SOME AEROBIC BLASTOCLADIOMYCOTA AND CHYTRIDIOMYCOTA CAN SURVIVE BUT CANNOT GROW UNDER ANAEROBIC CONDITIONS

Frank H. Gleason[1,3], Peter M. Letcher[2] and Peter A. McGee[1]

[1.] School of Biological Sciences A12, University of Sydney, 2006 Australia.
[2.] Department of Biological Sciences, University of Alabama, Tuscaloosa, AL 35487 USA.
[3.] Corresponding author; Email frankjanet@ozemail.com.au

Abstract

In the present study twenty-two chytrids isolated aerobically from soils in Australia were tested for ability to grow or to survive under strict anaerobic conditions. These fungi were previously assigned to the orders Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales by molecular techniques. None of the isolates grew in liquid growth media under strict anaerobic conditions. However, all twenty-two isolates survived in liquid growth media under strict anaerobic conditions for relatively short periods of time, which can occur periodically in the soil. Three of these isolates produced acid during growth in the presence of air indicating the capacity for lactic acid fermentation. Most members of the orders Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales are considered to be obligate aerobes. Based on previous growth studies only two genera in these orders have been classified as facultative anaerobes. In contrast all members of the order Neocallimastigales are obligate anaerobes.

Key words: soil chytrids, Chytridiomycota, anaerobic growth, anaerobic survival, acid production.

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Introduction

Chytrids are true fungi that produce zoospores usually with a single posterior whiplash flagellum. These microorganisms are currently placed into two closely related phyla, the Blastocladiomycota and Chytridiomycota and six orders, the Blastocladiales, Chytridiales, Monoblepharidales, Neocallimastigales, Rhizophydiales and Spizellomycetales (James *et al*. 2000, Barr 2001, James *et al.* 2006, Letcher *et al*. 2006). Prior to the discovery of the Neocallimastigales, chytrids were thought to be primarily an obligately aerobic group of fungi (Gleason 1976, Barr 2001). Many chytrids in the orders Blastocladiales, Chytridiales, Monoblepharidales, Rhizophydiales and Spizellomycetales have been isolated from top soil and fresh water environments and a few from marine environments where the dissolved oxygen concentration is probably relatively high (Sparrow 1960, Karling 1977, Barr 1987, Whisler 1987, Barr 2001). In contrast the fungi in the Order Neocallimastigales have been isolated from the digestive systems of herbivorous vertebrates. All species in this order are considered to be obligate anaerobes (Orpin 1994, Trinci *et al*. 1994, Orpin & Joblin 1997, Rezaeian *et al*. 2004), that is, they cannot grow or survive in the presence of oxygen.

Nevertheless, fungi belonging to several genera in the Blastocladiales, Chytridiales and Monoblepharidales have been observed growing on substrates in stagnant water, mud and benthic sediments where the dissolved

oxygen concentration is probably relatively low (Sparrow 1960, Paterson 1967, Willoughby 1961, Karling 1977, Emerson & Natvig 1981, Whisler 1987, Dasgupta & John 1988, Steciow *et al*. 2001). For this reason some early researchers proposed that, based on observations in the field, at least a few of these fungi might be facultative anaerobes (Craseman 1954, Emerson & Robertson 1974, Emerson & Natvig 1981). Despite this, few chytrids growing in stagnant water, mud or benthic sediments have been isolated and grown in pure culture in the laboratory, and measurements of dissolved oxygen concentration were not available from the sites where they were observed. Their presence in stagnant water, mud and benthic sediments may be because these fungi can actually grow under anaerobic conditions or because they may just tolerate temporary anaerobiosis or reduced oxygen tensions. Furthermore, the capacity to grow or survive under anaerobic conditions may be more widespread among the Blastocladiales, Chytridiales, Monoblepharidales and Spizellomycetales than previously thought.

Isolates in only two genera of chytrids have been classified as facultative anaerobes in laboratory studies (Emerson & Cantino 1948, Cantino 1949, Craseman 1954, Craseman 1957, Emerson & Robertson 1974, Emerson & Natvig 1981, Lingle & Barstow 1983, Gleason & Gordon 1988, Gleason *et al*. 2002). These include four isolates from the genus *Blastocladia*, which grew under strict anaerobic conditions (Held *et al*. 1969, Gleason & Gordon 1988) and *Macrochytrium* (Craseman 1954), which grew under reduced oxygen tensions. In contrast, isolates in six other genera (*Allomyces*, *Catenaria*, *Chytridium*, *Cladochytrium*, *Rhizophlyctis* (*Karlingia*) and *Phlyctorhiza*) were unable to grow under anaerobic conditions or reduced oxygen tensions in the laboratory (Stanier 1942, Craseman 1954, Ingraham & Emerson 1954, Rothwell 1956, Goldstein 1960, Willoughby 1962, Nolan 1970). The methods used by Goldstein (1960), Rothwell (1956), Stanier (1942) and Willoughby (1962) did not adequately control the level of dissolved oxygen. Survival under strict anaerobic conditions has not been tested in any of the obligately aerobic chytrids.

Some members of the Chytridiomycota have the capacity for lactic acid fermentation in liquid media containing carbohydrates as substrates so that it is possible for them to produce ATP in the absence of oxygen. However the production of lactic acid during growth has been reported in only six genera in the Blastocladiales and Chytridiales: *Allomyces* (Ingraham & Emerson 1954, Turian 1960), *Blastocladia* (Emerson & Cantino 1948, Cantino 1949, Craseman 1957, Gleason & Gordon 1988), *Blastocladiella* (Cantino 1951, Cantino 1960), *Chytridium* (Craseman 1954), *Cladochytrium* (Willoughby 1962) and *Macrochytrium* (Craseman 1954). Nevertheless, lactic acid fermentation may also be possible in other genera.

Little is known about the ability of most soil chytrids to grow or survive under anaerobic conditions. The purpose of this research is to examine both growth and survival of soil chytrids under strict anaerobic conditions and to determine whether soil chytrids may continue to grow or survive in a dormant state without growth during periodic anaerobiosis.

Materials and Methods

Twenty-two isolates of chytrids listed in Table 1 were selected for the present study. The putative identity and origin of these isolates and some of the procedures used in the present study have been described previously (Gleason *et al.* 2004, Letcher *et al*. 2004a,b, Commandeur *et al.* 2005, Gleason *et al*. 2005, Gleason *et al*. 2006, Letcher *et al*. 2006). The fungi were isolated from natural and cropping soils collected near the surface and in contact with air at sites in New South Wales and Tasmania from three general types of habitat: (1) highly organic and/or clay, poorly aerated, high moisture content and poor drainage, (2) mixed organic and sandy, good aeration, often moist and good drainage and (3) mostly sandy, good aeration, usually dry and good drainage.

Preparation of inocula

All cultures were maintained on PYG agar medium (glucose 3.0 g L^{-1}, peptone 1.25 g L^{-1}, yeast extract 1.25 g L^{-1} and agar 20 g L^{-1}). The inocula for monocentric chytrids were prepared by flooding seven-day-old cultures growing on

Table 1. Survival of Chytrids isolated from soils in Australia after incubation under strict anaerobic conditions.

Fungus name	Order[1]	Survival[2] (days)		Fungus name	Order[1]	Survival[2] (days)	
		7	31			7	31
Allomyces arbuscula Allo Mar CW16	B	Y	Y	*Boothiomyces* sp. AUS 2	R	Y	Y
Catenaria anguillilae Poly Ad 2-0	B	Y	Y	*Terramyces* sp. AUS 3	R	Y	Y
Catenaria anguillilae Dec CC 4-10Z	B	Y	N	*Boothiomyces* sp. AUS 6	R	Y	Y
Spizellomyces sp. Mar Ad 2-0	S	Y	Y	*Boothiomyces* sp. AUS 7	R	Y	Y
Gaertneriomyces sp. Mar C/C2	S	Y	Y	*Boothiomyces* sp. AUS 8	R	Y	Y
Spizellomyces sp. Dec CC 4-10F	S	Y	N	*Boothiomyces* sp. AUS 9	R	Y	Y
Rhizophlyctis rosea AUS 13	S	Y	Y	*Boothiomyces* sp. AUS 12	R	Y	N
Powellomyces sp. AUS 16	S	Y	N	*Kappamyces laurelensis* AUS 15	R	Y	Y
Powellomyces sp. AUS 17	S	Y	N	*Cladochytrium* sp. AUS 11	C	Y	Y
Rhizophydium sp. Mar Ad 14	R	Y	Y	*Chytriomyces hyalinus* AUS 14	C	Y	Y
Rhizophydium sp. Mar R2	R	Y	N	*Chytriomyces hyalinus* Ob 3-8	C	Y	Y

[1] B: Blastocladiales, C: Chytridiales, R: Rhizophydiales, S: Spizellomycetales. [2] Y: Yes, N: No.

PYG agar in 25 mL Petri dishes with 5 mL of de-ionized water. After two hours the resulting zoospores and sporangia were mixed with a transfer loop. Approximately 0.5 mL of a mixture of zoospores and sporangia was inoculated into the sterile media in the serum bottles using a sterile syringe with an 18-gauge needle. The inocula for polycentric and hyphal forms were prepared by grinding cells grown in liquid or solid PYG media with a micropestle in a 1.5 mL microcentrifuge tube. 0.5 mL of the resulting slurry was inoculated into sterile media.

When inocula with only zoospores and recently encysted zoospores were needed, seven day old lawns instead of clumps of thalli were first prepared on the surface of the solid PYG media. The lawn was then flooded with 5 mL of de-ionized water. The thalli remained attached to the surface of the solid medium, so that the inocula consisted of only zoospores and recently encysted zoospores in de-ionized water. All inocula were examined for composition with the light microscope.

Growth in anaerobic media

The general procedures for testing for growth of fungi under strict anaerobic conditions have been described previously by Gleason and Gordon (1988, 1989) but were modified slightly for the present study. The liquid growth media contained glucose 3.0 g L^{-1}, peptone 1.25 g L^{-1}, yeast extract 1.25 g L^{-1}, K_2HPO_4 1.74 g L^{-1} (buffer), $MgSO_4$ 0.12 g L^{-1}, cysteine. HCl 1.00, 0.3 or 0.0 g L^{-1} and either resazurin for oxygen detection or brom cresol

Table 2. Complex liquid media used to test for growth under aerobic, reduced oxygen tension and strict anaerobic conditions.

Growth medium number	Environment	Cysteine (g L^{-1})	Resazurin	Brom cresol purple	Bubbled with oxygen-free nitrogen
1	Anaerobic	1.0	+	-	+
2	Anaerobic	0.3	+	-	+
3	Reduced oxygen	0	-	+	+
4	Aerobic	0	-	+	-

In anaerobic growth media 1 and 2 the redox potential was held below -100 mV with the addition of cysteine, a reducing agent. The oxygen tension of growth medium 3 was reduced below 0.1% by bubbling with high purity oxygen-free nitrogen. The oxygen tension of the aerobic growth medium 4 was not changed during preparation.

purple for acid detection at 1 mg L^{-1}. Fifty mL of media was dispensed into each 100 mL serum bottle (Alltech). The media were then bubbled with high purity nitrogen for at least one minute. The bottles were closed with butyl rubber stoppers and aluminium crimp seals without allowing air to enter. Bottles were sterilized at 121°C for 15 minutes.

The four liquid growth media listed in Table 2 were prepared to test for aerobic and anaerobic growth. The incubation time for the experiments with media 1, 2 and 3 was 22 days. The growth experiment with medium 4 was stopped at seven days due to the rapid growth of all chytrids except for *Catenaria* sp. Poly Ad 2-0 and *Cladochytrium* sp. AUS 11. The latter two cultures were incubated for 22 days because of slow growth. In addition, as a control for each experiment, the inoculum for each isolate was placed onto the surface of 25 mL of solid PYG medium with 2% agar in Petri dishes (medium 5) and incubated for seven days to test for viability. At first all growth experiments included only one replicate of each isolate, and then the growth experiments with medium 1 and medium 5 (control) were repeated twice with each isolate for 22 days. Growth experiments with *Allomyces arbuscula* Allo Mar CW 16, *Catenaria* sp. Poly Ad 2-0 and *Cladochytrium* sp. AUS 11 in medium 4 were repeated three times.

Growth was monitored visually for up to 22 days at 20°C. The appearance of new clumps or increase in volume of clumps of thalli was taken to indicate growth. None of the cultures were shaken. The pH of the medium was monitored by observing the color of brom cresol purple during growth in experiments with media 3 and 4 by the method of Emerson (1958). Brom cresol purple changes color from purple to yellow when the pH drops below pH 6.0. In addition the pH was measured with a pH meter at harvest in medium 4. The redox potential was monitored by observing the color of the redox indicator resazurin ($E0^1$= -42 mV) (Gleason & Gordon 1988). Resazurin changes color from colorless to blue when the culture medium is oxygenated.

Survival in anaerobic medium

In two separate experiments, 0.5 to 1.0 mL samples of a mixture of zoospores, zoospore cysts and thalli in de-ionized water were inoculated into the liquid growth medium 1 in serum bottles and onto the solid medium (medium 5) in Petri dishes (for the control) and incubated for 7 and for 31 days. Then the contents of the serum bottles were poured into 50 mL centrifuge tubes. Following centrifugation most of the liquid medium was removed from the cultures. The thalli with some of the remaining liquid medium were transferred onto solid PYG agar in Petri dishes (recovery medium, medium 5) and examined for resumption of growth during aerobic incubation for seven days at 20°C.

In a third experiment serum bottles containing the liquid growth medium 1 were inoculated with 1 mL of de-ionized water containing only zoospores and recently encysted zoospores of AUS 6, AUS 14, Mar Ad 14 and Mar Ad 2-0. These cultures were incubated for one or for seven days. The contents of each serum bottles were poured into two 50 mL sterile centrifuge tubes. The second 25 mL of growth media was split between three Petri dishes. A one mL sample from the bottom of each Petri dish was spread onto the surface of solid PYG

medium in a Petri dish (recovery medium). The cultures were examined for resumption of growth during aerobic incubation for seven days at 20°C. The number of colonies on the surface of the recovery medium in each Petri dish was recorded. Each colony was examined with the dissecting microscope to verify that it had developed from a single zoospore or zoospore cyst.

Results

Growth under aerobic, reduced oxygen and anaerobic conditions

None of the twenty-two fungi (Table 1) grew during incubation for 22 days in anaerobic liquid media 1, 2 and 3. All of the fungi grew well during incubation for 7 days in aerobic liquid medium (medium 4) and on the aerobic solid medium (medium 5, the control).

The color of brom cresol purple in medium 3 (anaerobic) remained unchanged during the 22-day incubation period in all isolates. In medium 4 (aerobic) the color of brom cresol purple did not change during the growth of all of the isolates during the 7-day incubation period except for *Allomyces arbuscula* Allo Mar CW 16, where the color of the medium changed from purple to bright yellow before the end of the 7-day incubation period. In separate experiments with medium 4 (aerobic) the slower growing isolates of *Cladochytrium* sp. AUS 11 and *Catenaria* sp. Poly Ad 2-0 caused the color of brom cresol purple to change from purple to bright yellow during growth between 8 and 22 days.

In the initial experiment with medium 4 the pH was measured after the 7 days with a pH meter and remained above 6.0 with all isolates except for *Allomyces arbuscula* Allo Mar CW 16. In separate experiments with medium 4 the pH of three cultures of *Allomyces arbuscula* Allo Mar CW 16, *Cladochytrium* sp. AUS 11 and *Catenaria* sp. Poly Ad 2-0 was measured with a pH meter at the end of the 7, 14 and/or 22 days. The mean pH values (± 1 standard deviation) for Allo Mar CW 16 were pH 5.9 ± 0.1 at 7 days and 4.8 ± 0.01 at 14 days. The mean pH values for AUS 11 were pH 5.7 ± 0.1 at 14 days and 4.9 ± 0.2 at 22 days. The mean pH values for Poly Ad 2-0 were pH 5.7 ± 0.1 at 14 days and 5.2 ± 0.03 at 22 days.

Survival under anaerobic conditions

All of the fungi resumed rapid growth immediately after being transferred to the recovery medium following incubation under anaerobic conditions for 7 days (Table 1). Sixteen of the fungi (*Allomyces arbuscula* Allo Mar CW 16, *Catenaria anguillilae* Poly Ad 2-0, *Spizellomyces* sp. Mar Ad 2-0, *Gaertneriomyces* sp. Mar CC2, *Rhizophlyctis rosea* AUS 13, *Chytriomyces hyalinus* AUS 14 and Ob 3-8, *Cladochytrium* sp. AUS 11, *Boothiomyces* sp. AUS 2, AUS 6, AUS 7, AUS 8, AUS 9, *Terramyces* sp. AUS 3, *Kappamyces laurelensis* AUS 15 and *Rhizophydium* sp. Mar Ad 14) also resumed growth following incubation under anaerobic conditions for 31 days (Table 1).

In experiments using only zoospores and zoospore cysts as the inoculum, AUS 6, AUS 14, Mar Ad 14 and Mar Ad 2-0 were incubated for one or for seven days under anaerobic conditions and then transferred to recovery medium. In two separate experiments no colonies were obtained for Mar Ad 2-0 after anaerobic incubation for one and for seven days. With the other fungi after incubation for seven days under aerobic conditions the mean number of colonies (± 1 standard deviation) per plate was 13 ± 3 for AUS 6, 20 ± 1 for AUS 14, and 0 for Mar Ad 14.

Discussion

None of the isolates in the present study grew under strict anaerobic conditions in media 1 and 2 or under reduced oxygen tensions in medium 3 during the 22-day incubation period. Thus the growth of these soil chytrids appears to be very sensitive to low dissolved oxygen concentrations. The results reported in the present study with medium 3 are consistent with data previously reported for other isolates in several genera of chytrids (Stanier 1942; Craseman 1954; Ingraham & Emerson 1954; Rothwell 1956; Goldstein 1960; Willoughby 1962; Nolan 1970). Furthermore, the data support the hypothesis that most members of the Chytridiales, Blastocladiales, Rhizophydiales and Spizellomycetales are obligate aerobes.

Survival under strict anaerobic conditions had not been tested previously in any of the obligately aerobic chytrids. However all of the

isolates in the present study survived incubation for seven days and most isolates for 31 days under strict anaerobic conditions. Thus these fungi appear to tolerate periodic anaerobiosis, enabling continuation in the soil through periods of flooding or similar perturbations.

Although we could not observe growth visually under anaerobic conditions during the 21-day incubation time, it is possible that extremely slow growth may have occurred in some isolates. However, it is more likely that all of these chytrids became dormant at the onset of anaerobiosis and resumed growth later after the re-introduction of oxygen. The capacity of these chytrids to survive anaerobiosis beyond 31 days is unknown.

The stage in the life cycle that is resistant to anaerobic conditions is not known. Zoospores of all fungal structures are probably the least tolerant of stressful conditions. Zoospores and/or recently encysted zoospores of *Boothiomyces* sp. AUS 6 and *Chytriomyces hyalinus* AUS 14 but not *Rhizophydium* sp. Mar Ad 14 or *Spizellomyces* sp. Mar Ad 2-0 survived anaerobic conditions for seven days. The mature thallus however appears likely to be the structure that is most resistant to the lack of oxygen.

The method used in the present study to insure the maintenance of strict anaerobic conditions (with a redox potential below -100 mV) appeared to be satisfactory. Even with the introduction of a small amount of oxygen in the inoculum, the resezurin did not change color from colorless to blue during incubation.

None of the isolates in the present study were strongly fermentative and facultatively anaerobic like *Blastocladia* (Gleason & Gordon, 1988). Three isolates in the present study, *Allomyces* Allo Mar CW 16, *Cladochytrium* AUS 11 and *Catenaria* sp. Poly Ad 2-0 released enough acid into the growth medium with 10 mM phosphate buffer to lower the pH below 6. The fact that fungi in two of these genera are strong acid producers has been reported previously by Ingraham & Emerson (1954), Turian (1960) and Willoughby (1962). This indicates that at least three of the isolates used in the present study can ferment glucose to lactic acid, which is a process that does not require oxygen.

The reasons for the inability of soil chytrids to grow under low oxygen tensions or strict anaerobic conditions are not known. It is possible that these fungi cannot synthesize essential molecules in the absence of oxygen, that essential nutrients are not provided by peptone and yeast extract in the medium and/or that these fungi lack the metabolic machinery to produce enough ATP by fermentation for biosynthesis. In contrast, rumen fungi grow well in synthetic media under strict anaerobic conditions (Orpin 1994). It is interesting to note that the cells of one isolate of *Allomyces* pre-grown aerobically can continue to ferment carbohydrates but cannot grow under anaerobic conditions (Ingraham & Emerson 1954). This suggests that it is possible for some chytrids to produce enough ATP by fermentation to insure survival during short periods of anaerobiosis.

The purpose of the present study was to test chytrids isolated from aerated soils for the ability to grow and survive under anaerobic conditions. Because only a small number of isolates were tested, it cannot be concluded that facultatively anaerobic chytrids do not occur in aerated soils. Furthermore, it is possible that if the isolation procedures were carried out under strict anaerobic conditions, facultatively and obligately anaerobic chytrids could be found in samples of compacted soil, stagnant water, mud and benthic sediments. Special procedures and equipment may need to be adapted for isolations of potentially anaerobic chytrids, such as enrichment with reduced dissolved oxygen concentrations (Whisler 1987) and anaerobic growth chambers (Theodorou *et al*. 1994, Trinci *et al*. 1994, Orpin & Joblin 1997, Rezaeian *et al*. 2004).

The chytrids used in the present study were isolated from various soil types and climates in Australia. All twenty-two chytrids appeared to survive but could not grow during short periods of anaerobiosis. The capacity to tolerate anaerobiosis appears to be similar in chytrids isolated from clay and sand textured soils and hot and cold climates. While mature sporangia are the most likely survival

structure, some chytrids may also survive as zoospores or zoospore cysts. Without growth it is possible that only small quantities of nutrients are needed for survival through these periods of anaerobiosis. The mechanisms underlying responses to anaerobiosis in soil chytrids remain to be elucidated.

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