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Trehalase Activity and Cyclic AMP Content During Early Development of *Mucor rouxii* Spores

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Incubation of *Mucor rouxii* sporangiospores in complex medium under aerobic conditions resulted in a transient 20-fold increase in trehalase activity. Maximum activity was reached after 15 min. Simultaneously, the cyclic AMP (cAMP) content increased approximately eightfold, reaching a maximum within 10 min. Increases in trehalase activity and cAMP content were also observed under anaerobic conditions (CO_2). The extent of trehalase activation and the changes in cAMP content, during both aerobic and anaerobic incubation, varied with the medium used. Trehalase was activated in vitro by a cAMP- and ATP-dependent process. An even faster activation was obtained when cAMP was replaced by the catalytic subunit of beef heart protein kinase. The coincidence of, and the correlation between, increased cAMP contents and trehalase activities support the involvement of a cAMP-dependent phosphorylation in the in vivo regulation of trehalase activity.

Germination of fungal spores is accompanied by a dramatic increase in a variety of metabolic activities such as respiration and protein and RNA synthesis. The earliest described event during germination of heat-activated spores and during acetate activation of the dormant spores of *Phycomyces blakesleeanus* is a very rapid increase in cyclic AMP (cAMP) content and trehalase activity (27a). This increase in trehalase activity results in high internal levels of hexosephosphates and hexosebisphosphates (27), among which fructose-2,6-bisphosphate is a potent stimulator of fungal phosphofructokinases (25). Similar increases in trehalase activity and cAMP content have been described during germination of yeast ascospores (17, 18) and during glucose-induced growth of stationary-phase yeast cells (24).

To investigate whether these changes are due to the relief of the dormant state or are more generally associated with early spore germination, trehalase activity and cAMP levels were determined in spores of *Mucor rouxii*. Unlike the spores of the related fungus *P. blakesleeanus*, *M. rouxii* spores will germinate without any pretreatment whenever supplied with a suitable culture medium.

M. rouxii is a dimorphic fungus, growing as yeast cells under a CO_2 atmosphere or as mycelium under air. Therefore, determinations were done on both aerobically and anaerobically (CO_2) incubated spores.

Since cAMP-dependent phosphorylation and concomitant activation of trehalase are suggested for the enzyme from yeast (6, 12, 22, 28, 29) and from *P. blakesleeanus* (26, 27a), we sought to verify whether in vitro activation could also be obtained with the *Mucor* enzyme.

MATERIALS AND METHODS

Organism. *M. rouxii* (CBS 416.77) was grown on defined medium (2) supplemented with 0.2% casein hydrolysate and 1.5% agar. Sporangiospores, harvested in distilled water, were washed several times by centrifugation and used as inoculum.

Culture media. Complex liquid medium (YPG) contained 0.3% yeast extract, 1% peptone, and 2% glucose (1). Minimal medium (MMG) had the following composition: 10 mM KH_2PO_4, 10 mM glutamate, and 2% glucose. In some experiments, minimal medium without glucose (MM) was used. The pH of all media was adjusted to 4.5.

For germination experiments under anaerobic conditions, 25-ml Erlenmeyer flasks (one for each sample) were closed with a rubber stopper and flushed with CO_2 by means of two hypodermic needles to remove all air. Erlenmeyer flasks with center wells were used to keep spore inocula and germination media separated until flushing with CO_2 was completed. The incubation was started by tilting the flask to mix spores and medium. The spore suspensions were incubated in a reciprocating shaker at 25°C.

Prolonged incubation in YPG medium yielded mycelium (aerobic) or yeast cells (anaerobic) exclusively. Aerobic and anaerobic incubation in MMG resulted in delayed and incomplete development. Without glucose (MM), no germination was observed.

Determination of cAMP content. At the times indicated in the figures, two samples were taken. One sample was used for cAMP extraction as described previously (27a). cAMP was measured by the binding protein assay described by Tovey et al. (20), using a cAMP assay kit (Amersham International). Spores of the second sample were washed, suspended in 0.5 N NaOH, and boiled for 5 min. The protein content of the supernatant was determined by the Lowry method.

Trehalase assay. At appropriate times, a sample was taken and centrifuged for approximately 10 s at 10,000 × *g*, suspended in distilled water, and centrifuged for another 10 s. After the spores were suspended in 0.5 ml of ice-cold 10 mM phosphate buffer with 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (pH 7.0), the suspension was transferred to a test tube (11-mm diameter) containing 1.2 g of glass beads (0.5-mm diameter) and homogenized by vortexing for 1 min. The homogenate was centrifuged for 2 min at 10,000 × *g*. Trehalase activity was measured as described before (19). Protein content was determined by the Lowry method. Specific activity is expressed as nanomoles of glucose liberated per minute per milligram of protein.

In vitro activation of trehalase. Spores were suspended in 10 mM phosphate buffer (pH 6.5)–1 mM phenylmethylsulfonyl fluoride–1 mM dithiothreitol and transferred to a

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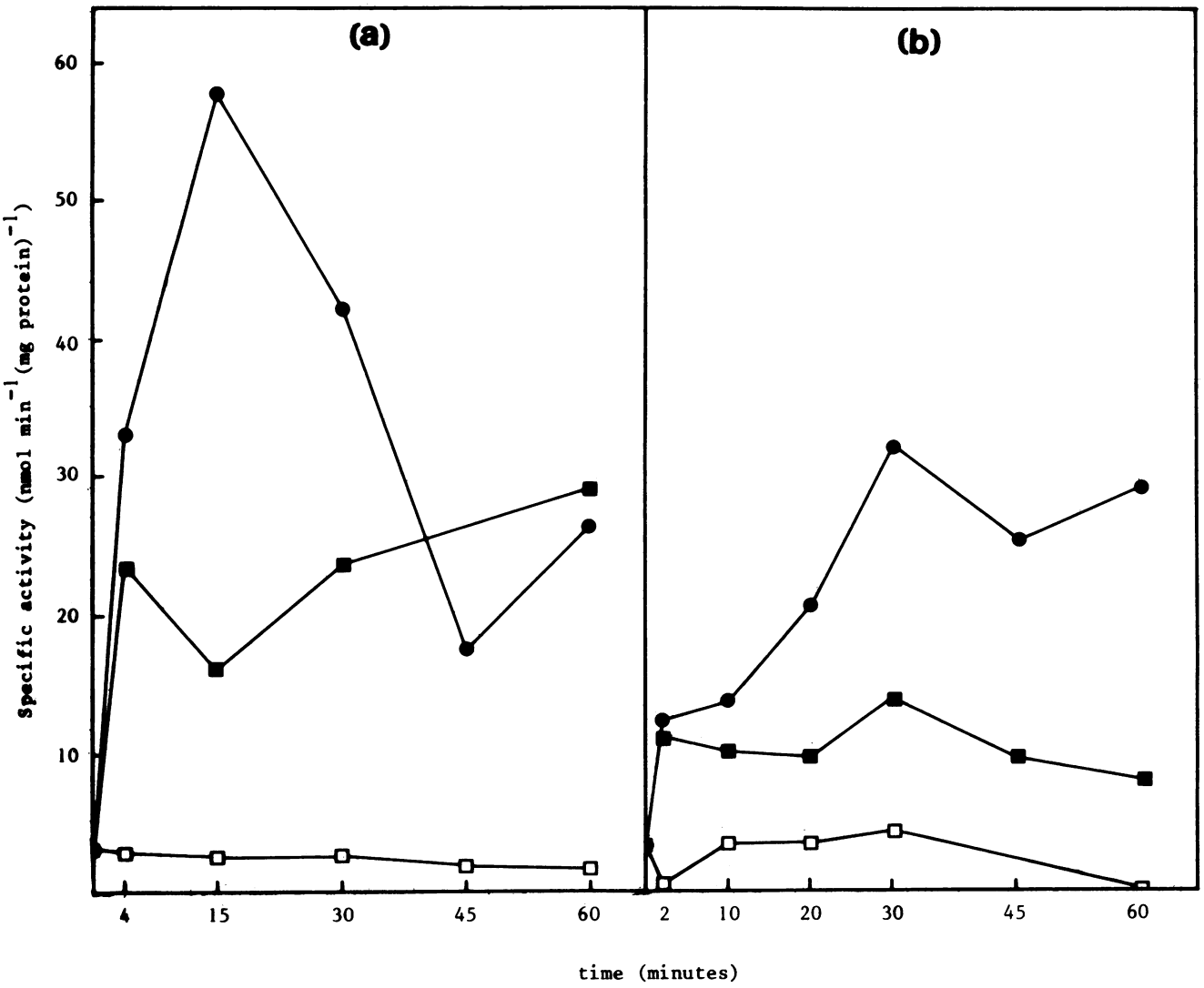

FIG. 1. Specific activity of trehalase during aerobic (a) and anaerobic (b) incubation of *M. rouxii* spores in YPG medium (●), MMG (■), or MM (□).

precooled 7-ml Teflon container containing 5 g of glass beads (0.5-mm diameter). The spores were homogenized by vibration in a microdismembrator (Braun, Melsungen GFR) for 3 min at an amplitude of 10 mm. The crude extract was centrifuged for 10 min at 100,000 × *g*, and the supernatant was desalted on a Sephadex G-25 column equilibrated with extraction buffer. For in vitro activation, the extract was incubated at 25°C in the presence of 1.8 mM $MgSO_4$, 1 mM theophylline, ATP, and cAMP at various concentrations. In some experiments, cAMP was replaced by 10 μg of the catalytic subunit of beef heart protein kinase (Sigma Chemical Co.) ml^{-1}. At appropriate times, 20 μl of the incubation mixture was taken to determine trehalase activity as described above. All experiments were repeated with consistent results in the trends shown.

RESULTS

Trehalase activity. Sporangiospores of *M. rouxii* do not germinate when suspended in water. Nevertheless, they are not dormant since germination readily occurs when the spores are suspended in a rich medium or, like the spores of *Mucor racemosus* (21), in a minimal medium containing glucose.

Since germination of the spores occurs only when adequate substrate is present in the medium, it was rather surprising to find that the activity of trehalase increased approximately 10-fold during the first minutes after suspension of the spores in minimal growth medium (Fig. 1a). Moreover, this increase in trehalase activity was dependent on the presence of glucose (the product of trehalase activity) in the minimal medium, since no increase was found when glucose was omitted (Fig. 1a). An even more pronounced but partially transient increase in trehalase activity was found when the spores were suspended in a complex medium. In this case, a nearly 20-fold increase in activity was found after 15 min, after which the activity declined to a value comparable to that under MMG conditions (Fig. 1a).

Measurements made during anaerobic incubations of spores also showed increased activities of trehalase. In complex medium, a 10-fold increase in trehalase activity was

found. In minimal medium, the increase was only threefold, and no increase in activity occurred in minimal medium without glucose (Fig. 1b).

In vitro activation of trehalase. The rapid activation of trehalase after addition of culture medium to the spores suggested that the activity of the enzyme could be regulated by a posttranslational modification such as phosphorylation-dephosphorylation, as suggested for the trehalase from yeasts and *P. blakesleeanus* (see above).

The trehalase from *M. rouxii* spores was activated nearly 10-fold by an in vitro preincubation in the presence of Mg, ATP, and cAMP (Fig. 2). The dependence of activation on the concentration of cAMP was clearly in the physiologically relevant range, since half-maximal activation was obtained at ca. 0.5 μM cAMP (Table 1). ATP concentration was optimal at 0.32 mM ATP. Higher concentrations yielded apparently less activation (data not shown), but this was due to the inhibitory effect of ATP on the trehalase activity in the subsequent assay. Indeed, as in *P. blakesleeanus* (23), trehalase activity was inhibited by concentrations of ATP less than 1 mM (data not shown). No in vitro activation was found in the absence of ATP, whereas in the absence of added cAMP a slight stimulation was found (Table 1).

That the activation of trehalase was the result of cAMP-dependent phosphorylation of the enzyme was further suggested by the fact that similar and even faster activation was obtained by incubation of the extract with Mg, ATP, and the catalytic subunit of beef heart protein kinase (Fig. 2).

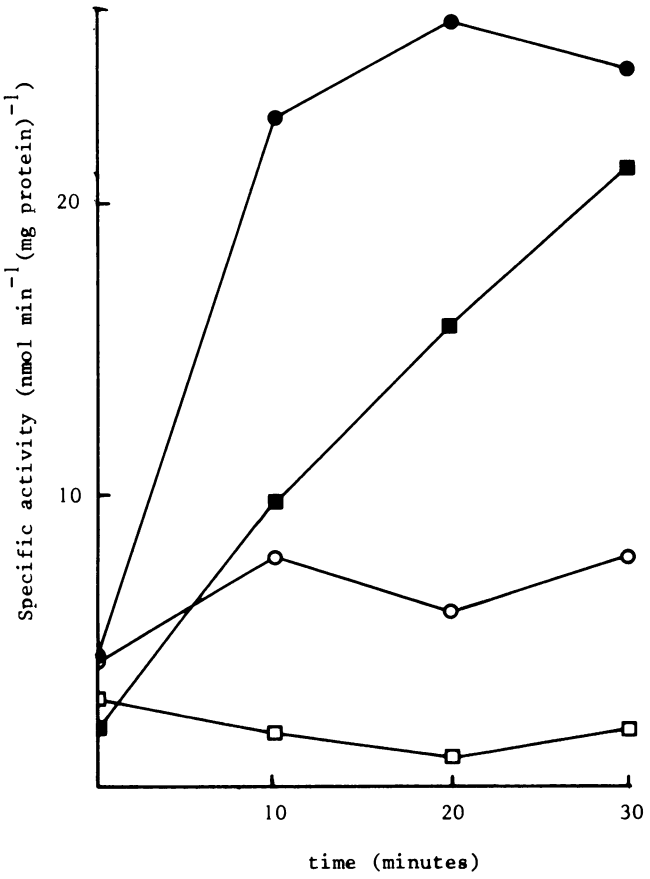

FIG. 2. Increase of trehalase activity during incubation of a desalted extract of *M. rouxii* spores at 25°C in the presence (closed symbols) or absence (open symbols) of 0.32 mM ATP. Symbols: □,■, with 20 μM cAMP; ○,●, without cAMP but with 10 μg of the catalytic subunit of beef heart protein kinase ml^{-1}.

TABLE 1. Effects of ATP and cAMP concentration on the activation of trehalase in a desalted spore extract after incubation for 30 min at 25°C with 1.8 mM $MgSO_4$ and various additions

Addition to spore extract	Sp act (nmol of glucose min^{-1} mg of protein^{-1})	Activation ratio (sp act/2.5)
None (time zero)	2.5	1
20 μM cAMP	2.0	0.8
20 μM cAMP + 0.016 mM ATP	4.8	1.9
20 μM cAMP + 0.048 mM ATP	10.4	4.2
20 μM cAMP + 0.16 mM ATP	15.1	6.0
20 μM cAMP + 0.32 mM ATP	21.3	8.5
0.32 mM ATP	5.4	2.2
0.32 mM ATP + 0.2 μM cAMP	6.6	2.6
0.32 mM ATP + 0.6 μM cAMP	15.3	6.2
0.32 mM ATP + 2.0 μM cAMP	20.3	8.1
0.32 mM ATP + 6.0 μM cAMP	18.7	7.5

cAMP in germinating spores. If the in vivo activation of trehalase is due to a mechanism similar to that of the in vitro process, one would expect an increase in cAMP content during early germination. After aerobic incubation in YPG medium, an eightfold increase in cAMP content occurred during a 10-min period; thereafter, the cAMP content decreased to approximately threefold the original level. In MMG, a less pronounced increase occurred (approximately twofold), whereas no significant changes were found in the absence of glucose (MM) (Fig. 3a). Similarly, under a CO_2 atmosphere, the cAMP content increased rapidly but only two- to threefold, and no difference was found between results in YPG and MMG (Fig. 3b).

DISCUSSION

Trehalase and germination. Activation of trehalase is a very early phenomenon in the development of *M. rouxii* spores, occurring during the first minutes after incubation of the spores in culture medium. In the related fungus *P. blakesleeanus*, an equally large and rapid increase in trehalase activity occurs during early germination. In *P. blakesleeanus*, however, this phenomenon occurs only after the spores have been activated by chemicals or by a heat treatment, and no changes occur when untreated spores are incubated in culture medium (27a).

Trehalase activation in stationary-phase yeast cells (24), yeast ascospores (18), and activated *Phycomyces* spores (19) is stimulated by glucose in the medium. As shown by our results, this is also the case for *M. rouxii* spores.

This paradoxical activation of trehalase (a nutrient-producing enzyme) by other nutrients could have a selective advantage for the spores. Breakdown of the endogenous reserves and concomitant germination would occur only when sufficient nutrients were available in the surrounding medium to give the spore a reasonable chance to reach maturity.

Mechanism of trehalase activation. The data described support a cAMP-mediated phosphorylation and concomitant activation of *M. rouxii* trehalase in vitro. The in vitro process is dependent on physiological concentrations of ATP and cAMP. cAMP (and endogenous protein kinases) can be replaced by the (cAMP-independent) catalytic subunit of

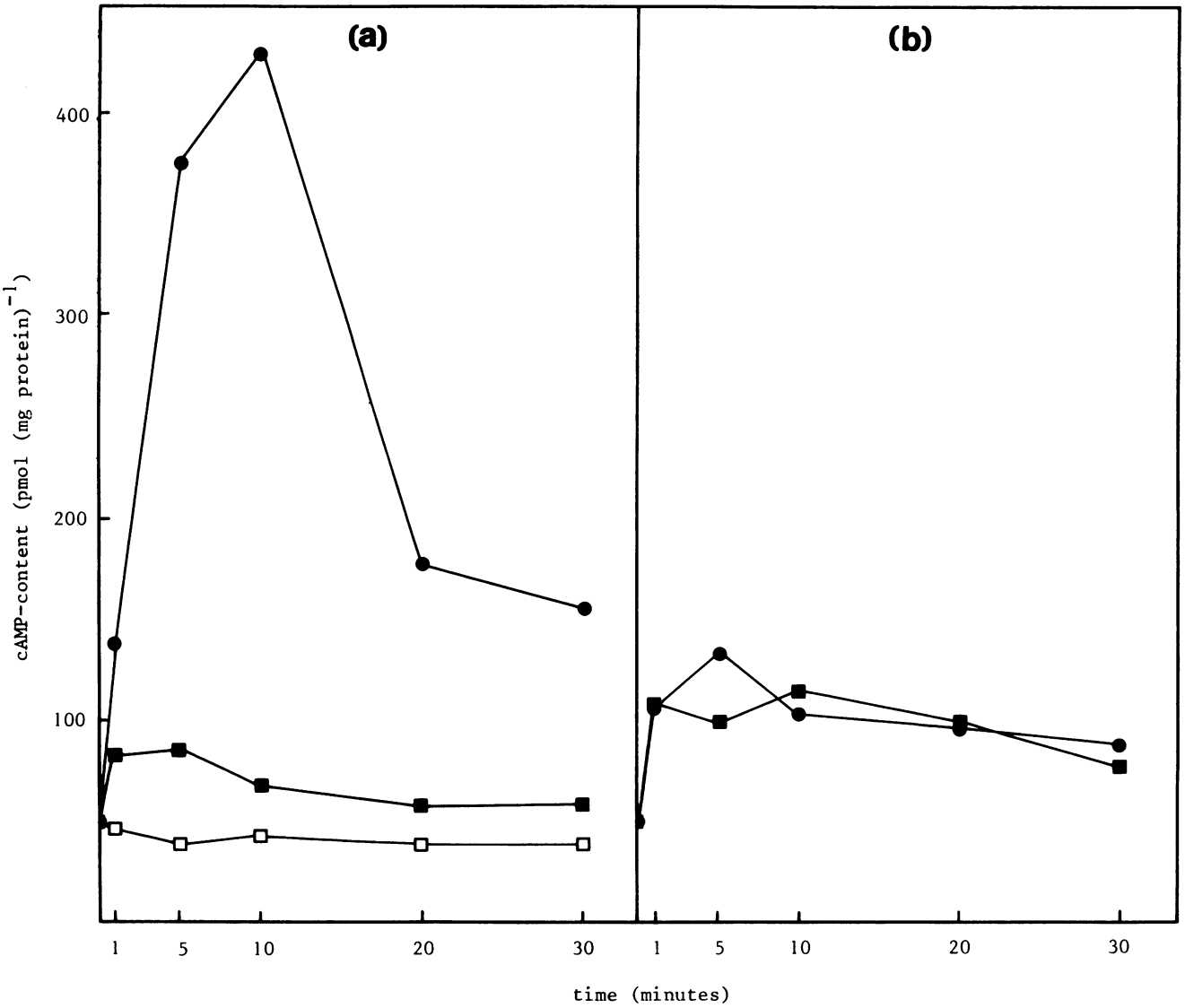

FIG. 3. Changes in cAMP content during aerobic (a) and anaerobic (b) incubation of *M. rouxii* spores in YPG medium (●), MMG (■), or MM (□).

beef heart protein kinase. That a phosphorylation is also involved in the in vivo activation of trehalase is suggested by the coincidence of, and the correlation between, rises in cAMP content and trehalase activity under various conditions. Therefore, the trehalase of *M. rouxii* can be classified as one of the so-called regulatory trehalases (17) which have only been described in another Mucoralean fungus, *P. blakesleeanus* (26, 27a), and in the yeast species *Saccharomyces cerevisiae* (6, 12, 22, 24, 28, 29) and *Pichia pastoris* (17).

cAMP and spore germination. The accumulation of cAMP and the stimulation of trehalase activity in different media appear to be correlated with the germination of the spores. Highest values for both cAMP and trehalase were obtained in complex medium (YPG) allowing complete germination; intermediate values were obtained in MMG, in which partial germination occurred; no increase was found in MM, in which no germination took place. The concept of cAMP as a hunger signal, as described from bacterial and animal physiology (16), certainly does not apply to *M. rouxii* spore germination, and probably not to other fungal regulatory processes.

Long-term changes in cAMP content in aerobically germinating *M. rouxii* spores have been described (3), but apparently the short-term effect reported here has been overlooked. The same comment could apply to changes in cAMP levels reported for *Mucor genevensis*, *Mucor mucedo* (10), and *M. racemosus* (14).

cAMP and anaerobiosis. cAMP has also been implicated in the regulation of mycelial versus yeast-like morphology, and changes in cAMP content have been correlated with morphological changes in dimorphic fungi (5, 7–9, 11, 13, 15). In *M. rouxii*, three to four times higher cAMP levels have been reported for anaerobically grown yeast cells as compared with aerobically grown mycelium (13). During hyphal budding of the polymorphic fungus *Aureobasidium pullulans*, a transient increase in cellular trehalose and in trehalase activity has been found (4). Our data for anaerobically

developing spores showed a generally smaller level of cAMP and trehalase activity as compared with aerobically grown cells during the first hour of growth.

In summary, rapid but transient increases in cAMP content occur during early germination of *M. rouxii* spores. The activity of trehalase could be controlled by the concentration of cAMP, as shown by the in vitro and in vivo results. These short-term variations in cAMP content might affect not only trehalase activity but also various other enzymatic activities that are important during fungal spore germination.

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