JOURNAL OF BACTERIOLOGY, Apr. 1980, p. 196-201
0021-9193/80/04-0196/06$02.00/0

Vol. 142, No. 1

S-Adenosylmethionine Levels and Protein Methylation During Morphogenesis of *Mucor racemosus*

J. ROBERTO GARCIA, WILLIAM R. HIATT, JULIUS PETERS,† AND PAUL S. SYPHERD*

Department of Microbiology, College of Medicine, University of California, Irvine, California 92717

The intracellular level of S-adenosylmethionine increased as the yeast-phase cells of *Mucor racemosus* were induced to convert to hyphae. This increase correlated well with the time course of the conversion in cell type and was independent of the metabolic changes caused by the shift to aerobic conditions. There was no significant change in the intracellular level of spermidine, a polyamine synthesized from putrescine and the propylamine group of S-adenosylmethionine. Spermine was not detected. An examination of protein methylation revealed an increase in the methylation of total protein during the shift in cell type and possible qualitative as well as quantitative changes in specific basic proteins.

The dimorphic phycomycete *Mucor racemosus* produces two vegetative cell types during its life cycle: budding yeasts and branched hyphae. The transition from one cell type to another in *M. racemosus* and other species of *Mucor* has been studied extensively and shown to be affected by various environmental factors such as the gaseous environment (4, 5), glucose concentration of the medium (3, 7), and the levels of cyclic AMP (17, 18). It has also recently been shown by Sypherd et al. (21) that methionine or one of its metabolites may be a key compound during morphogenesis. In that particular study, it was reported that a class of morphological mutants (*coy* for conditional yeast) required high levels of methionine for the transition in cell type after being shifted to an aerobic environment. Methionine, however, was not required for the growth of this class of mutants.

The present study was undertaken to investigate the role of methionine in the dimorphism of *M. racemosus*. Methionine may be utilized for protein synthesis or converted to its activated form, S-adenosylmethionine (SAM), which in turn serves as the methyl-group donor during the methylation of most macromolecules or as a precursor in the synthesis of cystathionine, cysteine, or other such compounds. SAM also serves as the propylamine donor during polyamine biosynthesis. In light of this, we have examined the relative size of intracellular SAM pool levels during the morphological transition and begun a systematic examination of methylated compounds that may be important during morphogenesis. The polyamine pools were also examined.

† Present address: Biology Programs, The University of Texas at Dallas, Richardson, TX 75080.

MATERIALS AND METHODS

Organism and medium. The organism used throughout this study was *M. racemosus* (*M. lusitanicus*) ATCC 1216B. Sporangiospores, which served as the inoculum in all experiments, were prepared from mycelia grown on YPG agar. After incubating the plates for 5 to 7 days at room temperature, we added a small amount of sterile distilled water to each bottle, scraped the sporangiospores gently from the mycelial mat with a glass rod and washed them several times with sterile water. The semi-defined growth medium used in all experiments was that described by Peters and Sypherd (19) supplemented with 0.5% peptone (9a).

Culture conditions. Anaerobic cultures (budding yeasts) were routinely prepared by inoculating a flask containing the semi-defined medium with 1×10^5 sporangiospores per ml. The culture was then placed in a 28°C water bath gyratory shaker for a period of 18 to 20 h. CO_2 gas was constantly bubbled through the culture to maintain an anaerobic environment. A shift in vegetative cell type (yeast to hyphae) was effected by changing the gaseous environment from CO_2 to air. The change in morphology occurred over a 2- to 3-h period after the shift to air.

Measurement of relative levels of intracellular SAM. Samples (5 ml) of yeast- and hyphal-phase cultures, previously labeled with L-[^{35}S]methionine (600 Ci/mmol) at a final concentration of 1 μCi/ml, were removed at specified time intervals during the shift and filtered through membrane filters. The cells collected on the filters were washed with cold medium and placed into cold 1.5 N perchloric acid for 1 h. The cell extract was then centrifuged (12,000 × *g*, 10 min) to remove the insoluble material and neutralized (to a pH of 2 to 3) with cold 2 M $KHCO_3$. The resulting white precipitate was removed by centrifugation. The supernatant, containing the SAM, was lyophilized and subsequently dissolved in 100 μl of 0.15 M acetate buffer (pH 5). Any insoluble material still present at this stage was removed by centrifugation. A sample of each extract, along with authentic SAM, was applied

to cellulose thin-layer chromatography plates (20 by 20 cm) and resolved in one dimension in a solvent system containing ethanol, water, and acetic acid (65:34:1). The plates were dried and sprayed with ninhydrin (UV-absorbing compounds were located before spraying). The SAM in the extracts was located by calculating the R_f value of a SAM standard and identifying ninhydrin-positive, UV-absorbing compounds which comigrated with the standard. Autoradiography was then performed and used to authenticate SAM (R_f of the putative radioactive SAM in the extract was identical to that of authentic SAM) and to delineate the area of the radioactive compound on the plate. This compound was scraped from the plate and placed into a scintillation counting solution consisting of PPO (2,5-diphenyloxazole; 0.4%) and POPOP {1,4-bis-[2]-(5-phenyloxazolyl)benzene; 0.01%} in toluene. The radioactivity in the sample was measured by liquid scintillation spectroscopy. The identity of the suspected SAM in the extracts was also authenticated by paper chromatography (on Whatman no. 1 chromatography paper), employing a solvent system consisting of butanol, acetic acid, and water (60:15:25), and by identifying it as a sulfur-containing compound after spraying the plate or paper with a platinum reagent (22). The method of Lowry et al. (13) was used to quantitate total cellular protein.

Measurement of total protein methylation levels. Aliquots (5 ml) of yeast- and hyphal-phase cultures were removed and labeled with L-[^{35}S]methionine (0.1 μCi/ml) and L-[*methyl*-^{3}H]methionine (1 μCi/ml) for 1.5 h. The specific activity of the tritiated methionine was 27 Ci/mmol. Cycloheximide (200 μg/ml) was also added at the onset of the labeling period to stop protein synthesis. After the labeling period an equal volume of 10% trichloroacetic acid was added to the samples, and these samples were then heated at 90°C for 30 min. The hot trichloroacetic acid-precipitable material was collected on glass fiber filters, treated to remove lipids (10), and placed into the scintillation counting solution previously described to determine the amount of radioactivity. The ratio of ^{3}H to ^{35}S counts served as an indicator of the level of protein methylation during the morphological transition.

Two-dimensional electrophoresis of methylated polypeptides. Budding yeasts grown under CO_2 or hyphae 3 h after the shift were labeled with 40 μCi of [*methyl*-^{3}H]methionine per ml in the presence of 400 μg of cycloheximide per ml for 1 h. Cells were harvested by filtration, washed with distilled water, and broken by grinding under liquid N_2. Ground material was suspended in buffer containing 0.01 M Tris-hydrochloride (pH 7.4), 5 mM $MgCl_2$, 50 μg of phenylmethylsulfonyl fluoride per ml, and 50 μg each of RNase and DNase, incubated for 15 min on ice, and then lyophilized. Lyophilized material was extracted with a solution of 0.5% sodium dodecyl sulfate, 9.5 M urea, 5% β-mercaptoethanol and 0.2% ampholytes (LKB) (pH 3.5 to 10) for 15 min at room temperature, followed by an equal volume of a solution containing 9.5 M urea, 4% Triton X-100, 1.6% ampholytes (pH 5 to 7), 0.4% ampholytes (pH 3.5 to 10), and 5% β-mercaptoethanol for an additional 15 min at 25°C. Any remaining debris was removed by centrifugation at 480 × *g* for 10 min at room temperature, and the supernatant was stored at −20°C. Of cellular protein, 95 to 99% was solubilized by this procedure.

Two-dimensional gel electrophoresis was performed as described by O'Farrell et al. (16). Non-equilibrium pH gradient gels used in the first dimension were 120 mm and contained 1% each of ampholytes (pH 6 to 8 and 8 to 9.5). Electrophoresis in the first dimension was for 3.5 h at 400 V. The resolving portion of the second-dimension sodium dodecyl sulfate-polyacrylamide gels was 14 cm in length, and the gels were prepared, electrophoresed, and stained as described previously (9). Gels were destained in 10% isopropanol-10% acetic acid, processed for fluorography (6), and exposed to preflashed (12) Kodak XR-5 X-ray film at −70°C.

Measurement of the intracellular level of polyamines. Cell samples (25 ml) taken at various times during the transition in morphology were collected on membrane filters, washed with cold 20 mM phosphate buffer (pH 7.2), and then placed into 2 ml of 0.2 N perchloric acid for 1 h. After the extraction period the samples were centrifuged (17,500 × *g*, 15 min) to pellet the cell debris, and the supernatant was lyophilized. All glassware that came in contact with the polyamines was treated with triethylchlorosilane (5% in toluene) before use to prevent the adsorption of the polyamines to the glass. The lyophilized samples were then dissolved in 400 μl of 0.5 M Na_2CO_3 (pH 9.8) and dansylated by adding 200 μl of dansyl-chloride (0.02% in acetone). The dansylation was allowed to proceed in the dark for 2 h. The dansylated derivatives were extracted with benzene, resolved in activated silica gel plates with a solvent system consisting of cyclohexane and ethyl ether (1:9), and immediately quantitated by measuring fluorescence intensities on a scanning densitometer (20). The fluorescence of the dansylated derivatives was enhanced by spraying the thin-layer chromatography plates with a 2:1 mix of isopropanol-triethanolamine before scanning.

RESULTS

Levels of SAM. Cells used for SAM measurements were grown in semi-defined medium and allowed to go through yeast-to-hyphae morphogenesis by a shift from CO_2 to air. Samples of the culture were removed at intervals throughout the shift and processed as detailed in Materials and Methods. The intracellular levels of SAM increased threefold, as shown in Fig. 1, reached a high point 2 h after the shift, and remained at that level for 2 to 3 h after the shift. The time course of the increase correlated well with the morphogenetic change of the organism since the majority (70 to 80%) of the budding yeast initiated germ tube formation during the 1- to 2-h interval after the shift to air. Thereafter, the remaining yeast cells initiated germ tubes and those with germ tubes continued to extend the hyphae and form branches perpendicular to the central hyphal element.

We tested the possibility that the increase in

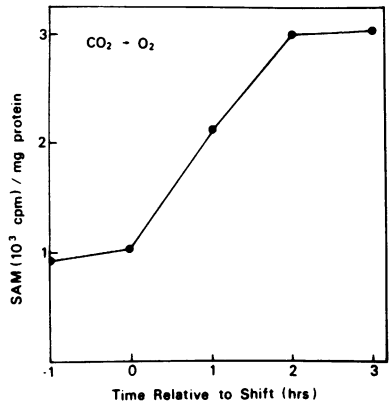

FIG. 1. *Intracellular concentration of SAM during an air-mediated shift in vegetative cell type (yeast → hyphae). Aliquots (5 ml) of yeast- and hyphal-phase cultures which had been labeled with L-[^{35}S]-methionine were removed at specific time intervals throughout the shift and processed as described in Materials and Methods. The cells were grown in semi-defined medium and labeled 3 h before removing the first yeast-phase aliquot.*

SAM levels was due to the onset of aerobic metabolism. This was done by inducing hyphal development by shifting CO_2 yeast cells to 100% N_2 (14). All other experimental conditions were identical to those utilized in the CO_2-to-air shifts. The N_2 atmosphere allowed for the transition in cell type under entirely anaerobic conditions (14). The intracellular concentration of SAM again increased to a level similar to that seen during CO_2-to-air shifts (Fig. 2). In addition, the kinetics seen in the CO_2-to-N_2 shift also correlated well with the morphogenetic shift from yeast to mycelia.

Intracellular concentration of polyamines. Because of the increase in intracellular SAM levels, we began a systematic examination of compounds, such as polyamines and methylated proteins, whose synthesis or modification depends on the availability of SAM. Our rationale was that the observed increase in SAM might be necessary to support an increase in polyamine biosynthesis or the methylation of macromolecules.

Figure 3 shows that the concentration of putrescine increased about fivefold during the conversion of yeast cells to hyphae; spermidine, however, showed no significant increase. Spermine was not detected, which is in agreement with the findings of Nickerson et al. (15).

Protein methylation. We examined the kinetics of protein methylation and qualitative or quantitative changes in the methylation of specific proteins during the conversion of yeast cells to hyphae. The data from the total protein methylation experiments are presented in Fig. 4 and 5. The data paralleled those seen with SAM; the methylation of proteins increased after the shift to air, reached a maximum 2 h after the shift, and generally followed the time course of the morphogenetic change. It should be pointed out that the threefold increase in protein methylation was within the same range as the increase in SAM levels. When morphogenesis was in-

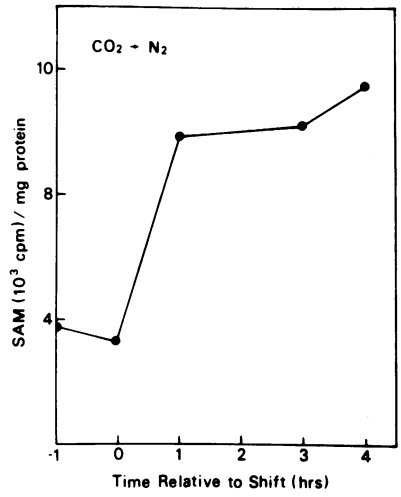

FIG. 2. *Intracellular concentration of SAM during an N_2-mediated shift in vegetative cell type (yeast → hyphae). Aliquots (5 ml) of yeast- and hyphal-phase cultures which had been labeled with L-[^{35}S]methionine were removed at specified time intervals throughout the shift and processed as described in Materials and Methods. The cells were grown in semi-defined medium and labeled 14 h before removing the first yeast-phase aliquot.*

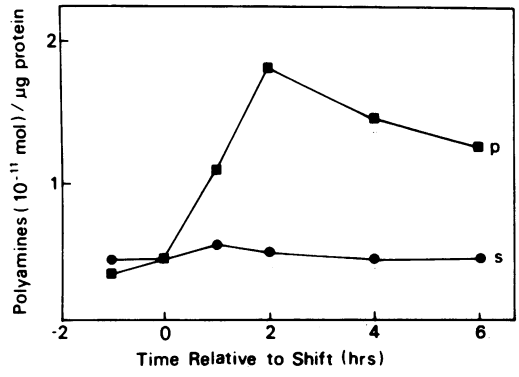

FIG. 3. *Intracellular concentration of polyamines during an air-induced shift in vegetative cell type (yeast → hyphae). Aliquots (25 ml) of yeast- and hyphal-phase cultures were removed at specific time intervals during the shift and processed as detailed in Materials and Methods. The cells used in this experiment were grown in the defined medium of Peters and Sypherd (19). s, Spermidine; p, putrescine.*

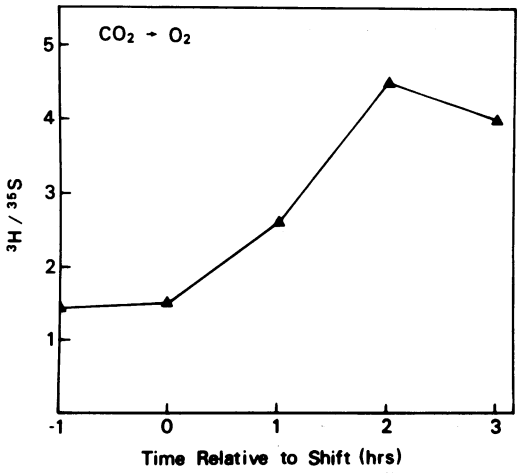

FIG. 4. *Protein methylation during an air-induced shift in cell type (yeast → hyphae). Aliquots (5 ml) of yeast- and hyphal-phase cultures were removed at specific time intervals, pulse-labeled with L-[^{35}S]- and L-[methyl-^3H]methionine in the presence of cycloheximide, and then processed as described in Materials and Methods. The ratio of ^3H to ^{35}S served as an indicator of protein methylation relative to protein synthesis.*

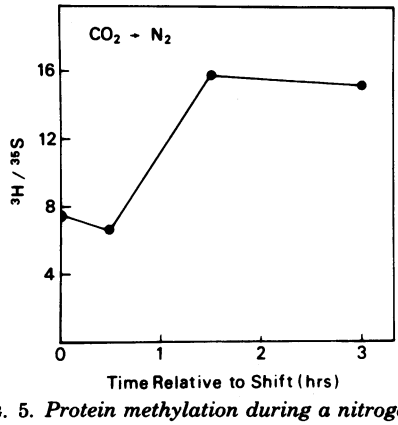

FIG. 5. *Protein methylation during a nitrogen-induced shift in vegetative cell type (yeast → hyphae). Aliquots (5 ml) of yeast and hyphal cultures were removed at specified time intervals, pulse-labeled with L-[^{35}S]- and L-[methyl-^3H]methionine in the presence of cycloheximide, and then processed as described in Materials and Methods. The ratio of ^3H to ^{35}S served as an indicator of protein methylation.*

duced by CO_2-to-N_2 shifts, the peak of the increase in protein methylation occurred approximately 1 h before the peak observed in CO_2-to-air shifts. The same was also true of the SAM data and the transition in cell type. It was determined that cycloheximide inhibited protein synthesis by 98% over controls.

Specific proteins, methylated during the yeast and hyphal phases, were identified by two-dimensional gel electrophoresis. Figure 6 is a composite of the gel patterns of basic methylated proteins from yeast cells produced under CO_2 (Fig. 6A) and hyphae grown in air (Fig. 6B). Methylated polypeptides with a pI of less than 6 were not detected (data not shown). Equal amounts of protein were applied to the first-dimension gel after extracting total cellular protein as described in Materials and Methods. The gel patterns show quantitative changes in the methylation of several basic proteins during the transition of yeasts to hyphae. The data do not reveal whether the increased methylation is due to increased levels of particular polypeptides or more rapid turnover of the labeled methyl groups. The data do, however, substantiate the results in Fig. 4 and 5, showing an overall increase in the level of methylation of proteins. A control experiment performed with L-[^{35}S]methionine instead of L-[*methyl*-^3H]methionine demonstrated that there were no detectable proteins labeled during the pulse period when cycloheximide was added to the culture.

DISCUSSION

It is clear from the data presented in Fig. 1 and 2 that the intracellular levels of SAM increased during the conversion of budding yeasts to hyphae. The increase began as early as 1 h after the shift to air, reached a maximum at 2 h and remained at that level for several hours after the shift. The relative level of SAM, at its highest point, represented a threefold increase over that seen when the culture was in CO_2. Differences in the SAM content of the different vegetative cell types have also been reported in other dimorphic fungi (1, 2). Balish and Svihla (2), for example, reported that a mycelial mutant of *Candida albicans* had a higher intracellular concentration of SAM than did the yeast-phase strains that were examined. A subsequent study by Balish (1), however, reported a decrease in the SAM pool during the medium-induced conversion of yeast to hyphae in *C. albicans*.

It is also interesting to note that the kinetics of the increase in *M. racemosus* correlate well with the time course of the conversion in vegetative cell type. The majority of yeast had initiated germ tube biogenesis 2 h after the shift to air. Thereafter, what occurs is primarily elongation of the preexisting germ tubes and formation of branches. This correlation also held true when cells were shifted from CO_2 to N_2 (Fig. 2) and demonstrated that the increase in SAM was dependent on morphology and not the gaseous environment. Yeast cells shifted to N_2 (Fig. 2) showed similar increase in SAM levels but reached a maximum approximately 1 h earlier

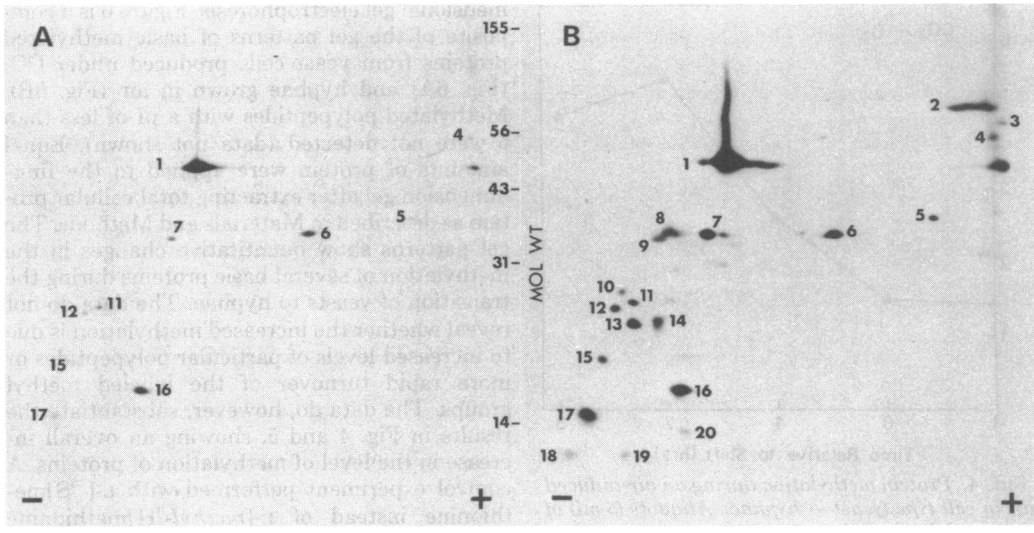

FIG. 6. *Fluorographs comparing polypeptides methylated by Mucor yeast and mycelia. Cells grown as yeast under CO_2 or mycelia 3 h after replacement of CO_2 with air were labeled, extracted, and subjected to two-dimensional gel electrophoresis as described in Materials and Methods. Equal amounts of total protein were applied to first-dimension gels from each sample. (A) Yeast-methylated polypeptides, 12,400 cpm of hot trichloroacetic acid-precipitable material applied to gel. (B) Polypeptides methylated by aerobic mycelia, 94,000 cpm of hot trichloroacetic acid-precipitable material applied to gel. Molecular weights were estimated from the mobilities of RNase, DNase, rabbit muscle actin, and the subunits of Bacillus subtilis RNA polymerase (9). Migration in the first dimension was toward the negative electrode.*

than the cells shifted from CO_2 to air. The time course of germ tube formation in cultures transferred from CO_2 and N_2 was also advanced approximately 1 h. We conclude that the increase in SAM levels is a correlate of morphogenesis and not an artifact due to changes caused by aerobic environment. There was no change in the SAM levels in experiments with budding yeasts kept under CO_2 (data not shown).

The search for changes in the levels of intracellular compounds that depend on SAM for their synthesis did not support the idea that the increased levels of SAM were needed to support an expansion in polyamine pools. The data in Fig. 3 show an increase in putrescine but fail to demonstrate any significant change in spermidine, which is synthesized from putrescine and the propylamine group of SAM.

The data obtained from the protein methylation experiments (Fig. 4–6), in contrast to the polyamine data, show the positive correlations of elevated SAM levels and increases in protein methylation. General quantitative changes are illustrated in Fig. 4 and 5. Notice that the kinetics of the increase in the level of general protein methylation paralleled and were of the same magnitude as the increases in the level of SAM. In an effort to corroborate the observed increases in protein methylation, the major methylated protein (protein no. 1) shown in Fig. 6 was cut from gels representing yeast- and hyphal-phase cultures. This protein was then eluted and hydrolyzed to identify and quantitate the methylated residues. The level of methyllysine and dimethyllysine, the only methylated residues in this protein, increased more than twofold during the shift in cell type. In addition, it was shown that the increase in protein methylation was not due to an aerobic environment (Fig. 3) but instead appeared to be a correlate of morphogenesis. Moreover, the increase in methylation, as well as the increase in SAM, occurred at a time when the majority of yeast cells were engaged in germ tube formation. The experiments using CO_2-to-N_2 shifts further strengthened this idea because it allowed us to advance, by approximately 1 h, the time course of the increases. When the time course of germ formation was examined, it was discovered that this particular parameter had also been accelerated to where it again correlated with the increases in SAM and protein methylation.

The specific proteins affected by the changes in methylation during a shift in cell type are shown in Fig. 6. The question of whether the methylation of these proteins was essential for the change from yeast to hyphae can only be answered by further experimentation. It must be determined, for example, whether an increase in the degree of methylation involves new or pre-

viously methylated residues on the protein molecule (e.g., methyl-group turnover). In addition, if the evidence indicates that the increase involves the same type of amino acid residue(s), then it must be determined whether this reaction involves new peptides or merely a quantitative change in support of an increase in the rate of synthesis of that particular protein. The involvement of protein methylation in the control of cellular processes is not without precedent. Kondoh et al. (11) recently reported a methyl-accepting protein required for chemotaxis in *Escherichia coli*, and Dilberto et al. (8) presented evidence which suggests that protein methylation may be involved in the process of exocytosis. The question dealing with the involvement of these proteins in the morphogenesis of *M. racemosus* can be answered by experiments involving morphogenic mutants or by inhibitors of SAM synthetase or protein methylases. The characterization of the *coy* mutant, mentioned in the Introduction, should provide some definitive answers about the involvement of SAM and protein methylation during the shift in vegetative cell type.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 23999 from the National Institute of General Medical Sciences.

We thank Carol Katayama for technical assistance in portions of this work.

LITERATURE CITED

1. **Balish, E.** 1973. Methionine biosynthesis and S-adenosylmethionine degradation during an induced morphogenesis of *Candida albicans*. Can. J. Microbiol. **19**:847–853.
2. **Balish, E., and G. Svihla.** 1966. Ultraviolet microscopy of *Candida albicans*. J. Bacteriol. **92**:1812–1820.
3. **Bartnicki-Garcia, S.** 1968. Control of dimorphism in *Mucor* by hexoses: inhibition of hyphal morphogenesis. J. Bacteriol. **96**:1586–1594.
4. **Bartnicki-Garcia, S., and W. J. Nickerson.** 1962. Nutrition, growth, and morphogenesis of *Mucor rouxii*. J. Bacteriol. **84**:841–858.
5. **Bartnicki-Garcia, S., and W. J. Nickerson.** 1962. Induction of yeastlike development in *Mucor* by carbon dioxide. J. Bacteriol. **84**:829–840.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
7. **Clark-Walker, G. D.** 1972. Development of respiration and mitochondria in *Mucor genevensis* after anaerobic growth: absence of glucose repression. J. Bacteriol. **109**: 399–408.
8. **Dilberto, E. J., O. H. Viveros, and J. Axelrod.** 1976. Subcellular distribution of protein carboxymethylase and its endogenous substrates in the adrenal medulla: possible role in excitation-secretion coupling. Proc. Natl. Acad. Sci. U.S.A. **73**:4050–4054.
9. **Hiatt, W. R., and H. R. Whiteley.** 1978. Translation of RNAs synthesized in vivo and in vitro from bacteriophage SP82 DNA. J. Virol. **25**:616–629.
9a. **Inderlied, C. B., R. L. Cihlar, and P. S. Sypherd.** 1980. Regulation of Ornithine decarboxylase during morphogenesis of *Mucor racemosus*. J. Bacteriol. **141**:699–706.
10. **Kanfer, J., and E. P. Kennedy.** 1963. Metabolism and function of bacterial lipids. J. Biol. Chem. **238**:2919–2922.
11. **Kondoh, H., C. B. Ball, and J. Adler.** 1979. Identification of a methyl-accepting chemotaxis protein for the ribose and galactose chemoreceptors of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **76**:260–264.
12. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335–341.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Mooney, D. T., and P. S. Sypherd.** 1976. Volatile factor involved in the dimorphism of *Mucor racemosus*. J. Bacteriol. **126**:1266–1270.
15. **Nickerson, J. W., L. D. Dunkle, and J. L. Van Etten.** 1977. Absence of spermine in filamentous fungi. J. Bacteriol. **129**:173–176.
16. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.
17. **Paveto, C., A. Epstein, and S. Passeron.** 1975. Studies on cyclic adenosine 3',5'-monophosphate levels, adenylate cyclase and phosphodiesterase activities in the dimorphic fungus *Mucor rouxii*. Arch. Biochem. Biophys. **169**:449–457.
18. **Paznokas, J. L., and P. S. Sypherd.** 1975. Respiratory capacity, cyclic adenosine 3',5'-monophosphate, and morphogenesis of *Mucor racemosus*. J. Bacteriol. **124**: 134–139.
19. **Peters, J., and P. S. Sypherd.** 1978. Enrichment of mutants of *Mucor racemosus* by differential freeze-killing. J. Gen. Microbiol. **105**:77–81.
20. **Seiler, N.** 1971. Identification and quantitation of amines by thin-layer chromatography. J. Chromatogr. **63**:97–112.
21. **Sypherd, P. S., M. Orlowski, and J. Peters.** 1979. Models of fungal dimorphism: control of dimorphism in *Mucor racemosus*, p. 224–227. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
22. **Toennies, G., and J. J. Kolb.** 1951. Techniques and reagents for paper chromatography. Anal. Chem. **23**: 823–826.