JOURNAL OF BACTERIOLOGY, Nov. 1973, p. 797-802

Vol. 116, No. 2
Printed in U.S.A.

Germination of *Bacillus megaterium* Spores After Various Extraction Procedures

JAMES C. VARY

Department of Biochemistry, University of Illinois at the Medical Center, Chicago, Illinois 60612

Received for publication 2 August 1973

The initiation of germination of *Bacillus megaterium* QM B1551 spores, grown in supplemented nutrient broth, has been studied. The initiation properties depend on buffer concentrations and the particular batch of spores. Initiation in L-alanine, KBr, calcium dipicolinate, or in buffer alone increases as a function of the spore age; whereas initiation in glucose, L-leucine, or L-proline remains relatively constant. Extraction of spores with alkali, sodium dodecyl sulfate-dithiothreitol, or lithium diiodosalicylate removes variable amounts of dipicolinic acid, hexosamine, and protein. These extracted spores are still capable of initiation and, in some cases, initiation is stimulated. However, extraction of spores with 8 M urea-10% mercaptoethanol inhibits subsequent initiation.

Several compounds such as glucose, certain amino acids, nucleosides, or ions can initiate the germination of bacterial spores (6). Some of these initiation agents may be metabolized (17), but it is not known how these agents, or products of their metabolism, start the degradation reactions of initiation. Methods have been described that extract proteins from the coats of dormant spores (1, 2, 4, 25). In some cases a change in the properties of initiation of germination has resulted (2, 20, 25). This suggests that the coat proteins may play a role in the mechanism of initiation.

Reported here are the initiation properties of *Bacillus megaterium* QM B1551 after extraction by several methods. The initiation of untreated spores has also been studied as a control because the properties of spores are dependent on their sporulation medium (12). Although Levinson and co-workers (8, 12, 13, 15) have extensively studied initiation in this strain, they have not investigated spores grown in supplemented nutrient broth (SNB). Yet, most of the biochemical data available on this strain are from Kornberg and co-workers (3, 22, 24) who did use SNB-grown spores but did not characterize the initiation properties of those spores. No attempt has been made to repeat all of the published work on this strain. However, some properties of this strain that may influence the interpretation of initiation data are reported here for SNB-grown spores.

MATERIALS AND METHODS

Organism. Spores of *B. megaterium* QM B1551 were grown in SNB as previously described (11). After the spores were washed and lyophilized, they were stored at room temperature and ambient relative humidity. References to weight of spores are then on a dry weight basis of lyophilized stocks.

Extraction procedures. Spores were suspended in the various reagents listed below at a final concentration of 10 mg/ml. After the indicated incubation time, the suspension was centrifuged at 5,000 × *g* for 10 min. The supernatant fluid (10 ml) was removed and dialyzed overnight at 0 C in 1.0 liter of water with one change of water after 3 h. The spores were washed at least six times with 1 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8), suspended in water and lyophilized.

SDS-DTT. Spores were suspended in a fresh solution of 0.5% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT), and 0.1 M NaCl (4). The pH was adjusted to 10.0 with NaOH, and the suspension was incubated at 37 C for 2 h.

NaOH. Spores were suspended in 0.1 M NaOH for 30 min at 0 C (25).

Urea. Spores were suspended in a fresh solution of 8 M urea and 10% 2-mercaptoethanol (pH 3) and incubated at 37 C (3) for 2.5 h.

LIS. Spores were suspended in 0.1 M lithium diiodosalicylate (LIS)-50 mM Tris (pH 7.5). After 15 min at 30 C, the suspension was diluted with two volumes of water and incubated an additional 10 min at 30 C (16).

Crude sonically treated suspension. Spores (200 mg) were suspended in 2.0 ml of 5 mM Tris (pH 8) and 2.7 g of glass beads. The suspension was cooled in a salt-ice bath and treated 10 times for 30-s each with a Branson W185D sonic oscillator at a setting of 5. The glass beads were removed by decantation, and the breakage, estimated by the aid of a microscope, was >95%.

Assays. Initiation of germination was measured

spectrophotometrically at 30 C on heat-activated spores (10 min at 60 C) as previously described (23). The percent decrease in absorbance was calculated after 20 min, and a 60 to 70% decrease represented > 90% initiation of germination (23). This was confirmed by observation under a phase-contrast microscope.

Heat resistance was determined by diluting 0.5 ml of a spore suspension (200 μg/ml) into 9.5 ml of water pre-equilibrated to 70 C. After incubation for 10 min at 70 C, the suspension was diluted and plated on SNB plates. The plates were incubated for 48 h at 30 C, and the colonies were counted. Lysozyme sensitivity was tested on suspensions of spores (200 μg/ml) in 5 mM Tris buffer (pH 8) by adding lysozyme to 10 μg/ml and measuring the decrease in absorbance at 660 nm for 30 min at 30 C.

Dipicolinic acid (DPA) (9, 19) and protein (14) were measured colorimetrically. Hexosamine was measured, after hydrolysis in 6 N HCl for 5 h at 100 C, by the method of Rondle and Morgan (18) with glucosamine as a standard.

Polyacrylamide gel electrophoresis. The gels were made with 7.5% acrylamide, 0.2% *N,N'*-methylenebisacrylamide, 0.1% *N,N,N',N'*-tetramethylenediamine, 0.15% ammonium persulfate, and 0.375 M Tris (pH 9.6). The marker dye was 0.025% bromophenol blue, and samples were applied in 20% glycerol. The upper buffer was 53 mM glycine-53 mM Tris (pH 8.5), and the lower buffer was 0.1 M Tris (pH 8.1). Electrophoresis was from negative to positive in a Buchler Polyanalyst at 3 MA/gel and 25 C. Gels were stained for 30 min with 0.0025% Coomassie Blue in 10% acetic acid-50% methanol and destained overnight with 7.5% acetic acid-5% methanol.

Materials. LIS and the reagents for polyacrylamide gels were purchased from Eastman Kodak Co. Coomassie Blue R250 was from Colab, and bromophenol blue was from Fisher Scientific. Lysozyme was from Pentex, and the glass beads were Superbrite type 100–5005 from 3M Co. Dipicolinic acid was from Aldrich Chemical Co., and all other reagents were from Calbiochem.

RESULTS

General properties. It was found that many preparations of spores could initiate germination in Tris buffer alone. This capability was dependent on the spore age and the concentration of buffer. There was some initiation (15% decrease in absorbance/20 min) in 50 mM Tris buffer (pH 8), but there was negligible initiation in 10 mM Tris or less (Table 1). As the age of the spores increased, the capability of initiation in Tris buffer increased while the concentration dependence decreased. The possibility that initiation in buffer was a result of unclean spores was tested by repeated washing both before and after heat activation, but no difference was observed. This initiation was not unique to Tris buffer since similar results were found with potassium phosphate (pH 8). Buffer initiation was not a universal finding because a few batches of spores did not initiate well in Tris even after 4 months of storage.

TABLE 1. *Initiation in Tris buffer*

Spore[a] age (days)	Percent decrease in absorbance/20 min[b]				
	0[c]	1	5	10	50
1	2	2	1	4	15
10	5	3	5	5	30
90	1	5	13	22	37

[a] Lyophilized spores were stored at room temperature for the indicated times.

[b] Spores were suspended in distilled water, heat activated for 10 min at 60 C, and diluted into water or Tris buffer (pH 8) at the indicated concentrations. The absorbance at 660 nm was measured at intervals during incubation at 30 C, and the percent decrease in absorbance was calculated after 20 min.

[c] Concentration (millimolar) of Tris.

Several commonly used initiation agents were tested on SNB-grown spores. Initiation occurred in glucose, L-leucine, L-proline, and SNB as previously described (5, 12, 23). In L-alanine, KBr, and Ca DPA, initiation was poor with freshly grown spores but improved with time of storage at ambient relative humidity and temperature. For instance, in one preparation of spores, 0.1 M L-alanine-5 mM Tris (pH 8) caused a 14% decrease in absorbance/20 min with spores 9 days old, but after storage for 14 days or 7 months the decrease in absorbance/20 min changed to 18 and 53%, respectively. Collectively, these results show that initiation is dependent on spore age, buffer concentration, and even the particular preparation of spores.

Extraction of spores. Spores were extracted with SDS-DTT, NaOH, urea or LIS. After treatment by any one of these methods, the spores appeared fully refractile under the phase-contrast microscope (Table 2). On a dry weight basis, they had similar heat resistance and retained most (>80%) of their DPA. Only the SDS-DTT and urea-extracted spores were sensitive to phase-darkening and lysis by lysozyme, indicating that coat proteins had been removed to make the peptidoglycan accessible to lysozyme (1, 4).

The ability of these extracted spores to initiate germination in glucose is shown in Fig. 1. Spores treated with NaOH, LIS, or SDS-DTT initiated similar to the control if compared on the basis of decrease in absorbance within 20 min. The spores treated with NaOH or SDS-DTT had slightly lower rates of loss in absorbance, possibly suggesting some impairment of

TABLE 2. *Properties of extracted spores*

Extraction method	Refractility[a]	Heat resistance[a, b] (CFU × 10^8/mg spore)	DPA[a] (μg/mg spore)	Lysozyme[a] sensitivity
Control	+	5.6	152	−
SDS-DTT	+	4.9	138	+
NaOH	+	5.9	128	−
Urea	+	5.8	124	+
LIS	+	5.6	−[c]	−

[a] Lyophilized spores (200 μg/ml) were suspended in distilled water and assayed for refractility, heat resistance, DPA, and lysozyme sensitivity as described in Materials and Methods.

[b] Heat resistance was calculated as colony-forming units (CFU) per milligram of spores.

[c] LIS interfered with the DPA assay and could not be completely removed from the spores.

initiation. However, with urea-treated spores there was a dramatic reduction in the ability to initiate germination in glucose (Fig. 1). This was confirmed by observation under the phase-contrast microscope. These spores eventually do initiate after several hours as expected since they are viable and heat resistant. Thus, the effect of the urea treatment is to increase the time required for the population of spores to initiate. Attempts to stimulate initiation by variable heat-activation times, the addition of Ca^{2+} or Mn^{2+} (20, 21), or addition of the dialyzed urea supernatant liquid were unsuccessful. A crude extract, which had been prepared by sonic treatment of untreated spores in 5 mM Tris (pH 8), was not effective in stimulating the initiation of urea-treated spores.

The ability of extracted spores to initiate germination (as judged by percent decrease in absorbance per 20 min) in other reagents was tested. Results similar to those for glucose were found for initiation in L-leucine and L-proline (Table 3). Initiation in L-alanine, KBr, or Ca DPA was poor in the control spores. These spores were 1 day old, but with storage the ability of the control spores to initiate in these three reagents improved (data not shown). When these 1-day-old spores were extracted with SDS-DTT, NaOH, or LIS, the initiation in L-alanine, KBr, or Ca DPA increased (Table 3). Thus, extraction with SDS-DTT, NaOH, or LIS appeared to mimic the effect of storage. However, the urea-treated spores initiated poorly in any of the reagents.

It should be noted that these data were obtained from a single batch of spores on which

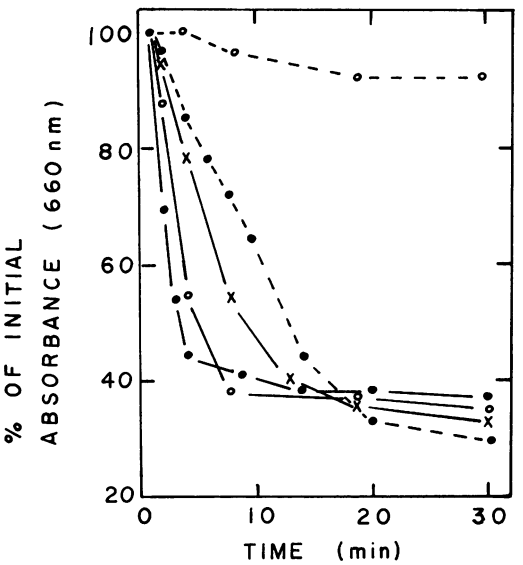

FIG. 1. *Glucose initiation of extracted spores. Heat-activated spores were diluted into 0.1 M glucose-5 mM Tris (pH 8) at 30 C. At intervals, the absorbance at 660 nm was determined, and the percentage of the initial absorbance was calculated for the purpose of normalization. In all cases, the decrease in absorbance in 5 mM Tris (pH 8) alone was < 10%. Untreated control (●——●); LIS (O——O); NaOH (×——×); SDS-DTT (●- - -●); Urea (O- - -O).*

TABLE 3. *Initiation of extracted spores*

Extraction method	Percent decrease in absorbance/20 min[a]					
	L-Leucine	L-Alanine	L-Proline	KBr	Ca DPA	Buffer
Control	44	7	41	14	4	1
SDS-DTT	62	11	54	55	12	0
NaOH	63	28	63	61	24	4
Urea	6	2	4	6	5	4
LIS	64	31	62	65	23	2

[a] Initiation of heat-activated spores was tested in 5 mM Tris (pH 8) with 7.6 mM L-leucine, 100 mM L-alanine, 8.7 mM L-proline, 40 mM KBr, or 10 mM Ca DPA. Percent decrease in absorbance was calculated after incubation for 20 min at 30 C.

the control initiation studies and all extractions were done after 1 day of storage. The initiation studies of the extracted spores were done 1 day later. This work has been repeated on several other batches of spores with essentially the same results. It might also be noted that the data in Fig. 1 and Table 3 are for the initiation of extracted spores that were heat activated prior to testing initiation. For each assay shown in Fig. 1 and Table 3, a control without prior heat activation was also tested, and in every assay the initiation was less. Thus heat activation was found to be required for initiation of the control and extracted spores.

After each method of extraction, the supernatant liquids were dialyzed. The amount of hexosamine and protein in each supernatant liquid was determined (Table 4). The hexosamine content of *B. megaterium* spores is 150 μg/mg of spores (L. Hsieh and J. C. Vary, unpublished data). Less than 6% was removed in a nondiffusible form by any extraction procedure. In each case, the extracted spores retained >90% of their hexosamine.

The total protein content of spores has been reported to be 330×10^{-15} g per spore (3) or about 33% of the spore dry weight. The batch of spores used in this study had 300 μg/mg of spores (determined on a crude sonically treated extract), and this value was used to calculate percent protein extracted. SDS-DTT, NaOH, or LIS extraction removed 4 to 46% of total spore protein without greatly reducing their capacity to initiate germination within 20 min. Yet the urea treatment, which removed only 26% of the protein, greatly altered initiation.

The extracted proteins were examined by polyacrylamide gel electrophoresis (Fig. 2). The SDS-DTT extraction shows a broad irregular band that migrates near the front of the gel

TABLE 4. *Extraction of protein and hexosamine from spores*

Extraction	Hexosamine extracted[a]		Protein extracted[a]	
	μg/mg of spore	Percent	μg/mg of spore	Percent
SDS-DTT	8	5.3	139	46
NaOH	1	0.7	12	4
Urea	1	0.7	79	26
LIS	4.2	2.8	18	6

[a] The supernatant liquids from the indicated extractions were dialyzed and then assayed for protein and hexosamine. Percent extracted was calculated from a value of 300 μg for total protein and 150 μg for total hexosamine in 1 mg of untreated spores.

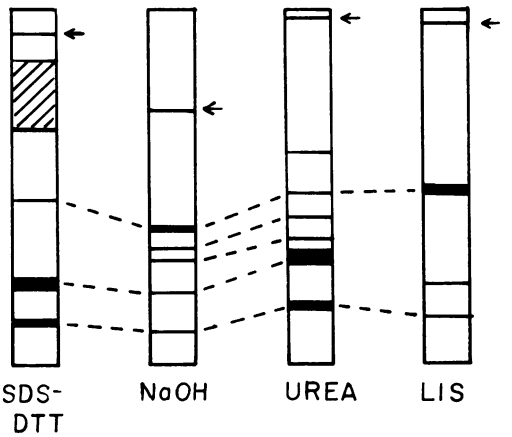

FIG. 2. *Electrophoresis of supernatant solutions. Each gel was loaded with about 30 μg of protein from the dialyzed supernatant solutions. The arrows show the position of the tracking dye after electrophoresis, and the dashed lines indicate approximate correlations of different bands between gels calculated by their mobilities relative to the tracking dye.*

which may be some low-molecular-weight material. Three distinct bands are found below. For NaOH, urea, and LIS extractions, the proteins resolve into five, six, and three bands, respectively, with none of the rapidly migrating material that was observed in the SDS-DTT supernatant. The mobilities of these bands were calculated relative to the tracking dye, and the dashed lines in Fig. 2 show the correspondence found between bands in different gels. These are approximate correlations (± 10%) and are shown only to aid in comparing the gels.

DISCUSSION

The properties of *B. megaterium* QM B1551 spores grown in SNB are very similar to those described by Levinson and co-workers (8, 12, 13, 15). The initiation of spores in buffer is probably similar to "ionic germination" (5, 6, 21). As predicted from Levinson's studies (8), these spores became activated by storage, but an additional effect was the increase in their capability to initiate in L-alanine, KBr, or Ca DPA. This change is not identical to "heat-induced reduction" of initiation described by Holmes et al. (7) because heat activation increased initiation, but it might have resulted from water vapor activation (8). Thus in addition to activation and lowering of the concentration requirements (8, 13) by aging, spores become less restrictive in the kinds of agents that will initiate germination. Although this information does not provide deeper insight into the bio-

chemistry of initiation, it emphasizes the importance of defining the age, buffer, and batch of spores when studying a particular initiation agent. Also it may provide an explanation for some of the discrepancies about initiation agents found in the literature (5, 12, 13, 15, 21, 23).

Treatment of *B. megaterium* spores with SDS-DTT, NaOH, or LIS extracted variable amounts of DPA, protein, and hexosamine. The extracted proteins, which could be resolved into three to five bands on polyacrylamide gels, may not be required for either heat resistance or the initiation of germination. The treated spores were mostly heat resistant, and they could initiate germination in any of the common reagents used for this strain. These three extraction procedures did increase the ability of fresh spores to initiate, which could be a result of increased permeability. Although this increase in initiation which is produced by extraction of the spores mimics the increase in initiation by storage of untreated spores, there is no evidence to suggest that the mechanisms are identical. Also this change does not appear to be a result of just activation since the extracted spores still required heat activation.

When spores are extracted with 8 M urea-10% mercaptoethanol (pH 3), 26% of the protein, but essentially no hexosamine, was removed. These spores were heat resistant, but initiation of germination was inhibited. The extracted proteins were resolved into six bands, five of which roughly correspond to bands found from other extraction procedures. It is possible that the protein in the sixth band is necessary for initiation. However, it was not possible to stimulate initiation by the addition of the dialyzed urea supernatant liquid or a crude sonically treated extract.

Although the morphology of extracted spores has not been examined by electron microscopy, the extracted proteins probably include the coat proteins (1, 4, 10, 25). If there are receptor sites and/or enzymes required for metabolism of initiation agents in the coats, one might expect these extracted spores to be incapable of initiation. These results and the results of others (1, 4, 25) show that the capacity to initiate is not eliminated. However, the coats may act as a permeability barrier since three of the extractions allowed them to initiate on a wider variety of compounds (see Table 3 and reference 25).

ACKNOWLEDGMENTS

The excellent technical assistance of Terri Stoesser is gratefully acknowledged.

This work was supported by an Institutional Grant from the American Cancer Society, National Science Foundation grant GB-36897, and Public Health Service grant GRSG #514.

LITERATURE CITED

1. Aronson, A. I., and P. C. Fitz-James. 1971. Reconstitution of bacterial spore coat layers in vitro. J. Bacteriol. **108**:571-578.
2. Cassier, M., and A. Ryter. 1971. Sur un mutant de *Clostridium perfringens* dormant des spores sans tuniques a germination lysozyme-dépendante. Ann. Inst. Pasteur **121**:717-732.
3. Chambon, P., E. J. Dupraw, and A. Kornberg. 1968. Biochemical studies of bacterial sporulation and germination. IX. Ribonucleic acid and deoxyribonucleic acid polymerases in nuclear fractions of vegetative cells and spores of *Bacillus megaterium*. J. Biol. Chem. **243**:5101-5109.
4. Fitz-James, P. C. 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105**:1119-1136.
5. Foerster, H. F., and J. W. Foster. 1966. Response of *Bacillus* spores to combinations of germinative compounds. J. Bacteriol. **91**:1168-1177.
6. Gould, G. W., and G. J. Dring. 1972. Biochemical mechanisms of spore germination, p. 401-408. *In* H. O. Halvorson, R. S. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D. C.
7. Holmes, P. K., E. H. Nags, and H. S. Levinson. 1965. Concurrent heat activation and suppression of *Bacillus megaterium* spore germination. J. Bacteriol. **90**:827-828.
8. Hyatt, M. T., and H. S. Levinson. 1968. Water vapor, aqueous ethyl alcohol, and heat activation of *Bacillus megaterium* spore germination. J. Bacteriol. **95**:2090-2101.
9. Jansen, F. W., A. J. Lund, and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26-27.
10. Kondo, M., and J. W. Foster. 1967. Chemical and electron microscope studies on fractions prepared from coats of bacillus spores. J. Gen. Microbiol. **47**:257-271.
11. Lammi, C. J., and J. C. Vary. 1972. Deoxyribonucleic acid synthesis during outgrowth of *Bacillus megaterium* QM B1551 spores, p. 277-282. *In* H. O. Halvorson, R. S. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D. C.
12. Levinson, H. S., and M. T. Hyatt. 1964. Effect of sporulation medium on heat resistance, chemical composition, and germination of *Bacillus megaterium* spores. J. Bacteriol. **87**:876-886.
13. Levinson, H. S., and M. T. Hyatt. 1970. Effect of temperature on activation, germination and outgrowth of *Bacillus megaterium* spores. J. Bacteriol. **101**:58-64.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265-275.
15. McCormick, N. G., F. Feeherry, and H. S. Levinson. 1972. Germination properties and proteins of spores of *Bacillus megaterium* QM B1551 mutants, p. 421-429. *In* H. O. Halvorson, R. S. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
16. Marchesi, V. T., and E. P. Andrews. 1971. Glycoproteins: isolation from cell membranes with lithium diiodosalicylate. Science **174**:1247-1248.
17. Prasad, C., M. Diesterhaft, and E. Freese. 1972. Initiation of spore germination in glycolytic mutants of *Bacillus subtilis*. J. Bacteriol. **110**:321-328.
18. Rondle, C. J. M., and W. T. J. Morgan. 1955. Determina-

tion of glucosamine and galactosamine. Biochem. J. **61**:586–589.
19. Rotman, Y., and M. L. Fields. 1968. A modified reagent for dipicolinic acid analysis. Anal. Biochem. **22**:168.
20. Rowley, D. B., and H. S. Levinson. 1967. Changes in spores of *Bacillus megaterium* treated with thioglycolate at a low pH and restoration of germinability and heat resistance by cations. J. Bacteriol. **93**:1017–1022.
21. Sacks, L. E. 1972. Influence of intra- and extracellular cations on the germination of bacterial spores, p. 437–442. *In* H. O. Halvorson, R. S. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
22. Setlow, P., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXII. Energy metabolism in early stages of germination of *Bacillus megaterium* spores. J. Biol. Chem. **245**:3637–3644.
23. Vary, J. C. 1972. Spore germination of *Bacillus megaterium* QM B1551 mutants. J. Bacteriol. **112**:640–642.
24. Vary, J. C., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXI. Temperature-sensitive mutants for initiation of germination. J. Bacteriol. **101**:327–329.
25. Waites, W. M., L. R. Wyatt, and B. Arthur. 1972. Effect of alkalai treatment on the germination and morphology of spores of *Clostridium bifermentans*, p. 430–436. *In* H. O. Halvorson, R. S. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.