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Relationship between Dimorphology and Respiration in *Mucor genevensis* Studied with Chloramphenicol

G. D. CLARK-WALKER

Department of Developmental Biology, Research School of Biological Sciences, Australian National University, Canberra, Australia

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Growth of *Mucor genevensis*, a facultatively anaerobic dimorphic mold, in high concentrations of chloramphenicol (4 mg/ml) leads to increased numbers of yeast-like cells and small club-like mycelial forms. This change in morphology is accompanied by a threefold increase in the mass doubling time, the loss of cyanide-sensitive respiration, and the development of cyanide-insensitive respiration. Associated with these changes is the absence of cytochromes aa_3 and *b* and the inability of the organism to utilize ethanol; in addition, mitochondria appear more numerous and have less internal membrane. A further inhibitory action of the antibiotic, other than eliminating functional mitochondria, appears likely since microaerobic cultures which lack respiratory ability have twice the mass doubling time in the presence of the drug. Although a small inhibition of amino acid incorporation by cytoplasmic ribosomes is found with a high chloramphenicol concentration, it is insufficient to account for the effect on growth of the microaerobic culture. The nature of this additional effect of chloramphenicol remains to be determined, but it has been shown that increasing the glucose concentration can partially reverse this action of the antibiotic. The effect of the drug on the morphology of the organism is not as dramatic as that of phenethyl alcohol in producing yeast-like forms. However, in view of the action of chloramphenicol in eliminating functional mitochondria in *M. genevensis* the suggestion that phenethyl alcohol exerts its effect in promoting yeast-like morphology by uncoupling oxidative phosphorylation should be re-examined.

During studies on the biogenesis of mitochondria in the facultatively anaerobic and dimorphic organism *Mucor genevensis*, it was noticed that aerobic batch culture in the presence of chloramphenicol resulted in the formation of increased numbers of yeast-like cells.

In a previous study with another facultatively anaerobic organism, *Saccharomyces cerevisiae*, it has been shown that chloramphenicol specifically inhibits in vivo mitochondrial protein synthesis, resulting in cells which no longer respire (2). Therefore, the possibility was considered that functional mitochondria play a role in the dimorphology of *M. genevensis* and are important in maintaining mycelial growth. This idea was seemingly substantiated by the suggestion that phenethyl alcohol exerts its effect in promoting yeast-like morphology in *M. rouxii* by acting as an uncoupling agent of oxidative phosphorylation (14). However, this communication describes results which indicate that the promotion of yeast-like morphology in *M. genevensis* by chloramphenicol cannot be concisely explained by an interference with mitochondrial function.

MATERIALS AND METHODS

The organism, culture medium, growth conditions, determinations of respiratory rate and dry weight, glucose estimation, absorption spectrum determination, and electron microscopy were as previously described (1, 6).

Additionally, chloramphenicol, at a concentration of 4 mg/ml in the 0.1 M sodium phosphate buffer used to measure respiration, produced no change in respiratory rate of cells after correction for the different oxygen capacity of the medium. A similar observation has been made with *Saccharomyces cerevisiae* (12).

Microaerobic culture conditions. For this study the 1% GSy0.1YE medium, described previously, was used (6). Since strict anaerobic culture of *M. genevensis* requires the presence of more yeast extract for sustained growth, the medium used in the present study was not exhaustively flushed with nitrogen after inoculation. Rather, the flasks containing the orga-

nism were sealed without further treatment after inoculation. These cultures are referred to as microaerobic.

Analysis of metabolic products. Ethanol and acetate were estimated in culture supernatant fractions with a Shimadzu gas chromatograph GC-1C by applying a sample directly to a Porapak Q column (6 ft by 1/8 inch [1.8 m by 4 mm], 120 mesh; Waters Associates Inc., Framingham, Mass.) operated at 180 C with 30 ml of N_2 per min and 40 ml of H_2 per min as carrier gas mixture. To estimate succinate and lactate, a 1-ml sample of the culture supernatant fluid was dried, and the residue was taken up in 0.2 ml of Sil-prep (Applied Sciences Laboratories Inc., State College, Pa.), and 2 to 5 μliters was applied to a column (6 ft by 1/8 inch) of 5% OV 101 (dimethyl silicone) on 100-mesh Varaport 30 (Applied Sciences Lab. Inc). operated on a temperature program of 4 C/min starting at 80 C using 60 ml of N_2/min 40 ml/min of H_2 as carrier gas mixture. The compounds in the culture supernatant fluids were identified and estimated by comparison with the retention times of known samples.

Amino acid incorporation. Ribosomes and pH 5 fraction enzymes were prepared from exponentially growing *M. genevensis* in 1% GSy0.1YE medium. The organism, in 5 liters of medium, was harvested at a cell density of 0.4 mg (dry weight)/ml by filtration and washed with a small quantity of ribosome isolation buffer (RIB) containing 20 mM tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride (pH 7.4), 2 mM mercaptoethanol, 5 mM magnesium acetate, and 10 mM KCl. The mycelium was suspended in a small volume of RIB, and the cell contents were extracted by shaking the mycelium with 1-mm diameter glass beads for 30 s in a Braun homogenizer cooled by expanding CO_2. Cell debris was removed by centrifugation at 2,000 × *g* for 5 min; mitochondria and small particles removed at 10,000 × *g* for 20 min; and ribosomes were collected by centrifugation at 100,000 × *g* for 90 min. Excess moisture was removed from the ribosomal pellet with tissue paper, and the pellet was resuspended in a minimal volume of RIB for use in the amino acid incorporation assay without further purification. The supernatant fraction from the high-speed centrifugation was brought to pH 5.2 by the gradual addition of 1 N acetic acid. After standing for 10 min at 0 C, the pH 5 precipitate was collected by centrifugation at 2,000 × *g*, washed with 5 ml of water, and suspended in 5 ml of RIB. The pH was adjusted to 7.5 by the addition of 0.1 N KOH, and a small amount of insoluble material was removed by centrifugation. Small samples, 0.3 ml of both the pH 5 fraction and the ribosomes, were frozen in liquid N_2 and stored at −20 C. Dry weight of the pH 5 fraction was determined after precipitation by ethanol, and dry weight of the ribosomes was estimated at 260 nm using an optical density of 1.0, equivalent to 74 μg (dry weight) of ribosomes per ml (H. Naora, personal communication).

For amino acid incorporation, the following constituents were present in 0.5 ml: Tris-hydrochloride (pH 7.4), 34 mM; KCl, 2 mM; NH_4Cl, 100 mM; Mg acetate, 6 mM; mercaptoethanol, 2.4 mM; adenosine triphosphate (ATP), 1 mM; guanosine triphosphate (GTP), 0.2 mM; phosphoenol pyruvate, 5 mM; pyruvate kinase, 30 μg; C^{14}-leucine, 1 μCi; ribosomes, 455 μg; and pH 5 fraction, 290 μg. The mixture was incubated at 30 C, and 0.1-ml fractions were transferred to 2.5-cm Whatman no. 3 filters for processing. After 30 s for the solution to be absorbed by the filter, the protein was precipitated by immersing the filter in 7% trichloroacetic acid in 33% (vol/vol) ethanol for 1 h at room temperature. The filter was washed for 5 min in 5% trichloroacetic acid before being transferred to fresh 5% trichloroacetic acid at 85 C for 15 min. After this time, the filters were transferred through two changes of 5% trichloroacetic acid and then placed in vials and heated for 2 h at 110 C to remove moisture. Radioactivity remaining on the filters was counted in a Beckman LS-250 liquid scintillation counter with 10 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(4 methyl-5-phenyloxazolyl)-benzene per vial.

Chloramphenicol at a high concentration was added to the amino acid incorporation mixture in two ways. For the system containing 2.25 mg/ml final concentration, the constituents of the incubation mixture minus the ribosomes and pH 5 fraction were dissolved directly in a solution containing 4 mg of chloramphenicol per ml. At the higher concentration of 5 mg/ml, the chloramphenicol was added to the final incubation mixture in 25 μliters of ethanol. This amount of ethanol had no detrimental effect on the amino acid-incorporating system.

L(+)-threo isomer of chloramphenicol. The L(+)-threo isomer of chloramphenicol was prepared from L(+)-threo-1-*p*-nitro phenyl-2-amino-1,3-propanediol and methyl dichloracetate by a published procedure (3, 11). The compound had a melting point of 152 C (chloramphenicol 152 C) and $[\alpha]_D^{25}$ 19° (chloramphenicol plus 19°).

Chemicals. Chloramphenicol and the precursor of the isomer were generous gifts from Parke-Davis & Co., Sydney, Australia. Antimycin was obtained from Cal-biochem., Los Angeles, Calif.; C^{14}-leucine (specific activity 40 μCi/μmol was from the Radio Chemical Centre, Amersham, England; and pyruvate kinase, phosphoenol pyruvate mono potassium salt, ATP, and GTP were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

In the absence of chloramphenicol, long branched filaments 4 to 12 μm in diameter are present, and towards the end of exponential growth some arthrospores are formed at the ends of hyphae (Fig. 1). In the presence of chloramphenicol, there are about equal numbers of short hyphae with swollen tips and budding yeast-like cells (Fig. 2). The yeast-like cells can be as large as 30 μm in diameter. In contrast to aerobic culture, in the absence of chloramphenicol no large clumps of intertwined mycelium are observed in cultures grown in the presence of high concentrations of chloramphenicol.

The growth and respiration of *M. genevensis* in the presence of 4 mg of chloramphenicol per

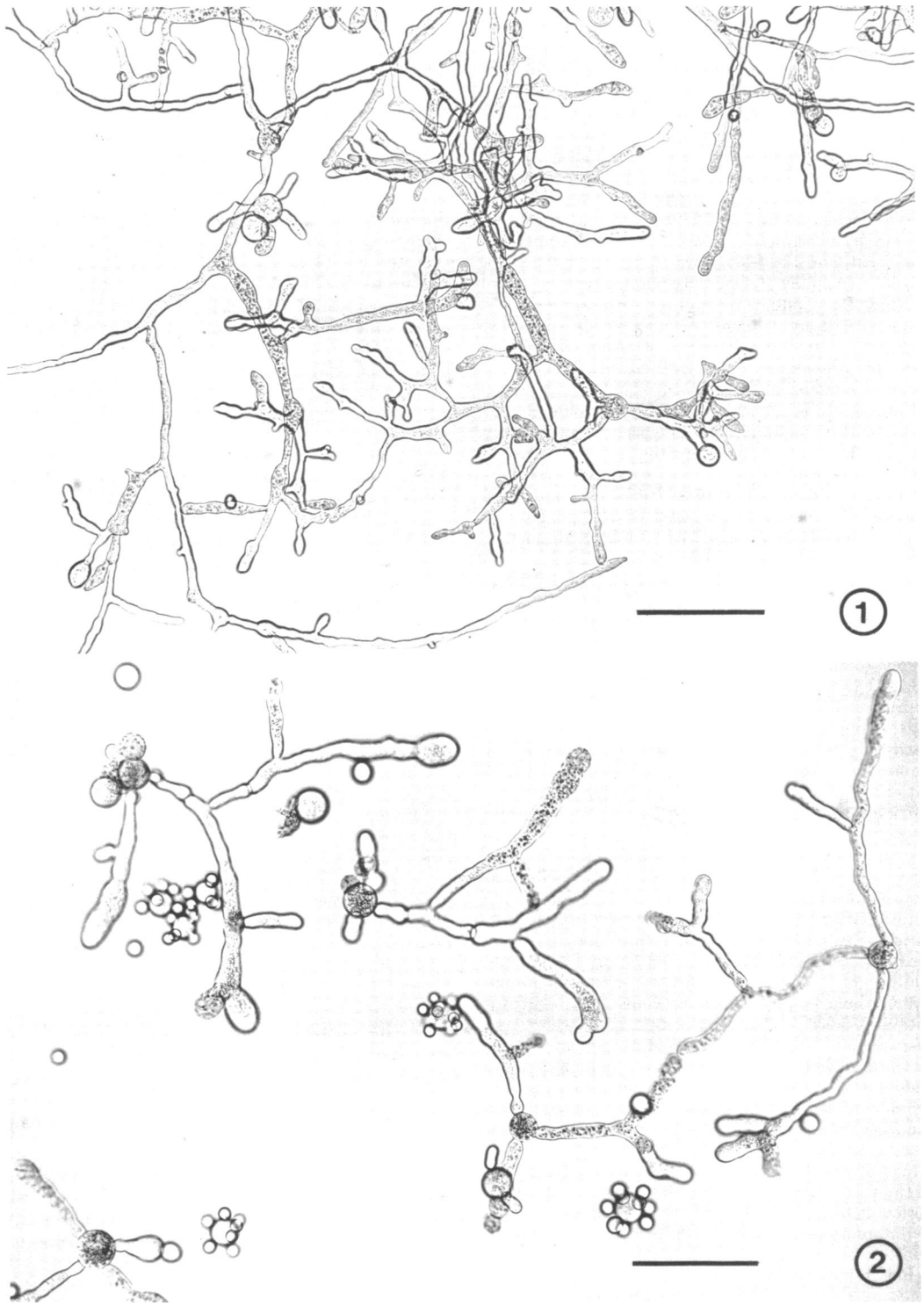

FIG. 1 and 2. *Light micrographs of M. genevensis grown aerobically in 1% GSy0.1YE medium to early stationary phase in the absence and presence of 4 mg of chloramphenicol per ml, respectively. The bar represents 100 μm.*

ml (close to a saturated solution) is shown in Fig. 3. At this concentration of antibiotic the mass doubling-time of the organism is 6 h, and the final cell yield, when the glucose is exhausted, is only 1.1 mg (dry weight) per ml. This is in contrast to a doubling time of 2 h and a cell yield of 4.0 mg/ml in the absence of the antibiotic (1) (Table 1).

The respiratory rate of the cells grown in 4 mg of chloramphenicol per ml is about half that obtained in the absence of the antibiotic (Fig. 3 and 4). Additionally, this respiration is completely insensitive to 1 mM potassium cyanide or 10 μg of antimycin per ml throughout the growth cycle.

A high concentration of chloramphenicol is necessary to bring about these effects on growth rate and respiration because at lower concentrations of antibiotic the mass doubling time is progressively shortened and final cell yields are increased (Table 1 and Fig. 4). In addition, at chloramphenicol concentrations below 4 mg/ml a cyanide-sensitive component is present in the respiration although the cyanide-insensitive respiration remains about the same. By contrast the L(+)-threo isomer of chloramphenicol had no effect on growth rate, morphology, or respiration even at a concentration of 4 mg/ml.

The effect of chloramphenicol on the growth rate can be partially reversed by 10% glucose in the medium (Table 1) although this concentration of glucose does not alter the growth rate of the organism in the absence of the drug.

Under microaerobic conditions, the mass doubling time of the cells in the 1% GSy0.1YE medium is 3 h, but the final yield is the same as in the aerobic culture grown in the presence of 4 mg of chloramphenicol per ml. The doubling time of microaerobically grown cells in the presence of 4 mg of chloramphenicol per ml is 7 h; however, under these conditions the cells stop growing before utilizing the available glucose. The respiration rate of microaerobically grown cells is 15 to 20 ng atoms of O_2 per min per mg dry weight, and 50% of this residual respiration is inhibited by 1 mM KCN. In the presence of 4 mg of chloramphenicol per ml, the respiratory rate is only 5 to 10 ng atoms of O_2 per min per mg dry weight and this respiration is insensitive to KCN or antimycin.

Succinate and lactate were found at constant levels in culture supernatants of *M. genevensis* during the growth cycle (Table 2), and on some

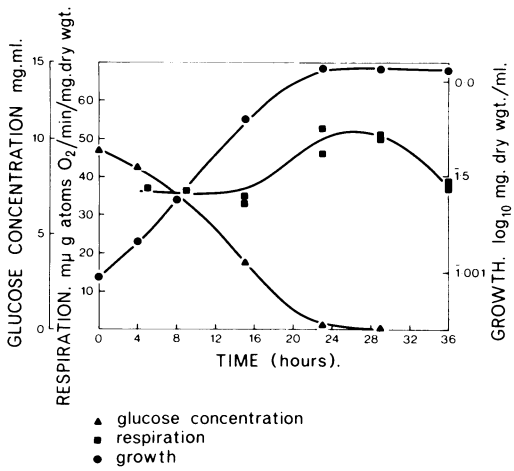

FIG. 3. *Respiration* (■) *and glucose concentration* (▲) *during aerobic growth* (●) *of M. genevensis in 1% GSy0.1YE medium in the presence of 4 mg of chloramphenicol per ml.*

TABLE 1. *Effect of chloramphenicol on the aerobic and microaerobic growth rate and cell yield of M. genevensis*

Gas phase	Glucose (mg/ml)	Chloramphenicol (mg/ml)	Mass doubling time (h)	Final cell yield (mg dry wt/ml)
Aerobic	10	0	2.0	4.0
Aerobic	10	0.5	2.5	2.2
Aerobic	10	1.5	4.1	1.3
Aerobic	10	4.0	6.0	1.1
Aerobic	100	0	2.0	4.0[a]
Aerobic	100	4.0	3.8	1.8[a]
Microaerobic	10	0	3.0	1.1
Microaerobic	10	4.0	7.0	0.4[a]

[a] Glucose still present.

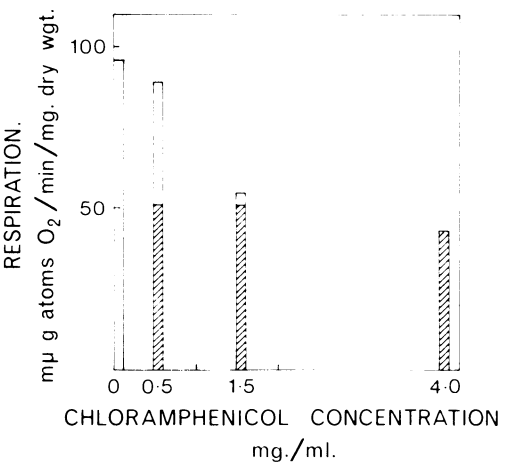

FIG. 4. *Respiration of exponentially growing M. genevensis from 1% GSy0.1YE medium containing the indicated levels of chloramphenicol. The hatched area of the bars represents cyanide-insensitive respiration.*

TABLE 2. *Metabolites in culture supernatant fluids of M. genevensis grown in 1% GSy0.1YE medium*

Growth conditions	Cell yield (mg dry wt/ml)	Conc of metabolite (mM)		
		Ethanol	Succinate	Lactate
Aerobic	0.48	0	35	2.85
	1.3	1.7		
	2.8	10.9	30	3.65
Aerobic + 4 mg of chloramphenicol per ml	0.08	3.7	64	3.15
	0.38	30.3	59	3.15
	1.1	101	42	4.45
Microaerobic	0.20	13.3		
	0.60	65		
	1.1	97		

occasions a small amount of acetate (less than 1 mM) was also detected. In the presence of chloramphenicol, a twofold increase in succinate concentration was noted, whereas lactate levels remained the same as in the absence of the drug. In contrast, during normal aerobic growth, ethanol levels remained low but sometimes rose slightly towards the end of the growth cycle if excessive clumping of the mycelium took place (as was the case with the experiment recorded in Table 2). However, in the presence of chloramphenicol or under microaerobic conditions ethanol accumulates throughout the growth cycle.

The absorption spectrum of *M. genevensis* in the absence of chloramphenicol showed peaks at 604 and 550 nm and a shoulder at 560 nm corresponding to the absorption bands of cytochromes aa_3, *c*, and b, respectively (Fig. 5). In the presence of 0.5 mg of chloramphenicol per ml, the cytochrome aa_3 peak at 604 nm is reduced in comparison to cytochrome *c* and is entirely absent from the spectrum at the higher concentration of antibiotic. The shoulder at 560 nm, corresponding to cytochrome *b*, is also significantly reduced in the presence of 4 mg of chloramphenicol per ml.

The appearance of *M. genevensis* grown to early stationary phase in the absence of chloramphenicol is shown in Fig. 6. This section of a hypha contains nuclei, mitochondria with clearly defined cristae, vacuoles, small vesicles, lipid granules, endoplasmic reticulum close to the cell wall, and unstained granules (presumably carbohydrate).

The ultrastructures of a swollen hypha and yeast-like cells from a culture grown to early stationary phase in the presence of 4 mg of chloramphenicol per ml showed a noticeable change in the appearance and number of mitochondria which now occupy a large proportion of the cell (Fig. 7, 8). In addition to the increase in number of mitochondria in chloramphenicol-

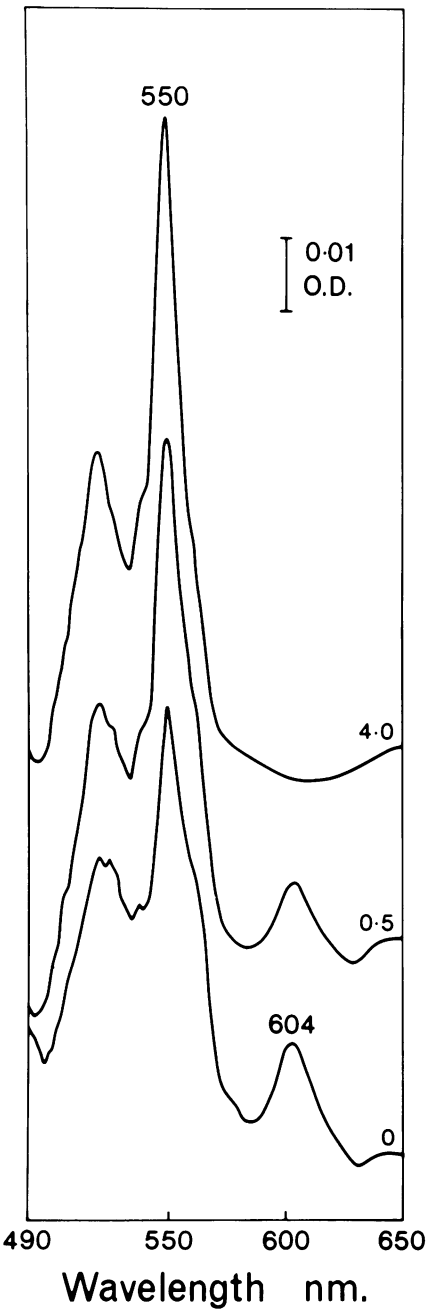

FIG. 5. *Absorption spectra of M. genevensis grown aerobically in 1% GSy0.1YE medium in the absence and presence of 0.5 and 4.0 mg of chloramphenicol per ml. Cell densities in the cuvettes were 0 (10.5 mg dry wt/ml), 0.5 (12.0 mg dry wt/ml), and 4.0 (18.5 mg dry wt/ml).*

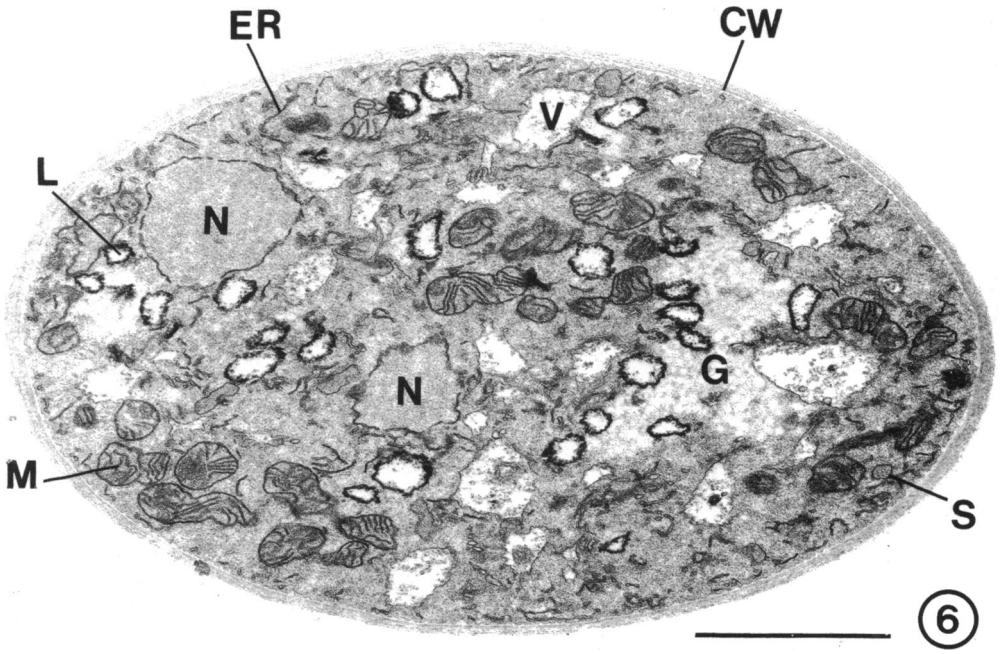

FIG. 6. *Section of a hyphal filament from early stationary-phase aerobically grown M. genevensis showing nuclei (N) endoplasmic reticulum (ER), lipid granules (L), mitochondria (M), vacuoles (V), single membrane vesicles (S), cell wall (CW), and glycogen-like granules (G). The bar represents 2 μm.*

grown cells, the internal structure of these mitochondria appears disorganized. Structures which lack cristae membranes can be seen and are distinguishable from the single membrane vesicles by being larger; in addition, internal membranes are present in some of these forms.

The leucine incorporated into trichloroacetic acid-insoluble material with cytoplasmic ribosomes from *M. genevensis* (Table 3) is about one-quarter of that obtained with the best preparations from yeast under similar circumstances (8). In contrast to *S. cerevisiae*, however, the amino acid incorporation by *M. genevensis* ribosomes is relatively insensitive to cycloheximide (Table 3). With very high concentrations of chloramphenicol a small inhibition of amino acid incorporation is found.

DISCUSSION

Although *M. genevensis* is able to grow fermentatively in the absence of functional mitochondria (1) and with a mass doubling time only 50% less than aerobically grown cells, its growth rate is, nevertheless, sensitive to chloramphenicol. This result is in contrast to that obtained with *S. cerevisiae* where it has been found that chloramphenicol does not alter the initial growth rate, although the second part of the diauxic growth is eliminated (2). However the interpretation of the effect of chloramphenicol on yeast is simplified by a glucose repression of mitochondrial function in this organism. Furthermore, it is known from both in vivo and in vitro studies with yeast that chloramphenicol specifically inhibits mitochondrial protein synthesis (2, 8). Therefore, it appears that mitochondria do not contribute to the initial growth of yeast as long as sufficient glucose is present to repress their functional formation. Such is not the case with *M. genevensis*. Glucose does not repress the formation of respiratory ability in this organism (1) nor, it seems, does it repress the functional contribution of mitochondria to the initial growth rate. This observation is supported by the fact that ethanol does not markedly accumulate during normal aerobic growth and that chloramphenicol, which eliminates cyanide-sensitive respiration, does reduce the initial growth rate of aerobic cultures. Likewise, chloramphenicol has been found to reduce the growth rate of other organisms such as *Candida parapsilosis* (7), *Rhodotorula glutinis* (13), *Pythium ultimum* (10), *Polytomella caeca* (9), and *Tetrahymena pyriformis* (15), but in all cases these are obligate aerobes.

In addition to eliminating functional mitochondria in *M. genevensis*, a further inhibitory action of the antibiotic on this organism appears likely. For example, microaerobic cultures

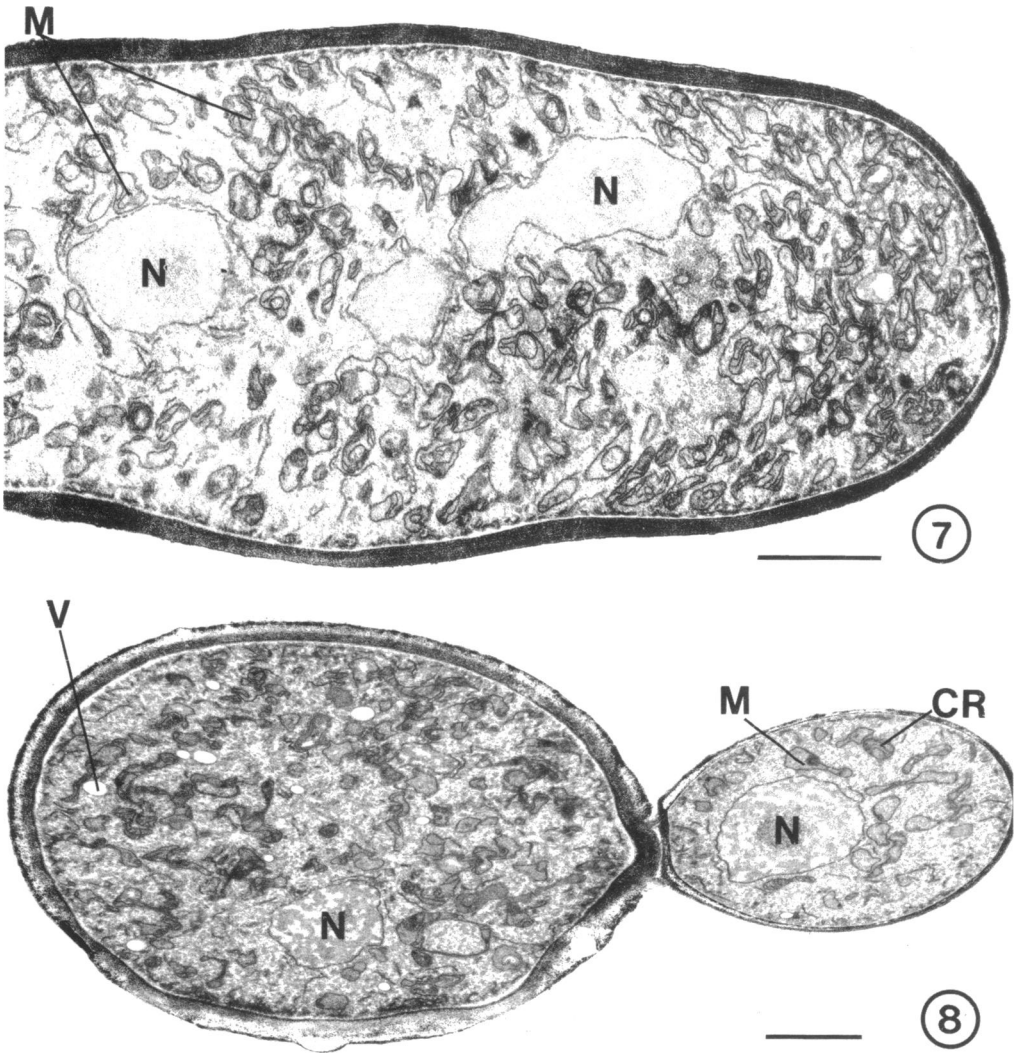

FIG. 7 and 8. *Sections of a club-like hyphal tip and a budding yeast-like cell from early stationary-phase aerobically grown M. genevensis cultured in the presence of 4 mg of chloramphenicol per ml. The sections show numerous mitochondria-like structures (M) some with cristae (CR), nuclei (N), vacuoles (V), and thick cell walls. The bars represent 2 μm.*

without chloramphenicol grow better than aerobic cultures in the presence of the drug and, moreover, chloramphenicol reduces still further the growth rate of microaerobic cells. If the only effect of the antibiotic was to prevent the formation of functional mitochondria, it might have been expected that aerobic growth in the presence of the drug would resemble a microaerobic culture. Since this was not the case, an explanation was sought by studying the effect of the antibiotic on cytoplasmic protein synthesis. Although a small inhibition of amino acid incorporation is noted at the highest concentration of antibiotic, it is felt that this result is insufficient to explain the halving of the growth rate of chloramphenicol-cultured cells in comparison to a microaerobically grown culture. The mechanism underlying this secondary effect of chloramphenicol on the growth rate of *M. genevensis* remains to be determined; however it must be noted that a high concentration of glucose causes a partial restoration of growth rate in the presence of the antibiotic. This suggests that the antibiotic may act by either inhibiting glucose transport or glycolysis, since its effect is competed against by increasing the glucose concentration.

Although chloramphenicol eliminates cyto-

TABLE 3. *Effect of chloramphenicol on amino acid incorporation by cytoplasmic ribosomes from M. genevensis*

Additions or omissions	Percentage of complete system
Complete system	100[a]
-Ribosomes	0
-pH 5 fraction	51
2 × pH 5 fraction	110
+7 mM (2.25 mg/ml) chloramphenicol	85
+15.5 mM (5.0 mg/ml) chloramphenicol[b]	82
+1 mM cycloheximide	69

[a] Represents 27.1 pmol of leucine incorporated per mg of ribosome per 20 min.
[b] Added in ethanol.

chromes aa_3 and *b* from cells and abolishes cyanide-sensitive respiration, it nevertheless does not lead to cells that completely lack respiratory ability, as in the case with *S. cerevisiae* (2). The residual respiration in antibiotic-treated cells, amounting to 40% of that present in controls, is insensitive to cyanide and antimycin. Chloramphenicol alone does not appear to induce this cyanide-insensitive respiration since cells grown under microaerobic conditions in the presence of the drug do not show an increased amount of this respiration. Moreover, the cyanide-insensitive respiration found in aerobic culture in the presence of chloramphenicol does not contribute significantly to the cells' metabolism since ethanol is not utilized and the yield of cells is not increased above that found in microaerobic culture.

Cyanide-insensitive respiration is not present in normal aerobic cultures of this organism although a small amount is found in anaerobically grown cells (1). However, in other fungi such as *C. parapsilosis* (7) and *Saprolegnia* sp. (16) it can represent 50 to 80% of the respiratory ability of these creatures. Moreover, with both organisms the percentage of this component of respiration increases as the cultures age. The above facts, together with the ability of chloramphenicol to produce cyanide-insensitive respiration in other organisms (7, 13) including HeLa cells (4), suggest that it arises as a result of mitochondrial deterioration.

Supporting evidence that mitochondria are disorganized by chloramphenicol is found in ultrastructure studies. In *M. genevensis* there is a large proliferation of mitochondrial forms which contain fewer internal membranes. Moreover, this observation is not complicated by glucose repression as it is in *S. cerevisiae* (2). This disorganization of mitochondrial structure by chloramphenicol, first reported in *S. cerevisiae*, now appears to be a general phenomenon both in other fungi (7, 10, 13) and mammalian cells (5); however, in *P. caeca* this does not seem to be the case, although the reason for this exception is obscure (9).

The effect of chloramphenicol on the morphology of *M. genevensis* is not as dramatic as that obtained with phenethyl alcohol on this and related species (14). Moreover, a complete reversion to yeast-like morphology in aerobic conditions in the presence of chloramphenicol is not obtained even at the highest concentration of the antibiotic. Two possible indirect causes of the effect of the drug on morphology, namely, ethanol and the presence of cyanide-insensitive respiration, have been considered. Both factors as causal agents of yeast-like morphology can be discounted because growth of *M. genevensis* in 10 mM ethanol is mycelial, and cyanide-insensitive respiration is not present in yeast-like cells grown in the presence of phenethyl alcohol.

It has been suggested that phenethyl alcohol promotes yeast-like morphology in *Mucor* because it stimulates fermentation and uncouples phosphorylation (14). However, this suggestion should be re-examined based on the effect of chloramphenicol in eliminating functional mitochondria while not leading completely to yeast-like forms. It may well be that both substances prevent the growth of normal mycelium by interfering with critical levels of high-energy molecules; alternatively they may act by directly interfering with the production of components necessary for hyphal wall formation.

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