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Isolation and Biochemical Analysis of *Mucor bacilliformis* Monomorphic Mutants

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Fourteen stable mutants of *Mucor bacilliformis* which grew yeastlike under both aerobic and anaerobic conditions were isolated after treatment of growing mycelium with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Biochemical characterization of the mutants included determination of growth in different carbon and nitrogen sources, determination of sensitivity of respiration to cyanide and salicylhydroxamate, analysis of cytochrome spectra, determination of glutamate dehydrogenases, glutamine synthase, and ornithine decarboxylase activities, and measurement of cyclic AMP levels. Data showed that all mutants were defective in some aspect of oxidative metabolism and had low levels of ornithine decarboxylase, whereas other characters were variable. It was concluded that morphological transition in *M. bacilliformis* is probably associated with mitochondrial functions and expression of ornithine decarboxylase, but may be independent of cyclic AMP and glutamate dehydrogenase levels. The importance of genetic studies in the analysis of dimorphism is stressed.

The yeast-to-hyphae morphological transition (dimorphism) is a well-documented phenomenon in *Mucor* species. Dimorphism is a freely reversible process that can be provoked by a variety of environmental conditions (1–3, 26). Most studies on *Mucor* spp. morphogenesis have dealt with the biochemical changes which occur during the dimorphic transition. In this regard, various parameters have been measured; among these we may cite respiratory activity (5, 9, 21, 24, 25, 27), carbon and nitrogen metabolism (1, 10, 13, 22, 26), intracellular levels of cyclic AMP (cAMP) (16, 20), and polyamine levels (18, 19, 26). However, the correlation of these parameters with morphogenesis is not clear, and we still lack a coherent picture of the molecular basis underlying the morphogenetic process.

Biochemical analysis of morphological mutants seems to be a useful approach toward distinguishing alterations closely associated with the dimorphic transition from those which have no correlation with morphogenesis, but rather only induction by changes in the environmental conditions in common. This approach, however, appears to have been hindered by the relative scarcity of available morphological mutants, as judged from reports in the literature. Storck and Morrill (25) were able to isolate respiratory-deficient mutants of *Mucor bacilliformis* which did not form hyphae when grown in air. Peters and Sypherd (unpublished data cited in reference 26) isolated a mutant from *M. racemosus* referred to as "*coy*" (conditional yeast) which formed hyphae when grown aerobically in complex media, but grew yeastlike on minimal media. The air-grown mutant formed hyphae on minimal media only when the media were supplemented with methionine. Because of the known role of methionine, via *S*-adenosylmethionine, in the synthesis of polyamines, the results led the authors to suggest that polyamines might play a role in the morphological interconversion. More recently, a filtration method for the enrichment of yeastlike mutants from *M. racemosus* was described by Borgia (P. T. Borgia, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, CC II 34, p. 176).

In the present paper we describe the isolation and biochemical characterization of 14 yeast morphological mutants independently isolated from *M. bacilliformis*. These mutants are all blocked in their ability to form hyphae in air.

MATERIALS AND METHODS

Organism and culture conditions. *M. bacilliformis* (NRRL 2346 or ATCC 12830) was kindly provided by Roger Storck, Rice University, Houston, Tex. Stock cultures of the organism were maintained on slants of YPG medium (3) with periodical transfers throughout the study. Large numbers of spores were obtained from 7- to 10-day-old aerobic cultures grown at 25°C in petri dishes containing the same medium. Spores were harvested with sterile distilled water, washed once

with water by low-speed centrifugation, and used to inoculate liquid medium. Erlenmeyer flasks containing 600 or 1,200 ml of liquid YPG (with 0.5% instead of 2.0% glucose) were inoculated with 10^6 spores per ml and incubated in a reciprocating water bath at 25°C either aerobically to obtain the mycelial form or under an atmosphere of nitrogen-CO_2 (70:30, vol/vol) to obtain the yeast phase. Incubation periods were variable depending on the experiment.

Mutagenesis. An adaptation of the method of mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine described for *Phycomyces* sp. by Cerda-Olmedo (4) was used. Accordingly, 12- to 24-h-old mycelium washed with sterile distilled water was resuspended in 50 mM Tris-maleate buffer (pH 7.0) containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) and incubated in a water bath shaker for 30 min at room temperature. The mutagen-treated mycelium was filtered, washed with water, placed over liquid medium, and allowed to sporulate. Spores were harvested as described above and used to inoculate petri dishes (about 200 per dish) containing solid YPG medium at pH 3. Plates were incubated for 7 to 10 days at 25°C to obtain discrete colonies.

Isolation of mutants. Experiments were carried out to select for auxotrophic, thermosensitive, or morphological mutants. Accordingly, sporulated colonies from YPG plates were replicated on plates containing either Vogel minimal medium (28) or YPG medium. After aerobic incubation for 24 h at 18 or 28°C, plates were searched for auxotrophic or thermosensitive mutants. Morphological mutants were isolated from either the wild-type strain or a lysine auxotroph obtained as described above. A number of colonies not showing the typical hyphal growth on YPG plates incubated under air were picked up, transferred to fresh YPG plates, and incubated aerobically for 24 to 48 h at 25°C. Colonies with a characteristic yeast morphology were selected, purified by repeated streaking on acid medium, and maintained on YPG slants. These yeastlike mutants were called "Lev" mutants (from Latin *levare*, to ferment, to leaven).

Growth on various carbon and nitrogen sources. The wild-type strain and mutants were transferred to plates of Vogel minimal medium (pH 4.5) containing niacin and thiamine (1 μg/ml) and supplemented with various carbon and nitrogen sources. After either aerobic incubation or incubation in anaerobic GasPak jars (BBL Microbiology Systems) for 48 to 72 h at 25°C, plates were observed for growth.

Measurement of oxygen uptake. A Yellow Springs Instrument model 65 oxygen polarograph equipped with a Clark electrode and connected to a Honeywell recorder model Electronic 19 was used to monitor respiratory activity of the strains. The electrode was attached to a water-jacketed glass chamber maintained at 26°C. A 4.5-ml amount of 10 mM $KNaHPO_4$ buffer (pH 6.5) containing 10 mM $MgCl_2$ was injected into the chamber with a syringe, and the concentration of oxygen was adjusted to 100% with the current amplifier. After checking for the absence of unspecific O_2 consumption, 0.5 ml of the cell suspension resuspended in the same buffer was added, and 1 min later glucose was injected to a final concentration of 2 mM and respiratory activity was recorded. The sensitivity of cell respiration to cyanide (1 mM) or salicylhydroxamic acid (SHAM; 150 μg/ml) was measured by addition of these compounds after glucose injection. Respiratory activity was expressed as microliters of oxygen consumed per minute per milligram of cell dry weight.

Cytochrome analysis. Aerobic cultures (16 to 18 h old) in liquid YPG medium were obtained as described above. Cells were harvested by filtration through a coarse-grade, sintered-glass filter equipped with a 5M membrane filter (pore size, 5 μm; Millipore Corp.), washed three times with 50 ml of 50 mM Tris-hydrochloride buffer (pH 7.0) containing 5 mM $MgCl_2$ and 1 mM EDTA (TME buffer), and resuspended in the same buffer. The following procedures were carried out at 2 to 4°C. Cells resuspended in TME buffer were mixed with 20 ml of 0.45- to 0.50-mm glass beads and broken for 45 s in a Braun model MSK cell homogenizer. The suspension was centrifuged at 1,000 × *g* for 5 min, the pellet was discarded, and the supernatant was centrifuged at 55,000 × *g* for 60 min to separate a mixed-membrane fraction, which was resuspended in TME buffer (about 4 to 8 mg of protein per ml) and used for the analysis of cytochromes. The differential absorption spectrum of cytochromes in the range of 500 to 630 nm was determined with a double-beam UV-VIS Aminco DW-2a spectrophotometer, using two aliquots of mixed-membrane fraction: a reference suspension fully oxidized with potassium ferricyanide and a sample suspension fully reduced with sodium dithionite. Quantitative data were expressed as peak height of the α-bands of the corresponding cytochromes over a basal line, per milligram of protein.

Measurement of cAMP. A reagent kit (Amersham Corp.) containing [^{3}H]cAMP (specific activity, 36 nmol/μCi) and the bovine cAMP-binding protein was used to measure the intracellular levels of cAMP by the competition method. The wild-type and mutant strains were grown for 13 h in YPG medium under aerobic or anaerobic conditions. Cells were harvested by low-speed centrifugation, washed with 50 mM Tris-hydrochloride buffer (pH 7.6) containing 4 mM EDTA (buffer A), and resuspended in the same buffer. A 2-ml aliquot of cell suspension was placed in a conical Corex tube, and the proteins were precipitated by heating for 3 min in a boiling-water bath. After centrifugation for 15 min at 2,000 × *g*, cAMP was determined in the supernatant. The assay mixture contained, in a total volume of 250 μl, 100 μl of cAMP-binding protein, 20,000 cpm of [^{3}H]cAMP, 50 μl of supernatant, and various amounts of unlabeled cAMP dissolved in buffer A. After 2 h of incubation at 2 to 4°C, 300 μl of 2% triethylaminoethyl-cellulose (Cellex-T; Bio-Rad Laboratories) in 10 mM Tris-hydrochloride buffer, pH 7.6 (buffer B), was added and incubation was continued for 5 min. The incubation mixtures were layered on columns (0.7 by 4 cm) of TEAE-cellulose and washed four times with 1.5 ml of buffer B. Protein-bound [^{3}H]cAMP was quantitatively eluted from the column with 3 ml of 1% sodium dodecyl sulfate and mixed with 10 ml of Aquasol (New England Nuclear). Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer.

GDH assay. Cells grown for 18 h under aerobic or anaerobic conditions were harvested by filtration as described for cytochrome analysis. They were washed with 0.2 M $KHNaPO_4$ buffer (pH 8.0), resuspended in the same buffer, and broken with glass beads as described above. The broken cell suspension was centrifuged for 15 min at 5,000 × *g*, the supernatant

was recovered, and 1 mM mercaptoethanol was added. Activities were measured in the direction of reductive amination of α-ketoglutarate, with the oxidation of NADH or NADPH measured at 366 nm. The assay mixtures contained, in a total volume of 1.0 ml, 50 to 100 μl of enzymatic extract, 200 mM ammonium chloride, and 30 mM potassium α-ketoglutarate in 200 mM $KHNaPO_4$ buffer, pH 8.0. Reaction mixtures without ammonium chloride or potassium α-ketoglutarate were used as controls. The reaction was started by addition of either NADH or NADPH at a final concentration of 0.125 mM, and the decrease in absorbance at 366 nm was recorded every 15 s. The difference in absorbance between the samples with and without substrates was taken as a measurement for NAD- or NADP-dependent glutamate dehydrogenase (GDH) activity. This was expressed as micromoles of NADH or NADPH oxidized per minute per milligram of protein.

Glutamine synthase assay. Cells grown for 18 h in Vogel minimal medium under aerobic or anaerobic conditions were harvested by filtration, washed with 50 mM Tris-hydrochloride buffer (pH 7.2), resuspended in the same buffer, and broken with glass beads. The suspension was centrifuged at 27,000 × *g* for 30 min, and the supernatant was recovered and assayed for glutamine synthase activity as reported by Quinto et al. (23). Activity was expressed as absorbance of hydroxamate at 546 nm, and specific activity was activity per milligram of protein.

ODC assay. Cultures (16 h old) of the wild type grown either aerobically or anaerobically or mutant strains grown aerobically in YPG medium were harvested by filtration as described above. Cells were washed with 10 mM $KHNaPO_4$ buffer (pH 7.2) containing 1.0 mM dithiothreitol, 0.5 mM EDTA, and 2 μM pyridoxal phosphate, resuspended in the same buffer, and broken with glass beads as already described. The suspension was centrifuged at 25,000 × *g* for 10 min, and the supernatant was recovered and assayed for ornithine decarboxylase (ODC) activity. This was measured by the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine. Accordingly, an assay mixture containing 0.2 M Tris-hydrochloride buffer (pH 8.3), 0.8 mM EDTA, 0.4 mM pyridoxal phosphate, and 300 μl (1 to 10 mg of protein) of the enzyme sample in a total volume of 0.5 ml was deposited in the outer chamber of a 10-ml Erlenmeyer flask equipped with a center well, into which a piece of filter paper (1 by 1.5 cm) soaked with 20% sodium hydroxide was placed to capture the released CO_2. Flasks were sealed with rubber stoppers. The reaction was started by injection of 0.016 μmol (about 0.2 μCi) of [1-^{14}C]ornithine through the stopper and allowed to proceed for 30 min at room temperature. At this time, 100 μl of 0.05 M sodium bicarbonate (as carrier) and 100 μl of 3 N HCl were injected through the stopper, and the flasks were left undisturbed for 60 min to permit CO_2 absorption by NaOH. Papers were taken out, dried at 60°C, and placed into vials containing 20 ml of nonaqueous solvent {0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene and 2.0 g of 2,5-diphenyloxazole dissolved in 1,000 ml of toluene}, and radioactivity was counted with a liquid scintillation spectrometer. ODC activity was expressed as nanomoles of CO_2 liberated per hour per milligram of protein.

Protein determination. Protein was measured by the method of Lowry et al. (17), with crystalline bovine serum albumin as the standard.

RESULTS

Isolation of mutants. The screening of several thousand colonies of *M. bacilliformis* isolated and analyzed in the manner described under Materials and Methods allowed the detection of one lysine-requiring auxotroph and one conditional mutant which grew as mycelium at 18°C and yeastlike at 28°C. For the isolation of morphological mutants, both a wild-type strain and a lysine-requiring mutant were used: 48 of the total wild-type colonies and 37 of the total Lys^- colonies exhibited a typical yeast growth under air. Most of these morphological mutants reverted to the parental phenotype after successive transfers during a period of 3 to 7 weeks. Only 8 of the 48 Lys^+ and 6 of the 37 Lys^- strains behaved as stable mutants, retaining the property of growing yeastlike under air after repeated transfers.

Utilization of various carbon and nitrogen sources. The wild-type strain incubated under air utilized all carbon sources tested: glucose, maltose, citrate, and succinate. Under anaerobiosis, it only grew with glucose. Similar results have been previously described for *M. rouxii* by Bartnicki-Garcia and Nickerson (3). All morphological mutants were unable to utilize maltose, citrate, and succinate when incubated in air; they only grew with glucose. The wild-type strain used either ammonium salts or glutamate as a nitrogen source when grown aerobically, whereas anaerobic cultures did not metabolize glutamate. All of the mutants cultivated under either condition utilized both nitrogen sources. The reason for this behavior remains unknown, but it is possibly due to a phenotypic response. That is, germination of sporangiospores may not occur in anaerobiosis with glutamate as nitrogen source, but once spores had germinated, glutamate would sustain growth in anaerobiosis. Similarly, all Lev mutants were resistant to crystal violet, whereas the wild type was sensitive when sporangiospores were used as the inoculum but resistant when grown yeasts were used instead.

Respiratory activity. Respiratory activity of mutants with glucose as substrate was very high, at least 50% of the respiratory activity of the wild type (Table 1). However, differences between the wild type and the mutants were observed when sensitivity of respiration to cyanide and SHAM was measured. Respiration of the wild type was completely resistant to SHAM, whereas respiration of the mutants was almost completely abolished by the inhibitor. On the other hand, respiratory activity of the wild type was completely sensitive to cyanide, whereas respiration of most of the mutants was resistant

TABLE 1. Respiratory activity of wild-type and morphological mutants of *M. bacilliformis* and effect of inhibitors[a]

Strain	Respiratory activity[b]	Inhibition (%) by:	
		Cyanide	SHAM
Wild type	2.23	100	0
Lev1	1.75	0	100
Lev6	0.92	15	71
Lev7	1.14	8.4	83
Lev9	1.40	0	100
Lev10	1.24	0	100
Lev11	1.43	0	85
Lev13	1.44	36	96
Lev14	1.73	0	100
Lys,Lev17	2.23	86	100
Lys,Lev18	2.32	0	83
Lys,Lev19	2.15	0	100
Lys,Lev20	2.37	0	100
Lys,Lev21	1.75	0	100
Lys,Lev22	2.94	35	100

[a] Cells resuspended in 5 ml of 10 mM $KHNaPO_4$ buffer (pH 6.5) containing 10 mM $MgCl_2$ were incubated at 26°C with 2 mM glucose, and respiratory activity was recorded as described in the text. Inhibitors were added after glucose injection.

[b] Expressed as microliters of oxygen consumed per minute per milligram of cell dry weight.

to the compound. The respiration of only five mutants was partially sensitive to cyanide, the most obvious case being that of mutant Lys^-, Lev17, whose respiration was inhibited almost completely by cyanide. The various patterns of sensitivity to inhibitors are shown in Fig. 1.

Cytochrome spectra. Aerobically grown wild-type *M. bacilliformis* displayed the normal cytochrome spectrum of eucaryotic organisms, already described by Storck and Morrill (25) with absorption peaks at 605, 560, 552, and 530 nm corresponding to the α-bands of cytochromes aa_3, *b*, and *c* and the β-band of cytochrome *b*, respectively. Cytochrome spectra of mutants were classified into six different types (Table 2): (i) normal; (ii) with displaced α-band of cytochrome aa_3; (iii) with decreased or absent cytochrome aa_3; (iv) with decreased and displaced α-band of cytochrome aa_3; (v) with decreased cytochrome *b*; and (vi) lacking cytochromes aa_3 and *b*. Typical spectra of these classes are shown in Fig. 2.

cAMP levels. As mentioned above, changes in the intracellular concentration of cAMP have been correlated with *Mucor* spp. morphogenesis (16, 20). In the present study we measured the intracellular concentration of this nucleotide in cell-free extracts obtained from the parental and mutant strains as described in Materials and Methods. Results are shown in Table 3. It can be seen that anaerobically grown wild-type cells contained 2.5 times more intracellular cAMP than the corresponding aerobically grown mycelium. These results are in agreement with the findings of Larsen and Sypherd (16), who obtained similar results with *M. racemosus*. Aerobically grown mutants Lev9, Lev11, and Lys^-,Lev20 exhibited higher cAMP levels than the parental cells, whereas the rest of the mutants gave values comparable to those detected in the aerobically grown wild type.

Glutamine synthase and GDH activities. Glutamine synthase and GDH are enzymes that catalyze key reactions which couple carbon and nitrogen metabolism. These enzymes play both anabolic (glutamine synthase and NADP-dependent GDH) and catabolic (NAD-dependent GDH) roles. Peters and Sypherd (22) found that in *M. racemosus* similar, but very low, levels of NADP-dependent GDH were present in both carbon dioxide-grown yeast cells and air-grown hyphae. In contrast, aerobic mycelium contained higher levels of the NAD-dependent enzyme than anaerobically grown yeasts. We measured the levels of both NAD- and NADP-dependent GDHs present in the wild type and some selected monomorphic mutants (Table 4). It can be seen that activity of the NAD-dependent enzyme was considerably higher in aerobically than in anaerobically grown wild-type cells. On the other hand, NADP-dependent activity present in carbon dioxide-grown yeasts was twice that observed in air-grown mycelia. Except for strains Lev9 and Lev11, which showed levels of NAD-dependent activity similar to those found in anaerobically grown yeasts, all of the mutants showed values comparable to those of the air-grown mycelium. Levels of NADP-dependent GDH activity were higher only in mutants Lev1, Lev11, and Lev14, whereas the rest of the strains showed activities similar to those of air-grown mycelium. Growth of the wild type and five selected mutants in synthetic medium with glutamate as nitrogen source not only did not increase levels of NAD-dependent GDH, but in fact decreased them (Table 5). This result was unexpected since the NAD-dependent enzyme has been considered to play a catabolic role. Comparison of the levels of NAD-dependent GDH present in aerobic versus anaerobically grown cells revealed that, although most morphological mutants contained levels of the enzyme comparable to those of the aerobic wild type, its regulation by the aerobic-anaerobic shift was lost, since anaerobic growth produced only a slight decrease in the levels of the enzyme (Table 5). Levels of glutamine synthase were low in complex medium and highest in synthetic medium with glutamate as nitrogen source (not shown), but they were only slightly affected by anaerobic growth in either the wild

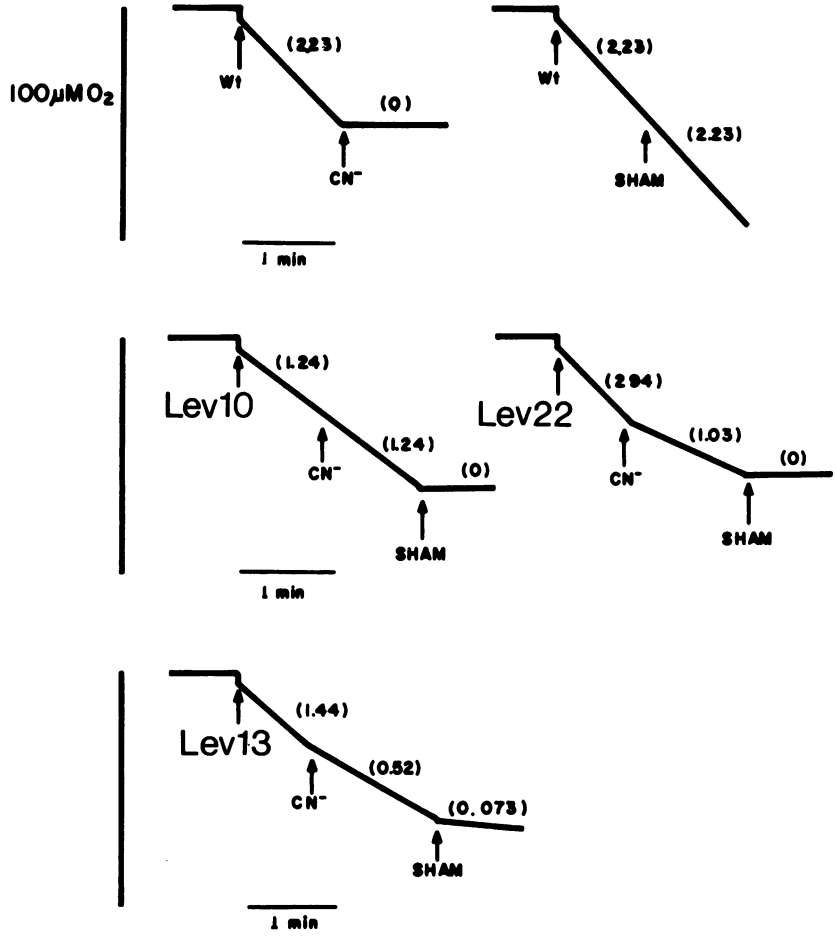

FIG. 1. Effect of cyanide and SHAM on respiratory activity of wild type (wt) and morphological mutants of *M. bacilliformis*. Respiratory activity of cell suspensions in 10 mM $KHNaPO_4$ buffer, pH 6.5, with 2 mM glucose as a substrate was monitored as described in the text. The respiration inhibitors cyanide (CN^-; 1 mM) and SHAM (150 μg/ml) were added where indicated by arrows. Numbers in parentheses refer to specific respiratory activity.

type or morphological mutants. Representative data appear in Table 6.

ODC activity. The polyamines spermine, spermidine, and putrescine are naturally occurring polyvalent cations which play a very important regulatory role in mammalian cells. Rapidly growing cells have a higher content of polyamines than non-proliferating cells (11), and the activity of ODC, the key enzyme involved in the biosynthesis of polyamines, is dramatically increased in response to a number of growth stimuli (14). Peters and Sypherd (cited in reference 26) analyzed the changes in the levels of polyamines after yeast-to-hyphae transition in *M. racemosus*. Their results indicated a close correlation between the morphogenetic change and the appearance of the three polyamines. Later on, Inderlied et al. (12) reported a 30- to 50-fold increase in ODC activity after yeast-to-hyphae transition of *M. racemosus*. In the present study, we measured ODC activity in the wild type and several morphological mutants. We observed that wild-type aerobically grown cells contained higher ODC activities than cells grown in anaerobiosis and that all of the mutants analyzed contained even lower levels of the enzyme than the anaerobically grown wild type (Table 7).

DISCUSSION

Several lines of evidence indicate that the morphological mutants isolated in this study have alterations in their oxidative metabolism. First, all mutants failed to utilize nonfermentable substrates such as citrate or succinate as the

TABLE 2. Spectral characteristics of cytochromes from wild-type and morphological mutants of *M. bacilliformis*[a]

Spectral type	Characteristics	Strains
1	Normal	Wild type, Lys$^-$,Lev17
2	Normal levels; displaced α-band of cytochrome *a*	Lev6, Lys$^-$,Lev22
3	Decreased or absent cytochrome *a*	Lev1, Lev11
4	Decreased and displaced α-band of cytochrome *a*	Lev7
5	Decreased cytochrome *b*	Lev10, Lev13, Lys$^-$,Lev18, Lys,Lev21
6	Absence of cytochromes *a* and *b*	Lev9, Lev14, Lev19, Lys$^-$,Lev20

[a] Cytochrome spectra of strains analyzed as described in the text were divided into six types according to the alterations observed.

sole carbon source. Second, with the exception of mutant Lys$^-$,Lev17, respiration of all mutants was resistant to cyanide but sensitive to SHAM. Similar findings have been reported in *Neurospora crassa* with altered mitochondrial functions due to either mutation or growth in the presence of chloramphenicol or antimycin A. Under these conditions there is an alternate respiratory pathway which is insensitive to cyanide and antimycin A but susceptible to SHAM (6, 7, 15). Third, all mutants (except Lys,Lev17) showed altered cytochrome spectra, although only four mutants (Lev9, Lev14, Lev19, and Lys$^-$,Lev20) showed cytochrome spectra similar to that of the respiratory-deficient mutant of *M. bacilliformis* isolated by Storck and Morrill (25). These latter mutants containing only cytochrome *c* resemble *petite* mutants of *Saccharomyces cerevisiae* (8, 29) and *poky* mutants of *N. crassa* (29). The rest of the mutants showed different alterations in their cytochrome spectra. It is interesting that mutant Lys$^-$,Lev17, which showed a normal cytochrome spectrum, also had cyanide-sensitive respiration. It is likely that this mutant is altered in the mechanism of energy transduction, i.e., ATPase, or the proton motive force conservation, or else in an enzyme from the tricarboxylic acid cycle.

Our results show that high levels of cAMP are not obligatorily linked to yeast morphology since only 3 of the 14 morphological mutants analyzed contained high intracellular levels of this nucleotide comparable to those found in the anaerobically grown wild type. Paznokas and Sypherd (21) reached a similar conclusion when they examined the levels of cAMP in yeast and hyphal cells of *M. racemosus* grown under different nitrogen flows.

Nitrogen metabolism has been considered an important factor in *Mucor* spp. morphogenesis since yeast growth is more fastidious than mycelial growth in regard to nitrogen source requirement. Peters and Sypherd (22) studied the in vivo regulation of NAD-dependent GDH during *M. racemosus* morphogenesis and found that the yeast-to-hyphae transition was accompanied by

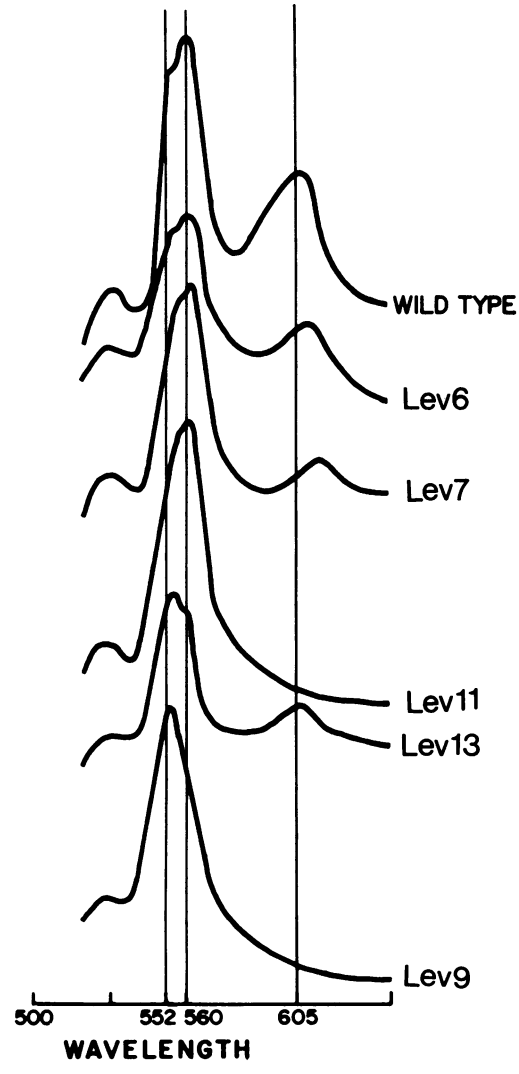

FIG. 2. Representative scans of the various cytochrome spectra found in *M. bacilliformis* wild type and morphological mutants. Differential spectra of mixed-membrane fraction samples fully reduced with sodium dithionite versus reference suspensions fully oxidized with potassium ferricyanide were obtained as described in the text.

TABLE 3. Intracellular levels of cAMP in wild type and morphological mutants of *M. bacilliformis*[a]

Strain	pmol of cAMP per mg of protein
Wild type, aerobic	3.88
Wild type, anaerobic	9.77
Lev1	4.00
Lev6	2.46
Lev7	2.66
Lev9	13.30
Lev10	2.75
Lev11	16.00
Lev13	4.68
Lev14	5.02
Lys,Lev17	4.66
Lys,Lev18	6.34
Lys,Lev19	4.86
Lys,Lev20	15.70
Lys,Lev21	3.17
Lys,Lev22	7.51

[a] The intracellular concentration of cAMP in aerobically or anaerobically grown wild type and aerobically grown mutants was determined in protein-free cell extracts in the manner described in the text.

an increase in the enzyme activity which preceded germ tube appearance. This increase was prevented by cAMP with concomitant suppression of the morphological change. These results led the authors to conclude that a close correlation existed between morphogenesis and NAD-dependent GDH expression in *M. racemosus*. In the present study, only two yeast mutants (Lev9 and Lev11) showed low levels of NAD-dependent GDH activity and, interestingly, they possessed high intracellular levels of cAMP. These results suggest that changes in the NAD-dependent GDH levels, rather than being morphology associated, are related to cAMP concentration. The observation that the aerobic to anaerobic shift did not significantly decrease the levels of NAD-dependent GDH activity in the mutants may indicate that the expression of the enzyme does not necessarily depend on the presence of oxygen but rather on the functional state of mitochondria. It was also interesting to observe that NAD-dependent GDH activity decreased when the wild type and five selected mutants were grown in synthetic medium with glutamate as nitrogen source. Growth under these conditions should have induced higher levels of NAD-dependent GDH activity, taking into account the reported catabolic function of this enzyme in nitrogen metabolism. Levels of glutamine synthase were also unaffected by growing the wild type and mutants under anaerobic conditions. Considering these data and the extremely low levels of NADP-dependent GDH, the involve-

TABLE 4. Activity of NAD- and NADP-dependent GDH in wild type and morphological mutants of *M. bacilliformis*[a]

Strain	Sp act[b]	
	NAD-GDH	NADP-GDH
Wild type, aerobic	2.24	0.012
Wild type, anaerobic	0.37	0.023
Lev1	1.45	0.045
Lev9	0.34	0.017
Lev10	1.40	0.010
Lev11	0.46	0.056
Lev14	2.58	0.051
Lys,Lev17	1.56	0.016
Lys,Lev18	1.77	0.013
Lys,Lev20	1.98	0.103

[a] The activity of NAD- and NADP-dependent GDH was measured in cell-free extracts obtained from aerobically or anaerobically grown wild-type and aerobically grown mutants as described in the text.

[b] Expressed as micromoles of NADH or NADPH oxidized per minute per milligram of protein.

TABLE 5. Effect of anaerobiosis and culture medium on synthesis of NAD-dependent GDH by wild type and morphological mutants of *M. bacilliformis*

Strain	Sp act[a]			
	Aerobiosis		Anaerobiosis	
	Complex[b]	Minimal[c]	Complex	Minimal
Wild type	2.90	1.42	0.21	0.28
Lev6	3.87	0.64	1.72	0.78
Lev11	0.17	—[d]	0.19	—
Lev10	5.41	2.21	2.91	1.10
Lev14	2.81	1.52	1.15	0.46
Lev20	0.84	0.77	0.29	0.35

[a] Expressed as micromoles of NADH oxidized per minute per milligram of protein.

[b] YPG medium.

[c] Minimal medium with 0.1% yeast extract and glutamate as nitrogen source.

[d] —, No growth.

TABLE 6. Glutamine synthase activity in wild type and morphological mutants of *M. bacilliformis*[a]

Strain	Sp act[b]	
	Aerobiosis	Anaerobiosis
Wild type	0.180	0.132
Lev6	0.168	0.126
Lev10	0.246	0.204
Lev14	0.348	0.164
Lev20	0.428	0.191

[a] Glutamine synthase activity was measured in cell-free extracts obtained from aerobic or anaerobic cultures of wild-type and mutant strains, as described in the text.

[b] Expressed as absorbancy of hydroxamate at 546 nm per 60 min per milligram of protein.

TABLE 7. ODC activity in wild type and morphological mutants of *M. bacilliformis*[a]

Strain	Sp act[b]
Wild type, aerobic	3.48
Wild type, anaerobic	1.03
Lev1	0.17
Lev9	0.04
Lev10	0.02
Lev14	0.09
Lys$^-$,Lev17	0.05
Lys$^-$,Lev18	0.08
Lys$^-$,Lev20	0.28

[a] ODC activity was determined in cell-free extracts prepared from 18-h-old aerobic or anaerobic cultures of wild type or aerobic cultures of mutants as described in the text.

[b] Expressed as nanomoles of CO_2 liberated per hour per milligram of protein.

ment of this enzyme in ammonia assimilation is highly unlikely. Rather, it may be suggested that this process involves either NAD-dependent GDH or the GOGAT-glutamine synthase system.

Another enzyme examined in this study because of its reported value as a morphogenetic indicator was ODC, the enzyme responsible for putrescine formation. We found that young cultures of aerobic mycelium exhibited high ODC activities, whereas carbon dioxide-grown wild type and all of the morphological mutants analyzed contained very low levels of the enzyme. Inderlied et al. (12) reported that the yeast-to-hyphae transition in *M. racemosus* was concomitant with an increase in ODC activity which preceded germ tube emergence.

From the data presented here the following conclusions can be drawn. (i) There is a correlation between functional mitochondria and the morphological transition; i.e., all monomorphic mutants were defective in some aspects of oxidative energy metabolism. (ii) Alterations in the concentrations of cAMP and NAD-dependent GDH either are not in the main morphogenetic pathway or represent early steps in the same, since only a limited number of mutants showed abnormal levels. This alternative will be answered only by complementation studies among mutants. (iii) There is a close correlation between NAD-dependent GDH and cAMP levels. Mutants with altered enzyme values had higher values of the nucleotide. (iv) There is a relationship between cell morphology and ODC expression, since all monomorphic mutants analyzed had low levels of the enzyme. However, the possibility that its expression depends on some mitochondrial function cannot be discarded. (v) Genetic approaches will be very useful in the future for studying the morphogenetic phenomenon in *Mucor* species.

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