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Protein Synthesis During Fungal Spore Germination

II. Aminoacyl-soluble Ribonucleic Acid Synthetase Activities During Germination of *Botryodiplodia theobromae* Spores[1]

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The specific activities of 13 aminoacyl-soluble ribonucleic acid (sRNA) synthetases were measured at various time intervals during the germination of *Botryodiplodia theobromae* conidiospores. The enzyme activities were low or absent in ungerminated spores, and they increased rapidly as germination proceeded. When extracts of the ungerminated spores were prepared with mortar and pestle, very little or no enzyme activity was detected. When the extracts were prepared with a mechanical homogenizer, however, they exhibited some enzyme activity, although less than did the extracts from germinated spores. Enzyme activities from germinated spores were approximately the same, regardless of the method of preparation. The enzyme fraction from ungerminated spores prepared with a mechanical homogenizer could also stimulate incorporation of phenylalanine into polyphenylalanine in the presence of ribosomes, polyuridylic acid, and sRNA, although the activity was approximately only 15 to 20% that of a similar enzyme fraction from germinated spores. It is concluded that ungerminated spores of *B. theobromae* contain active aminoacyl-sRNA synthetases and transfer enzymes, although the activities are low when compared to germinated spores.

Among the most striking and significant features of spore germination of most fungi are the initiation of the synthesis of protein and its rapid increase (3, 14, 18, 20). Therefore, an investigation that compares the protein-synthesizing components of germinated and ungerminated spores may provide some information about the cellular control mechanisms that are involved in spore germination. A previous paper in this series (19) described the characteristics of an in vitro polyuridylic acid (poly U)-directed phenylalanine-incorporating system prepared from germinated conidiospores of the mycelial fungus *Botryodiplodia theobromae* Pat. The activities of the enzyme and ribosome fractions from germinated and ungerminated spores were compared, and it was concluded that the ribosomes from the ungerminated spores have about 25% the activity of those prepared from germinated spores. However, the enzyme fraction from the ungerminated spores was essentially inactive.

The inability of the enzyme fraction from the ungerminated spores of *B. theobromae* to stimulate incorporation of phenylalanine into polyphenylalanine could be due to an enzyme deficiency or to low specific activity at one of several steps in the protein synthesis process. It is well established that the first step in the synthesis of protein involves an activation of an individual amino acid by its corresponding aminoacyl-soluble ribonucleic acid (sRNA) synthetase enzyme and the subsequent transfer of the activated amino acid to its specific sRNA (10).

This paper reports an investigation of the possibility that the phenylalanyl-sRNA synthetase reaction is limiting in the enzyme fraction from ungerminated spores. In addition, the effect of several reaction parameters on phenylalanyl-sRNA and leucyl-sRNA synthetases, and the activities of 11 additional aminoacyl-sRNA synthetase enzymes are reported for extracts that were prepared at several stages of spore germination.

MATERIALS AND METHODS

Materials. Yeast sRNA (stripped) was obtained from General Biochemicals Corp., Chagrin Falls, Ohio. Uniformly labeled ^{14}C-L-phenylalanine (366 μc/μmole), ^{14}C-L-proline (204 μc/μmole), ^{14}C-L-isoleucine (234 μc/μmole), ^{14}C-L-lysine (237 μc/μmole), ^{14}C-L-leucine (251 μc/μmole), ^{14}C-L-valine (190 μc/μmole), ^{14}C-L-arginine (234 μc/μmole), ^{14}C-L-

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methionine (187 μc/μmole), ^{14}C-L-serine (122 μc/μmole), ^{14}C-L-alanine (215 μc/μmole), ^{14}C-L-glutamic acid (195 μc/μmole), ^{14}C-L-aspartic acid (167 μc/μmole), and ^{14}C-glycine (116 μc/μmole) were obtained from New England Nuclear Corp., Boston, Mass. The suppliers of other materials that were used in these studies, and the techniques for growth, harvest, and germination of the *B. theobromae* spores were identical to those described previously (19).

Preparation of extracts. Cell-free extracts were obtained by grinding the spores with mortar and pestle, and the soluble enzyme fractions and ribosomes were separated from the extracts according to techniques already described (19). However, in some instances the enzyme fractions were prepared with a Braun model MSK mechanical homogenizer (Bronwill Scientific Co., Rochester, N.Y.). When this instrument was used, 3 to 10 g of spores (quantity depended upon germination stage) and 15 to 30 g of 1-mm glass beads were combined with 15 ml of extraction buffer, following the recommendations of Rick et al. (11). This mixture was shaken for 60 sec at 4,000 oscillations per min while being cooled by carbon dioxide. The enzyme fractions were always treated with protamine sulfate (0.17 mg/ml of enzyme solution) for 30 min to precipitate endogenous sRNA. After this treatment, the enzyme solution was centrifuged and passed through a column of Sephadex G 25. The protein fraction was either assayed immediately or stored in samples under liquid nitrogen.

Assay for aminoacyl sRNA formation. The standard assay contained (in a final volume of 0.75 ml) 100 μmoles of tris(hydroxymethyl)aminomethane (Tris; *p*H 7.8, 10 μmoles of magnesium chloride, 2.5 μmoles of potassium fluoride, 10 μmoles of adenosine triphosphate (ATP), 0.5 mg of yeast sRNA, 0.3 μc of ^{14}C-amino acid, and 30 to 200 μg of enzyme protein. The concentration of added enzyme was such that a doubling of the enzyme concentration produced a doubling of the aminoacylation reaction. The reaction mixtures were incubated in conical glass centrifuge tubes at 20 C for 10 min, and the reactions were terminated by the addition of trichloroacetic acid to a final concentration of 12%, 20 μmoles of the appropriate unlabeled amino acid, and 0.5 mg of bovine albumin. The tubes were placed in ice for 60 min and then centrifuged; the precipitates were transferred onto cellulose nitrate filters (HA Millipore filter, 25 mm in diameter, 0.45 μm pore size), and washed three times with 10 ml of 7% trichloroacetic acid. The filters were dried and counted as previously described (19). The results are expressed as pmoles of aminoacyl sRNA formed per mg of protein per 10 min. The values given are corrected for zero-time reactions. All experiments were conducted at least twice, and usually three times.

Other determinations. When phenylalanine incorporation into polyphenylalanine was determined, a ribosomal assay system similar to that previously described was used (19). Protein was determined according to the method of Lowry et al. (9), with bovine albumin as a standard.

RESULTS

Requirement for phenylalanyl-sRNA and leucyl-sRNA formation. The effects of several parameters on phenylalanyl-sRNA and leucyl-sRNA formation were determined initially by use of an enzyme fraction that was prepared from spores germinated for 11 hr. The data in Table 1 indicate that a *p*H range of 7.8 to 8.4 was optimal for the acylation of phenylalanyl-sRNA, and that a *p*H range of 7.2 to 7.8 was optimal for the acylation of leucyl-sRNA. The data in Fig. 1 demonstrate the time course of the reactions for incubation temperatures of 20 and 36 C. The acylation of phenylalanyl-sRNA was linear for a longer period of time at the lower incubation temperature. Very little acylation of leucyl-sRNA took place at a temperature of 36 C. At a temperature of 20 C, the acylation of leucyl-sRNA was linear for at least 10 min. Under the present assay conditions, 8 and 5 μmoles of magnesium chloride were optimal for the acylation of phenylalanyl-sRNA and leucyl-sRNA, respectively (Fig. 2). In data not shown, the optimal requirements for potassium fluoride or potassium chloride were not as stringent for phenylalanyl-sRNA and leucyl-sRNA acylation, since good activity was obtained at potassium concentrations between 0 and 10 μmoles per assay, with a slight decrease in activity at higher potassium concentrations. The system did contain approximately 3 μmoles of potassium even at zero potassium concentration, because this ion was present in the standard buffers that were used in the preparation of the enzyme.

The effect of ATP concentration on phenylalanyl-sRNA and leucyl-sRNA acylation is reported in Table 2. As expected, the reactions were completely dependent on added ATP, with a maximum of 7 to 10 μmoles of ATP per assay for both amino acids. The reactions were mark-

TABLE 1. *Effect of pH on the acylation of sRNA by ^{14}C phenylalanine and ^{14}C leucine*

*p*H[a]	Aminoacyl-sRNA (pmoles) formed per mg of protein per 10 min	
	Phenylalanine	Leucine
6.0	153	18.4
6.6	286	74.4
7.2	437	249
7.8	587	239
8.4	627	70
9.0	455	24

[a] Tris-maleate buffer used for *p*H 6.0, 6.6, and 7.2. Tris-HCl buffer used for *p*H 7.8, 8.4, and 9.0.

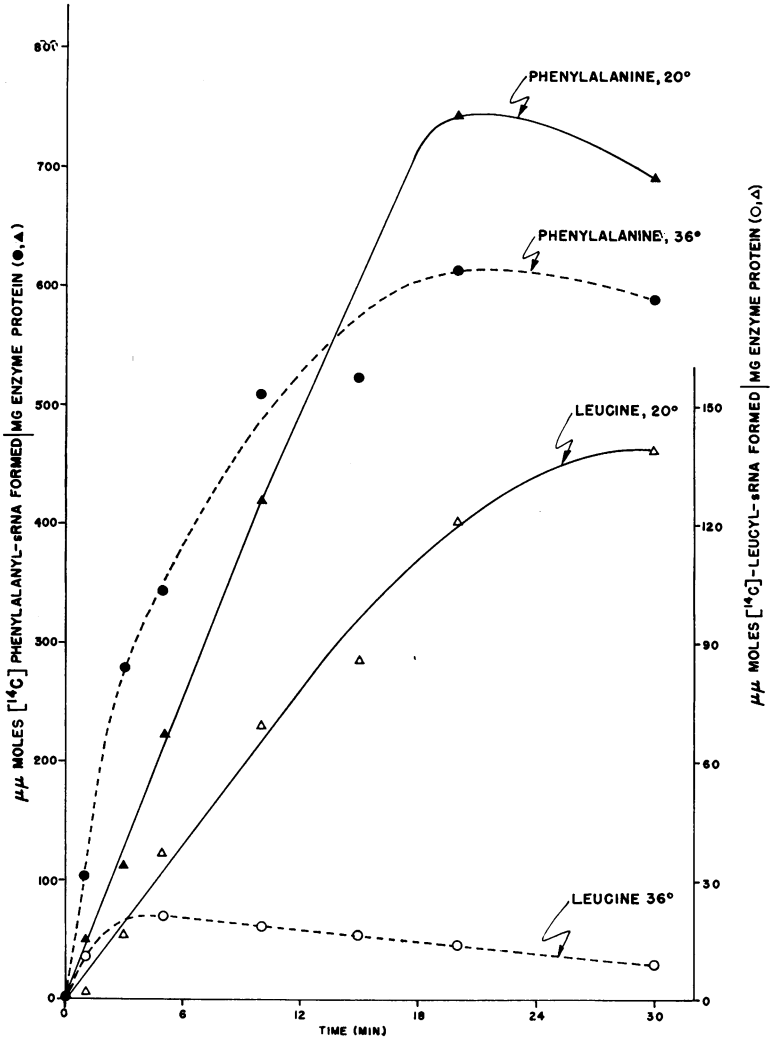

FIG. 1. *Effect of time on the formation of phenylalanyl-sRNA and leucyl-sRNA at 20 and 36 C.*

edly inhibited at ATP concentrations greater than 10 μmoles per assay. The addition of several concentrations of mercaptoethanol to the assay mixture had essentially no effect on the acylation reactions; however, the enzyme extracts were prepared in buffers that contained mercaptoethanol. Puromycin and cycloheximide, two antibiotics reported to inhibit the polymerization of amino acids into protein (but not the initial acylation reaction), had no effect on this reaction. In addition, if the reaction mixtures were boiled after the addition of trichloroacetic acid, all of the precipitable radioactivity was eliminated. Therefore, the radioactivity recovered in the assays was not due to polypeptide synthesis.

Enzyme activity during germination. The principal purpose of this investigation was to attempt to explain the previous inability to obtain an active enzyme fraction from ungerminated spores that could synthesize polyphenylalanine from phenylalanine in an in vitro ribosomal system. Therefore, the activities of phenylalanyl-sRNA synthetase and of 10 other aminoacyl-sRNA synthetases were determined from extracts that were prepared at various stages of germination. Under the growth conditions that were used, the spores began to form germ tubes after 4 hr, and germination reached a maximum of about 90% after 11 hr. The dry weight of the spores remained constant for the first 6 to 8 hr of

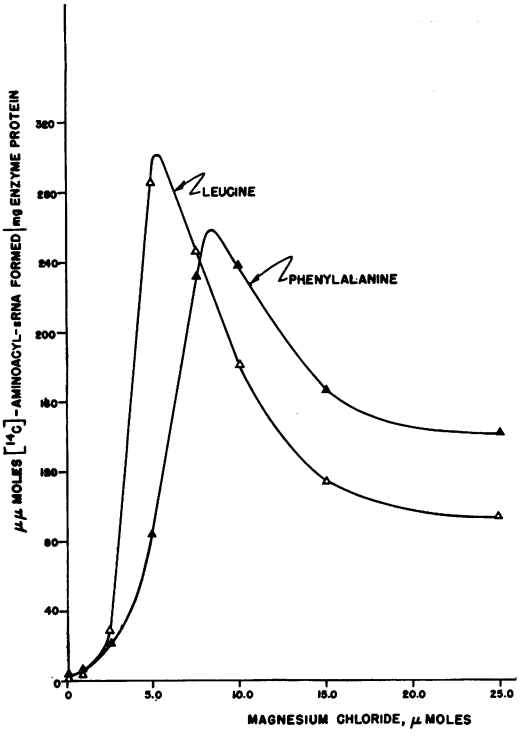

FIG. 2. *Effect of magnesium chloride on the formation of phenylalanyl-sRNA and leucyl-sRNA.*

incubation, after which it began to increase until there was approximately a 1.4- to 2-fold increase in dry weight at 11 hr (19).

The data reported in Table 3 are from assays of extracts, at various stages of germination, that were prepared by grinding the spores with mortar and pestle. It is evident that, except for arginyl-sRNA synthetase, the specific activities of all the enzymes were essentially undetectable in extracts from ungerminated spores. The specific activities of most of the aminoacyl-sRNA synthetases increased dramatically between 2 and 8 hr after the spores were added to the germination medium. Very poor or no aminoacyl-sRNA synthetase activity was obtained at any stage of germination for glycine, glutamic acid, and aspartic acid.

Since it was possible that the enzyme fraction from the ungerminated spores contained an inhibitor of the acylation reaction, mixed enzyme studies were tried with the two amino acids, phenylalanine and leucine. However, concentrations of up to 500 μg of enzyme from the ungerminated spore had essentially no effect on the activity of 115 μg of the enzyme fraction from the germinated spores. Therefore, it was concluded that the lack of aminoacyl-sRNA activity in the enzyme fraction from the ungerminated spores was not due to a general inhibitor, such as ribonuclease.

At that point of the study, we began using a Braun model MSK mechanical homogenizer to disrupt the spores for our cell-free extract preparations. The data reported in Table 4 result from assays of extracts at various stages of

TABLE 2. *Effect of ATP on the acylation of sRNA by ^{14}C-phenylalanine and ^{14}C-leucine*

ATP (μmoles) per assay	Aminoacyl-sRNA (pmoles) formed per mg of protein per 10 min	
	Phenylalanine	Leucine
0	21	1.4
0.5	244	28
1.0	289	40
4.0	495	149
7.0	642	206
10.0	531	234
12.5	118	149
15.0	30	90
20.0	11	

TABLE 3. *Activities of aminoacyl-sRNA synthetases at various periods of spore germination*[a]

Time of harvest (hr)	Percentage of spores germinated	Aminoacyl-sRNA (pmoles) formed per mg of protein per 10 min												
		Phenylalanine	Leucine	Valine	Arginine	Proline	Lysine	Methionine	Isoleucine	Serine	Alanine	Glutamate	Aspartate	Glycine
0	0	3.3	0	0	123	0	0	0.1	0	0	0	0	0	0
2	0	6.2	7.2	0	162	0	0	2.2	0	0	2.1	0	0	0
5	21	389	143	7.3	293	0	106	144	14	2.7	19	0.8	0	0
8	62	467	170	79	484	3.5	225	321	16	3.6	28	1.1	0	0
11	90	446	239	40	461	4.1	404	368	31	7.9	27	1.3	0	0

[a] Cell-free preparations were made by grinding with mortar and pestle.

TABLE 4. *Activities of aminoacyl-sRNA synthetases at various time periods of spore germination*[a]

Time of harvest (hr)	Percentage of spores germinated	Aminoacyl-SRNA (pmoles) formed per mg of protein per 10 min												
		Phenylalanine	Leucine	Valine	Arginine	Proline	Lysine	Methionine	Isoleucine	Serine	Alanine	Glutamate	Aspartate	Glycine
0	0	114	55	3.4	104	0.1	21	15.4	0.7	1.6	0.5	0	0	0
2	0	180	85		120	1.2	34	32.1	5.2		7.1	0	0	0
5	21	406	219	32.4	320	4.8	310	95		7.6	28.5	0	0.2	0
8	62	589	187	66.8	570	5.5	494	120	5.0	6.8	35.6	0	1.1	0
11	90	480	286	76.0	447	4.7	464	242	8.7	8.9	41.9	0	1.8	0

[a] Cell-free preparations were made by homogenizing in a Braun MSK mechanical homogenizer.

germination that were prepared by homogenizing the spores in a Braun homogenizer. It is obvious that higher activities were obtained for most of the amino acids from ungerminated spores when this instrument was used as a means of making extracts than when they were prepared with mortar and pestle. Again, however, the specific activities of all of the enzymes increased during spore germination. At present, there is no plausible explanation for enzyme activity having been obtained from ungerminated spores when the extracts were prepared with a Braun homogenizer, while essentially no activity occurred when the extracts were prepared by grinding with mortar and pestle. It is all the more interesting because enzyme extracts prepared from spores germinated for 11 hr had roughly the same specific activity, regardless of how they were prepared. One possibility is that the aminoacyl-sRNA synthetases are more tightly bound to a particulate fraction in the ungerminated spores than they are in the germinated spores, and that the more vigorous breakage achieved by the mechanical homogenizer releases the enzymes. However, experiments to test this possibility were inconclusive.

Since activity for phenylalanyl-sRNA synthetase was obtained in extracts from the ungerminated spores when prepared with the Braun homogenizer, this enzyme fraction was tested for its ability to stimulate incorporation of ^{14}C-phenylalanine into polyphenylalanine in vitro by use of a ribosomal system, as described previously for *B. theobromae* (19). The enzyme fraction from the ungerminated spores that was prepared by homogenization in the Braun MSK was much more active than the spore extracts that were prepared by grinding with mortar and pestle (Table 5). However, the ability of an enzyme fraction from ungerminated spores to stimulate incorporation of phenylalanine into polyphenylalanine was still less than that of an enzyme fraction from germinated spores (Table 5).

TABLE 5. *Ability of supernatant fluid (105,000 × g) from germinated and ungerminated spores to stimulate incorporation of ^{14}C-phenylalanine*

Ribosomes[a]	Supernatant fluid (105,000 × g)	Incorporation of ^{14}C-phenylalanine (pmoles) per assay
Germinated		3.0
None	Germinated (MP)[b]	3.3
Germinated	Germinated (MP)	398.0
None	Germinated (MSK)[c]	2.9
Germinated	Germinated (MSK)	524.0
None	Ungerminated (MP)	2.8
Germinated	Ungerminated (MP)	4.2
None	Ungerminated (MSK)	1.4
Germinated	Ungerminated (MSK)	86.0

[a] When added, ribosomes were equivalent to 0.33 mg of RNA per assay; the supernatant fluid enzymes from the germinated and ungerminated spores were equivalent to 0.150 mg of protein per assay.

[b] Supernatant fluid (105,000 × g) prepared by grinding with mortar and pestle.

[c] Supernatant fluid (105,000 × g) prepared by homogenizing in the Braun mechanical homogenizer.

DISCUSSION

In this study, only one set of conditions was used for assaying all of the aminoacyl-sRNA synthetases, but from both our data and that reported by Rubin et al. (13), it is becoming clear that different synthetase enzymes have different requirements for optimal activity. The amount of aminoacyl-sRNA formed is dependent upon enzyme activity and the rate of hydrolysis of the end product, which varies with different amino-

acyl-sRNA preparations (21). The relatively short assay time of 10 min and the incubation temperature of 20 C that were used in these experiments were chosen to minimize the effect of this degradation. It is possible that a specific aminoacyl-sRNA synthetase has different requirements for activity when it is prepared at different stages of spore germination. However, experiments in our laboratory indicate that the optimal magnesium and ATP concentrations for phenylalanyl-sRNA formation with the extract from ungerminated spores were essentially identical to those with an enzyme fraction from germinated spores. Another possibility is that the enzyme fraction from the ungerminated spores is charging a different sRNA molecule(s) than the one being charged by the enzyme fraction from germinated spores, in a manner similar to that reported with *Neurospora crassa* enzyme and *Escherichia coli* sRNA (2). An enzyme preparation from *N. crassa* could charge at least three chromatographically distinct sRNA molecules from *E. coli* with phenylalanine, only one of which was identical to the phenylalanyl-sRNA recognized by the *E. coli* enzyme. With the assay procedure that was used in this investigation, we could not detect this possibility.

Barnett and Brown (1) have demonstrated recently that mitochondria from *N. crassa* contain specific synthetases and sRNA molecules for various amino acids, and that these enzymes and sRNA molecules are different from the ones that are present in the cytoplasm. Although the differential centrifugation procedures used in the present experiments would remove all of the whole mitochondria, it is possible that some mitochondrial breakage did occur.

However, fairly good activity was obtained for the synthetases of all of the amino acids tested except for glutamic acid, aspartic acid, and glycine; the reason for this is not known. Several experiments, such as the use of unfrozen enzyme, unfrozen sRNA, endogenous sRNA, slightly altered assay conditions, etc., were performed specifically to obtain activity with these amino acids. It is most likely that these enzymes or specific sRNA molecules are extremely labile, or altered in some way. Since yeast sRNA was used in these experiments, another possibility is that these particular aminoacyl-sRNA synthetases from *B. theobromae* are unable to recognize the appropriate yeast sRNA molecules.

One can conclude from the present study that the specific activities of the aminoacyl-sRNA synthetases increased during germination. This trend was obtained regardless of how the cell-free extracts were prepared. However, an active enzyme fraction from ungerminated spores was obtained only when the spores were homogenized in the Braun homogenizer. This enzyme fraction from ungerminated spores could also stimulate incorporation of phenylalanine into polyphenylalanine when used with poly U and an active ribosomal system, although the activity was less than that obtained with a similar enzyme fraction from germinated spores (Table 5).

With the demonstration in ungerminated spores of both soluble enzymes and ribosomes (19), which are active in stimulating incorporation of phenylalanine into polyphenylalanine, one has to conclude that the ungerminated spores contain at least some of the apparatus for synthesizing protein if sRNA and a messenger RNA (mRNA), such as poly U, are provided. Therefore, the mechanism that allows germinating spores to initiate the synthesis of protein may be the formation of mRNA. Data comparing the ribosomal profile of germinated and ungerminated spores of *N. crassa* and *Aspergillus oryzae* suggest this possibility since only monoribosomes could be isolated from the ungerminated spores, while polyribosomes appeared during spore germination (6, 7, 8). More recently, however, Staples et al. (17) have demonstrated polyribosomes in ungerminated uredospores of *Uromyces phaseoli*, an obligate parasite whose spores can produce germ tubes in culture but are unable to continue growth. The sedimentation coefficients of the monoribosomes and the nucleotide composition of ribosomal RNA were essentially the same in ascospores, conidiospores and hyphae of *N. crassa* (4, 5). Furthermore, ribosomes isolated from ungerminated spores and hyphae of *N. crassa* were similar serologically, and the ribosomal proteins isolated from the two fungal stages behaved similarly on acrylamide gel electrophoresis (12). However, the biological activities of the ribosomes obtained from the various stages of spore germination were not reported.

It is interesting to compare our data with that of Staples et al. (15, 16), who are investigating in vitro protein synthesis during uredospore germination of *U. phaseoli*. No differences in the biological activities of the ribosomes and protein-synthesizing enzymes prepared from ungerminated and germinated spores were observed when poly U was employed as mRNA. This is in contrast to the results from our laboratory with the facultative parasitic fungus *B. theobromae*, where about a three- to four fold increase in ribosomal activity (19) and a five- to sixfold

increase in enzyme activity occurred with germination. The activity of the in vitro phenylalanine incorporating system from ungerminated spores of *B. theobromae* is approximately the same as that of the *U. phaseoli* system. This is interesting because when spores of *U. phaseoli* are germinated in culture, they do not exhibit a net synthesis of protein, although some protein turnover does occur (18), whereas those of *B. theobromae* show a two- to fourfold increase in protein during the time period studied (Van Etten, *unpublished data*).

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