Loubradou *et al.*, 1997, 1999). Several of the remaining clones implement low-efficiency non-respiratory pathway(s) during catabolism, and are thus indicative of mitochondrial respiratory deficiency in the sterile cultures. These components include pyruvate decarboxylase (AJ274332), xylulose-5-phosphate phosphoketolase (Xpk, CN808491), alcohol dehydrogenase (CN808912), acyl-CoA dehydrogenase (CN808313) and non-respiratory P450 cytochromes (AJ274003 and CN808111). Pyruvate decarboxylase converts pyruvate into acetaldehyde. The latter is also produced from ethanol (a product of Xpk) by alcohol dehydrogenase (Flikweert *et al.*, 1996). Upregulation of

Table 1. Comparison of destruxin (Dtx) production and endocellular cAMP level between wild-type and sector cultures

Values are mean $\pm$ SD.

Culture	Dtx A (mg l ⁻¹)	Dtx B (mg l ⁻¹)	Dtx E (mg l ⁻¹)	cAMP (pmol mg ⁻¹)
V275	29.80 $\pm$ 2.21	16.32 $\pm$ 2.29	45.74 $\pm$ 0.33	82.08 $\pm$ 7.09
V275Sec	2.37 $\pm$ 0.51	1.70 $\pm$ 0.32	8.45 $\pm$ 1.02	26.87 $\pm$ 1.35
V245	8.16 $\pm$ 1.84	4.92 $\pm$ 1.00	11.41 $\pm$ 1.36	34.42 $\pm$ 0.93
V245Sec	0	0	1.70 $\pm$ 0.22	27.83 $\pm$ 1.46

Fig. 2. Transcriptional profiling and characterization. (A) Entire transcriptional profiles of the sector cultures, (B) functional distribution of significantly differentiated genes and (C) clusters of genes that varied in expression between cuticle-containing medium and SDB, or between the sectors. Gene functional categories in (A) are as described previously (Wang *et al.*, 2005). Cut., cuticle.

Table 2. Differentially upregulated genes identified by the SAM test

A few other genes with different expression profiles are shown.

Accession no.	Fold change of V275Sec			Fold change of V245Sec			Best match	E value	Description	Organism
	Cuticle	SDB	cAMP	Cuticle	SDB	cAMP				
AJ273662	7.64	2.85	5.31	6.82	5.20	3.92	AAA33589	8.91E-32	30 kDa heat-shock protein	<i>Neurospora crassa</i>
AJ273036	6.17	2.18	4.26	7.17	3.88	3.34	AAR36902	1.00E-16	Small heat-shock protein	<i>Chaetomium globosum</i>
AJ273210	1.55	1.13	1.86	1.47	1.82	1.57	S55900	3.02E-31	DNAJ-like (Hsp40) protein homologue	<i>Schizosaccharomyces pombe</i>
AJ274186	2.96	1.53	2.59	2.32	3.28	2.85	AAB97626	1.00E-136	Mod-E, heat-shock protein of the Hsp90 family	<i>Podospira anserina</i>
CN809338	2.33	1.74	5.10	2.55	5.65	4.41	P31540	6.00E-83	Heat-shock protein HSP98	<i>Neurospora crassa</i>
CN809267	2.02	1.44	2.38	1.61	3.73	2.79	EAA59007	1.00E-87	Mod-E, heat-shock protein 90 homologue	<i>Aspergillus nidulans</i>
AJ273534	1.96	1.87	3.41	3.99	3.89	2.89	AAA82183	1.00E-49	70 kDa heat-shock protein	<i>Neurospora crassa</i>
AJ274192	1.66	1.68	2.63	4.09	4.26	3.19	AAD08909	2.00E-39	Heat-shock protein 70	<i>Trichophyton rubrum</i>
CN809024	1.58	1.60	2.41	3.22	4.22	2.68	EAA70431	1.00E-108	Heat-shock 70 kDa protein (HSP70)	<i>Gibberella zeae</i> PH-1
CN808249	3.79	2.53	3.60	1.09	1.20	1.58	AAF66098	7.00E-95	Putative cyanide hydratase	<i>Leptosphaeria maculans</i>
AJ274332	1.64	4.08	5.41	1.24	4.04	3.41	AAD16178	1.00E-59	Pyruvate decarboxylase	<i>Aspergillus oryzae</i>
CN808912	1.04	4.36	3.81	1.19	2.14	2.30	XP_328193	1.00E-122	Alcohol dehydrogenase I	<i>Neurospora crassa</i>
CN808313	3.63	1.61	2.18	1.41	2.81	2.36	NP_948628	2.00E-62	Acyl-CoA dehydrogenase	<i>Rhodospseudomonas palustris</i>
AJ274003	1.02	1.85	2.37	1.12	1.83	1.58	CAA06156	1.00E-36	Cytochrome P450 monooxygenase	<i>Zea mays</i>
CN808111	12.27	8.55	6.12	4.67	1.02	1.23	BAA11408	6.00E-99	Cytochrome P450nor1	<i>Cylindrocarpus lichenicola</i>
AJ272720	1.70	1.73	2.51	1.03	2.58	2.58	CAA28860	3.02E-25	Cytochrome c ₁ , haem protein precursor	<i>Neurospora crassa</i>
AJ274200	2.07	1.29	2.27	4.14	3.11	2.22	CAC18796	2.00E-42	Proline oxidase	<i>Emmericella nidulans</i>
CN808016	1.96	3.79	4.37	1.04	3.20	3.48	CAD15482	1.00E-12	Putative signal peptide protein	<i>Ralstonia solanacearum</i>
CN808339	1.91	2.59	3.23	1.40	2.41	2.61	AAK69534	1.00E-40	CAP20-like protein	<i>Blumeria graminis</i>
CN808315	3.61	4.38	6.17	9.50	14.57	6.08	T49444	2.00E-24	Related to lustrin A	<i>Neurospora crassa</i>
AJ274277	4.74	16.29	12.82	2.81	20.16	20.72	CAB97468	1.00E-25	Related to spore-coat protein SP96 precursor	<i>Neurospora crassa</i>
CN808142	1.23	2.25	2.11	1.44	1.65	1.65	BAB33421	4.00E-60	Putative senescence-associated protein	<i>Pisum sativum</i>
CN808094	2.83	1.31	1.30	1.20	1.12	1.23	NP_588321	2.00E-32	Putative Zn-protease	<i>Schizosaccharomyces pombe</i>
CN808832	1.11	1.47	1.67	1.08	1.55	1.19	AAA58585	2.00E-09	Haemolysin	<i>Acanthamoeba polyphaga</i>
CN807921	3.29	3.64	2.19	1.57	1.10	1.12	EAA68216	3.00E-47	Hypothetical protein FG10442	<i>Gibberella zeae</i> PH-1
CN808510	2.27	2.63	3.98	1.65	3.57	3.39	EAA70132	4.00E-24	Hypothetical protein FG09906	<i>Gibberella zeae</i> PH-1
CN808392	2.04	2.65	3.83	1.40	4.17	3.24	Q06177	5.00E-3	STF2-like protein YLR327c	<i>Saccharomyces cerevisiae</i>
CN808510	2.27	2.63	3.98	1.65	3.57	3.39	P16965	1.00E-3	ATPase stabilizing factor 15 kDa protein	<i>Saccharomyces cerevisiae</i>
AJ273832*	1.19	1.24	1.78	-1.22	1.17	1.41	AAH63355	2.00E-12	Thioredoxin domain containing protein 5	<i>Xenopus tropicalis</i>
AJ272796*	-1.29	1.64	1.33	-1.92	1.68	1.50	AAD02822	1.00E-124	Mitogen-activated protein kinase kinase	<i>Cryphonectria parasitica</i>
CN808780*	1.30	1.48	2.08	-1.17	1.09	1.73	CAB71146	9.00E-12	Putative serine/threonine protein kinase	<i>Homo sapiens</i>
CN808491*	3.43	4.30	5.05	-3.30	2.49	2.53	NP_699578	0	Xylulose-5-phosphate phosphoketolase	<i>Brucella suis</i>
CN809572*	2.24	1.18	2.45	-2.12	1.74	1.97	AAP68979	8.00E-77	β -tubulin	<i>Gibberella zeae</i> PH-1
CN808948*	1.69	1.55	2.36	-1.06	1.41	1.60	NP_015428	1.00E-73	20S proteasome beta-type subunit Pre2p	<i>Saccharomyces cerevisiae</i>
AJ272756*	1.37	1.31	1.86	-1.18	1.12	1.45	CAA26308	5.01E-66	Cytochrome c reductase iron-sulfur subunit	<i>Neurospora crassa</i>
CN808936*	-1.31	1.29	1.87	-1.50	1.02	1.39	AAD53944	2.00E-08	25 kDa cell death inducing protein elicitor	<i>Pythium aphanidermatum</i>

*Not identified as positively regulated genes in the SAM test, but showing upregulation in both sectors when the fungi were grown in SDB.

acyl-CoA dehydrogenase, but not acetyl-CoA synthetase, in both sectors suggests that cytosolic acetyl-CoA will be mainly used for lipid biosynthesis, rather than being transported to mitochondria for the TCA cycle (Flikweert *et al.*, 1996). This is also consistent with upregulation of a C-4 methyl sterol oxidase (CN808291) and a fatty acid hydroxylase (CN808272) (Fig. 2C, Table 2). The upregulation of a coproporphyrinogen oxidase (CPO) (CN808455) also implies that the sterile cultures are under strong oxidative stress, as it is an essential enzyme involved in the haem biosynthetic pathway (an alternative oxidative pathway) (Amillet *et al.*, 1995; Rosenfeld & Beauvoit, 2003).

Ageing/senescence genes

A second feature of the sterile cultures is upregulation of several genes previously reported as being involved in ageing/senescence in *Podospora*, *Aspergillus* and *Neurospora* species (Griffiths, 1992; Bertrand, 2000). These include a 'senescence-associated' protein (CN808142), a haemolysin (CN808832) and a zinc-dependent protease (CN808094). The down-regulation of cytochrome *c* oxidase (COX, AJ272726) (Supplementary Table S1) observed in both V245Sec and V275Sec also occurred in non-functional mitochondria from senescent *P. anserina* (Borghouts *et al.*, 1997). In senescent *Neurospora* strains with mitochondrial mutations, cytochrome *c* is in large excess (Griffiths, 1992), and its release from mitochondria activates caspase-9, the initiator of apoptosis (Sreedhar & Csermely, 2004). Thus, upregulation of cytochrome *c*₁ (AJ272720) in V245Sec and V275Sec (Table 2) is consistent with the established relationship between respiration, production of ROS and fungal lifespan (Dufour *et al.*, 2000; Osiewicz & Scheckhuber, 2002). The upregulation of genes encoding a haemolysin, a Zn protease and a proteasome component (CN808948) (Table 2) suggests that sector production is connected with autophagy (Klionsky & Emr, 2000; Shintani & Klionsky, 2004), which is also connected with ageing (Camougrand *et al.*, 2004).

Genes involved in cell-structure reorganization

Patterns of gene expression indicate extensive cell-structure reorganization during sectorization. Down-regulated genes included those for the hydrophobins (AJ274156 and CN809178, Fig. 2A), and we observed that the sector cultures were easily wettable (data not shown), consistent with loss of hydrophobins (Kamp & Bidochka, 2002). Conversely, several structural genes were upregulated in sectors, including an orthologue of *Neurospora* lustrin A (CN808315), the spore-coat protein SP96 (AJ274277) and β -tubulin (CN809527) (Table 2). Upregulation of spore-coat protein SP96 suggests that the gene-expression profile leading to spore production is not completely blocked in sterile cultures. However, as well as being a structural component, SP96 helps coordinate cell-wall synthesis (Srinivasan *et al.*, 2000; Metcalf *et al.*, 2003), and an altered pattern of expression could thus contribute to defects in sporulation.

Genes involved in signal transduction

Sterile sectors may employ regulatory modulators of growth, metabolism and/or development that differ from the wild-type strains. Differentially upregulated genes included a proline oxidase (AJ274200) and a CAP20-like protein (CN808339) (Table 2). Endocellular proline levels inversely control cell division (Maggio *et al.*, 2002), and CAP20 has been found to play an important role in the control of development in *Colletotrichum gloeosporioides* (Hwang *et al.*, 1995). A mitogen-activated protein (MAP) kinase kinase (AJ272796) and a serine/threonine protein kinase (CN808780) were also upregulated in both sectors. The MAP kinase kinase of *Candida albicans* is essential to the oxidative stress response (Arana *et al.*, 2005). Upregulation of AJ272796 indicates that the sectors were, at least in part, adapting to circumstances of oxidative stress.

Genes differentially expressed on insect cuticle and SDB

Genes specifically upregulated on insect cuticle but not in SDB include the chymotrypsins AJ273081 and AJ273663 (> fivefold for V275Sec and V245Sec). Wild-type *M. anisopliae* strains sharply upregulate proteases such as chymotrypsins, trypsin, carboxypeptidases and subtilisins in cuticle media (Freimoser *et al.*, 2003). In contrast, the sector cultures down-regulate subtilisins, a carboxypeptidase (AJ272919) and a trypsin-like protease (AJ274008) (Fig. 2C). Differential regulation of proteases by sectors suggests that they are under the control of different pathways and may possess other functions. Genes down-regulated on cuticle but upregulated in SDB compared to the wild-type include a catalase (CN808348) and a flavohaemoglobin (CN808105) that function in oxidative stress responses (Rosenfeld & Beauvoit, 2003). Upregulation of the carboxypeptidase (Fig. 2C), a homologue of yeast carboxypeptidase Y (CpY), in sectors grown in SDB is consistent with its putative role against the effects of oxidative damage (Martinez *et al.*, 2003).

Transcriptional differences between the two sector cultures in response to cAMP

Although there were no evident phenotypic differences between V275Sec and V245Sec, expression patterns suggest that they were physiologically quite distinct. Compared to V245Sec, V275Sec had higher expression levels of many genes during growth on insect cuticle or SDB $\pm$ cAMP (Figs 2A and 3A–C), and the *t* test identified 181 (~10% of total) that were differentially regulated. These were principally distributed in the cell metabolism (26.0%), protein metabolism (23.8%) and cell structure and function (13.3%) categories (Fig. 3D). Since the addition of cAMP could restore the conidiation ability of V245Sec but not V275Sec, the effects of cAMP on gene expression were compared between the sectors grown in different media (Fig. 4). Only a sporulation protein, SPO72 (AJ272728), was upregulated upon the addition of cAMP in both sectors.

Fig. 3. Transcriptional profiling and characterization. Differences between V275Sec (blue) and V245Sec (red) grown in (A) media containing insect cuticle, (B) SDB, (C) SDB amended with 10 mM cAMP. (D) Functional distribution of differentially regulated genes between V275Sec and V245Sec.

Deletion of *Spo72* blocks the initiation of sporulation in *Saccharomyces cerevisiae* (Briza *et al.*, 2002).

A cluster of genes was up- or down-regulated in one strain only. Thus, addition of cAMP led to significant repression of several genes in V275Sec that were not repressed in V245Sec (Fig. 4A, B). These include a glyoxal oxidase (CN809393), a β -1,3-exoglucanase (AJ273011), a cell-wall protein (CN808187) and a transmembrane protein (AJ274188). The upregulation of a glutathione *S*-transferase (CN808753, from -1 -fold to 2.6 -fold) and a flavohaemoglobin (AJ273710, from -2.2 -fold to 1.6 -fold) in V275Sec indicates that the addition of cAMP increased the detoxification and nitrosative stress response abilities of V275Sec (Liu *et al.*, 2000). However, in V245Sec, flavohaemoglobin AJ273710 was more highly upregulated (>6 -fold) in SDB with or without cAMP (Supplementary Table S1). The specific upregulation of a cell-death-resistant protein Cid1 homologue in V275Sec (AJ273722, from -1.7 -fold to 3.4 -fold) upon the addition of cAMP (Fig. 4A, C) implies that

V275Sec may be at increased risk of progressing into mitosis before the completion of DNA replication (Wang *et al.*, 1999). In addition, V275Sec upregulated a clathrin adapter complex medium chain (AJ27236) (Fig. 4C), involved in cargo capture and membrane-protein trafficking during endocytosis/autophagy (Huang *et al.*, 1999). This implies differences between sectors in their organelles/proteins autophagy programmes.

Genes specifically upregulated in V245Sec include a homologue of yeast Ssn6 (AJ274057, 2.8 -fold in V245Sec and -1.3 -fold in V275Sec) that is essential for derepression of yeast sporulation-specific proteins (Schultz & Carlson, 1987; Friesen *et al.*, 1997). Thus, the up-regulation of AJ274057 in V245Sec may be a precondition for restoring its ability to produce conidia. NADPH oxidase has been shown to be a source of ROS in *A. nidulans* (Lara-Ortiz *et al.*, 2003). The upregulation of a NADPH oxidase (CN807950) in V245Sec thus presented an additional oxidative pathway in V245Sec that is absent in V275Sec.

Fig. 4. Effect of cAMP on gene expression. Genes differently expressed by (A) V275Sec and (B) V245Sec during growth in SDB or SDB plus 10 mM cAMP and (C) clusters of genes that differ between V275Sec and V245Sec during growth in SDB plus cAMP.

Table 3. Fold-change comparison for selected genes between RT-PCR and microarray analysis

Gene	Fold change for:	
	RT-PCR	Microarray
<i>Pr1A</i>		
V275Sec	-6.39 ± 0.83	-2.35 ± 0.21
V245Sec	-7.25 ± 0.37	-2.31 ± 0.22
<i>Pr1B</i>		
V275Sec	-10.24 ± 1.12	-2.84 ± 0.35
V245Sec	-28.66 ± 2.34	-16.90 ± 0.96
<i>Chy1</i>		
V275Sec	10.58 ± 0.82	5.47 ± 0.65
V245Sec	1.50 ± 0.25	5.43 ± 0.23
<i>Try1</i>		
V275Sec	-3.63 ± 0.63	-1.64 ± 0.18
V245Sec	-5.49 ± 0.24	-1.16 ± 0.26
<i>Ste1</i>		
V275Sec	-4.71 ± 0.21	-1.43 ± 0.09
V245Sec	-6.50 ± 0.25	-1.49 ± 0.31

RT-PCR validation

The fold-changes in microarray analysis had a significant correlation ($r=0.94$, $P<0.01$) with the results from RT-PCR examination (Table 3), confirming a high level of accuracy for microarray analysis. However, consistent with our previous analysis, the expression ratios were

underestimated in microarray analysis compared with RT-PCR (Freimoser *et al.*, 2005).

Confirmation of oxidative stress in the sector cultures

The microarray data indicating that both sector cultures are under strong oxidative stress were confirmed by the NBT reduction assay (Fig. 5). Also, consistent with the microarray analysis, which showed that the addition of exogenous cAMP did not result in down-regulation of oxidases, the sterile cultures were similar in their abilities to reduce NBT to form purple/blue formazan in either SDB or SDB amended with 10 mM cAMP (Fig. 5B, C). The production of ROS is concentrated in mycelial tips (Fig. 5E, F) compared to the older mycelia (Fig. 5G). The same results were obtained using the cultures grown on solid SDA medium.

mtDNA alterations in the sterile cultures

Enzyme digestion of mtDNA resulted in similar patterns but with slight band shifting between the wild-type and the sterile cultures (Fig. 6). The V275Sec mtDNA bands were slightly larger and the V245Sec bands smaller than their wild-type counterparts. This rules out dramatic mtDNA rearrangements, such as large fragment deletions or the transposable-element insertions observed in senescent cultures of *Neurospora* (Seidel-Rogol *et al.*, 1989) and *Podospora* (Borghouts *et al.*, 2000). The band shifting implies the existence of smaller mtDNA modifications/

Fig. 5. Sector cultures are under strong oxidative stress. (A) Wild-type culture vacuum-filtered with NBT solution for 20 min; (B) sector culture grown in SDB filtered with NBT; (C) sector culture grown in SDB plus 10 mM cAMP filtered with NBT. (D–F) Microscopic images of (D) the wild-type mycelia, (E) sector grown in SDB and (F) in SDB plus cAMP, in each case filtered with NBT and mounted in 30 % glycerol. (G) ‘Old’ sector mycelia have no NBT-reducing activity. Bar, 10 μ m. Note that both sectors and their wild-type cultures show the same sample profile; herein only V275 and V275Sec images are presented for simplicity.

Fig. 6. Restriction enzyme analysis of mitochondrial DNA digested with (A) *Hind*III or (B) the CpG methylation-sensitive enzyme *Hpa*II. M, 1 kb DNA ladder; W, wild-type; S, sector culture. DNA was separated on a 1.0% agarose gel.

alterations; these will be the subject of further studies. The mtDNA from both the wild-type and sterile cultures was digested in similar fashion with *Hpa*II (Fig. 6B), indicating that DNA methylation patterns had not altered.

DISCUSSION

While phenotypic deterioration of fungi is notorious, little is known concerning its molecular mechanisms. In this study, we demonstrate that sectorial degenerative cultures of the filamentous fungus *M. anisopliae* are under strong oxidative stress and show syndromes regarded as typical of ageing, including the upregulation of cytochrome *c* and the down-regulation of COX. The down-regulation of COX as well as an NADH dehydrogenase (AJ273910) suggests that the respiratory chain in the sectors is impaired or not fully functional (e.g. Joseph-Horne *et al.*, 2001). In addition, the activity of COX is copper-dependent and cellular copper homeostasis is a major cause of fungal senescence (Borghouts *et al.*, 2000, 2001). Consistent with this, a low-affinity copper transporter, CN808791, is down-regulated in both sectors in all the growth media, and a high-affinity copper transporter (AJ273995) is also down-regulated during growth in SDB (Supplementary Table S1). In mammalian cells/tissues, the relationship between cAMP and ageing has been widely reported (e.g. Bowles, 1998; Gerhold *et al.*, 2005). In this respect, common molecular machinery that results in ageing may occur throughout eukaryotes and the reduction in cAMP accumulation in the sectors suggests a new area for research on fungal ageing.

Overall, sectors were characterized by extensive down-regulation of gene expression. Fig. 7 summarizes the physiological alterations predicted by these changes in gene expression. The increase of ROS production in the sterile

cultures is expected to trigger/mediate global physiological changes. ROS accumulation could damage proteins, DNA and membranes, and is a major determinant of lifespan (e.g. Sohal & Weindruch, 1996; Balaban *et al.*, 2005). However, the degenerative *M. anisopliae* cultures do not die even after being transferred for more than 50 generations. This could result from the protection imparted by fungal deoxidation responses such as catalase, as well as from HSPs. This may be why sectorization increases the lifespan of basidiomycetous fungi (Gramss, 1991).

The trigger(s) for fungal degenerative sectorization and accumulation of intracellular ROS were not determined. Sectorization could trigger oxidative stress in cells, or conversely the accumulation of cellular ROS could induce sectorization. The factors which have been reported to be involved in sectorization, such as RNA virus infection, were not evident in the wild-type or sector cultures (data not shown). Methylation of mtDNA was not observed in *M. anisopliae* sectors. Fungal cultures undergoing senescence may demonstrate mtDNA rearrangements and deletions (Griffiths, 1992; Bertrand, 2000), but similarly severe mtDNA reorganization was not found in *M. anisopliae*. Lack of such impairment is consistent with the sectors' displaying signs of ageing without actually dying.

The involvement of HSPs has also been well documented in mammalian cell senescence (reviewed by Sreedhar & Csermely, 2004) but has not been reported before in senescent fungi. HSPs as 'stress proteins' are induced by a variety of stressful stimuli in fungi, including changes in membrane fluidity (Maresca & Kobayashi, 1993). The changes predicted by gene-expression patterns in major cell-wall components such as hydrophobins could easily impact on the membrane structure. More directly, a C-4 methyl

Fig. 7. Schematic presentation of physiological responses during fungal sectorization. Up arrows, increase; down arrows, decrease.

sterol oxidase is upregulated in sterile cultures. This gene is involved in the biosynthesis of ergosterol, an essential component contributing to the fluidity of fungal-cell plasma membranes (Bard *et al.*, 1996).

The sterile cultures upregulated three major classes of HSP: small HSPs (AJ273036, AJ273662 and AJ273210), HSP70 cognates (AJ273534, AJ274192 and CN80809024) and HSP90 cognates (AJ274186, CN809267 and CN809338) (Table 2). Protection by HSP chaperones includes prevention of protein aggregation, refolding of misfolded proteins, retention of mitochondrial integrity and blocking DNA damage (Sreedhar & Csermely, 2004). In addition, some HSPs are also involved in signal transduction to regulate morphogenic variation (Rutherford, 2003). For example, differential expression of an HSP70 gene has been observed during transition from the mycelial to the yeast form in the human pathogenic fungus *Paracoccidioides brasiliensis* (Da Silva *et al.*, 1999). Consequently, HSPs could be involved in the morphological change from dense-sporulating to fluffy-mycelial type. On the other hand, *Metarhizium* HSP90 cognates AJ274186 and CN809338 are the homologues of vegetative incompatibility suppressors in *Podospora* and *Aspergillus*, respectively. Together with the down-regulation of a homologue to *Neurospora* Het-C (CN808914), the data imply that the mycelia of sterile cultures lose tight control of non-self recognition (Sarkar *et al.*, 2002; Glass & Kaneko, 2003). This could facilitate the transmission and proliferation of dysfunctional

mitochondria throughout the mycelial network, as observed in other senescent fungi (Debets *et al.*, 1994; Bertrand, 2000).

Heterokaryon-incompatibility-triggered cell death can induce autophagic pathways to degrade cytosolic proteins and organelles (Pinan-Lucarre *et al.*, 2003). The upregulation of autophagy-associated proteins (CN808094 and CN808832, Table 2) in the sterile cultures indicates that autophagic pathways were triggered in the sectorial *Metarhizium* cultures (Fig. 7). The involvement of HSPs in chaperone-mediated autophagy (Salvador *et al.*, 2000; Agarraberes & Dice, 2001) is consistent with HSPs being involved in multiple functions in the sterile cultures.

Despite similar visible phenotypes, microarray data revealed that 181 of 1730 genes were differentially expressed between the two sector cultures, and the addition of exogenous cAMP only rescued sporulation in V245Sec. The wild-type strains V275 and V245 have previously been revealed to have chromosome-length polymorphisms (Wang *et al.*, 2003) and to differ in Dtx production (Wang *et al.*, 2004). This study shows that there are also significant differences in transcriptional responses between the two strains. Some genes, such as *fluffy*, the hydrophobin-encoding gene *eas* and the clock-controlled gene *cgc-2*, are essential for conidiation (Rermsamran *et al.*, 2005). Mutations in any of these genes could result in sterile cultures. However, in view of their high frequency of occurrence and the fact that V245Sec

conidiation can be rescued, it is unlikely that fungal sectorization is caused by spontaneous gene mutation.

Growth in host-related medium did not result in the recovery of wild-type characteristics by the sterile cultures, suggesting that 're-juvenilization' does not readily occur. These data may help to alleviate the long-standing dispute as to whether passage through insect hosts can (e.g. Hayden *et al.*, 1992) or cannot (Fargues & Robert, 1983) restore the virulence of attenuated strains of insect pathogenic fungi.

In conclusion, we demonstrated that extensive gene down-regulation occurred in sterile cultures of *M. anisopliae* in comparison with the wild-type strains, and that the sectors are under strong oxidative stress. Overall, fungal sectorization shows similar changes in transcriptional profiles to those of ageing. Besides the loss of conidiation ability, the degenerative cultures may reorganize their cell structure and employ additional signal-transduction pathways for physiological adaptation. The pathways involved in 'self-rescue' include components responsible for deoxidation, HSP chaperones that could protect protein and DNA from oxidative damage, and mechanisms for disposing of damaged proteins/organelles (Fig. 7). Similarities between the mammalian ageing process and fungal degenerative sectorization, such as cAMP production, oxidative stress and the involvement of HSPs, suggest that fungi may provide a good model for studies of ageing. Indeed, further studies are required to identify the trigger(s) for spontaneous sectorization/degeneration, the level of mitochondrial dysfunction and the extent to which this involves alterations in mtDNA as well as genomic DNA.

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