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Effects of Ethylenediaminetetraacetate and Chloramphenicol on Mitochondrial Activity and Morphogenesis in *Mucor rouxii*

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The present study demonstrates the importance of mitochondrial activities in controlling *Mucor rouxii* morphogenesis. The respiratory capacity of the spores of this facultatively anaerobic, dimorphic fungus becomes repressed if germination and growth take place in the absence of oxygen. The level of activity of mitochondrial enzymes such as cytochrome oxidase and malate dehydrogenase is lower in the anaerobic yeastlike cells than it is in ungerminated spores and in aerobic hyphae, but the reverse is true for glycolytic enzymes such as pyruvate kinase and alcohol dehydrogenase. Following exposure to air, yeastlike cells convert into hyphae after a lag period corresponding to aerobic adaptation. Anaerobic cultures grown in the presence of ethylenediaminetetraacetate (EDTA) at a concentration of 10^{-4} M exhibit hyphal morphology. These cells, which are fully adapted to anaerobic fermentation, nevertheless have potentially active mitochondria with the same levels of respiratory enzymes as ungerminated spores. These cells are able to grow immediately after aeration, without an adaptation lag. Evidence is presented which indicates that the morphogenetic effect of EDTA is not the result of elimination of free metals. Additional evidence proving mitochondrial control of morphogenesis in *M. rouxii* is that chloramphenicol (4 mg/ml) induced the formation of respiratory-deficient, yeastlike cells in aerobic cultures.

Mucor rouxii, like other *Mucor* species, is a facultative anaerobe. Its growth with or without oxygen is accompanied by striking changes in morphology. Without oxygen, spores germinate into spherical yeastlike cells which reproduce by budding. If oxygen is present, they develop into typical hyphae. This phenomenon represents an example of dimorphism (1). It has been demonstrated that many aspects of cell metabolism are involved in the expression of dimorphism, but the molecular basis of this phenomenon remains undefined. Considerable evidence has been accumulated which suggests that mitochondrial functions are needed for hyphae formation. For example, growth is yeastlike even in the presence of oxygen for a respiratory-deficient mutant (15), or for the wild type in the presence of chemical agents which impair or uncouple respiration (16). Under these conditions, the respiratory quotient results are altered and a high level of glycolysis is commonly observed. On the other hand, filamentous growth can be obtained in anaerobic atmospheres provided the chelating agent ethylenediaminetetraacetate (EDTA) is added to the growth medium. This observation, which was made by Bartnicki-García and Nickerson (2), led them to suggest that such an effect was produced by EDTA through the elimination of certain cations such as Zn^{2+} which are required in yeastlike morphogenesis. The fact that filamentous growth, which otherwise occurs only in the presence of oxygen, can also be obtained under anaerobic conditions if EDTA is present in the medium prompted us to investigate further the mode of action of this agent to gain a better understanding of the phenomenon of dimorphism.

MATERIALS AND METHODS

M. rouxii NRRL 1894, a strain originally from C. W. Hesseltine (Northern Utilization Research and Development Division, Peoria, Ill.), was kindly provided by Roger Storck (Rice University, Houston, Tex.).

The spores of *M. rouxii* were produced and stored as described by Haidle and Storck (4). The synthetic medium (BG) of Bartnicki-García and Nickerson (2) was used throughout all experiments. Thiamine and nicotinic acid were added to this medium for anaerobic growth. The above medium was supplemented with EDTA (10^{-4} M) or chloramphenicol (4 mg/ml) when indicated. Anaerobic incubation was done under a CO_2 atmosphere. The spore inoculum was 5×10^5 spores/ml of medium for anaerobic cultures, and 10^5 spores/ml for aerobic growth. Culture flasks were incubated at 27 C on a reciprocating shaker at 120 strokes/min.

Estimation of growth rate. Anaerobic cultures, with or without supplement, were grown for various periods, harvested aseptically by filtration on membrane filters (pore size, 0.45 μm; Millipore Corp.), rinsed with sterile distilled water, and inoculated in fresh BG medium and reincubated as before but in an aerobic atmosphere. Samples of the culture were taken each hour and processed for determination of total protein (7).

Respiratory determinations. O_2 uptake and CO_2 liberation were determined at 27 C by conventional manometric techniques (17) in a Warburg respirometer.

Preparation of extracts and crude mitochondrial fractions. After grinding the cells in a chilled mortar with twice their wet weight of dry, cold sand for 4 to 6 min, a homogeneous paste was obtained. The paste was buffered with 0.1 M tris(hydroxymethyl)-aminomethane buffer, pH 7.9, containing 0.25 M sucrose, 5 mM Mg^{2+}, and 0.15% bovine serum albumin. The resulting homogenate was centrifuged twice at $1,000 \times g$ for 10 min in a Sorvall RC2-B centrifuge to remove sand, cell debris, and nuclei. The resulting supernatant fraction was centrifuged at $27,000 \times g$ for 90 min to separate a crude mitochondrial fraction from the crude supernatant fraction.

Enzymatic assays. Cytochrome oxidase activity was determined by the method of Smith (13). Specific activity of the enzyme is expressed as units of activity per milligram of protein. For malate dehydrogenase assay, the method of Ochoa (10) was used. Specific activity is expressed as for cytochrome oxidase. Before the mitochondrial enzymes were assayed, the crude mitochondrial fraction was treated with 1% octylphenoxypolyethoxy-ethanol (Triton X-100). Pyruvate kinase was assayed by the 2,4-dinitrophenylhydrazone method (8), and alcohol dehydrogenase was determined by the method of Bonnichsen (3). Protein was determined according to Lowry et al. (9).

RESULTS

Effect of EDTA on cell morphology and growth. Bartnicki-García and Nickerson (2) showed that *M. rouxii* spores produce branched filaments under anaerobic conditions in a medium supplemented with 10^{-4} M EDTA. They suggested that EDTA acted by complexing metal ions which were required for the development of yeastlike cells. If this is the case, the same phenomenon should be obtained by lowering the concentration of the salt components of the medium or by systematically eliminating each of these.

Such a possibility was tested, but, as shown in Table 1, growth was always filamentous under anaerobic conditions in the presence of EDTA and yeastlike in its absence. This was so even when germinating spores were pretreated with a high (10^{-2} M) EDTA concentration to remove traces of metals and then washed and incubated anaerobically in metal-free media. It was noticed that if Zn^{2+} was omitted the growth yield was low, especially in the presence of EDTA. This phenomenon might be attributed to interference with the activity of glycolytic

TABLE 1. *Effects of EDTA and of the atmosphere of incubation on the morphology and growth of M. rouxii in metal-deficient media*

Medium	Growth condition[a]		
	O_2	CO_2	CO_2 + EDTA (10^{-4} M)
Synthetic medium (BG)[b]	F	Y	F
BG with salts diluted 1,000-fold	F	Y	F
BG minus Fe^{2+}	F	Y	F
BG minus Zn^{2+}	F	Y	F (±)
BG minus Mn^{2+}	F	Y	F
BG minus Cu^{2+}	F	Y	F
BG minus Mg^{2+}	F (A)	Y	F (A)
BG minus Fe^{2+}, Cu^{2+}	F	Y	F
BG minus Fe^{2+}, Zn^{2+}	F	Y	F (±)
BG minus Fe^{2+}, Mn^{2+}	F	Y	F
BG minus Cu^{2+}, Mn^{2+}	F	Y	F
BG minus Cu^{2+}, Zn^{2+}	F	Y	F (±)
BG minus Mn^{2+}, Zn^{2+}	F	Y	F (±)
BG minus Fe^{2+}, Cu^{2+}, Mn^{2+}	F	Y	F
BG minus Fe^{2+}, Cu^{2+}, Zn^{2+}	F	Y	F (±)
BG minus Fe^{2+}, Zn^{2+}, Mn^{2+}	F	Y	F (±)
BG minus Zn^{2+}, Mn^{2+}, Cu^{2+}	F	Y	F (±)
BG minus Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}	F	Y	F (±)

[a] Morphology and growth were observed after 48 h of development at 27 C. F = filamentous growth; Y = yeastlike growth; (A) = abnormal morphology; (±) = poor development. (Zn^{2+}-deficient cultures usually gave 60 to 70% of the cell mass of the control.)

[b] Glucose, 20 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 1.8 mg; $(NH_4)_2SO_4$, 2.4 g; $FeSO_4 \cdot 7H_2O$, 1.0 mg; $MnSO_4 \cdot H_2O$, 0.3 mg; $CuSO_4 \cdot 5H_2O$, 0.4 mg; casein hydrolysate (vitamin-free), 2.0 g; thiamine, 1.0 mg; nicotinic acid, 1.0 mg; and water to 1,000 ml.

enzymes, some of which are activated by Zn ions (5). Therefore, it appears unlikely that the morphogenetic effect of EDTA is due to unavailability of free metals. Instead, it appears that the active agent is EDTA itself. We cannot conclude, however, whether the active form is free EDTA or an EDTA-metal complex.

Effect of EDTA on the growth kinetics after exposure to air. Anaerobically grown yeastlike cells convert to filaments and form a mycelium after exposure to air (4). We observed that the time which elapses between the onset of aeration and that of exponential growth is proportional to the age of the anaerobic culture. This is shown in Fig. 1, where it can be observed that the lag previous to exponential growth is directly related to the length of the anaerobic incubation and that the time required for doubling the cell protein increased from 2.2 h to 4.2 h when a 15 h old culture was compared to one 35 h old. It should be noted that regardless of the size of the lag the rate of growth was the same for all cultures. In contrast, EDTA-treated anaerobic cultures behave differently; as shown in Fig. 2, exponential growth started immediately after aeration, even for a 40-h

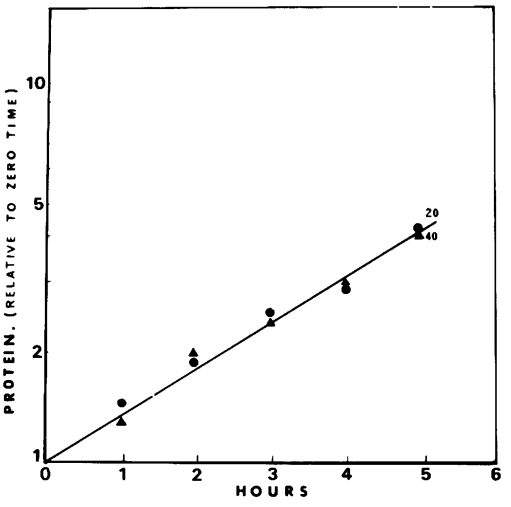

FIG. 2. *Growth of cells grown anaerobically in the presence of 10^{-4} M EDTA, after exposure to air. The cultures were incubated under a CO_2 atmosphere during 20 h (●) or 40 h (▲). After the anaerobic incubation, the cells were harvested by filtration, resuspended in fresh medium without EDTA, and incubated aerobically. Growth was determined as described in Fig. 1.*

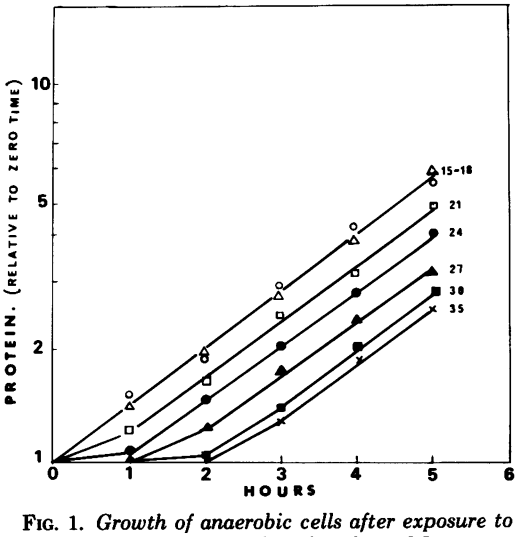

FIG. 1. *Growth of anaerobic cells after exposure to air. The cultures were incubated under a CO_2 atmosphere for the number of hours indicated at the top of each curve. The cells were harvested by filtration, resuspended in fresh medium, and incubated aerobically. Care was taken to have the same amount of cells, in all cases, at the beginning of aeration. The rate of growth was estimated by measuring total protein every hour, in a portion of the culture. The experimental points represent the ratio between the amount of protein at a given time and the amount at zero time, plotted semilogarithmically.*

anaerobic culture. The simplest explanation for these results is to assume that the respiratory system of the spores was repressed by anaerobiosis, and therefore the lag which is observed corresponds to the period needed for aerobic adaptation. EDTA appears somehow to protect the respiratory capacity of the cells from being lost in the absence of oxygen, since EDTA-treated cultures grow actively without a noticeable delay after being aerated.

Development of respiration of EDTA-treated cells after exposure to air. Oxygen uptake and CO_2 liberation were determined manometrically for cells grown anaerobically with or without EDTA. The measurements were made 30 min after exposing the cultures to air. The results in Fig. 3 show that the cells which were grown anaerobically for 45 h had a slower rate of establishment of the specific oxygen uptake than those which were grown in the same situation for only 15 h (Fig 3A). On the other hand, EDTA-treated cells maintained an unchanged rate of establishment of oxygen uptake regardless of the period of anaerobiosis (Fig. 3b). Furthermore, the O_2-CO_2 ratios (Fig. 3B and C) were higher in the EDTA-treated cells than in those grown without the agent; this fact suggests a more oxidative type of carbohydrate degradation in the former.

Effect of EDTA on the activity of mito-

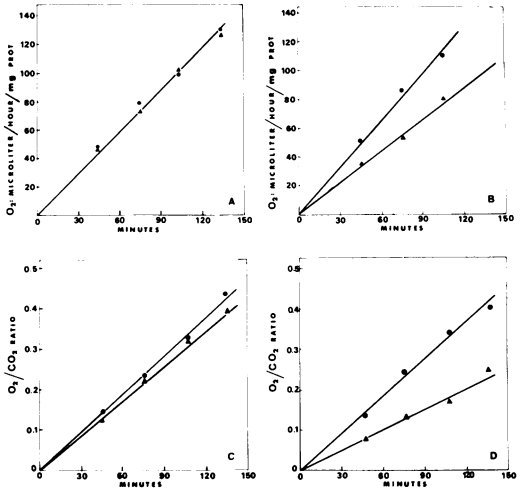

FIG. 3. *Appearance of respiratory capacity after exposure to air in cells grown anaerobically in the presence (●) or absence (▲) of EDTA. Respiratory rates were determined manometrically at 27 C by measuring O_2 uptake and CO_2 liberation 30 min after aeration of the cultures. (A) Evolution of the specific oxygen uptake in a 15-h-old culture; (B) same as A, in a 45-h-old culture; (C) evolution of the O_2-CO_2 ratio in a 15-h-old culture; (D) same as C, in a 45 h-old culture.*

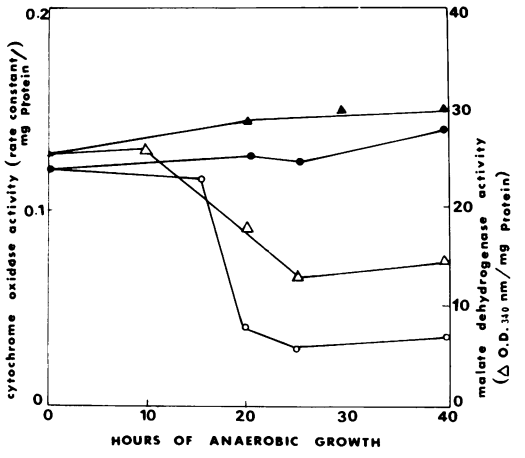

FIG. 4. *Effect of EDTA on the specific activity of mitochondrial enzymes as a function of time of anaerobic growth. (○) Cytochrome oxidase, no EDTA added; (●) cytochrome oxidase, 10^{-4} M EDTA; (△) malate dehydrogenase, no EDTA added; (▲) malate dehydrogenase, 10^{-4} M EDTA. Enzymatic activities were measured in a crude mitochondrial fraction as described in Materials and Methods.*

chondrial enzymes. The possibllity that the differences in growth behavior and respiratory capacity observed between EDTA-treated and untreated anaerobic cultures were also associated with enhanced levels of respiratory enzymes was investigated. Cytochrome oxidase and malate dehydrogenase activities were determined in 27,000 × *g* pellets obtained from ungerminated spores and from anaerobic cells grown with or without EDTA for various time periods. It can be observed in Fig. 4 that during the first 10 to 15 h of anaerobic growth the specific activity of the two enzymes remained constant, at a level comparable to that present in ungerminated spores; up to this time, no differences were noticeable between EDTA-treated and untreated cultures. After 15 h, the level of cytochrome oxidase and malate dehydrogenase diminished to one-fourth and one-half of the initial values, respectively, in the untreated cells; on the other hand, EDTA-treated cells conserved the initial specific activities of mitochondrial enzymes throughout the anaerobic incubation.

These results suggest that the effect of EDTA on morphology, growth, and respiration of *M. rouxii* might be explained by assuming that the cells treated with this agent are somehow protected from anaerobic de-differentiation; therefore, at the time of exposure to air these cells are able to grow faster and respire more efficiently since they possess a more competent chondrioma than those which developed in the absence of EDTA. It is worthwhile mentioning that after anaerobic de-differentiation has occurred the cells become insensitive to EDTA. This was shown by adding the agent after 20 h of anaerobic development; no filaments, but yeastlike cells, developed in the culture.

Effect of chloramphenicol on the activity of mitochondrial and glycolytic enzymes. To demonstrate further the involvement of mitochondrial activity in the morphogenetic processes of *M. rouxii*, we inoculated spores in BG medium with saturating amounts of chloramphenicol (4 mg/ml) and incubated them aerobically. The antibiotic induced yeastlike growth (Fig. 5), and its effect was potentiated if the concentration of glucose in the medium was raised to 5%; at lower concentrations of glucose, there was mainly growth inhibition, and a few percent of the spores formed abnormal hyphae.

The enzymatic characteristics of chloramphenicol-treated cells are compared in Table 2 to those of ungerminated spores, anaerobic cells treated or not with EDTA, and aerobic mycelium. The effect of EDTA on the activity of respiratory enzymes was as described above, but the level of glycolytic enzymes was similar to that of anaerobic yeastlike cells and was, therefore, not affected by this agent. Chloramphenicol-treated cells exhibited a low cyto-

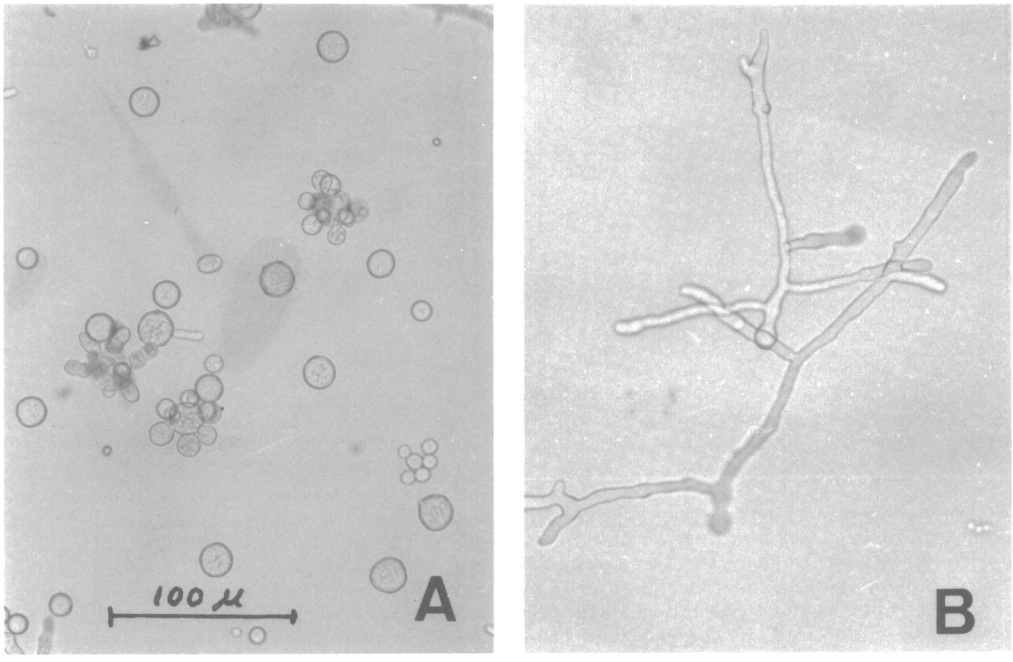

FIG. 5. *Effects of chloramphenicol and EDTA on the morphology of Mucor rouxii. (A) The cells were grown aerobically in BG medium supplemented with 4 mg of chloramphenicol/ml; glucose concentration was 5%. (B) The cells were grown anaerobically in BG medium supplemented with 10^{-4} M EDTA; glucose concentration was 2%. Pictures were taken after 25 and 40 h of development, respectively.*

TABLE 2. *Specific activities of mitochondrial and glycolytic enzymes in Mucor rouxii grown anaerobically with or without EDTA (10^{-4} M), or aerobically with or without chloramphenicol (4 mg/ml), as compared to that present in ungerminated spores*

Type of cell[a]	Cytochrome oxidase[b]	Malate dehydrogenase[c]	Pyruvate kinase[d]	Alcohol dehydrogenase[c]
Ungerminated spores	0.120	24	2.3	5.0
Mycelium (aerobic)	0.340	60	0.9	3.9
Yeastlike cells (anaerobic)	0.025	15	4.8	19.5
Yeastlike cells (aerobic, chloramphenicol)	0.014	68	3.2	5.9
Mycelium (anaerobic, EDTA)	0.140	33	5.2	14.6

[a] Aerobic cultures were harvested after 24 h of growth and anaerobic cultures after 48 h.

[b] Expressed as the rate constant per milligram of protein.

[c] Expressed as the change in optical density at 340 nm per minute per milligram of protein.

[d] Expressed as micromoles of pyruvate per minute per milligram of protein.

chrome oxidase activity and enhanced levels of pyruvate kinase and alcohol dehydrogenase; no noticeable effect at the level of malate dehydrogenase was observed. The observed effect of chloramphenicol on *M. rouxii* mitochondrial enzymes is in agreement with what was reported by Howell et al. (6) for a *Neurospora crassa* mutant.

DISCUSSION

The availability of molecular oxygen is the determining factor which induces a germinating spore of *M. rouxii* to develop into a yeastlike or a filamentous body. Considerable evidence has accumulated showing that apical growth and differentiation of mycelial structures in *Mucor*, as well as in other fungi, are often associated with aerobic metabolism. In view of that, it was proposed that filamentous morphology in fungi should be considered as "a morphogenetic expression of the Pasteur effect" (16).

The evidence presented in this work demonstrates that the mitochondrial structures existing in the spore of *M. rouxii* will lose their functionality if germination and growth occur in an anaerobic atmosphere; this is an example of the well-known "repression by anaerobiosis" (12). As a result of such repression, cells which were grown anaerobically had a slower rate of respiratory induction and lower activity of mitochondrial enzymes than ungerminated spores and aerobic mycelium. This phenomenon is

accompanied by the necessity for a period of adaptation, after exposure to air, before the anaerobic organism is able to take advantage of its respiratory machinery for active growth.

In the presence of EDTA, not only the morphology of the cultures resembled that which is observed aerobically, but the respiratory system appeared unaffected by anaerobiosis. A situation similar to that of EDTA-treated *M. rouxii* cells was reported for *Saccharomyces cerevisiae* grown anaerobically in the absence of Zn^{2+} (11), or with galactose instead of glucose (13). Under such conditions, however, the higher respiratory capacity exhibited by the cells, compared to that of controls, was due to an extremely fast induction of the respiratory system, because when cycloheximide was added before aeration no differences were observed between cultures grown with or without Zn^{2+}, or with galactose instead of glucose (11). The addition of cycloheximide (200 μg/ml) to EDTA-treated *M. rouxii* cells before aeration did not significantly change the results shown in Fig. 4 and Table 2. Therefore, the enhanced respiratory activity of these cells cannot be the result of a rapid induction during the period of harvesting of the cultures.

Our results do not permit us, so far, to suggest a reasonable interpretation of the mode of action of EDTA. We can only state that the effect of this agent could not be reproduced just by eliminating the various metallic components of the medium, and therefore it should be concluded that EDTA itself must be the active agent for the induction of filamentous morphogenesis under anaerobic conditions. It was previously demonstrated that the effect of EDTA on growth and morphogenesis of *M. rouxii* was progressively abolished by increasing the concentration of some divalent cations (2). This suggests that an EDTA-metal complex is inactive, but it does not permit us to decide whether the active form of EDTA is free EDTA or a complex formed with a different metal, since in both alternatives the active form should disappear if the equilibrium is shifted toward the formation of an inactive complex.

Our work provides evidence of the involvement of mitochondria in the morphogenetic processes of *M. rouxii* by demonstrating that inhibition of mitochondrial protein synthesis by chloramphenicol induces yeastlike growth under aerobic conditions. In contrast, the filamentous cells obtained under anaerobic conditions in the presence of EDTA possessed potentially active mitochondria. Although at the present time the evidence tends to suggest that yeastlike morphogenesis is a response of the organism to any situation which favors a high level of fermentation, it should be kept in mind that the status of cell respiration is mainly a consequence of mitochondrial activity. Therefore, the possibility cannot be discarded that morphogenesis in *Mucor* could be controlled through some other type of mitochondrial-cytoplasmic interaction. Otherwise, it might be difficult to explain EDTA-induced filamentation, which takes place in an obligately fermentative environment.

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