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Function of S-Adenosylmethionine in Germinating Yeast Ascospores

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Germination and outgrowth of ascospores of *Saccharomyces cerevisiae* 4579 require both methionine and adenine, whereas leucine is only required for outgrowth. The methionine requirement may be satisfied by S-adenosylmethionine, but this sulfonium compound will not substitute for adenine. Between 30 and 70 min of protein synthesis is initially required for the completion of germination in strain 4579. The inhibition of S-adenosylmethionine synthetase by trifluoromethionine prevents both germination and protein synthesis. During the initial stages of germination, the S-adenosylmethionine synthetase, S-adenosylmethionine decarboxylase, and transfer ribonucleic acid methyltransferases increased significantly, indicating that polyamines and/ or the methylation of transfer ribonucleic acid are required for the initiation of germination.

Germination and outgrowth of yeast spores provide an excellent biological system for the study of biochemical events associated with the initiation of mitosis. Conditions for the germination of yeast ascospores have been thoroughly established (13, 14, 17, 18, 20), yet very little information exists concerning the biochemical and genetic processes necessary for the transition of a spore of *Saccharomyces cerevisiae* into a vegetative cell (14, 15, 25). As defined by Tingle et al. (25), germination is the transition period from a refractile to a nonrefractile spore, and outgrowth is the development of a budded cell from a phase-dark cell.

Numerous reports have linked methylated bases, polyamines, and their precursor, S-adenosylmethionine, to rapid growth processes (1, 4, 16, 24). Both the synthesis of polyamines and the methylation of ribonucleic acid (RNA) have been implicated as regulatory mechanisms for the control of cellular growth (1, 9, 11). In eucaryotes, polyamines and methylated bases have been shown to be closely correlated with the initiation of transcription and translation. In addition to its roles in these processes, S-adenosylmethionine also can serve as a source of methionine (21) and adenine (6) in *S. cerevisiae*.

Our present experiments deal with the nutritional requirements for S-adenosylmethionine for the germination and outgrowth of *S. cerevisiae* ascospores.

MATERIALS AND METHODS

Organisms, culture conditions, and measurement of uptake and incorporation. A diploid *S. cerevisiae* prototroph (ATCC 7752) and a heterothallic diploid, 4579, auxotrophic for adenine, methionine, uracil, and leucine, were used in this study. The preparation of a single spore population was as described by Tingle et al. (25). Spores were germinated in a succinic acid synthetic medium (SSM) as described by Sebastian et al. (19). Germination and outgrowth were monitored by spectrophotometry of the change in absorbance at 600 nm (13) and by phase-contrast microscopy. A normal ascospore suspension contained about 95% light-refractile spores. Budding was determined by direct microscopic observation.

The uptake of radioactive compounds was determined by the withdrawal of 0.5- to 1.0-ml samples from the germination medium. After filtration on membrane filters (0.45-μm pore size), the cells were washed with 10 ml of cold water (4°C) containing 1% Tween 80; the filters were then dried and the radioactivity was determined. The incorporation of [^{14}C]leucine (2 μCi/μmol) into material precipitated by hot trichloroacetic acid was used as a measure of protein synthesis (12). Samples of 0.5 ml were removed from the culture at specified intervals after the addition of the isotope and added to 1.0 ml of cold 10% trichloroacetic acid containing carrier substrate (100 μg/ml). The samples were heated at 100°C for 15 min, and the precipitates were collected on glass-fiber filters, washed four times with 5 ml of 5% cold trichloroacetic acid containing 50 μg of carrier substrate per ml, and then washed twice with 5 ml of 95% cold ethanol. The filters were dried in an oven at 65°C, and the radioactivity was counted in 5 ml of Triton X-toluene (1:2) in 0.5% PPO (2,5-diphenyloxazole) by using a Beckman LS 250 liquid scintillation spectrometer. The incorporation of [^{14}C]uracil (2 μCi/μmol) into material precipitated by cold trichloroacetic acid was used to follow nucleic acid synthesis. Samples were harvested and added to 1.0 ml of cold 10% trichloroacetic acid con-

taining 100 μg of carrier substrate per ml. The precipitates were collected and further processed as described for leucine incorporation (12). The incorporation of [^{14}C]uracil into deoxyribonucleic acid throughout germination and outgrowth was also determined (12). However, during the germination and outgrowth periods, virtually no [^{14}C]uracil was found in deoxyribonucleic acid that had been precipitated with NaOH. Therefore, the incorporation of [^{14}C]uracil into nucleic acids was assumed to indicate incorporation into RNA.

Enzyme extracts, assays, and compounds. At specified intervals throughout germination and outgrowth, cells were harvested by centrifugation at 10,000 × *g* for 10 min. The pellets (1 to 3 g, wet weight) were washed twice with saline (0.85% NaCl), suspended in saline (1:1, wet wt/vol), layered over glass beads (0.45 to 0.5 mm in diameter) in a precooled homogenizer bottle, and subjected to mechanical agitation at a frequency of 4,000 cycles per min with a Bronwill cell homogenizer, model MSK. After the cells were homogenized by three 10-s treatments at 5-s intervals with a continuous flow of liquid CO_2, the homogenate was recovered from the beads by suction. Cell debris was removed by centrifugation, twice for 10 min at 12,000 × *g*. The supernatant fluid was dialyzed for 16 h at 4°C against 1,000 volumes of distilled water.

S-adenosylmethionine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from *S*-adenosyl-L-[^{14}COOH]methionine as described by Pegg (10). Methionine adenosyltransferase activity was assayed by measuring the conversion of [$^{14}CH_3$]methionine into S-adenosyl-L-[$^{14}CH_3$]methionine as described by Holcomb and Shapiro (5). *S*-adenosylmethionine:homocysteine methyltransferase was assayed as described by Shapiro (21), except that a Dowex 50 Na^+ column instead of an Li^+ column was used to isolate the product, [$^{14}CH_3$]-methionine. 5'-Methylthioadenosine nucleosidase was assayed by the method of Ferro et al. (3), and transfer RNA (tRNA) methyltransferase activity was determined by the method of Sharma et al. (23). For each enzyme, 1 U of activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol of product in 1 min.

Protein was determined by the method of Lowry et al. (8). 5'-[$^{14}CH_3$]methylthioadenosine was prepared from *S*-adenosyl-L-[$^{14}CH_3$]methionine (3). Other compounds were obtained from commercial sources: *S*-adenosylmethionine and *S*-adenosylhomocysteine, Boehringer Mannheim; *S*-adenosyl-L-[$^{14}CH_3$]methionine and *S*-adenosyl-L-[^{14}COOH]-methionine, Amersham Searle; trifluoromethionine, Cyclo-Chemical Co.; and *Escherichia coli* B tRNA, General Biochemicals.

RESULTS

Some differences between *S. cerevisiae* strains 7752 and 4579 were noted in regard to germination and outgrowth of their ascospores. Spores of the prototrophic strain, 7752, germinated more rapidly than did spores of the auxotrophic strain, 4579 (Fig. 1). The maximum decrease in light absorbance occurred after about 1.5 h in strain 7752 and after 3.0 h in strain 4579. The change in light absorbance was concomitant with a phase microscopic darkening of the spores. Although strain 7752 germinated more rapidly than strain 4579, the latter demonstrated a more rapid rate of outgrowth; after 7 h both strains had reached approximately the same absorbance. During the outgrowth period of both strains, initial bud formation began shortly after the maximal decrease in absorbance was attained (Fig. 1).

Auxotrophic requirements. Since strain 4579 requires both adenine and methionine, which are precursors of *S*-adenosylmethionine, the auxotrophic requirements of this strain were studied during germination and outgrowth. In the absence of either adenine or methionine, neither germination nor outgrowth occurred. The omission of extracellularly supplied leucine, however, was not essential for germination, although the loss in absorbance was less than that observed in leucine-supplemented cultures. Leucine was required, however, for completion of outgrowth

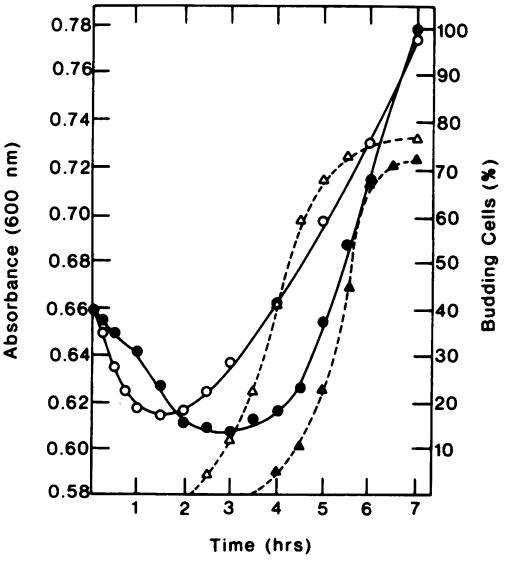

FIG. 1. *Kinetics of germination and outgrowth of spores of S. cerevisiae strains 7752 and 4579, and formation of buds during outgrowth. Spores of strain 7752 were germinated in SSM medium, and spores of strain 4579 were germinated in SSM medium supplemented with 20 μg each of adenine, uracil, leucine, methionine, histidine, and threonine per ml. Symbols: ○, absorbance of strain 7752; ●, absorbance of strain 4579; △, percentage of buds of strain 7752; ▲, percentage of buds of strain 4579.*

and bud formation, as expected from the auxotrophic requirement for this amino acid.

Since adenine and methionine were essential for germination and outgrowth of strain 4579, S-adenosylmethionine and related compounds were examined as substitutes. The methionine auxotrophic requirement was replaced by either S-adenosylmethionine or homocysteine, but not by S-adenosylhomocysteine, S-methylmethionine, cysteine, 5'-methylthioadenosine, or homoserine (Fig. 2). The purine requirement, however, was found to be specific for adenine; neither S-adenosylmethionine, S-adenosylhomocysteine, adenosine, nor 5'-methylthioadenosine supplementation could replace adenine. Separate experiments on the uptake of these compounds into spores of strain 4579 showed in each instance that their inactivity was not due to permeability factors. This is especially obvious from our observation that S-adenosylmethionine was the compound taken up the least, yet it supported germination and outgrowth in the absence of methionine while other related compounds were inactive.

Protein synthesis. Because of the methionine requirement, the role of protein synthesis during germination and outgrowth was examined (Fig. 3). When cycloheximide (100 μg/ml) was added to the germination medium at T_0 or T_{30}, neither germination nor protein synthesis occurred. Apparently at least 30 min of protein synthesis was required for germination of spores of strain 4579. The addition of the antibiotic at either 70 or 90 min, although inhibiting leucine incorporation, did not prevent germination. Outgrowth, however, did not occur when cycloheximide was added to the medium at each of the tested times. The data indicate

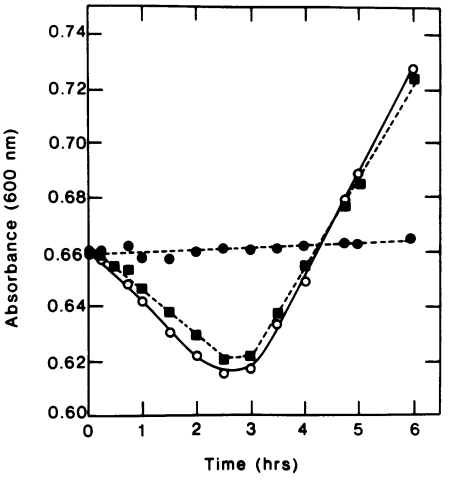

FIG. 2. *Kinetics of germination and outgrowth of spores of strain 4579 with the substitution of S-adenosylmethionine or S-adenosylmethionine-related compounds for the methionine requirement. Spores were germinated in SSM medium supplemented with 20 μg each of adenine, uracil, leucine, histidine, and threonine per ml. Each supplement was added to the medium at a final concentration of 0.2 mM. Symbols: ○, plus S-adenosylmethionine; ■, plus homocysteine; ●, no supplement or plus either S-adenosylhomocysteine, S-methylmethionine, cysteine, 5'-methylthioadenosine, or homoserine.*

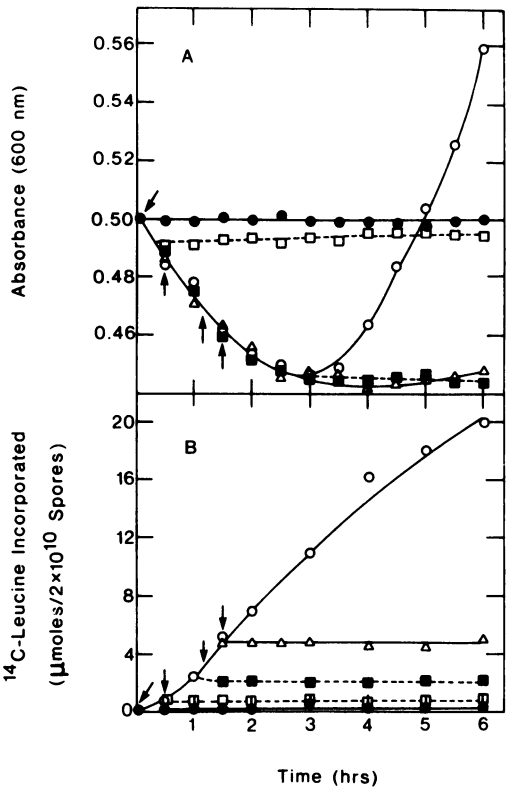

FIG. 3. *Effect of cycloheximide on (A) germination and outgrowth and on (B) protein synthesis in spores of strain 4579 during germination and outgrowth. Spores were germinated in the presence and absence of cycloheximide in SSM medium supplemented with (A) 20 μg each of adenine, uracil, leucine, methionine, histidine, and threonine per ml or (B) 20 μg each of adenine, uracil, methionine, histidine, threonine, and [^{14}C]leucine (0.2 μCi/ml) per ml. Cycloheximide (100 μg/ml) was added to the medium at 0, 30, 60, and 90 min. At the designated times, (A) absorbance readings were taken at 600 nm; (B) samples of 0.5 to 1 ml were withdrawn, and the trichloroacetic acid-precipitable counts were determined. Arrows indicate the time at which the inhibitor was added. Symbols: ○, no cycloheximide; ●, cycloheximide added at zero time; □, cycloheximide added at 30 min; ■, cycloheximide added at 70 min; △, cycloheximide added at 90 min.*

that for the completion of germination an initial period of protein synthesis of between 30 and 70 min is necessary. It should be mentioned that the prevention of yeast spore germination by cycloheximide was not observed in all strains of yeast tested by us.

S-Adenosylmethionine. Since S-adenosylmethionine could replace the methionine auxotrophic requirement, the effect of inhibitors of the enzyme that converts methionine and adenosine 5'-triphosphate into S-adenosylmethionine (methionine adenosyltransferase) was examined. Trifluoromethionine, an inhibitor but not a substrate of methionine adenosyltransferase, was added to the germination medium at 10^{-5} M (Fig. 4). Germination and outgrowth were not observed in trifluoromethionine-supplemented cultures (Fig. 4). de Robichon-Szulmajster and co-workers (2) observed that the inhibition by trifluoromethionine was, to a certain extent, overcome by the addition of methionine. Thus, the small decrease in absorbance is probably due to the methionine supplementation. The data suggest that, in the absence of S-adenosylmethionine biosynthesis, germination and outgrowth do not occur. It is evident from the data in Fig. 4 that the extracellular addition of trifluoromethionine also results in the inhibition of protein synthesis.

Enzyme activities during germination and outgrowth. To investigate the role that S-adenosylmethionine (7, 22) may play in the initiation of yeast spore germination, several enzymes involved in S-adenosylmethionine metabolism were investigated throughout germination and outgrowth. Table 1 shows the activities of S-adenosylmethionine:homocysteine methyltransferase, S-adenosylmethionine decarboxylase, 5'-methylthioadenosine nucleosidase, and tRNA methylase in dialyzed extracts at designated times during germination and outgrowth. Of the enzymes assayed, only the S-adenosylmethionine:homocysteine methyltransferase showed elevated activity in the ungerminated spores. The activity of this enzyme also remained at about the same level throughout the entire period. Three enzymes showed significant increases after the first hour of ger-

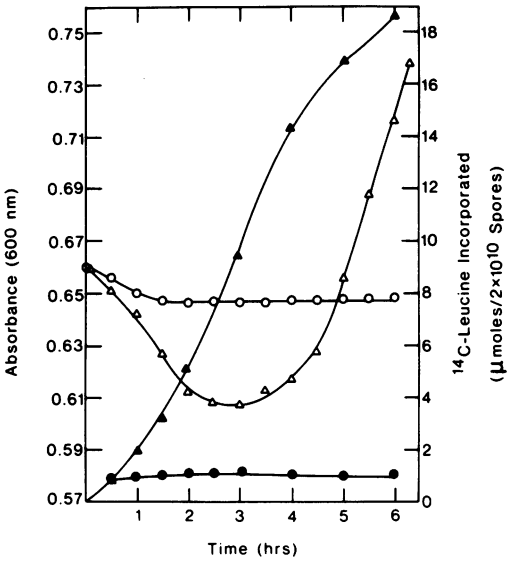

FIG. 4. *Effect of trifluoromethionine on germination, outgrowth, and protein synthesis of spores of strain 4579. Spores were germinated in the presence (○) and absence (△) of 10^{-5} M trifluoromethionine. The medium was supplemented as stated in the legend of Fig. 3. [^{14}C]leucine (0.2 μCi/ml) was supplemented to the medium in the presence (●) and absence (▲) of 10^{-5} M trifluoromethionine, and its incorporation into material precipitated by hot trichloroacetic acid was determined as described in the text.*

TABLE 1. *Enzyme activities during germination and outgrowth*[a]

Enzyme	Enzyme activity[b]					
	T_0[c]	T_1	T_2	T_3	T_4	T_5
S-adenosylmethionine: homocysteine methyltransferase	2.71	2.62	2.80	2.70	2.60	2.75
Methionine adenosyltransferase	35	158	154	200	614	758
S-adenosylmethionine decarboxylase	12	32	42	48	48	72
tRNA methyltransferase	0.6	5.0	6.2	5.7	5.8	5.6
5'-Methylthioadenosine nucleosidase	18	22	37	112	107	120

[a] Spores were germinated in SSM medium, and extracts were prepared as described in the text.

[b] Enzyme activity for S-adenosylmethionine:homocysteine methyltransferase is expressed as milliunits per milligram of protein. All other enzyme activities are expressed as microunits per milligram of protein.

[c] Time (hours) in germination medium.

mination: methionine adenosyltransferase, S-adenosylmethionine decarboxylase, and tRNA methyltransferase. The methionine adenosyltransferase showed an early 4.5-fold rise at the onset of germination, followed by a second dramatic increase after the first 3 h. By 5 h, the methionine adenosyltransferase level had increased 20-fold compared to the T_0 value. The S-adenosylmethionine decarboxylase activity increased steadily throughout the entire period of incubation; its level increased 2.5-fold during the first 2 h. The most pronounced increase appeared in the tRNA methyltransferase activity, which reached its maximal activity by the first 2 h, with about an eightfold increase over the T_0 value. The 5'-methylthioadenosine nucleosidase level demonstrated its greatest alteration between 2 and 3 h, during which time the activity increased threefold.

DISCUSSION

The kinetics of germination and outgrowth of *S. cerevisiae* ascospores appear to be strain specific, which is in agreement with similar observations of Savarese (17). The finding, however, that the supplementation of methionine and adenine is required for germination of a strain auxotrophic for these compounds has not been reported. Rousseau and Halvorson (15) observed that the free amino acid pools of yeast spores contain relatively large amounts of leucine, whereas the methionine pool is very low. This observation supports our finding that leucine supplementation is not required for germination.

Knudsen et al. (6) demonstrated that an adenine mutant of *S. cerevisiae* was able to utilize S-adenosylmethionine or S-adenosylhomocysteine as a source of adenine for vegetative growth. These compounds were degraded after entering the cell, and the adenine moiety was reutilized. In regard to germination, however, the present results demonstrate that neither S-adenosylmethionine nor S-adenosylhomocysteine allows an adenine auxotroph to germinate in the absence of adenine. Since both of these compounds enter the spores, it is apparent that neither of them is degraded to recycle the purine moiety. In agreement with this is our observation that the 5'-methylthioadenosine nucleosidase, which catalyzes the formation of adenine, was not present in significant quantities in the early phase of the germination process.

S-adenosylmethionine, however, was capable of substituting for methionine. In vegetative cells, S-adenosylmethionine may act as the precursor of methionine either by transmethylations producing S-adenosylhomocysteine, which is degraded to adenosine and homocysteine, the latter being methylated to form methionine, or by the direct methylation of homocysteine to form methionine and S-adenosylhomocysteine by the S-adenosylmethionine: homocysteine methyltransferase. Our data indicate that the latter mechanism is probably the source of methionine because extracellularly supplied S-adenosylhomocysteine does not support germination in the absence of methionine, whereas homocysteine does support germination. The observation that the S-adenosylmethionine:homocysteine methyltransferase is present in high levels in the ungerminated spore also supports this hypothesis.

An analysis of the sequence of macromolecule synthesis during germination and outgrowth revealed that the synthesis of proteins occurred first; it was followed rapidly by the synthesis of RNA and much later by deoxyribonucleic acid synthesis (15). Our data indicate that in strain 4579, 30 to 70 min of protein synthesis is essential for the completion of germination. Inhibition of germination by trifluoromethionine, an inhibitor of the methionine adenosyltransferase, indicates that the biosynthesis of S-adenosylmethionine may be necessary for germination. Of the enzymes examined during germination and outgrowth, the methionine adenosyltransferase, S-adenosylmethionine decarboxylase, and tRNA methyltransferase increased significantly during the initial stages of germination, which corroborates the importance of S-adenosylmethionine. A close relationship between tRNA methylation, polyamines, and the onset of rapid growth processes has been reported (1, 4, 16, 24). Our data suggest that these biosyntheses are essential for the initiation of germination of yeast spores.

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