Environmental Control of the Respiratory System in the Dimorphic Fungus *Mucor rouxii*

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Spores of *Mucor rouxii* had low levels of respiratory activity partially sensitive to cyanide and salicylhydroxamic acid (SHAM). They contained a full set of cytochromes but in low concentrations, especially cytochromes *aa*₃. Aerobic growth induced a rapid increase in respiratory activity resistant to SHAM but fully sensitive to cyanide, and in the levels of cytochromes, mainly cytochromes *aa*₃. These processes were subject to catabolite repression. Incubation under anaerobic conditions, independent of cell morphology, favoured the persistence of SHAM-sensitive respiration, and the maintenance of low levels of *b* and *c* cytochromes. No cytochromes *aa*₃ were formed under these conditions. CO-difference spectra suggested the presence of cytochrome *o* in higher levels in aerobic than in anaerobic cells.

INTRODUCTION

Several *Mucor* species are characterized by their dimorphism, i.e. they are able to grow as mycelium or as a yeast-like form according to the prevailing growth conditions, notably O₂ tension and the type and concentration of the carbon source (see reviews by Bartnicki-Garcia, 1963; Sypherd *et al.*, 1978; Inderlied *et al.*, 1985; Ruiz-Herrera, 1985). In general, an anaerobic environment and high concentrations of fermentable carbon sources are associated with yeast-like growth, whereas mycelial growth requires aerobiosis and low concentrations of fermentable or non-fermentable carbon sources.

Storck & Morril (1971) isolated a respiratory mutant of *Mucor bacilliformis* which lacked both *b* and *a* cytochromes, and which failed to grow as mycelium under aerobic conditions. We have isolated a significant number of yeast monomorphic mutants from *M. bacilliformis* which are unable to grow on non-fermentable carbon sources. These mutants exhibit cyanide-resistant respiration and alterations in their cytochrome pattern (Ruiz-Herrera *et al.*, 1983). Other observations also link mitochondrial development to morphogenesis in *Mucor* species. Rogers *et al.* (1974) observed that a reduction in the oxygen tension led to the disappearance of the cytochrome *b* band from *Mucor genevensis*, concomitant with the development of yeast-like morphology. Addition of chloramphenicol favoured the yeast-like form in *M. rouxii* (Zorzopolus *et al.*, 1973), but not in *M. genevensis* (Rogers *et al.*, 1974). In both organisms the drug inhibited the expression of cyanide-resistant respiration.

Unfortunately, there are no systematic studies of the cytochrome composition of *Mucor* species grown under different conditions, and how this is related to the respiratory capacity of

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Abbreviation: SHAM, salicylhydroxamic acid.

this fungus. We have examined the cytochrome patterns and the development of respiratory capacity of *M. rouxii* during spore germination and outgrowth as either hyphae or yeast-like cells in order to examine their possible relationships with fungal morphology. Some comparative data on *M. bacilliformis* are also reported.

METHODS

Strains. *Mucor rouxii* IM 80 (ATCC 24905) and *Mucor bacilliformis* NRRL 2346 (ATCC 12830) were used.

Media and growth conditions. Strains were maintained on slants of YPG medium (Bartnicki-Garcia & Nickerson, 1962). Spores were obtained from 4-d-old (*M. rouxii*), or 7-d-old (*M. bacilliformis*) aerobic cultures grown at 28 °C (*M. rouxii*) or 24 °C (*M. bacilliformis*) in Roux bottles containing solid YPG medium. Spores were harvested with sterile distilled water, washed twice by low-speed centrifugation, resuspended in sterile distilled water and counted with a haemocytometer. When respiration or cytochrome spectra of ungerminated spores were measured, spores were harvested with a solution containing 100 µg cycloheximide ml⁻¹ and 200 µg chloramphenicol ml⁻¹, and washed and resuspended in a solution of the same composition. Freshly harvested spores (5×10^5 ml⁻¹) were inoculated in 1200 ml of the defined medium described by Bartnicki-Garcia & Nickerson (1962) with casein hydrolysate and different carbon sources, contained in 4 litre Erlenmeyer flasks, and incubated at 28 °C (*M. rouxii*) or 24 °C (*M. bacilliformis*) either aerobically or anaerobically. Aerobiosis was provided by sparging the cultures with air; anaerobiosis was provided by sparging with a mixture of N₂/CO₂ (70:30, v/v) or with N₂ alone. In the latter case, the N₂ was passed through a stainless steel tube containing copper filings heated at 500 °C. The gas was cooled by sparging in a water-trap maintained on ice, and passed through 50 ml of a solution containing 0.025 M-methyl viologen and 0.5 mg Na₂S₂O₄ ml⁻¹, in order to detect any contamination with O₂. Results from experiments in which the methyl viologen became oxidized (colourless), were discarded. Unless otherwise indicated *M. rouxii* was grown aerobically for 7–9 h and anaerobically for 13–20 h, and *M. bacilliformis* was grown aerobically for 13–14 h and anaerobically for 18–20 h.

Cell harvest and determination of respiratory activity. Cells were harvested by centrifugation and resuspended in one-tenth their volume of fresh medium. Respiration was measured with an oxygen electrode (YSI) in a 2.0 ml plastic chamber maintained at 25 or 30 °C for *M. bacilliformis* or *M. rouxii* respectively. Respiratory activity was calculated as µl O₂ consumed per 10⁷ cells or per mg protein per min. KCN (1.0 mM) or SHAM (1.23 mM) were tested as inhibitors of respiratory activity.

Cytochrome analysis. Cells were harvested by centrifugation and washed twice with a solution containing 100 µg cycloheximide ml⁻¹ and 200 µg chloramphenicol ml⁻¹. The cells were finally resuspended in 50 mM-Tris/HCl, pH 7.4, containing 5 mM-CaCl₂ and 5 mM-MgCl₂ (TCM buffer), and broken for 4 min (for 30 s periods) in a Braun homogenizer. Extracts were centrifuged at 1100 g for 5 min to remove whole cells and cell walls, and the supernatant was centrifuged in a 70 Ti Beckman rotor at 165000 g (r_{av}) for 50 min to sediment the mixed-membrane fraction; this was resuspended in TCM buffer containing 50% (v/v) glycerol and kept at –70 °C until used.

Difference spectra (reduced minus oxidized) were measured at either room temperature (1.0 cm path-length cuvettes) or at 70 K (2.0 mm path-length cuvettes) in a SLM-Aminco Midan II spectrophotometer. Samples were oxidized by flushing with air or by addition of ammonium persulphate, or reduced with sodium hydrosulphite. To obtain the CO-difference spectra of dithionite-reduced samples, CO was sparged through the samples for about 3 min before the spectra were recorded at room temperature. A slit-width of 2.0 nm was used in all experiments.

Quantitative determination of cytochromes was done as described by Escamilla & Benito (1984). Since the amount of cytochromes in ungerminated spores was too low to be measured accurately in spectra obtained at room temperature, their relative amount was measured as follows: photocopies of spectra at liquid-nitrogen temperature were obtained, and basal lines were drawn between minima of the bands of the cytochrome peaks, which were cut out and weighed. Specific activities and cytochrome concentrations are based on the amount of protein present in the mixed-membrane fraction.

Protein determination. This was done by the Lowry method.

RESULTS

Development of respiratory capacity during germination of sporangiospores

Respiratory activity of ungerminated sporangiospores from both *M. rouxii* and *M. bacilliformis* was very low and partially sensitive to cyanide and SHAM. After incubation in an appropriate medium under aerobic conditions, respiratory activity remained relatively constant and equally sensitive to SHAM for some time, then it increased exponentially, and simultaneously became completely sensitive to cyanide, whereas SHAM sensitivity

Fig. 1. Development of respiratory activity and sensitivity to cyanide during germination of *M. rouxii* sporangiospores. Spores were incubated aerobically as described in Methods. At various times samples were recovered and respiratory activity was measured (○). Sensitivity of respiration to cyanide (●) or SHAM (▲) was determined. The arrow indicates the time when the first germ tubes were observed.

Table 1. *Effect of chloramphenicol and cycloheximide on the development of respiratory activity in M. rouxii*

Incubation time (h)	Protein synthesis inhibitor	Respiratory activity ($\mu\text{l O}_2$ per min per 10^7 spores)	Percentage inhibition by:		
			KCN (11.9 mM)	SHAM (21.23 mM)	Both
0	None	0.36	8	33	100
5	None	1.13	100	0	100
5	Chloramphenicol (200 $\mu\text{g ml}^{-1}$)	0.97	86	32	100
5	Cycloheximide (100 $\mu\text{g ml}^{-1}$)	0.39	100	32	100
5	Chloramphenicol + cycloheximide	0.11	82	41	100

disappeared. Fig. 1 shows data for *M. rouxii*. The behaviour of *M. bacilliformis* was similar, but the process was slower (not shown). Full development of cyanide-sensitive respiration preceded germ-tube emergence. *M. bacilliformis* spores incubated at 30 °C can go through the first stages of germination, but do not produce germ tubes (Storck & Morril, 1977). Under these conditions, however, full cyanide-sensitive respiration was developed.

We determined the effect of inhibitors of protein synthesis on the development of respiratory activity in sporangiospores from both *M. rouxii* and *M. bacilliformis* incubated under aerobic conditions. In the presence of chloramphenicol, SHAM-sensitive respiration was not completely abolished. Cycloheximide inhibited both spore germination and increase in respiratory activity, but respiration became more sensitive to cyanide than to SHAM. In the presence of both chloramphenicol and cycloheximide, respiratory activity decreased, but sensitivity to SHAM underwent the same changes observed with either of the above (Table 1).

Changes in cytochrome pattern during sporangiospore germination

Sporangiospores of *M. rouxii* isolated in the cold and in the presence of both chloramphenicol and cycloheximide had a cytochrome spectrum indicative of the presence of cytochromes *b*, *c* and *aa*₃. However the relative amount of cytochromes *aa*₃ compared to the other cytochromes was very low (Table 2). After aerobic incubation in growth medium, cytochrome concentration (based on cell protein) increased rapidly. The most noticeable increase was of cytochromes *aa*₃ relative to cytochromes *c* and *b* (Table 2).

Table 2. *Cytochrome development during spore germination and outgrowth under aerobic conditions*

Incubation time (h)	Stage	Cytochrome concentration*		
		<i>c</i>	<i>b</i>	<i>aa</i> ₃
0	Sporangiospores	1.48 (8.7)	0.83 (4.9)	0.17 (1.0)
4	Swollen spores	10.89 (2.3)	5.70 (1.2)	4.70 (1.0)
5	Germlings	9.13 (1.2)	5.45 (0.7)	7.45 (1.0)

* Arbitrary units (see Methods). Numbers in parentheses represent ratios taking the *aa*₃ value as 1.0.

Table 3. *Respiratory activity of M. rouxii cells grown under different conditions*

Carbon source and concentration (% w/v)	Growth atmosphere	Morphology	Respiratory activity [$\mu\text{l O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$]	Percentage inhibition by:		
				KCN (1 mM)	SHAM (1.23 mM)	Both
Glucose (0.5)	Air	Mycelium	42.1	100	0	100
Glucose (2.0)	Air	Mycelium	31.0	100	0	100
Glucose (8.0)	Air	Mycelium	19.0	65	15	80
Glucose (2.0) + phenethyl alcohol (0.2)	Air	Yeast-like	24.9	100	0	100
Glycerol (2.0)	Air	Mycelium	93.0	—	—	—
Succinate (2.0)	Air	Mycelium	58.4	—	—	—
Glucose (0.5)	N ₂	Mycelium	13.0	59	25	84
Glucose (2.0)	N ₂	Yeast-like	11.0	37	42	77
Glucose (2.0)	N ₂ /CO ₂	Yeast-like	9.8	21	73	73

—, Not measured.

Effect of oxygen and carbon source on development of respiratory activity

Sporangiospores of *M. rouxii* were incubated in growth medium supplemented with various carbon sources. Cells were harvested and their respiratory activity was measured. Results (Table 3) showed that under aerobic conditions, the greater the amount of glucose present in the growth medium, the less the specific respiratory activity. At low concentrations of glucose (0.5 and 2%), respiratory activity was completely resistant to SHAM and sensitive to cyanide. The same results were obtained even when phenethyl alcohol was added to the culture medium to force the development of yeast-like forms under aerobic conditions (Terenzi & Storck, 1969). At high glucose concentrations (8%), respiration was partially sensitive to cyanide, SHAM, and interestingly, to both, indicating the appearance of a third O₂-consuming pathway. As originally described by Bartnicki-Garcia (1963), the yeast-like form was obtained when the medium was sparged with a mixture of N₂/CO₂, or with N₂ alone when the glucose concentration was high, whereas sparging with N₂ in the presence of 0.5% glucose induced the formation of germlings. In all cases, respiratory activity of these anaerobically grown cells was very low and partially sensitive to cyanide, SHAM or to both, although suggestive quantitative differences were observed (Table 3). Aerobic growth in the presence of non-fermentable carbon sources gave rise to the highest specific respiratory activity (Table 3).

Effect of oxygen and carbon source on the development of different cytochrome systems

Cells grown under aerobic conditions contained cytochromes *c*, *b* and *aa*₃, but their relative concentrations were influenced by the nature and concentration of the carbon source (Table 4). As observed for respiratory activity, excess glucose produced a decrease in the concentration of cytochromes. Use of non-fermentable carbon sources, such as glycerol or succinate, gave rise to the highest concentrations of cytochromes. Addition of phenethyl alcohol to a glucose-

Table 4. Cytochrome concentration in *M. rouxii* cells grown under aerobic conditions

Carbon source and concentration (% w/v)	Morphology	Cytochrome concentration [pmol cytochrome (mg protein) ⁻¹]				
		<i>c</i>	<i>b</i>	<i>aa</i> ₃	<i>a</i> ₃ -CO*	<i>o</i> -CO*
Glucose (0.5)	Mycelium	89.0	122.0	27.5	32.0	52.0
Glucose (2.0)	Mycelium	17.2	46.1	5.3	17.9	10.8
Glucose (8.0)	Mycelium	17.3	23.0	4.5	4.0	13.0
Glucose (2.0) + phenethyl alcohol (0.2)	Yeast-like	20.0	57.0	0.1	0.1	47.0
Glycerol (2.0)	Mycelium	171.0	132.0	49.0	99.0	144.0

* Calculated from CO-difference spectra.

Table 5. Cytochrome concentrations in *M. rouxii* cells grown under anaerobic conditions

Carbon source and concentration	Growth atmosphere	Morphology	Cytochrome concentration [pmol (mg protein) ⁻¹]				
			<i>c</i>	<i>b</i>	<i>aa</i> ₃	<i>a</i> ₃ -CO*	<i>o</i> -CO*
Glucose (0.5)	N ₂	Mycelium	6.8	17.7	ND	ND	10
Glucose (2.0)	N ₂	Yeast-like	2.6	9.2	ND	ND	15
Glucose (2.0)	N ₂ /CO ₂	Yeast-like	2.2	7.6	ND	ND	20

ND, Not detected.

* Calculated from CO-difference spectra.

containing medium induced yeast-like development, and decreased significantly the levels of cytochromes *aa*₃, but not those of cytochromes *c* and *b*.

Cells grown under anaerobic conditions contained no cytochromes *aa*₃ whatever their morphology (yeasts or mycelium). Levels of *c* and *b*, but not of *o* cytochromes (see below) were affected by the concentration of glucose and the presence of CO₂ (Table 5). In general, levels of cytochromes *c* and *b* were very low compared to those from aerobic cells, but the least affected was cytochrome *b* which was predominant in spectra of anaerobic cells.

Cytochrome *o* deserves a special mention. This terminal oxidase, originally described in bacteria (see Smith, 1961), has since been found in several eukaryotic systems (Kronick & Hill, 1974; Edwards & Chance, 1982; Scott *et al.*, 1983), including a yeast (Salmon & Poole, 1980) and a filamentous fungus (Drabikowska *et al.*, 1974), but had not been previously reported in Zygomycetes. It was tentatively identified in both *M. rouxii* and *M. bacilliformis* by its CO-difference spectra (Fig. 2), in cells grown under aerobic or anaerobic conditions. Its role in the respiratory activity of *Mucor* remains unknown, but the fact that its concentration was raised by both aerobic growth and non-fermentable carbon sources (or low glucose) suggests that it may play a role as a terminal oxidase.

DISCUSSION

Our results show the extreme plasticity of the respiratory system from *Mucor* species, which responds drastically to environmental conditions. Aerobically grown mycelium contains the normal set of cytochromes present in eukaryotic mitochondria and displays cyanide-sensitive respiration, whereas anaerobic cells have low levels of *c* and *b* cytochromes, lack cytochromes *aa*₃, and their respiration is partially sensitive to cyanide and SHAM. Both aerobic and anaerobic cells also contain cytochrome *o*. The type and quantity of the energy source also affect respiration and cytochrome levels.

Ungerminated sporangiospores have low specific respiratory activities which upon germination increase rapidly. Similar observations have been made in other fungi (Brambl,

Fig. 2. CO-difference spectra of *M. rouxii* and *M. bacilliformis* cells. 1, *M. rouxii* mycelium grown aerobically with 2% (w/v) glucose; 2, *M. rouxii* yeasts grown under N_2/CO_2 with 2% glucose; 3, *M. rouxii* mycelium grown aerobically with 2% (w/v) glycerol; 4, *M. bacilliformis* mycelium grown aerobically with 0.5% glucose.

1975; Kobilinski & Beattie, 1977; Stade & Brambl, 1981). Ungerminated sporangiospores of *M. rouxii* contain mitochondria with an apparently normal morphology (Bartnicki-Garcia *et al.*, 1968), but their respiratory pathway is still incompletely organized, since respiration is partially sensitive to cyanide and SHAM. The SHAM-sensitive pathway, which utilizes an alternative route (Lambowitz & Slayman, 1971), is lost during aerobic, but not anaerobic germination. Whether SHAM-sensitive respiration plays a role during the initial stages of spore germination remains debatable. Thus, SHAM addition inhibits the respiratory activity of *Botriodiplodia theobromae* spores (Brambl, 1980), but does not affect their germination. On the other hand, SHAM is a potent inhibitor of both the respiratory bypass (Shepherd *et al.*, 1978), and germ-tube formation in *Candida albicans* blastospores (Shepherd *et al.*, 1980). Mutants of *Neurospora crassa* affected in cytochrome oxidase exhibit the SHAM-sensitive respiratory pathway (Edwards & Kwiecinski, 1973; Bertrand & Werner, 1979). Stade & Brambl (1981) described that SHAM had only a slight effect on spore germination of these mutants, whereas cyanide inhibited their germination as well as that of wild-type spores. The demonstration that cytochromes aa_3 were indeed present in the mutant spores led the authors to suggest that the normal cytochrome pathway was indispensable for spore germination. Similar conclusions were reached by Brambl (1975) for *B. theobromae*.

Inhibition of cytoplasmic protein biosynthesis by cycloheximide blocked development of respiratory activity and growth of the spores. In contrast to previous observations (Zorzopulos *et al.*, 1973), chloramphenicol did not affect either process. It is possible that mitochondrial autogenous proteins are accumulated although not organized in the spores. Their synthesis may

be required at a later period as has been described for *B. theobromae* (Brambl, 1975). Sensitivity to SHAM, however, is not lost in the presence of chloramphenicol. In *N. crassa*, Lambowitz & Slayman (1971) and Edwards & Rosenberg (1976) have shown that inhibition of mitochondrial protein biosynthesis induces the SHAM-sensitive respiratory pathway suggesting that a mitochondrial gene product is responsible for the regulation of this pathway.

From our results it becomes clear that respiratory activity is not strictly associated with fungal morphogenesis in *Mucor*. Anaerobic yeast-like cells and hyphae obtained by alterations in the atmospheric environment and carbon source concentration had the same cytochrome composition and their respiration was equally sensitive to SHAM and cyanide. On the other hand, aerobic yeast-like cells obtained by growth in the presence of phenethyl alcohol showed cyanide-sensitive respiration, and contained the same relative amounts of cytochromes *c* and *b* but not *aa*₃, in agreement with a previous report (Terenzi & Storck, 1969). The observation that yeast-like mutants are affected in respiratory functions (Storck & Morrill, 1971; Ruiz-Herrera *et al.*, 1983) suggests that there must exist an association between mitochondrial functions and hyphal growth, but clearly this function is not respiration. Whether the key mitochondrial role is to synthesize ATP, or to compartmentalize specific biosynthetic reactions as suggested by Inderlied *et al.* (1985), remains to be determined.

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