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Germination of Myxospores from the Fruiting Bodies of *Myxococcus xanthus*

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Germination of myxospores from fruiting bodies of *Myxococcus xanthus* was examined under a light microscope as well as by analyzing the incorporation of [^{3}H]uracil into the RNA fraction. Efficient germination was observed in 0.2% Casitone containing 8 mM $MgSO_4$ and 1 mM $CaCl_2$ at 30 C. Under this condition, spherical myxospores were converted into rod-shaped vegetative cells within 5 to 6 h. The germination was severely inhibited in the presence of 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor, indicating that a serine protease(s) is required for the myxospore germination. EGTA (1 mM) also completely blocked germination, indicating that Ca^{2+} plays an important role in myxospore germination. In 1% Casitone without added Mg^{2+} and Ca^{2+} or 0.2% Casamino Acids with 8 mM $MgSO_4$ and 1 mM $CaCl_2$, myxospores lost their refractility under a phase microscope, while no RNA synthesis took place within 6 h, as judged by the incorporation of [^{3}H]uracil. A group of proteins were found to be specifically synthesized during an early stage of germination. In addition, a new major spore-associated protein with a size of 41.5 kDa became detectable in the spore shell fraction 3 h after germination. The present results demonstrate that myxospore germination occurs in at least two steps: the loss of myxospore refractility, followed by an outburst of metabolic activities. The first step can occur even in the absence of energy metabolism, while the second step was blocked by rifampin, EGTA, and protease inhibitors.

Myxococcus xanthus, a gram-negative soil bacterium, displays spectacular morphogenesis, forming multicellular fruiting bodies upon depletion of nutrients (see references 2 and 18 for review). Within the fruiting body, rod-shaped vegetative cells are converted into spherical myxospores, which become desiccation and heat resistant (19). During fruiting body formation, a number of specific proteins have been shown to be induced (6, 7, 13). More recently, a number of eukaryotic-like protein serine/threonine kinases have been found in *M. xanthus* (20), one of which was shown to be induced upon sporulation (12).

When myxospores are reexposed to a nutrient-rich condition, they germinate to convert to vegetative cells. During the germination process, the sequential disappearance of myxospore-specific characteristics has been demonstrated in the following order: heat resistance, resistance to sodium dodecyl sulfate (SDS), spore refractility, and spherical shape (3). Factors required for germination have also been investigated for both myxospores from fruiting bodies (3) and glycerol-induced spores (14). The latter spores are known to be formed even in a rich liquid medium in the presence of glycerol and have been shown to be substantially different from myxospores from fruiting bodies in terms of structures observed under an electron microscope, metabolic activities, and heat and SDS resistance (see reference 18 for review).

In the present work, we investigated the germination of myxospores from fruiting bodies by monitoring RNA synthesis. Under the best germination condition used, active RNA synthesis began 2 h after the initiation of germination. We found that Ca^{2+} as well as a serine protease or proteases is required for myxospore germination. A group of proteins were found to be specifically synthesized during the early stage of germination. Interestingly, protein S, a well-characterized major spore coat protein (7), disappeared by being degraded during the early stage of germination, while a new major protein with a size of 41.5 kDa became detectable in the spore shell fraction 3 h after germination. The results indicate that a series of events occur during myxospore germination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* FB(DZF1) was used for the preparation of myxospores. Cells were grown at 30°C with vigorous aeration in Casitone-yeast extract medium (5). For fruiting body formation, clone fruiting (CF) agar plates were used as described previously (6).

Preparation of myxospores. An exponentially growing culture (100 to 150 Klett units) was centrifuged at 4000 × *g* for 10 min at 4°C. The cell pellet thus obtained was resuspended in 1/25 volume of 10 mM Tris-HCl (pH 7.6) containing 8 mM $MgSO_4$. The concentrated cell suspension was spotted on CF agar plates (5 μl per spot, 144 spots per plate), and the plates were incubated for 7 days at 30°C. Fruiting bodies were harvested by gentle scraping of the surface of agar plates and were suspended in cold distilled water. The suspension of fruiting bodies was then sonicated four times for 15 s each to disrupt vegetative cells. The myxospores were then washed five to six times with cold distilled water to remove cell fragments. The myxospore preparation thus obtained was checked for contamination of vegetative cells under a phase microscope. After confirmation that the content of vegetative cells was less than 0.1%, the myxospores were again precipitated by centrifugation at 12,000 × *g* for 10 min. The pellets were well drained and stored at −70°C until used.

Germination conditions. In all cases, myxospores were incubated at 30°C with continuous shaking at a density of (1 to 5) × 10^8 spores per ml.

RNA synthesis. RNA synthesis was examined as previously described (9, 17). [5,6-^{3}H]uracil (ICN Biomedical, Inc.) was used at 0.1 to 0.5 μCi/ml. Samples (0.15 ml each) were taken at various intervals, and incorporation was stopped by the addition of 2 volumes of 10% cold trichloroacetic acid. After 60 min of incubation at 4°C, the samples were collected on glass fiber filters, washed three times with 2 ml of 5% trichloroacetic acid containing 1 mM uracil, and washed three times with ethanol. Radioactivity on the filters was measured with a liquid scintillation counter.

Protein synthesis during early germination. Myxospores were suspended at a density of 5 × 10^8/ml in 1% Casitone with 8 mM $MgSO_4$ (1C-Mg) and 0.5 mM $CaCl_2$ and incubated at 30°C. At 1-h intervals after the initiation of germination, 1 ml of the myxospore suspension was labeled with 100 μCi of TRANS ^{35}S LABEL (1.090 Ci/mmol; ICN Biomedical, Inc.) for 40 min. After labeling,

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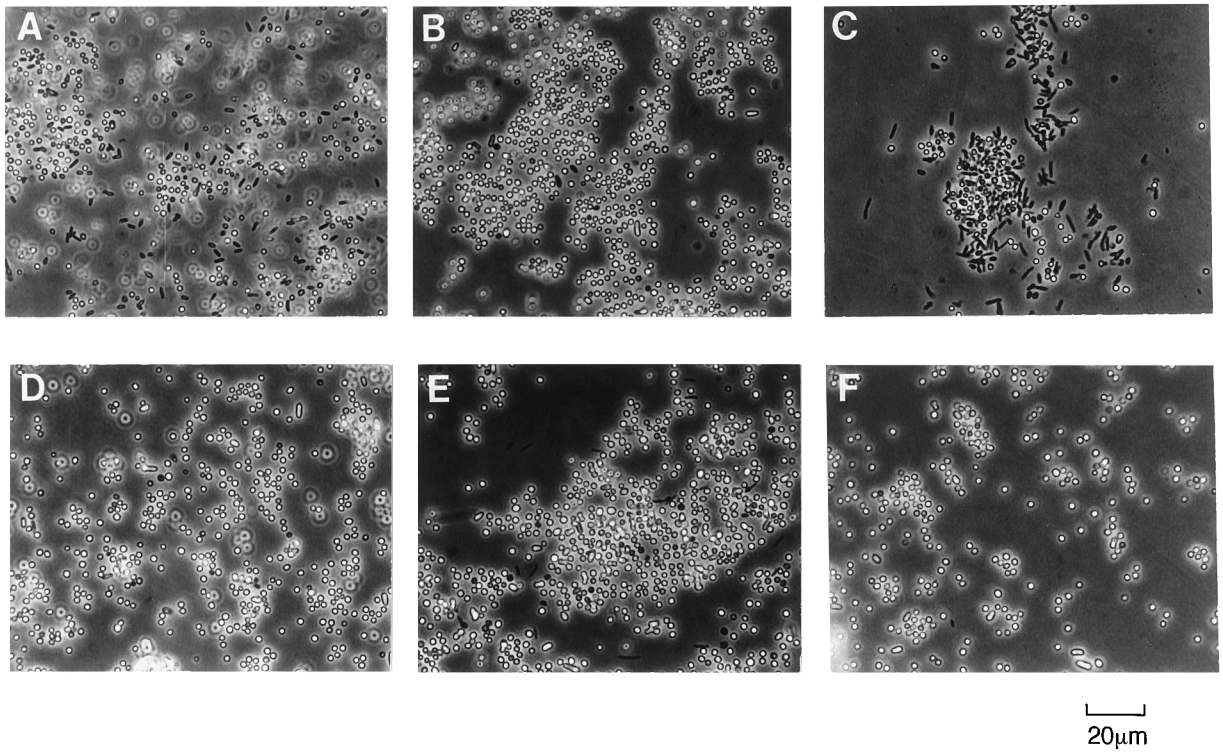

FIG. 1. Germination of myxospores under various conditions. Photographs were taken under a phase light microscope. Myxospores, prepared as described in Materials and Methods, were germinated under the following conditions. (A) 1C-Mg. (B) 1/5C-Mg. (C) 1/5C-MgCa. (D and E) 1/5CA-MgCa. (F) Distilled water. Photographs were taken after 5 h of incubation for A, B, C, and D; 24 h for E; and 10 h for F at 30°C, respectively.

myxospores or cells collected by centrifugation were washed, suspended in 50 μl of TM buffer (described below), and disrupted by sonication with 25 μl of glass beads (<100 μm) 20 times for 30 s each at 30-s intervals. Extracted materials were mixed with 2× loading buffer, boiled for 2 min, and analyzed by SDS-polyacrylamide (17.5%) gel electrophoresis (PAGE).

Isolation of myxospore shells. Myxospores were incubated in 1C-Mg at 30°C. At 1-h intervals, myxospores and cells were collected from 1 ml of suspension by microcentrifugation for 5 min, washed once with 10 mM sodium phosphate buffer (pH 7.6), and resuspended in 50 μl of 10 mM Tris-HCl (pH 7.6) containing 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA (TM buffer). A mixture of germinating spores and cells was disrupted by sonication four times for 30 s each. Unbroken spores and spore shells were isolated by microcentrifugation at 5,000 × *g* for 10 min and washed twice with 1.5 ml of cold TM buffer. The pellet was suspended in 20 μl of loading buffer (2% SDS, 80 mM Tris-HCl [pH 6.8], 10% glycerol), boiled for 2 min, and analyzed by SDS-PAGE.

RESULTS

Changes in morphology of myxospores during germination. There are at least two steps in morphological events occurring during myxospore germination. First, the refractility of myxospores observed under a phase microscope is lost, which is always accompanied by the induction of germination (3). Under proper conditions, metabolic activities are then resumed in the myxospores which have lost refractility. Eventually the spherical spore shells are broken, from which rod-shaped vegetative cells emerge. In this paper, we reexamined conditions for myxospore germination, which are required not only for the induction of germination, as judged by the loss of refractility, but also for the outgrowth of vegetative cells, as judged by the initiation of RNA synthesis together with morphological observation under a microscope.

Figure 1 shows morphological changes in myxospores prepared from 7-day-old fruiting bodies under various conditions. The numbers of different cell types in each picture were counted after the images were enhanced with an Image Master DTS (Pharmacia LKB) and are summarized in Table 1. When myxospores were incubated in 1% Casitone at 30°C for 5 h, the change in the turbidity of the spore suspension as measured at 560 μm (14) was little and turbidity was almost identical to that of the spore suspension in distilled water incubated at 30°C for 5 h. However, when myxospores were incubated in 1C-Mg (defined as CT medium in reference 10), the turbidity dropped by approximately 70% during the incubation. Under this condition, more than 70% of the myxospores lost refractility and more than 30% were elongated into rod-shaped structures (Fig. 1A).

Next we examined the effect of Ca^{2+}, which was shown to stimulate myxospore germination (3). Since the addition of 1 mM $CaCl_2$ to 1% Casitone resulted in the formation of precipitates which were considered to be calcium-phosphate, 0.2% Casitone was used for experiments. When myxospores were

TABLE 1. Induction of myxospore germination in different media

Panel in Fig. 1	Germination medium (h postincubation)[a]	% contents of cell type		
		Refractile	Nonrefractile (stage I)	Rod shaped (stage II)
A	1C-Mg (5)	28	38	34
B	1/5C-Mg (5)	37	59	4
C	1/5C-MgCa (5)	19	12	68
D	1/5CA-MgCa (5)	45	52	3
E	1/5CA-MgCa (24)	31	63	6
F	Water (10)	84	14	1

[a] Photographs taken at the time indicated in parentheses were used to count numbers of different cell types after enhancement of the images with Image Master DTS (Pharmacia LKB).

incubated in 0.2% Casitone supplemented with 8 mM $MgSO_4$ (1/5C-Mg), germination proceeded much more slowly than in 1C-Mg. After 5 h of incubation at 30°C, about 60% of myxospores lost refractility, but only 4% of the myxospores elongated into rod-shaped structures (Fig. 1B). On the other hand, when myxospores were incubated at 30°C for 5 h in 1/5C-Mg supplemented with 1 mM $CaCl_2$ (1/5C-MgCa), almost all myxospores lost refractility and more than 60% of myxospores were already converted into rod-shaped vegetative-like cells (Fig. 1C).

It has been reported that more than 90% of myxospores were germinated in 0.2% Casamino Acids supplemented with 1 mM $CaCl_2$ after 10 h of incubation at 33°C, as judged by the loss of the refractility (3). We also tested myxospore germination in 0.2% Casamino Acids supplemented with both 8 mM $MgSO_4$ and 1 mM $CaCl_2$ (1/5CA-MgCa). More than 50% of the myxospores indeed lost refractility after 5 h of incubation at 30°C (Fig. 1D); however, even after prolonged incubation (24 h), only a minor population of myxospores were converted into vegetative-like cells (Fig. 1E). To examine whether myxospores are able to lose refractility without nutrients, myxospores were incubated in distilled water. About 15% of the myxospores lost refractility within a 10-h incubation (Fig. 1F), and more than 50% lost refractility after a 24-h incubation at 30°C (data not shown). However, no myxospores were converted into vegetative-like cells even after 24 h of incubation. Together, it seems apparent that myxospore germination always accompanies the loss of spore refractility under a phase microscope at a very early state, which occurs even without the addition of nutrients. However, in order for myxospores to further commit to the next step, which permits cellular outgrowth by conversion from spherical spores to rod-shaped vegetative-like cells, more stringent conditions are required, including the presence of Ca^{2+} and Mg^{2+} ions as well as tryptic digests of casein (Casitone) but not acid hydrolysates of casein (Casamino Acids). Consistent with this notion, the addition of energy poisons such as sodium azide and 2,4-dinitrophenol to 1C-Mg did not prevent the loss of refractility of myxospores but blocked cellular outgrowth (not shown).

RNA synthesis during germination. On the basis of the results obtained above, RNA synthesis was examined during germination under different conditions, by measuring the incorporation of [^{3}H]uracil into the acid-precipitated fraction. As shown in Fig. 2, in 1C-Mg, substantial uracil incorporation was initiated after 2 h of incubation at 30°C, while little incorporation was observed in 1/5C-Mg. However, as expected from the experiments described above (Fig. 1C), when 1/5C-Mg was supplemented with 1 mM $CaCl_2$, uracil incorporation was even higher at 6 h of incubation than that in 1C-Mg. Uracil incorporation in 1C-Mg was completely blocked in the presence of 10 μg of rifampin per ml, indicating that [^{3}H]uracil was indeed incorporated into the RNA fraction. We also tested RNA synthesis of myxospores incubated in 1/5CA-MgCa. As shown in Fig. 2, [^{3}H]uracil was equally incorporated during the first 2-h incubation in 1/5CA-MgCa as was the case with 1/5C-MgCa. However, in contrast to myxospores incubated in 1/5C-MgCa, there was no further increase in [^{3}H]uracil incorporation after the 2-h incubation. This result is consistent with the earlier observation that myxospores incubated in 1/5CA-MgCa lost refractility but did not proceed to cellular outgrowth (Fig. 1D and E).

When [^{35}S]methionine and [^{35}S]cysteine incorporation into the acid-insoluble fraction in 1C-Mg with 0.5 mM Ca^{2+} was examined, a pattern of incorporation similar to that of [^{3}H]uracil was obtained, in which for the first 2 h there was very little incorporation of [^{35}S]methionine and [^{35}S]cysteine, followed by a sudden increase in incorporation (not shown).

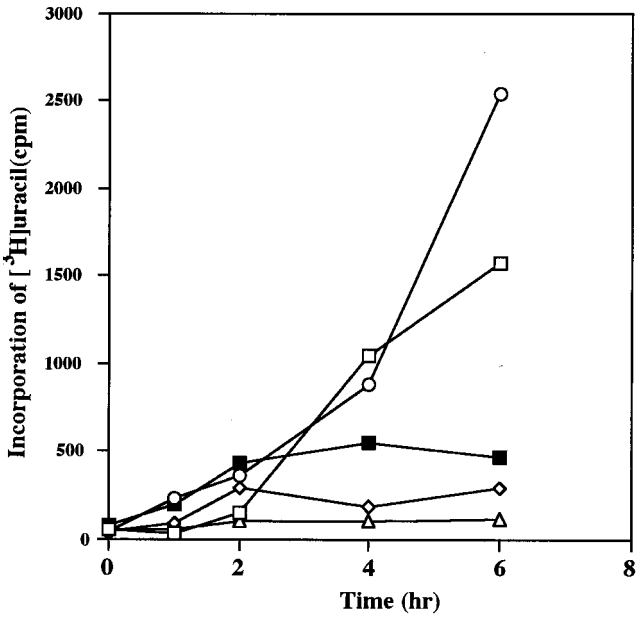

FIG. 2. RNA synthesis during myxospore germination. RNA synthesis was measured by [^{3}H]uracil incorporation as described in Materials and Methods. Samples were taken at the time points indicated in the figure. □, 1C-Mg; ◇, 1/5C-Mg; ○, 1/5C-MgCa; ■, 1/5CA-MgCa; △, 1C-Mg with rifampin.

Effect of EGTA on myxospore germination. The results described above clearly indicate that Ca^{2+} plays an important role in myxospore germination. In order to further confirm this notion the effect of the addition on EGTA, a Ca^{2+}-specific chelator, was examined on RNA synthesis in 1C-Mg medium. As shown in Fig. 3, RNA synthesis was inhibited as the concentrations of EGTA increased. At 1 mM EGTA, RNA synthesis was almost completely blocked. The inhibitory effect of EGTA was unlikely to be due to the deficiency of Mg^{2+}, since Mg^{2+} existed at a concentration of 8 mM in all experiments. When 0.5 mM $CaCl_2$ was added in addition to 1 mM EGTA, approximately 60% of RNA synthesis was recovered (Fig. 3). These results clearly demonstrated that Ca^{2+} is indispensable during the process of myxospore germination.

Effect of protease inhibitors on myxospore germination. It has been shown that protease and lytic enzymes are associated with the events occurring during the germination of spores from *Bacillus cereus* (1) and *Bacillus megaterium* KM (4). In order to test whether a protease(s) is also required for myxospore germination, phenylmethylsulfonyl fluoride (serine protease inhibitor [8]), tosyl phenylalanyl chloromethyl ketone (TPCK; chymotrypsin-like protease inhibitor [16]), and tosyl lysyl chloromethyl ketone (TLCK; trypsin-like serine protease inhibitor [15]) were tested during germination. As shown in Fig. 4, RNA synthesis in 1C-Mg measured by [^{3}H]uracil incorporation was severely inhibited in the presence of 1 mM phenylmethylsulfonyl fluoride (more than 70% inhibition) or in the presence of 0.1 mM TPCK but not in the presence of 0.1 mM TLCK. It should be noted that these inhibitors showed no inhibitory effect on the growth of vegetative cells at the concentration used (not shown).

The results described above indicate that a chymotrypsin-like protease(s) is involved in the germination process. In order to examine whether proteolytic degradation of spore-specific proteins could be observed, proteins associated with spore

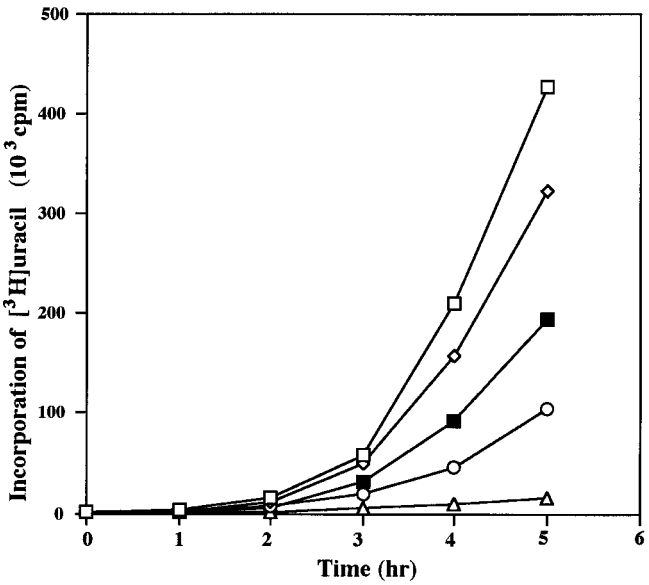

FIG. 3. Effect of EGTA on RNA synthesis during myxospore germination. Myxospores prepared as described in Materials and Methods were germinated in 1C-Mg at 30°C with addition of various concentrations of EGTA. RNA synthesis with [^{3}H]uracil was measured at the time points indicated in the figure as described in Materials and Methods. □, 1C-Mg. Note that 0.1 (◇), 0.5 (○), and 1 (△) mM EGTA and 0.5 mM EGTA plus 0.5 mM $CaCl_2$ (■) were added to 1C-Mg.

shells during myxospore germination were analyzed by SDS-PAGE. Myxospores were germinated in 1C-Mg at 30°C, and samples were taken at the time intervals indicated in Fig. 5. The samples were disrupted by sonication, and the spore shells thus formed were collected by low-speed centrifugation. During this procedure, the internal soluble fraction and the membrane dissociated from the spore shell remained in the supernatant. Note that unbroken spores were collected together with the spore shells. The low-speed precipitates were suspended in loading buffer, boiled in a boiling water bath, and subjected to SDS-PAGE. As shown in Fig. 5, at earlier time points, protein S, the major spore coat protein assembled on the surface of myxospores in the presence of CA^{2+} ions (7), is evident in the low-speed-centrifuged spore shell fraction (lanes 1 and 2). However, 2 h after the initiation of germination, the density of the protein S band became reduced with the concomitant appearance of a number of lower bands (lane 3). At 3 h (lane 4), protein S almost disappeared, and the lower bands persisted until the 4-h time point (lane 5). This result may be due to the existence of a protease, which digests protein S during germination.

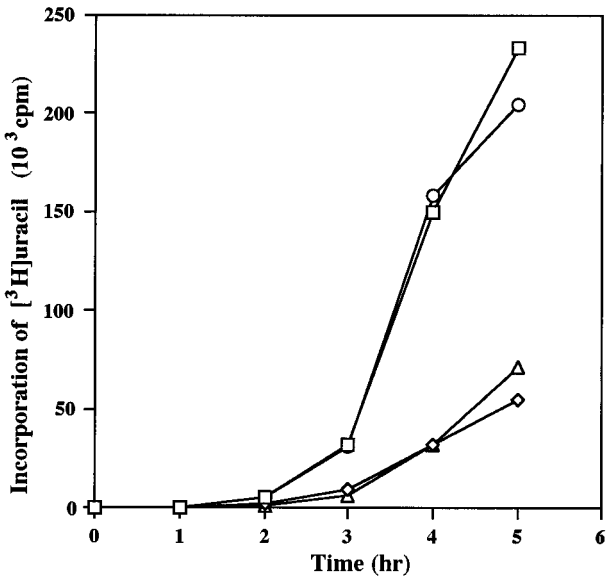

FIG. 4. Effect of protease inhibitors on RNA synthesis during myxospore germination. Myxospores, prepared as described in Materials and Methods, were germinated in 1C-Mg in the presence of 1 mM phenylmethylsulfonyl fluoride (◇), 0.1 mM TLCK (○), and 0.1 mM TPCK (△). RNA synthesis was measured by [^{3}H]uracil incorporation as described in Materials and Methods.

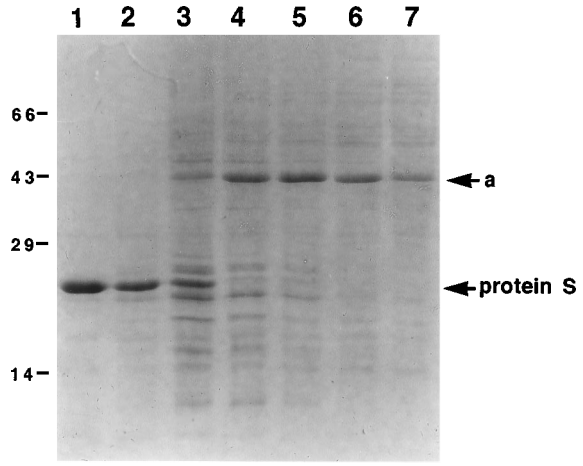

FIG. 5. SDS-PAGE of the spore shell fraction. Germinated spores, as described in Materials and Methods, were harvested at various time points and disrupted by sonication. The spore shell fraction was collected by centrifugation. The pellet was solubilized in 2× sample loading buffer and analyzed by SDS-PAGE. The numbers at the left-hand side indicate molecular masses (in kilodaltons) of the markers. Arrow a indicates the position of protein W. Lanes 1 to 7 represent samples taken at 0, 1, 2, 3, 4, 5, and 6 h of incubation after initiation of germination, respectively.

Interestingly, a novel spore-associated protein was identified (position *a*, Fig. 5). This protein, with an apparent molecular mass of 41.5 kDa, became detectable at 2 h (lane 3) and was most prominent between the 3- and 5-h time points (lanes 4 to 6). As judged from the intensity of the band stained by Coomassie brilliant blue, the amount of the protein appears to be 50% that of protein S. The amount of the protein started to decrease 6 h after germination (lane 7). The protein, now designated protein W, may be tightly associated with an internal component(s) of myxospores so that it could not be detectable in the myxospores isolated from fruiting bodies. During germination, myxospores losing refractility also lose their rigid structure, as judged by the fact that they become sensitive to heat and SDS treatments (3). This critical step was likely to happen between 2 and 3 h after germination initiation, where the myxospores became fragile and easily disintegrated by sonication. Protein W thus became extractable from spore shells by SDS. It is also possible that protein W is chemically attached to a spore component and that the linkage is cleaved during germination. Recently, the gene for protein W has been cloned in our laboratory. The characterization of the gene and its mutants will provide insights into the role of this protein.

Patterns of protein synthesis during myxospore germination. During myxospore germination, the expression of specific

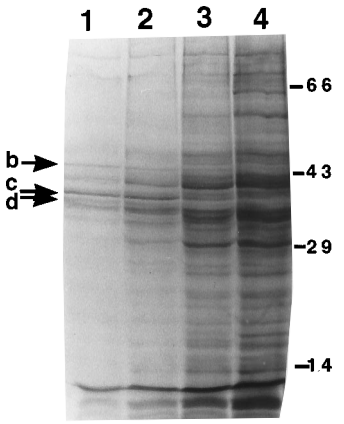

FIG. 6. Pattern of protein synthesis during myxospore germination. Myxospores, prepared as described in Materials and Methods, were germinated in 1C-Mg–0.5 mM Ca medium at 30°C. At 1-h time intervals, germinating spores were labeled for 40 min at 30°C with 100 μCi of TRANS ^{35}S LABEL (ICN Biomedical, Inc.). At the end of the incubation, the cells were harvested and disrupted by sonication with glass beads as described in Materials and Methods. The samples were mixed with 2× loading buffer and analyzed by SDS-PAGE. Lanes 1 to 4 represent samples labeled 1, 2, 3, and 4 h after germination, respectively. Arrows b, c, and d indicate positions of bands specifically labeled during early germination.

genes may be required for germination. In order to identify such genes, proteins were labeled with TRANS ^{35}S LABEL for 40 min at 1-h intervals after the initiation of germination in 1C-Mg supplemented with 0.5 mM $CaCl_2$. Samples were collected at each time point as indicated in Fig. 6 and disrupted by sonication with glass beads as described in Materials and Methods. Extracted materials were solubilized in loading buffer and analyzed by SDS-PAGE. Several proteins were synthesized immediately after initiation of germination (lane 1, Fig. 6). [^{35}S]methionine and [^{35}S]cysteine incorporation continued to increase in these protein bands, as shown in Fig. 6. In contrast, three proteins, indicated by arrows b, c, and d, were synthesized only at early time points (lanes 1 and 2, Fig. 6), synthesis of which became diminished 3 h after the initiation of germination. These proteins may play important roles in early events of spore germination.

DISCUSSION

A number of conditions have been shown to be able to initiate myxospore germination, as judged by the loss of refractility observed under a phase microscope (3). In the present paper, we demonstrated that the loss of myxospore refractility is not necessarily followed by the burst of RNA synthesis and by further morphological changes from spherical spores to rod-shaped vegetative cells.

The second step to occur after 2 to 3 h of incubation requires rather stringent conditions, including Mg^{2+} and Ca^{2+} ions. Interestingly, 0.2% Casitone could not be replaced with 0.2% Casamino Acids for the second step, indicating that amino acids in Casamino Acids are not effectively utilized as carbon sources as polypeptides in Casitone. Alternatively, Casamino Acids may lack a factor(s) existing in Casitone which is required for spore germination.

During the second step, a large number of specific gene expression and biochemical reactions are considered to be coordinated, dictating the precise events required for myxospore germination. In the present study, we found induction of specific proteins at a very early stage of germination and a requirement for a chymotrypsin-like serine protease for germination. It is interesting that *B. megaterium* contains a spore-specific protease which works specifically during germination to degrade spore-specific proteins (11). It has been also suggested that for this activity the protease requires Ca^{2+} (11). It remains to be determined whether the protease of *M. xanthus* requires Ca^{2+} ions.

We also found a 41.5-kDa protein associated with myxospores. This major spore-specific protein has not been identified previously because it appears to be associated with the internal structure of myxospores. Therefore, it became detectable only when myxospores became fragile during germination, so that they could be easily disintegrated by sonication. This protein, designated protein W, may play an important role in the structure and stability of myxospores. Biochemical and genetic analyses of this protein currently in progress in our laboratory will shed light on its function.

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