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Respiratory Capacity, Cyclic Adenosine 3',5'-Monophosphate, and Morphogenesis of *Mucor racemosus*

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A variety of cultural conditions were examined to determine the relationship between respiratory capacity and the growth of *Mucor racemosus* in the yeast and mycelial form. The results show that both yeasts and hyphae can develop when the respiratory capacity is low (e.g., in N_2). In addition, the yeast form of the fungus could be grown in air in the presence of cyclic adenosine 5'-monophosphate with high respiratory rates characteristic of air-grown mycelia. These results indicate that their is not an obligatory relationship between respiratory capacity and morphogenesis in *M. racemosus*. Low intracellular levels of cyclic adenosine 5'-monophosphate, however, were correlated with aerobic mycelial development, whereas yeast development under CO_2 was characterized by higher cyclic adenosine 5'-monophosphate levels.

Mucor racemosus is a dimorphic fungus, possessing the ability to grow either as budding yeasts or as branched mycelia, depending on the cultural conditions. The ease of inducing morphological transitions by manipulating the culture conditions makes this a useful system for studying the processes of microbial differentiation. In the case of *Mucor*, several environmental factors are known to influence the form of growth. In general, the organism grows as a budding yeast in 100% carbon dioxide (2) and grows in the mycelial form in air (1). Of the multitude of conditions that influence the transition between yeast and mycelial growth, only generalizations can be made about the physiological processes that lead to these transitions. Anaerobiosis, particularly in atmospheres of carbon dioxide and high levels of a fermentable hexose, lead to yeastlike development (2). Growth in air, low levels of hexose, or growth in nitrogen usually lead to the formation of mycelia. These conditions led us to speculate that cyclic adenosine 5'-monophosphate (cAMP) played a role in the regulation of morphogenesis in *M. racemosus*. We demonstrated that there was a correlation between the endogenous levels of cAMP and the morphological form; the yeast form containing higher levels of cAMP than the mycelial form. Furthermore, the addition of dibutyryl cAMP to air-grown mycelia resulted in yeastlike development (4).

Other forms of evidence suggest that there is a relationship between respiration, or respiratory capacity, and the ability of *Mucor* to grow in the mycelial form. For example, *M. rouxii* grows in the mycelial form in air but can be induced to form yeasts by the addition of phenethyl alcohol (11). The addition of chloramphenicol to air-grown cultures also results in the formation of yeasts (13), and a respiratory-deficient mutant of *M. bacilliformis* grows as a yeast in air (10). Such observations suggest that the biochemical mechanism which controls morphogenesis in *Mucor* is directly related to oxidative metabolism.

Because of the confusion surrounding the possible role of respiratory capacity on the one hand, and cAMP on the other, in the control of dimorphism, we carried out a series of experiments examining these two forms of regulation under a variety of conditions. We present here evidence that dimorphism can be uncoupled from the type of energy metabolism being utilized by the organism. We further show that intracellular cAMP levels correlate closely but not invariably with the form of the organism.

MATERIALS AND METHODS

Organism and culture conditions. *M. racemosus* (ATCC 1216B) was maintained in 0.3% yeast extract, 1.0% peptone (Bacto), and 2% glucose (YPG medium) in agar petri plates. For liquid culture, the YPG medium was adjusted to pH 4.5 with H_2SO_4. Spores were harvested in distilled water from 4- to 10-day-old cultures growing on agar plates. YPG medium was inoculated with 5×10^6 spores per ml and incubated at room temperature on a gyrotory shaker platform. Air cultures were incubated in cotton-stoppered

flasks. For growth under CO_2 and N_2, the cultures were continuously flushed with gas which had been first purified by passage through a hot copper catalyst. Cultures grown in CO_2 were flushed at 3 volumes of gas per volume of culture per min. Mycelia grown in N_2 were flushed at the rate of 1 volume of gas per volume of culture per min (D. Mooney and P. S. Sypherd, submitted for publication) with 20 ml of YPG medium in a 125-ml Erlenmeyer flask. Yeast cultures grown in N_2 were flushed at the rate of 3 volumes of gas per volume of cultures (D. Mooney and P. S. Sypherd, submitted for publication). Measurements of growth were performed by determining cell number, turbidity, or cellular protein as described by Larsen and Sypherd (4).

Morphological determinations. Morphology and cell number were assessed simultaneously by microscopy on a hemocytometer grid. Unit cell numbers were determined by the method of Larsen and Sypherd (4). When cultures contained only one differentiated morphological state, they are referred to as purely yeast or mycelia.

Cell extracts and analytical methods. For measurements on the intracellular concentration of cAMP, duplicate samples were removed from the culture and collected on membrane filters. The filters were then immersed in 2.5 ml of ice-cold 0.1 N HCl. Cells were resuspended from the filter by vigorous agitation. The suspensions were then frozen and thawed three times and cellular debris was removed by sedimentation. A 1.0-ml sample of the supernatant liquid was removed to another tube and evaporated to dryness at 60 C under a stream of air. The residue was then dissolved in 1.0 ml of 0.05 M sodium acetate buffer, pH 6.2. The cAMP content of the sample was determined without further purification by the radioimmune assay procedure of Steiner (1) with a kit supplied by Schwarz/Mann. As a control, 2×10^4 counts/min of [^{3}H]cAMP were added to selected samples before the filter was placed in the 0.1 N HCl. Recovery of the [^{3}H]cAMP was assessed by determining the amount of radioactivity which chromatographed with untreated authentic cAMP in a thin-layer system, with isopropanol-ammonium hydroxide-water (70:10:20) as solvent. Recovery of the [^{3}H]cAMP was routinely 85 to 90%.

Respiratory capacity was determined by measuring oxygen consumption with a Beckman oxygen electrode (12). Samples of 1 to 10 ml of culture were filtered onto membrane filters. The cells were then washed with fresh medium and suspended in 2.0 ml of fresh YPG medium, pH 4.5. This suspension was transferred to a 5.0-ml reaction vessel into which the oxygen electrode was inserted. Upon completion of the respiration measurements, the cell suspension was removed from the reaction vessel and filtered onto a membrane filter. The protein content of these cells was determined after suspending the cells from the filter. Samples usually contained 0.2 to 2.0 mg of protein. The oxygen content of the YPG medium was taken to be 480 ng-atom of O_2/ml. Respiratory capacity is reported as nanogram-atom of oxygen consumed per minute per milligram of protein. Cyanide sensitivity was measured by addition of potassium cyanide to a final concentration of 0.2 mM, after establishing a respiration rate.

Cellular protein was determined by collecting a sample of culture on membrane filters and placing the filters in a tube containing 3.0 ml of 1.0 N sodium hydroxide. The tube was agitated to wash the cells from the filter and then the filter was removed. The cell suspension was heated to 100 C for 20 min and the debris was removed by centrifugation. Protein analysis was performed on the supernatant by the method of Lowry (6) with bovine serum albumin as the standard.

RESULTS

Aerobic respiration, growth, and intracellular cAMP levels. When spores are inoculated into liquid YPG medium, they begin to swell and by 10 h, produce germ tubes. During this time, the protein content of the culture increases exponentially. Figure 1 shows that respiratory capacity begins to develop immediately in the germinating spores, reaching a maximum near midexponential growth and then declines as the cells approach the stationary phase. The intracellular levels of cAMP during the growth cycle (Fig. 1) also rise during the germination process, reaching a maximum at 4 to 6 h. After this time, the intracellular levels of cAMP drop to a minimum about the same time germ tube formation begins. The levels of cAMP remain low for the remainder of the growth cycle. Maximum levels of cAMP are routinely observed during the germination phase of growth, independent of the conditions

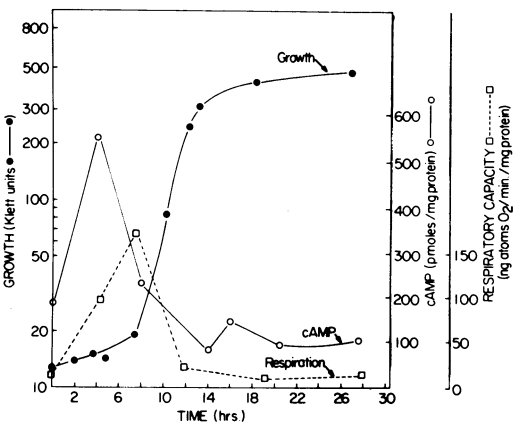

FIG. 1. *Respiratory capacity* (□) *and intracellular cAMP levels* (○) *during aerobic growth* (●) *of M. racemosus. The culture was inoculated with* 5×10^5 *spores/ml into YPG medium and incubated at 23 C on a rotary shaker. Samples (1 to 10 ml) were removed at indicated times, and respiration and cAMP were determined as described. Growth is expressed as milligrams of protein per milliliter of culture as determined by the method of Lowry (6).*

of germination. It should be pointed out that several repetitions of this experiment gave the same pattern of cAMP accumulation. However, the absolute levels of cAMP were found to vary as a function of the particular assay kit employed and with different batches of media and growth conditions. In all the experiments reported here, comparisons of cAMP levels were made to eliminate these variables between paired samples.

Respiratory capacity and intracellular cAMP levels during growth in CO_2. *M. racemosus* will grow in the yeast form in 100% CO_2, although growth is much slower than in air. The development of respiratory capacity and growth in carbon dioxide is shown in Fig. 2. As expected, the spores germinating in carbon dioxide developed lower respiratory capacity than those germinating in air. The respiratory capacity that was observed was predominantly cyanide sensitive (greater than 80%). As with air-germinated spores, the cAMP levels reached a maximum at 4 to 6 h and then declined during exponential growth. However, the cAMP levels during logarithmic growth were consistently higher for CO_2-grown cells than air-grown cells.

cAMP levels during yeast-mycelia transition. If spores are germinated in air and allowed to develop short germ tubes, then shifted to carbon dioxide, they stop growing immediately. After 6 to 8 h, cells begin producing yeastlike buds from the sides and tips of the hyphae. If growth is continued for 20 h after the shift to carbon dioxide, most of the population will consist of multipolar budding yeasts. Figure 3 shows that the intracellular cAMP levels increase threefold within 90 min after a shift

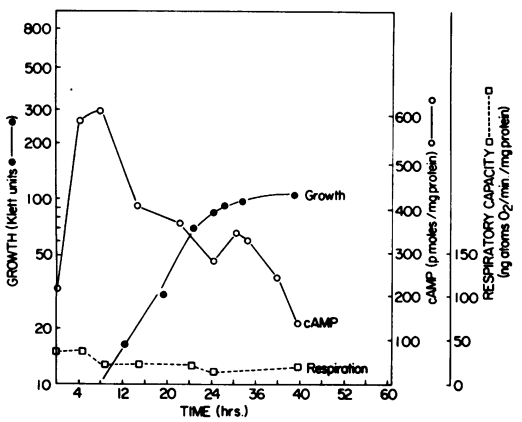

FIG. 2. *Respiratory capacity* (□) *and intracellular cAMP levels* (○) *during growth* (●) *of M. racemosus in 100% CO_2. Sampling procedures and assays were as described for Fig. 1.*

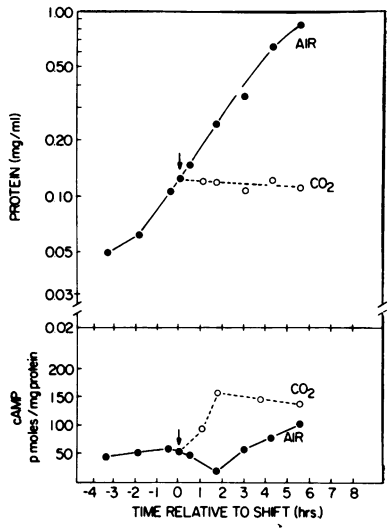

FIG. 3. *Intracellular cAMP levels of cultures shifted from air to CO_2. A 200-ml YPG culture was inoculated with 5 × 10^6 spores/ml and incubated in air on a rotary shaker overnight. Samples were removed at 30-min intervals for 3 h before being shifted to CO_2. At zero time, one-half of the volume remaining was transferred to a stoppered flask and flushed with 100% CO_2. Samples of both air* (●) *and CO_2* (○) *cultures were analyzed for protein by the method of Lowry (6) after extracting the samples with 0.1 N HCl. The extract of air* (●) *and CO_2* (○) *samples were analyzed for cAMP as described.*

from air to carbon dioxide and that the levels remain high for several hours. Cells which remain in air increase their cAMP levels only after they enter late exponential and stationary phase. Therefore, one immediate effect of encountering an atmosphere of carbon dioxide is the elevation of the cAMP levels in the cells. Thus, the increases in the cAMP concentration seem to be a prelude to the morphogenetic act leading to yeast formation. Conversely, as shown previously (4), the reduction of intracellular cAMP appears to be a prelude to the formation of mycelial cells.

Development of respiratory capacity in air and cAMP. Yeast cells formed either in carbon dioxide or nitrogen continue growing as yeasts when shifted to air and 3 mM dibutyryl cAMP. The growth rate under these conditions is similar to that of mycelia growing in air. Figure 4 shows that nitrogen-grown yeasts, when shifted to air, begin immediately to develop respiratory capacity in the presence or absence of dibutyryl cAMP. When no dibutyryl cAMP is present, the yeasts begin developing germ tubes in 1 h. In the presence of 3 mM dibutyryl cAMP, the cultures remain yeastlike but respiratory capac-

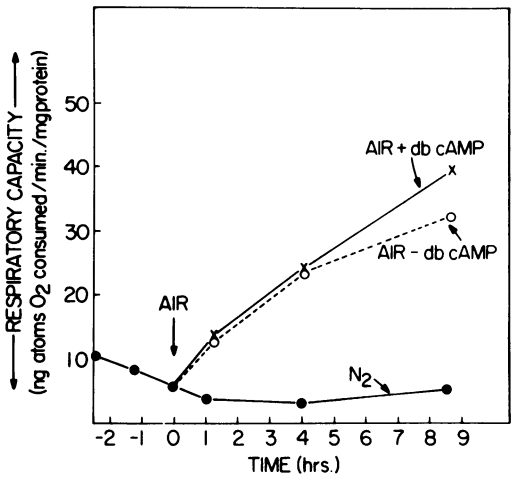

FIG. 4. *Respiratory capacity during aerobic adaptation of a nitrogen yeast culture* (●), *with* (×) *and without* (○) *3 mM dibutyryl cAMP. A 30-ml YPG culture was inoculated with 5 × 10⁶ spores/ml and incubated under 100% nitrogen at a flow rate of 3 volumes of gas per volume of culture liquid for 12 h. At zero time two 10-ml aliquots were transferred to cotton-stoppered flasks, one of which contained sufficient dibutyryl cAMP to make the final concentration 3 mM. Cells in this flask remained yeastlike through the growth in air. Respiratory capacity was determined on 1-ml aliquots which were placed in a 3-ml reaction vessel. A 1.0-ml amount of well-aerated growth medium was added, and oxygen consumption was assessed with a Clark-type oxygen electrode. At the end of the measurement the entire content of the reaction vessel was collected on a membrane filter (Millipore Corp.). The cells were washed off the filter with 1.0 N NaOH and assayed for protein by the method of Lowry (6). The protein content of the N_2 culture at 2 h before the shift was 0.058 mg/ml. Protein content at 8.5 h after the shift was 0.39 mg/ml.*

ity develops at the same rate as the mycelial culture. In both cases, respiratory capacity develops immediately, even in the presence of 2% glucose. These data show, therefore, that although cells growing in dibutyryl cAMP and air are yeastlike, they develop a respiratory capacity similar to that of mycelial cells. However, even in the presence of dibutyryl cAMP, a hexose is necessary for yeast development. If a hexose is not present, the cells develop germ tubes and become mycelial even in the presence of dibutyryl cAMP. Thus, cAMP is not itself sufficient to induce yeast morphology.

Formation of mycelia in nitrogen. The development of respiratory capacity in yeast cells growing in air and dibutyryl cAMP prompted us to search for conditions which might lead to mycelial growth without concomitant development of the respiratory chain. Since it had been reported previously that mycelial growth can be obtained with *M. rouxii* in pure nitrogen, we examined *M. racemosus* under these conditions. A more complete analysis of growth under 100% N_2 is soon to be published (D. Mooney and P. S. Sypherd, submitted for publication). As Fig. 5 shows, mycelia formed in N_2 does not contain appreciable levels of respiratory enzymes, as judged by the ability to take up O_2. Thus, not only can yeasts develop full respiratory capacity (Fig. 4), but mycelia need not be capable of respiration (Fig. 5).

Intracellular cAMP levels of N_2-grown mycelia developed essentially the same way as aerobic mycelia. That is, levels reached a maximum during germination and then the levels fell during subsequent exponential growth (Fig. 5).

Formation of yeasts in nitrogen. As indicated above, yeasts developed under N_2 when the gas was flushed through the culture at 3 to 4 volumes per min. Growth, respiratory capacity, and intracellular cAMP levels were examined under these conditions. Although development is purely yeastlike, each of the parameters measured (Fig. 6) was typical of mycelial growth in N_2 (Fig. 5). Thus, when cells are grown in N_2, whether in the yeast or mycelial phase, respiratory capacity is very low. Moreover, intracellular cAMP falls to low levels, characteristic of air-grown mycelia, during the exponential phase of growth.

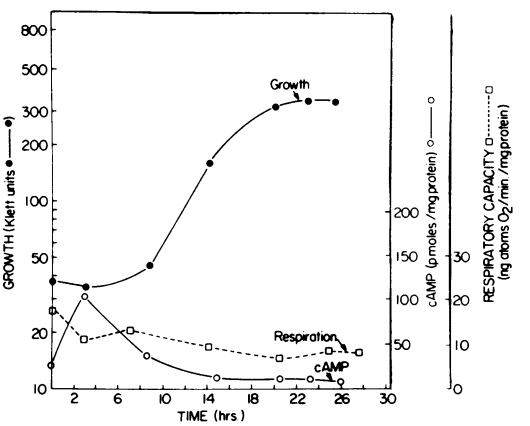

FIG. 5. *Respiratory capacity* (□) *and intracellular cAMP levels* (○) *during anaerobic growth (100% N_2)* (●) *of low flow mycelia of M. racemosus. Culture conditions and assay procedures were the same as described for Fig. 1 except that the culture was incubated under N_2 at a flushing rate of 0.5 volumes of gas/ml of culture.*

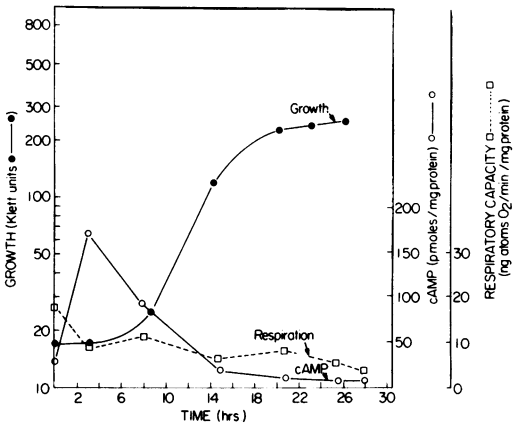

FIG. 6. *Respiratory capacity* (□) *and intracellular cAMP levels* (○) *during growth* (●) *of yeastlike M. racemosus. Conditions were the same as for Fig. 5 except flushing rate was 3 volumes of N_2/volume of culture fluid.*

DISCUSSION

This study has revealed two interesting features of the morphogenesis of *M. racemosus*. First, there is an apparent correlation between the germination process and the steady increase in intracellular cAMP regardless of the morphologic form which ultimately persists. This fact naturally leads to the suggestion that cAMP plays a central role in the differentiation processes which accompany spore germination. Secondly, the experiments reported provide strong evidence against the notion that dimorphism is regulated by mitochondrial activity or respiratory capacity.

Two sets of data convincingly separate the generation of respiratory capacity from the ability of *M. racemosus* to grow in one or the other form. On the one hand (shown in Fig. 4), if the organism grows in air and dibutyryl cAMP, it develops into yeast form. However, respiratory capacity is quite high. On the other hand, if the organism is growing N_2 in the mycelial form, respiratory capacity is quite low (Fig. 5). These two conditions are in contradistinction to the usual cases where yeasts develop anaerobically with the production of low respiratory capacity, while mycelia develop aerobically with the production of high respiratory capacity. When spores are germinated in air, they begin immediately to develop respiratory capacity and this process continues until sometime during midlogarithmic growth, after which respiratory capacity in these cells begins to decline. This decline is not due to a loss of activity but due to an increase in the cellular mass without further development of respiratory capacity. These observations are in agreement with Rogers and Clark-Walker's (8) observations with *M. genevensis*. As shown in the present study, when spores are germinated in an anaerobic environment in 2% glucose, they develop and maintain a low level of respiratory capacity which is 70 to 80% cyanide sensitive. This respiratory capacity apparently is mitochondrial since cell extracts contained a cyanide-sensitive cytochrome oxidase activity. The ratio of cytochrome oxidase activity to respiratory capacity was the same for both aerobic mycelia and aerobic yeast. Furthermore, we have found by electron microscope observation that yeasts grown in CO_2 have mitochondria with developed cristae. This has also been shown by Clark-Walker (3) and by Lindsey and Bartnicki-Garcia (5). Moreover, well-developed mitochondria are present in yeast cells grown in air and cAMP. This, of course, is in contrast to the yeast *Saccharomyces* which, under anaerobic conditions, has no demonstrable mitochondrial-type respiratory activity, and mitochondria with developed cristae are not observed in electronmicrographs (8).

The yeast form of *M. racemosus* develops under conditions which result in high glycolytic activity. High cAMP levels are correlated with glycolytic activity, but cAMP alone apparently is not sufficient for yeast formation. This is concluded from the fact that when cells are growing in air and cAMP, they form yeasts only when the carbon source is a hexose. If the carbon source is glycerol, succinate, or maltose, they grow in the mycelial form (unpublished experiments of A. Larsen). This suggests that control of mycelia-to-yeast development in *M. racemosus* is not due to a single compound, but the result of the interaction of several physiological processes. The precise role of cAMP in the control of dimorphism cannot be defined as yet.

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