troduced into the B-XIV titanium zonal rotor (*10*) between a sucrose gradient (26 to 52 percent) and 100 ml of a sucrose overlay (10 percent) and centrifuged 2½ hours at 39,000 rev/min. Figure 1 shows electron micrographs of the starting material and the banded fractions recovered from the rotor. Three distinct bands were present. A transitional area located between the second and third band (numbered from the top of the gradient) contained any small mitochondria which were present. This region (Fig. 1D) was discarded before combining the three bands remaining for DNA extraction.

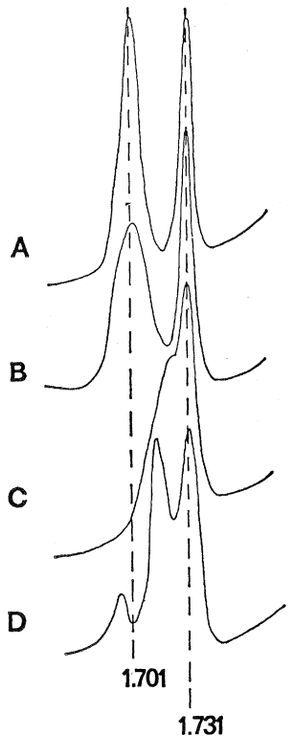

Fig. 2. Densitometer traces of DNA banded in cesium chloride in the model E ultracentrifuge equipped with ultraviolet optics. The materials were centrifuged 20 to 24 hours at 44,000 rev/min at 25°C. The band at 1.731 is a *Micrococcus lysodeikticus* marker. (A) DNA extracted from the pooled material described in Fig. 1. Note the absence of a satellite DNA peak. The band is destroyed by deoxyribonuclease but is not affected by ribonuclease. (B) The same material as (A) after brief sonication. Material treated in this manner was used for the subsequent heat denaturation and reannealing experiments. (C) The sonicated DNA after thermal denaturation at 100°C for 10 minutes in one-tenth concentrated SSC and quick cooling in ice. (D) DNA treated as in (C), then adjusted to standard SSC and incubated at 60°C for 24 hours. The small peak has renatured much as nuclear satellite would, although (A) indicates that satellite may be absent. Considerable reannealing has occurred in the major component, as evidenced both by band movement and sharpening of the component.

Figure 2 shows the cesium chloride banding characteristics of the DNA from the combined fractions, and the results of thermal denaturation and reannealing experiments with the material. The native density of the DNA is indistinguishable from both mitochondrial DNA and the mouse nuclear DNA major band. However, satellite DNA (*7*) comprising about 10 percent of the mouse nuclear DNA apparently is missing, because DNA from controls similarly tested show distinct sharp satellite bands at a density of 1.691. The absence of the satellite in addition to the metabolic activity of the DNA indicates that its presence is not from nuclear contamination. Material similar to nuclear satellite DNA was found in the reannealing experiment (Fig. 2D). A small component reannealed to a density of 1.697. However, since this large an amount of nuclear satellite DNA is clearly absent, it is probable that a new mouse satellite DNA has been demonstrated by the experiment.

In the reannealing experiment shown in Fig. 2, the microsome-associated DNA is not mitochondrial, because the latter anneals more readily and more completely. An intermediate buoyant density was found after 6 hours of incubation, an indication that the annealing process was still progressing at this time.

We have demonstrated a unique form of DNA associated with microsomes. The material is not mitochondrial. If it should come from nuclear contamination, it would represent a form of nuclear DNA heretofore unknown. The DNA appears metabolically active, and, based upon its reannealing characteristics, it is quite complex and contains potentially a considerable amount of genetic information.

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Blastocladia and Aqualinderella: Fermentative Water Molds with High Carbon Dioxide Optima

Abstract. *The uniflagellate aquatic phycomycete* Blastocladia ramosa *appears to be a facultative anaerobe.* Blastocladia pringsheimii *requires traces of oxygen. Growth of both species is no greater or only slightly greater at normal atmospheric oxygen pressure than under 0.2 percent oxygen pressure, but their growth is enhanced by the addition of 5 or 20 percent carbon dioxide. The cells of both species lack typical cristate mitochondria and contain only traces of cytochrome.* Blastocladia *resembles the biflagellate* Aqualinderella fermentans *in adaptation to an environment poor in oxygen and rich in carbon dioxide.*

The existence of a polyphyletic group of fermentative water molds adapted to anaerobic environments rich in CO_2 has been suggested (*1*). We now confirm the occurrence of such an ecological pattern among uniflagellate phycomycetes and provide further data about their biflagellate counterparts.

Fungi are generally regarded as obligate aerobes (*2*). In facultative anaerobes such as *Saccharomyces cerevisiae* (*3*) and *Mucor rouxii* (*4*) the removal of oxygen causes a loss of mito-

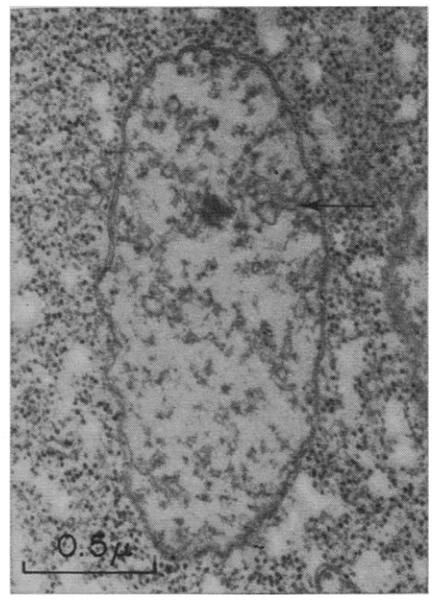

Fig. 1. Double-membraned vesicle in a germling of *B. ramosa* (isolate 54-8) (19 hours old), grown as described by Emerson (*11*). For details of specimen preparation, see Fuller and Calhoun (*12*). Arrow indicates possible site of invagination of inner membrane.

chondrial structure and function and a shift to fermentation. Oxygen has only minor, secondary effects on the biflagellate water mold *Aqualinderella fermentans* (Oomycetes, Leptomitales), a facultative anaerobe which is obligately fermentative (*1, 5*). *Aqualinderella fermentans* requires supplemental CO_2 and grows best in an atmosphere containing 20 percent of this gas, an amount detrimental to most fungi (*2*). This fungus also grows under tank CO_2. The uniflagellate water mold genus *Blastocladia* (Chytridiomycetes, Blastocladiales) has similar properties (*6–8*); *B. pringsheimii* and *B. ramosa* are obligately fermentative and require little or no oxygen. *Blastocladia pringsheimii* grows under tank CO_2 (*6*), but neither species requires supplemental CO_2. Both *Aqualinderella* and *Blastocladia*, growing as pustules covered with other microbial growth, have been obtained from stagnant water on fleshy fruit baits (*6, 9*); their natural habitat may therefore be low in O_2 and high in CO_2.

We examined the effects of O_2 and CO_2 on two isolates of *B. ramosa*, 54-8 and 54-14, and one isolate of *B. pringsheimii*, 51-26 (*10*). We found no requirement for O_2 in the first species, whereas the second required a trace amount. Both responded favorably to supplemental CO_2. Mitochondria and cytochromes either were absent or occurred in vestigial form in *Blastocladia* and in the type isolate of *Aqualinderella*.

Stocks of *Blastocladia* were maintained as described by Emerson (*11*). Medium GY5, containing glucose and yeast extract (*11*), was used unless otherwise indicated. Portions (150 ml) of the medium in 250-ml Erlenmeyer flasks were equilibrated with a given gas mixture in a sealed jar. The *p*H of the medium incubated with supplemental CO_2 was adjusted to 6.3, or to other values when appropriate, with NaOH. Flasks were rapidly inoculated with small tufts of young thalli (0.5 to 1 mg, dry weight) and returned to the jars. After being shaken gently for several hours to facilitate the final gas equilibration, the jars were kept stationary at 23°C until harvest. We harvested the cultures while the *p*H was still above the detrimental level of 4.5 (*6*). Anaerobic conditions were established by the "strict" method (see *1*, Table 6): the O_2 pressure in the jar was reduced by evacuation to 0.2 percent, which was then combined with H_2 with the aid of a palladium catalyst. Other methods used are described by Emerson and Held (*1*).

Both isolates of *Blastocladia ramosa* behaved as facultative anaerobes (Table 1). In the CO_2-supplemented atmosphere, aerobic growth with 20 percent O_2 was about 20 percent better than anaerobic or near-anaerobic growth. This increase might be related to an increased tendency to produce zoosporangia and to proliferate. The molar growth yield, the ratio of biosynthetic activity (milligrams, dry weight) to consumption of the energy source (millimoles of glucose), was also only slightly improved under aerobic conditions. Howvere, in a parallel series, when the atmospheres were not supplemented with CO_2 (0.03 percent CO_2), growth of *B. ramosa* was indifferent to O_2.

Blastocladia pringsheimii required trace amounts of O_2 (Table 1). The same amount of growth occurred in the presence of 0.2 and 20 percent O_2. The existence of the requirement for O_2 was difficult to demonstrate, since considerable growth took

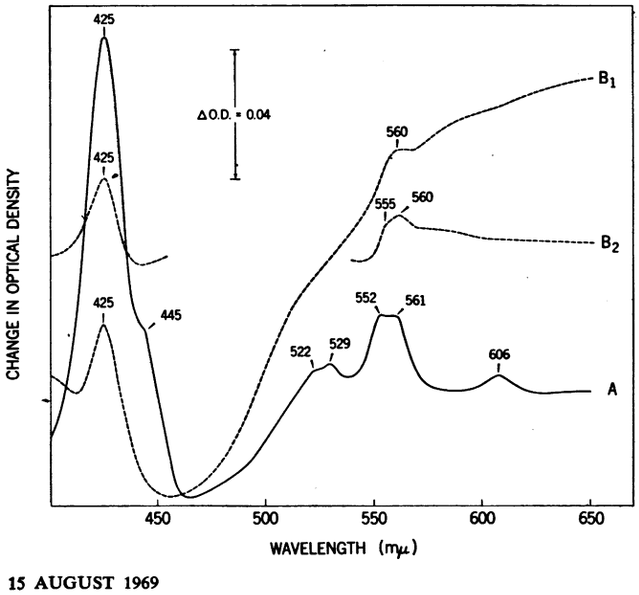

Fig. 2. Cytochromes in *Blastocladia ramosa*. Curve B_1 indicates the difference spectrum of homogenized mycelium [46 mg (dry weight) per milliliter of homogenate]; the difference is determined from that amount reduced by hydrosulfite minus that bubbled with oxygen, at room temperature. In curve B_2 the base line was adjusted to flatten the trough. Material was isolate 54-14, grown under air in a medium containing glucose, peptone, and yeast extract (*13*). Curve *A* is included for comparison, and indicates the full cytochrome system of the blastocladialean *Allomyces catenoides* (*14*), obtained with 19 mg (dry weight) per milliliter of homogenate. See Gleason and Unestam (*13*) for details of procedure. Addition of potassium ferricyanide to the oxidized cuvette did not alter the difference spectrum of *Blastocladia* above 500 mμ. Similar difference spectra were obtained with *B. pringsheimii*.

Table 1. Effect of oxygen on growth of *Blastocladia*. Two flasks were examined for each treatment. Inoculum for all treatments was grown anaerobically for 50 hours. The near-anaerobic H_2 atmosphere was obtained by omitting the catalyst from the anaerobic setup. The H_2 atmospheres contained about 1 percent N_2. *Blastocladia ramosa* was harvested 54 hours after inoculation; *B. pringsheimii*, 105 hours after inoculation. Glucose consumption was measured with Glucostat (Worthington).

Atmosphere (76 cm-Hg)			Milligrams (dry weight) per 150 ml			Molar growth yield (mg/mmole of glucose consumed)		
			B. ramosa		*B. pringsheimii*	*B. ramosa*		*B. pringsheimii*
Base	CO_2 (%)	O_2 (%)	Isolate 54-8	Isolate 54-14		Isolate 54-8	Isolate 54-14	
H_2	5	None	28.0	31.1	0	30.0	30.1	
H_2	5	0.2	29.1	32.5	39.6	32.6	32.4	38.0
N_2	5	0.2	27.8	30.6	39.8			
Air	5	20	33.1	36.0	38.7	37.9	34.9	41.2

place if exposure to air was prolonged even slightly above the minimum required for inoculation. This organism was not killed by anaerobic incubation. (The maximum period tested was 12 days.) Cultures that showed no development in anaerobic jars renewed their growth in air.

When 5 percent of the air in the jar was replaced by CO_2, growth of all three isolates increased (Table 2). The beneficial effect of 5 percent CO_2 varied quantitatively in successive trials with the same isolate, and the degree of growth promotion appeared to be inversely related to the size and vigor of the inoculum. Subsequent experiments demonstrated that growth was promoted by 5 percent CO_2 in anaerobic (for *B. ramosa*) and near-anaerobic (for both species of *Blastocladia*) atmospheres. At harvest, the *p*H of the medium of the CO_2-supplemented cultures was as low as, or lower than, the *p*H of the controls. Hence the effect of CO_2 was not due to the buffering action of bicarbonate but was probably nutritional. Growth under 20 percent CO_2 was usually similar to, or slightly better than, growth under 5 percent CO_2 (Table 2). Occasionally, this atmosphere produced considerable variability between duplicates of the same group.

The application of 80 or 99 percent CO_2 slowed growth of *Blastocladia* (Table 2). *Blastocladia ramosa* 54-8 showed the greatest sensitivity and in several instances failed to grow under 99 percent CO_2. Usually, the thalli of the initial inoculum were not killed by the high CO_2 tensions; in fact, they grew extremely large and produced many reproductive structures but were relatively late in releasing zoospores. Once discharge had taken place, proliferation and mass increase would rapidly follow. Under these high CO_2 pressures, *Blastocladia* formed dense clumps of thalli instead of the fluffy mats produced under lower pressures. The effect of 80 percent CO_2 was independent of the pressure of O_2. Inhibition of growth was slightly greater than that recorded in Table 2 when the *p*H was adjusted to 6.3 under 80 and 99 percent CO_2; presumably this was due to high concentrations of bicarbonate or sodium (or both).

Table 2. Effect of CO_2 on growth of *Blastocladia*. Two flasks were examined for each treatment. Inoculum for all treatments was grown under air for 2 days (*B. ramosa*) or 4 days (*B. pringsheimii*). Sodium hydroxide was added to autoclaved, cooled medium after it had been dispensed in flasks and before incubation for gas equilibration in the test atmosphere.

Atmosphere		NaOH added (*M*)	Initial *p*H	Growth [milligrams (dry weight) per 150 ml]					
				B. ramosa isolate 54-8		*B. ramosa* isolate 54-14		*B. pringsheimii*	
CO_2 (%)	O_2 (%)			At hour 48	At hour 156	At hour 48	At hour 84	At hour 96	At hour 156
0.03	21		6.3	21.5		19.7		11.0	
5	20	0.0025	6.3	34.0		34.6		37.1	
20	20	.0125	6.3	33.2		39.7		40.9	
80	20	.0125	5.9	9.5	22.3	17.6	51.4	2.7	54.1
80	0.2	.0125	5.9		18.1		54.0		50.2
99	.2	.0125	5.8	4.0	4.0	8.2	40.4	7.9	33.9

In a search for mitochondria by electron microscopy, only sparse, double-membraned vesicles, with a suggestion of invagination of the inner membrane, were observed in *B. ramosa* (Fig. 1) and *B. pringsheimii* grown under air. Similar vesicles were observed in both aerobically and anaerobically (with 5 percent CO_2) grown *Aqualinderella fermentans*. These structures resemble the rudimentary mitochondria of yeast cells whose respiration is genetically impaired or chemically inhibited (*3*) and may prove to be vestigial mitochondria.

Both species of *Blastocladia* contained only trace amounts of cytochrome, apparently of the b type (Fig. 2). No cytochromes were detected in *Aqualinderella fermentans* grown in medium GYS1W (*1*), in stationary culture, in air containing 5 percent CO_2. This may be similar to the loss of cytochromes in yeast with impaired respiration (*3*).

We measured manometrically the endogenous respiration of the mycelia of both *Blastocladia* species (*13*). We found some capacity for oxygen uptake, but its rate (Q_{O_2}), about 3.5 μl of O_2 per hour per milligram (dry weight), was much smaller than the value (about 24 μl) found in an aerobic blastocladialean (*15*).

With the exception of *Blastocladia* and *Aqualinderella*, members of the Blastocladiales and Leptomitales that have been investigated have cristate mitochondria (*16, 17*) and an oxidative energy metabolism with a complete cytochrome system (*13–16*). *Blastocladia* and *Aqualinderella* appear to have arisen in these two distantly related orders by parallel evolution in adaptation to their existence as obligate fermenters in stagnant, polluted waters. Even though considerable physiological differences exist between these two genera and also between the two *Blastocladia* species, there are three points of similarity: (i) excellent growth in the absence of oxygen or with only trace amounts of this gas, (ii) favorable response to atmospheric CO_2 pressures of 5 to 20 percent, and (iii) absence or near-absence of oxidative energy generation.

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Serum Elastase Inhibitor Deficiency and α_1-Antitrypsin Deficiency in Patients with Obstructive Emphysema

Abstract. *A decreased inhibition of pancreatic elastase has been detected in the serums of six patients with α_1-antitrypsin deficiency. Five have severe clinical and physiological pulmonary emphysema. This observation extends the defect of inhibition by serum to a second, biologically active proteolytic enzyme in this form of familial emphysema.*

In one type of dysproteinemia of human serum, α_1-antitrypsin, a main component of the α_1-globulin fraction of serum, is deficient (*1*). This dysproteinemia is associated with severe pulmonary emphysema beginning at an early age (*2*). In one family reported by Eriksson (*2*), the amounts of trypsin inhibitor in the serums of various members were either normal, moderately decreased (60 percent of normal), or greatly decreased (less than 10 percent of normal). Eriksson suggested that the concentration of α_1-antitrypsin was under genetic control. Cases have since been reported on the association between α_1-antitrypsin deficiency and familial, chronic, obstructive lung disease (*3–6*); these findings reinforce the concept of genetic transmission of the defect as an autosomal recessive with emphysema occurring only in the homozygotes. We now report that the serums of individuals with severe pulmonary emphysema which are deficient in α_1-globulin antitrypsin are also deficient in an inhibitor of pancreatic elastase.

The combined deficiencies were found in six patients [five white males (C.S., age 26; A.Sa., age 47; W.P., age 51; E.M., age 46; A.S., father of C.S., age 72) and one white female (M.A., age 35)]. With the exception of A.S., all patients have clinical and physiological evidence of pulmonary emphysema.

Inhibition of elastase was measured by exposure of a commercial elastin-orcein complex (Sigma) to bovine crystalline pancreatic elastase (Worthington) in the presence of serum, according to the method of Sachar *et al.* (*7*) as modified by Mandl *et al.* (*8*). Results are reported as units of elastase inhibited by 1 ml of undiluted serum, with 1 unit equivalent to the elastase activity releasing all the orcein from 1 mg of the elastin-orcein complex (*8*). In practice, values are converted from the inhibition observed at serum dilutions of 1 to 50.

We measured antitrypsin activity, by the method of Blackwood and Mandl (*9*), with crystalline trypsin (Tryptar, Armour) and a substrate of benzoyl-arginine naphthylamide hydrochloride (Mann Chemical) at *p*H 7.3. All tests were done in duplicate; serums were tested on more than one occasion, and a reference serum from a normal subject with normal inhibitory activity as well as uninhibited controls without human serum were tested each time.

Antibody to α_1-antitrypsin was made by immunizing rabbits with a normal human serum fraction containing the α_1-globulin obtained by elution from diethylaminoethyl-Sephadex columns. The amount of α_1-antitrypsin in the serums of the patients was measured by its reaction with prepared and commercial rabbit antibody to human α_1-antitrypsin (Hoechst Pharmaceutical) by Ouchterlony double diffusion on agar gel.

Three groups of patients were tested: (i) twenty (14 males and 6 females) normal, healthy adults (ages 20 to 65 years); (ii) nine subjects (three females, six males) with chronic obstructive lung disease (ages 44 to 67 years); and (iii) six subjects with α_1-antitrypsin deficiency.

The inhibition found at a dilution of 1:50 for serum tested against elastase and at a dilution of 1:60 against trypsin was plotted. Elastase inhibitor activity has been expressed as units of elastase inhibited per 1 ml of undiluted serum. Antitrypsin activity has been plotted as the number of milligrams of trypsin inhibited per milliliter of undiluted serum. There is no statistical difference in elastase inhibition or trypsin inhibition between the group of 20 normal subjects and the 9 subjects with chronic obstructive lung disease (Fig. 1). The average normal inhibitory activity per milliliter of serum is 55 ± 4 units of elastase and $1.27 \pm .08$ mg of trypsin. However, patients C.S, A.Sa., and W.P. all had 0 units of inhibition for elastase and inhibited 0.25 to 0.4 mg of trypsin. Patient M.A. inhibited 9 units of elastase and 0.3 mg of trypsin per milliliter of serum. The moderately deficient subjects E.M. and A.S. demonstrated 45 to 60 percent of the normal inhibitory activity for each enzyme.

The titer of α_1-antitrypsin against rabbit antiserum was less than 5 percent of normal for C.S., W.P., and A.Sa., and 25 percent of normal for A.S. and E.M.

All five subjects who demonstrated pulmonary emphysema had the following in common: (i) early age of onset, usually in the 3rd or 4th decade; (ii) absence of a history of cough and sputum either before or as a part of their current respiratory complaints; and (iii) radiographic evidence of hyperradiolucent lungs with bullae without cardiomegaly.

Pulmonary function tests (Table 1) indicate severe bronchial obstruction and a large total lung capacity, with a large functional residual capacity and residual volume. Diffusing capacity for carbon monoxide was reduced in A.S. and A.Sa. as measured by the steady-state method. With the exception of W.P., the arterial carbon dioxide tension was normal or low; arterial oxygen saturation was above 90 percent in each