JOURNAL OF BACTERIOLOGY, May 1979, p. 431-441
0021-9193/79/05-0431/11$02.00/0

Vol. 138, No. 2

Biochemistry of L-Proline-Triggered Germination of *Bacillus megaterium* Spores

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Received for publication 9 February 1979

The mechanism by which L-proline triggers germination in *Bacillus megaterium* QM B1551 spores was investigated. First, brief exposure of spores to L-proline, followed by dilution, was sufficient to trigger germination. Once germination was triggered, the spores continued initiation of germination and did not require high concentrations of L-proline. Triggering of germination was pH and temperature dependent. Second, enzymes for L-proline catabolism were absent in spores, and several non-metabolizable analogs of L-proline were effective trigger compounds. Third, triggering of germination occurred in the presence of inhibitors of proton motive force production, oxygen uptake, and metabolism. Fourth, uptake of L-proline occurred after the triggering of germination. These results argue that neither uptake nor metabolism of L-proline was necessary to trigger germination. Instead, L-proline probably causes a biophysical alteration in the spores that triggers the biochemical changes in germination.

A few specific compounds are capable of triggering the initiation of germination in *Bacillus megaterium* QM B1551 spores (19, 20). One of these compounds is L-proline (19), and we are attempting to understand the biochemical mechanism by which this compound causes spores to lose their dormant properties. Based on inhibitor studies, mutant analysis, and the presence of certain enzyme activities, several models have been proposed to explain triggering of germination that include the necessity of a proton motive force (7, 8) or metabolism of the trigger compound (24). Presented here is a study of the effect of various inhibitors on L-proline-triggered germination and an assessment of the necessity for metabolism of L-proline. Investigating L-proline-triggered germination offers several unique advantages, since there are only a limited number of known metabolic reactions for L-proline, L-proline catabolism is oxygen dependent, and a large number of non-metabolizable analogs are available.

In this report, the following definitions for germination will be used. Germination is the process by which dormant spores become irreversibly transformed into actively metabolizing vegetative cells. Triggering of germination is the process by which a compound reacts stereospecifically with a spore to start germination (10, 16). Previous studies with L-alanine have shown that triggering of germination requires only a brief exposure of heat-activated spores to L-alanine (10, 12, 28). After the L-alanine is rapidly removed, continued incubation of the spores in buffer showed that spores can continue to initiate germination as judged by absorbance loss or ^{45}Ca release. This observation has been termed triggering (10, 16) or commitment (28). Both terms are operationally the same, but we prefer the former because it is used in other developmental systems. Thus, triggering of germination requires only brief contact with the spores and then the L-alanine is no longer necessary. Preliminary results with *B. megaterium* QM B1551 spores suggested that L-proline triggered germination in a similar manner (D. P. Rossignol and J. C. Vary, Fed. Proc. 37:1611, 1978). This triggering reaction leads to initiation of germination (16), which is primarily a sequence of degradative reactions such as proteolysis (30, 32), peptidoglycan hydrolysis (14), calcium dipicolinate loss (14, 27), and lipid turnover (D. J. Ellar, personal communication). In *B. megaterium* QM B1551 there is a sequential loss in heat resistance, dipicolinic acid, absorbance of visible light, and refractility under the phase-contrast microscope (21). At or about the same time as absorbance loss, some biosynthetic events can occur such as ATP synthesis and reduction of pyridine nucleotides (6, 29), neither of which requires macromolecular synthesis. The above reactions probably represent the last steps in initiation of germination. The next stage, outgrowth, is characterized by RNA and protein synthesis and finally DNA synthesis (16).

We report here studies on the kinetics of L-proline-triggered germination, proline metabolism and transport, oxygen consumption, and pH

changes when L-proline triggers initiation of germination. Also, several inhibitors of metabolism and proton motive force uncouplers were used to assess the necessity of the above reactions. Some of these data have been reported in preliminary form (26; Rossignol and Vary, Fed. Proc. 37:1611, 1978).

MATERIALS AND METHODS

Organism and growth conditions. *B. megaterium* QM B1551 was the wild type, and strain JV-28 is a spontaneous streptomycin-resistant mutant previously described (37). Spores were grown on supplemented nutrient broth (SNB), harvested, and stored as previously described (14, 18). All references to spore weights are on a dry weight basis. Vegetative cells were grown as previously described (6) in SNB or a glucose minimal salts medium (18) containing L-proline (1 mg/ml) as the sole nitrogen source or sole carbon source. Lysates of these cells were made as previously described (6). Spores were disrupted by sonic oscillation or by lysis of sodium dodecyl sulfate-dithiothreitol-treated spores with lysozyme as previously described (34), except that 10 mM Tris-hydrochloride buffer (pH 8) was used. The lysates were stored on ice, or in some cases a supernatant fraction was obtained by centrifugation at 10,000 × *g* for 10 min and stored on ice.

Mutant isolation. Spores of *B. megaterium* that were unable to germinate on L-proline but could grow on L-proline as a carbon source were isolated by the following procedure. Vegetative cells of strain JV-28 were mutagenized with ethyl methane sulfonate, and the mutagenized cells were allowed to sporulate as previously described (37). Approximately 2 mg of mutagenized spores was heat activated in 0.1 ml of water for 10 min at 60°C, cooled on ice, added to 1.0 ml of 5 mM Tris buffer (pH 8) containing 2 mg of L-proline per ml, and incubated at 30°C for 1.5 to 3.5 h to allow germination. The suspension was then heated by dilution into 9 ml of water at 80°C, maintained at 80°C for 10 min, and cooled. After centrifugation at 5,000 × *g* for 10 min, the pellet was washed twice in water, suspended in 1.0 ml of water, and stored overnight at 4°C. The suspension was then heated at 60°C for 10 min, inoculated into 10 ml of SNB medium, and incubated at 30°C with shaking until growth and sporulation were complete as judged microscopically. The spores were harvested by centrifugation, washed twice as above, suspended in 2 ml of distilled water, and refrigerated.

The above cycle of initiating germination in L-proline with ~10^8 spores, followed by heating at 80°C and then sporulation in SNB, was repeated four successive times. This cycle was repeated three more times as described above, except that germination was in L-proline plus 750 μg of methicillin per ml for 16 h, followed by heating at 80°C and then sporulation in SNB as above. Finally, spores were diluted in water, plated on SNB agar plates containing streptomycin (100 μg/ml), and incubated overnight at 30°C. Eighty colonies were picked, suspended in water, and screened for germination and colony formation on SNB or minimal medium containing proline (1 mg/ml) as the sole carbon source. Spores from 51 colonies that did not germinate and therefore did not form colonies with proline as the sole carbon source were isolated, purified, and further screened to obtain the following phenotype. Heat-activated spores failed to form colonies on medium with proline as the sole carbon source, but vegetative cells did grow on that medium. Heat-activated spores or vegetative cells grew on glucose minimal salts medium or SNB and were streptomycin resistant. When spores were tested for germination, one mutant, JV-137, was unable to initiate germination on L-proline (see Results). Because of the lengthy selection procedure, an estimate of the mutation frequency cannot be made, nor have we measured the reversion frequency.

Assays. Losses in absorbance at 660 nm, heat resistance, and dipicolinic acid were measured with heat-activated spores (10 min at 60°C) as previously described (15), except that L-proline was used at indicated concentrations to trigger germination. The minimum exposure times to L-proline were measured by incubating spores (10 mg/ml) in 5 mM Tris (pH 8) plus 10 mM L-proline at 30°C, diluting samples 1:100 into 5 mM Tris buffer (pH 8) at 30°C, and, after 30 min, determining the absorbances at 660 nm. This dilution technique reduced the L-proline concentration to levels below that capable of triggering germination but allowed the completion of germination for spores that had been triggered (see Results). Published methods were used to assay Δ'-pyrroline-5-carboxylic acid (P5C) dehydrogenase (E.C. 1.5.1.2; ref. 4), P5C reductase (E.C. 1.5.1.2; ref. 35), proline dehydrogenase (3, 11), and protein (22). Proline oxidase (E.C. 1.4.3.2) was assayed in 55 mM $NaHCO_3$ at pH 9, the optimal pH for the vegetative cell enzyme, by two methods (4, 9), both of which gave comparable results. The enzyme assays were linear with protein concentration and time. For each assay the pH optimum was determined. Oxygen consumption and changes in pH were measured as previously described (25).

Uptake of L-proline during triggering of germination was assayed by preincubating spores (1 mg/ml) in 5 mM Tris (pH 8) and chloramphenicol (100 μg/ml) in the presence or absence of inhibitors for 5 min at 30°C, followed by the addition of L-[*U*-^{14}C]proline to a final concentration of 10 mM (0.093 μCi/μmol). Samples (100 μl) were removed and quickly diluted into 2.0 ml of 10 mM Tris (pH 8)–0.15 M NaCl–0.5 mM $MgCl_2$–10 mM L-proline and immediately filtered on a Millipore HAWP 0.45-μm filter. The tube was rinsed twice, and the filter was washed twice with 2.0 ml of the same buffer without L-proline. The filters were dried and counted as previously described (25). All values were corrected for background with a sample which had been preincubated exactly as above; at zero time, this sample was diluted into the above buffer containing 10 mM L-proline, L-[*U*-^{14}C]proline was added, and the suspension was immediately filtered and washed. In all cases, the control values were reproducible and represented <0.1 nmol of L-proline bound per mg of spores. This same background was obtained by other procedures such as filtering the spores before the addition of L-[*U*-^{14}C]proline, using 4 mM $HgCl_2$ to

inhibit triggering, or using dormant spores or even spores that had been boiled for 20 min.

To measure uptake of L-proline in germinated spores, heat-activated spores were allowed to completely initiate germination by incubation for 30 min in 5 mM Tris (pH 8), plus 10 mM L-proline and chloramphenicol (100 μg/ml). The spores were >95% phase dark. Spores were then washed three times in cold distilled water plus chloramphenicol, and uptake was monitored for 6 min after addition of washed spores to the transport assay medium used above, containing L-proline at the indicated concentrations. The uptake was linear from 2 to 6 min, from which the rates were calculated. Inhibitors were added as indicated.

Materials. D- and L-proline, hydroxy-L-proline, baikiain, allo-4-hydroxy-L-proline, chloramphenicol, L-thiazolidine-4-carboxylic acid, streptomycin, and P5C were from Calbiochem. *N,N'*-Dicyclohexylcarbodiimide (DCCD), tetrahydrofuran, cyclopentane carboxylic acid, and 2-thiophene carboxylic acid were from Aldrich; carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), L-proline amide, L-proline methyl ester, and all peptides were from Sigma. Methicillin was from Bristol Laboratories, Syracuse, N.Y. All other chemicals were reagent grade. Stock solutions of DCCD and CCCP were made in absolute ethanol. Solutions of L-proline and analogs (20 mM) were neutralized to pH 7 with NaOH, and the final concentration of added NaOH was less than 2 mM in all cases.

RESULTS AND DISCUSSION

Sequence of events during initiation of germination. Heat-activated spores were incubated in 10 mM L-proline and 5 mM Tris buffer (pH 8), and samples were rapidly diluted 100-fold into 5 mM Tris buffer (pH 8) at 30°C. The percent decrease in absorbance for each sample after 30 min of incubation in buffer was plotted versus the time of dilution (Fig. 1) and shows that, after 20 s of exposure to L-proline, fractions of the spore population had triggered germination as judged by absorbance loss. At 1.5 min about 50% of the spores had triggered (a 60% decrease in absorbance represents >95% phase darkening and loss in heat resistance; 6, 14, 15). Samples at 0, 10, or 20 s showed no loss in absorbance after dilutions, indicating that a 100-fold dilution was sufficient to reduce the proline concentration to levels that did not trigger germination; this was also confirmed by the fact that spores (100 μg/ml) not previously exposed to a high concentration of L-proline did not lose absorbance when 100 μM L-proline was added for 30 min. This experiment shows that only brief exposures to high concentrations of L-proline are necessary to trigger germination, as judged by loss in absorbance after dilution of the L-proline. Although we used dilution, similar results were obtained using filtration or centrifugation to lower the concentration of trigger compound. Also, we chose to use absorbance loss as the criterion for initiation of germination, for convenience and because it is a late event (15, 21), although similar results have been found by measuring the release of ^{45}Ca (28). Finally, the 30-min incubation time was chosen because >95% of the spores completed initiation of germination within 30 min (14, 15, 21, 34).

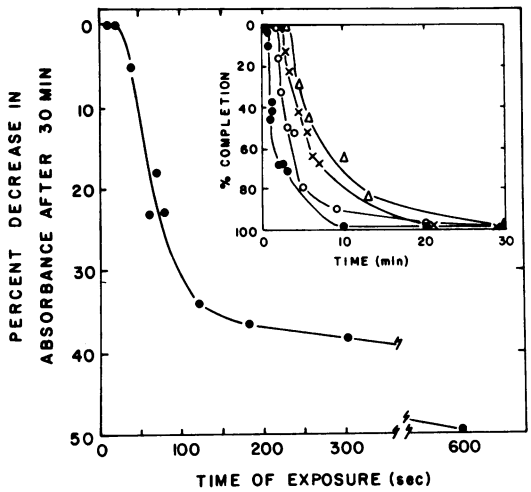

FIG. 1. *Sequence of events during triggering and initiation of germination. Heat-activated spores (10 mg/ml) were incubated at 30°C in the presence of 10 mM L-proline and 5 mM Tris (pH 8). At times indicated, samples were diluted, and after 30 min the absorbances were determined. The percent losses from the initial absorbance were plotted (●) as a function of the time of exposure to L-proline before dilution as described in the text. In the same experiment, loss in heat resistance (○), dipicolinic acid (×), and absorbance at 660 nm (△) were measured as described in the text (inset). These data and those in the main part of this figure were normalized to the total changes within 30 min and plotted as a percentage of these values. The 30-min time point represents greater than 80% triggering of germination. The total losses per 30 min for heat resistance, dipicolinic acid, and absorbance were 99%, 110 μg/mg of spore, and 54%, respectively.*

If the data in Fig. 1 are plotted on the same time scale and normalized for losses in heat resistance, dipicolinic acid, and absorbance, it is possible to compare the minimum exposure times that trigger germination with the sequence of other known events for the population of spores (inset, Fig. 1). At 30 s, when no heat-resistance, dipicolinic acid, or absorbance losses had occurred, 10% of the spores had been triggered for germination. From this comparison, it is apparent that triggering of germination for the population of spores occurred before other measurable events. This observation does not conflict

with a recent report that Ca^{2+} and dipicolinic acid are released almost immediately after the addition of L-alanine to *B. megaterium* KM spores (27). In that report, highly sensitive assays capable of detecting <1% loss of Ca^{2+} or dipicolinic acid were used, which is not possible for heat-resistance or absorbance measurements. The early Ca^{2+} and dipicolinic acid release could represent either a small fraction of the spores being triggered within 20 s, or the whole population losing about 0.1% of its Ca^{2+} and dipicolinic acid. Based on the slight differences in the ratio of Ca^{2+} to dipicolinic acid release at late times, Scott and Ellar suggested that the latter possibility might be true (27). However, the differences in ratios were very small and may be subject to considerable error at early times. Also, the time for 50% loss of total dipicolinic acid was approximately 8 min, comparable to our data (inset, Fig. 1). Since it is impossible to investigate the sequence of events on a single spore, we feel that it is more reliable to compare the sequence of events in the entire population; a convenient, technically feasible point of comparison is at 50% completion. It is important to note that spore populations are heterogeneous, and thus some small fraction that may trigger at early times may give misleading information, just as the small fraction of "super dormant" spores that will not trigger even after several hours are not representative of the bulk of the spore population. Therefore, when looking at the entire spore population, triggering of germination or "commitment" (28) occurs very early, after which the trigger compound is no longer necessary in high concentrations. The trigger reaction(s) is then followed by losses in heat resistance, dipicolinic acid, and absorbance.

By using this time for 50% triggering of germination, we have investigated the effects of pH and temperature. Optimal triggering of germination occurred between pH 7 and 8.5. Triggering of germination was proportional to temperatures between 13 and 46°C, but did not occur below 13°C, even after 1 h of exposure to L-proline. These data are similar to previous observations in other organisms (10, 12, 28). Interestingly, once germination was triggered, the completion of initiation of germination, as judged by absorbance loss, did occur at 4°C (data not shown), as was observed with *B. licheniformis* (10). We interpret these results to show that L-proline must interact with spores and go through some temperature-dependent reaction(s) that triggers germination, but that then the remaining reactions do not have a strict temperature dependency.

Since only a brief exposure of the spores to L-proline was sufficient to trigger germination, we have studied several possible mechanisms that might explain how L-proline could trigger germination. Based on work with other species and the strain studied here, possible mechanisms might involve metabolism of L-proline, production of a proton motive force, or stimulation of endogenous metabolism (2, 7, 8, 24).

Enzymes for proline metabolism. To investigate triggering of germination, we first studied the possibility that L-proline may be metabolized through known pathways of proline catabolism. Since spores grown in SNB were capable of triggering germination on L-proline, we measured enzymes for proline metabolism in SNB-grown spores. As shown in Table 1, crude extracts obtained from spores by either lysozyme treatment or sonication had no detectable proline oxidase activity ($<1.43 \times 10^{-5}$ μmol/min per mg of protein). The activity of P5C dehydrogenase was also below detection, whereas the specific activity of the proline anabolic enzyme, P5C reductase, was comparable to that found in vegetative cells. A fourth enzyme, proline dehydrogenase, previously reported in *Clostridium sporogenes* (3), was not detectable. The only other known proline catabolic enzyme is proline reductase; this enzyme was not studied in spores,

TABLE 1. *Activity of enzymes in proline metabolism*

Enzyme	Sp act (μmol/min per mg of protein)	
	Vegetative cells[a]	Spores[b]
Proline oxidase	0.018	$<1.43 \times 10^{-5}$
P5C dehydrogenase	20	$<7 \times 10^{-5}$
P5C reductase	0.030	0.031
Proline dehydrogenase	—	$<5 \times 10^{-5}$

[a] Vegetative cells were grown on L-proline as a sole nitrogen source, and lysates were made as described in the text. Proline oxidase was assayed in the crude lysate, and P5C dehydrogenase was assayed in the supernatant fraction from the lysate. However, P5C reductase was assayed in supernatant fractions from lysed cells grown in SNB medium.

[b] Spores were disrupted by sonic oscillation as described in the text. Proline oxidase and proline dehydrogenase were assayed in the crude extract, whereas P5C dehydrogenase and P5C reductase were assayed in the supernatant fraction. Each enzyme was also assayed from spore lysates prepared by lysozyme treatment of sodium dodecyl sulfate–dithiothreitol-extracted spores as described in the text, and the activities were the same. Spore extracts were also assayed for proline oxidase in 55 mM cacodylic acid at pH 6.5 (4) and Tris at pH 7.8 (9), and the results were the same.

since it is found to be active only with D-proline under anaerobic conditions (33). As a control, proline oxidase, P5C dehydrogenase, and P5C reductase were also measured in vegetative cells (Table 1). To measure proline oxidase and P5C dehydrogenase, it was necessary to grow the vegetative cells under conditions that should induce enzymes for proline catabolism (4). These assays were conducted only to show that the assays were valid for each enzyme and comparable to values obtained in other species of *Bacillus* (17) and *Salmonella* (4, 5).

The undetectable levels of L-proline catabolic enzymes in spores suggested that the metabolism of L-proline probably did not play a major role in triggering germination. This conclusion is further supported by results on oxygen consumption (see below).

Triggering of germination on proline analogs. To further study the possibility of proline degradation and the specificity of the trigger reaction, analogs of proline were tested for their ability to trigger germination (Table 2). Clearly, some but not all compounds similar in structure to L-proline were capable of triggering germination. The analogs are listed in the order of decreasing effectiveness as trigger compounds. The lower absorbance losses per 30 min for most analogs was a reflection of lower rates of absorbance losses per 30 min. NaCl was tested to show that sodium salts formed during neutralization were not responsible for the observed triggering. These compounds were also tested for their ability to serve as a sole carbon source for growth, and only L-proline and L-2-pyrrolidone-5-carboxylic acid were effective. The latter may have been metabolized by conversion to glutamate with 5-oxoprolinase activity (36), but this was not tested directly since the analog did not trigger germination. Not shown in Table 2 is the fact that L-proline methyl ester also triggered germination, but it was also a substrate for growth, suggesting that spores may contain an esterase activity that converts this analog to proline. As a further test for metabolism, two analogs (thiophene-2-carboxylic acid and cyclopentane carboxylic acid) were tested as substrates for the vegetative proline oxidase by oxygen uptake in crude extracts; they were found to be inactive (data not shown).

These data show that there is no correlation between the ability of a compound to trigger germination and its ability to support growth or to be metabolized. These studies also indicated some stereospecificity for the trigger compound. For instance, L-proline but not D-proline was effective, indicating a necessity for the L isomer. Also, the carboxyl group may be modified (L-proline amide), but not with a bulky substituent (L-proline-*t*-butyl ester or dipeptides). The number four position can be hydroxylated, but only in one configuration (hydroxy-L-proline, not allo-4-hydroxy-L-proline), or carbon number 4 may be substituted with sulfur (thiazolidine-4-carboxylic acid). An exhaustive analysis of all possible configurational alterations has not been done because there was a gradient of trigger effectiveness which did not correlate with specific molecular modifications. However, some of these structural requirements were exploited to

TABLE 2. *Initiation of germination on proline analogs*

Compound[a]	Decrease in absorbance per 30 min (%)	Utilization for growth as a sole carbon source[b]
Buffer	0	–
L-Proline	60	+
P5C	51	–
L-Proline amide	53	–
Thiazolidine-4-carboxylic acid	34	–
Tetrahydrofuran	31	–
2-Thiophene carboxylic acid	25	–
Pyrrolidine	21	–
Hydroxy-L-proline	21	–
Cyclopentane carboxylic acid	17	–
2-Furoic acid	13	–
Pyrrole-2-carboxylic acid	9	–
L-2-Pyrrolidone-5-carboxylic acid	9	+
Allo-4-hydroxy-L-proline	6	–
L-Proline-*t*-butyl ester	<1	–
D-Proline	<1	–
NaCl	<3	–

[a] Spores were suspended in distilled water, heat activated for 10 min at 60°C, and diluted to a final concentration of 200 μg/ml into 5 mM Tris buffer (pH 8) containing the indicated compound. The absorbance at 660 nm was measured at intervals during incubation at 30°C, and the percent decrease in absorbance was calculated after 30 min. All compounds were 20 mM except tetrahydrofuran, which was 14 mM, and allo-4-hydroxy-L-proline, which was 10 mM. In addition, 20 mM baikiain triggered 26% decrease in absorbance, and the following analogs and peptides were tested at 20 mM and found to trigger <10% decrease in absorbance per 30 min: dansyl-L-proline, L-prolyl-L-alanine, L-prolyl hydroxy-L-proline, L-prolyl-L-tyrosine, L-prolyl-L-phenylalanine, glycyl-L-proline, and L-prolyl-glycine.

[b] The ability of vegetative cells to grow on the indicated compound as a sole carbon source was tested by inoculating cells from an overnight glucose minimal salts culture into 1 ml of liquid minimal salts medium, containing proline or the indicated analog at 1 mg/ml, and incubating at 30°C for 48 h with shaking.

study the possibility that L-proline exerts its effects by interaction with a component of the transport system (see below).

L-Proline or analogs might exert their effect by stimulation of endogenous metabolism. It has been suggested that the production of a proton motive force might be part of the mechanism of initiating germination (7). We have tested this possibility with the use of proton motive force uncouplers and by directly measuring oxygen consumption and pH changes.

Oxygen consumption and pH changes. It was previously reported that the initiation of germination in *B. megaterium* KM spores by alanine plus inosine was accompanied by rapid oxygen consumption (39). Although oxygen uptake was insensitive to chloramphenicol, the necessity of this reaction in the sequence of events leading to initiation of germination was not investigated.

We found that during L-proline-triggered germination of *B. megaterium* QM B1551, the loss in absorbance at 660 nm preceded oxygen consumption by approximately 2 min (Fig. 2). The absence of detectable oxygen uptake during the first 4 min after the addition of L-proline was followed by a rapid uptake, reaching a maximum rate of 6.67 nmol/min per mg of spores at 8 min. This uptake is less than that previously reported (39), possibly as a result of the lack of an exogenous energy source with proline. This explanation is consistent with the observation that less reduction of NAD occurs during initiation of germination on non-metabolizable compounds than on metabolizable compounds such as D-glucose (29).

To further investigate the role of oxygen during initiation of germination, we studied the effects of several metabolic inhibitors. As shown in Table 3, 1 mM KCN had no effect on the absorbance loss, whereas it inhibited oxygen consumption by 56%. These results are consistent with previous findings (39). With DCCD and CCCP, almost complete inhibition of oxygen consumption occurred (83 and 97%, respectively), yet there was a 31 to 36% loss in absorbance. With DCCD, triggering of germination was complete as judged by the complete release of dipicolinic acid (data not shown). The same result was reported for glucose-triggered germination (38). The control with ethanol showed essentially no effect on absorbance loss or oxygen consumption. The presence of 1 mM $HgCl_2$ completely inhibited the uptake of oxygen while allowing the triggering of germination to occur (25).

The generation of a proton motive force may be assayed by measuring the decrease in the pH of the medium due to extrusion of intracellular protons (2). When spores were incubated in 4 mM Tris–0.1 M LiCl, without L-proline, there

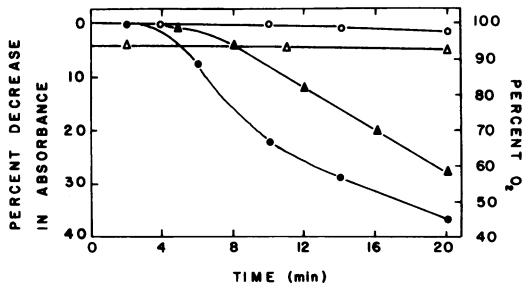

FIG. 2. *Oxygen uptake during initiation of germination of B. megaterium spores. Oxygen consumption in the medium was measured, as described in the text, with heat-activated spores (1 mg/ml) in 5 mM Tris (pH 8) in the presence (▲) or absence (△) of 10 mM L-proline. Absorbance was measured at 660 nm in an identical sample in the presence (●) or absence (○) of 10 mM L-proline. The lower limit of O_2 detection was <0.5% of saturation.*

TABLE 3. *Effect of inhibitors on oxygen uptake, pH drop, and initiation of germination*[a]

Inhibitor	Decrease in absorbance per 20 min[b] (%)	O_2 uptake[c] (nmol/min per mg of spores)	Decrease in pH (pH units/4 min)[d]
Heat-activated spores only	5	0	0.028
None	50	6.67	0.078
KCN	50	2.94	0.051
DCCD	36	1.06	0.036
CCCP	31	0.21	0.049
Ethanol	48	6.09	0.065
$HgCl_2$	16	0.00	0.023

[a] Assays were performed as described in the legends to Fig. 1 and 2. Heat-activated spores were preincubated for 5 min with inhibitors before the addition of 10 mM L-proline to start the reaction. All inhibitors were present at 1 mM final concentration and included 1% ethanol for DCCD and CCCP.

[b] Although spore concentrations in the assays for oxygen consumption and pH drop were different, percent decrease in absorbance varied less than 3%. In addition, the time for 50% maximal absorbance loss was 7 to 8 min in all cases except CCCP, which was 12 min. The time for 50% completion cannot be calculated for the control (line 1) or $HgCl_2$ (25).

[c] Oxygen uptake was calculated from a tangent drawn through the inflection point where maximal uptake occurred (within 5 to 15 min after addition of L-proline).

[d] A change in pH of 0.050 over 4 min (corrected for background) would correspond to 7.8×10^{11} protons per mg of spores.

was a slow change in pH (0.007 pH units/min). Although the cause of this slow pH change is unknown, it was reproducible and occurred with either heat-activated or dormant spores, neither of which triggered germination. A similar background was observed previously (6). However, after the addition of L-proline to heat-activated spores, there was a 45-s lag and then a rapid decrease in pH (Fig. 3). Because of the curvilinear pH decrease, the results are most easily expressed as change in pH per 4 min.

With KCN and CCCP, the pH decline was reduced slightly, but in the presence of DCCD a marked inhibition of the pH decline was observed, even though initiation of germination was triggered. The control with ethanol had little effect. These data suggest that the decline in pH during L-proline-triggered germination may be due in part to extrusion of protons as a result of endogenous metabolism. However, this pH decline may be reduced without stopping triggering of germination. Similar results were obtained with Na_2HAsO_4 (data not shown). Finally, the pH decrease in 1 mM $HgCl_2$ was reduced to background levels, yet the spores still triggered germination as discussed above. These data indicate that the triggering of germination may occur without concomitant oxygen uptake or proton motive force formation.

Uptake of L-proline. Another possible mechanism for the triggering of germination may be through the interaction of the triggering agent with a part of the transport system. The interaction of a chemosensory system with membrane proteins responsible for transport has been previously shown (1). We investigated this possibility by directly measuring the uptake of radioactive L-proline in germinating spores and studying the effect of competitive inhibitors.

To test for the necessity of transport in the sequence of events leading to germination, we simultaneously measured the accumulation of L-[*U*-^{14}C]proline and absorbance changes. At an L-proline concentration great enough to trigger rapid germination (10 mM), no transport was detectable until 3.5 min after the addition of L-proline (Fig. 4). At this time, absorbance loss (a "late" event as shown in Fig. 1) had already begun with a 15% loss. Thus, detectable L-proline transport did not occur prior to loss in absorbance.

Preincubation of spores for 5 min with 1 mM DCCD or CCCP inhibited uptake for the first 20 min (Fig. 4), but did not stop triggering of germination in this strain. It may be noted that these inhibitors were dissolved in ethanol, but after dilution the final concentration was 1% (vol/vol) and this amount of ethanol did not

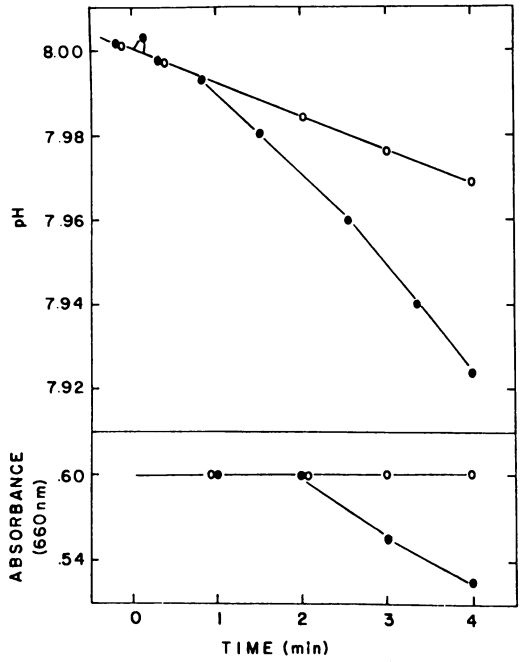

FIG. 3. *Decrease in pH during initiation of germination on L-proline. Heat-activated spores (3 mg/ml) were preincubated for 5 min in 4 mM Tris (pH 8.0)–0.1 M LiCl before the addition of L-proline dissolved in 0.1 M LiCl (pH 8.0). The pH and absorbance changes were measured in the presence (●) and absence (○) of L-proline as described in the text.*

inhibit either uptake or absorbance loss (data not shown). We have previously reported that 1 mM $HgCl_2$ blocked uptake of L-proline even though triggering of germination was complete as judged by dipicolinic acid loss (25). Thus, detectable proline uptake was not necessary prior to triggering of germination, but, after triggering, proline could be taken up. Although the mechanism of uptake after triggering of germination has not been characterized, it probably involves active transport based on the inhibitory effects of DCCD and CCCP.

These data do not rule out the possibility that loosely bound proline may have been present on the spore, or that a small amount of uptake (10^6 to 10^7 molecules per spore) may have occurred before triggering of germination. However, it is important to note that the filtration technique employed here is similar to that used by others (28) to remove essentially all of the trigger compound. Therefore, if one is interested in measuring how many molecules of trigger compound might be loosely bound, other techniques must be used. We are presently synthesizing an affinity label analog of L-proline which might be a more useful approach to the above question.

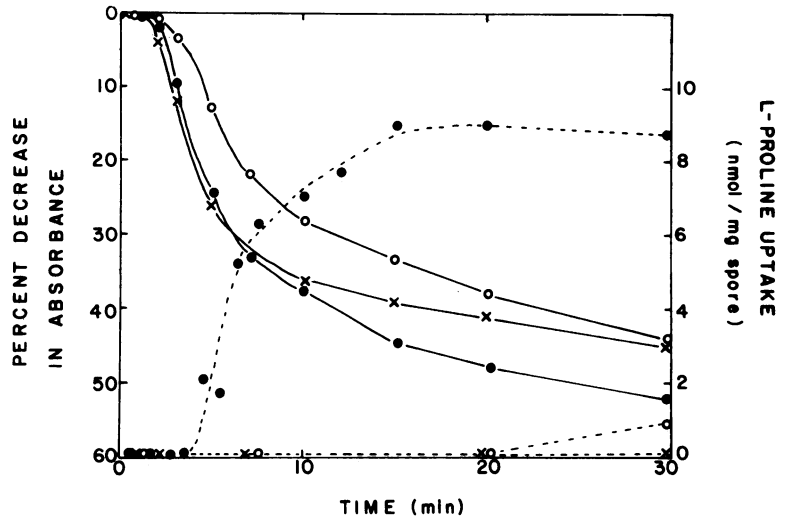

FIG. 4. *L-Proline uptake during triggering of spore germination. Heat-activated spores (1 mg/ml) were preincubated for 5 min at 30°C in 5 mM Tris buffer (pH 8) and chloramphenicol (100 μg/ml) with or without inhibitors, followed by the addition of L-[U-^{14}C]proline (0.093 μCi/μmol) to a final concentration of 10 mM. Losses in absorbance at 660 nm (solid lines) were determined for the control (●) and tubes containing 1 mM DCCD (○) or 1 mM CCCP (×). At intervals, samples were removed and the amount of L-[U-^{14}C]proline uptake was determined (broken lines) for the control (●) and tubes containing 1 mM DCCD (○) or 1 mM CCCP (×) as described in the text.*

Inhibition of uptake by proline analogs. To further assess a possible relationship between the mechanism of proline triggering and proline transport during outgrowth, heat-activated spores were allowed to completely initiate germination and were then assayed for L-[U-^{14}C]proline uptake as described in Materials and Methods. By these methods, L-proline uptake in germinated spores had an apparent K_m of 33 μM and a V_{max} of approximately 1.4 μmol/min per mg of spores (Fig. 5). The same results were obtained if the spores were triggered in 0.1 M glucose prior to measuring L-proline uptake. It may be noteworthy that the K_m for proline uptake in germinated spores was ~20-fold less than the concentration of proline (~0.6 mM) necessary to trigger 50% of the maximal absorbance loss. However, since measurements of absorbance loss per 30 min are not the same as catalytic rate measurements, the above comparison must be interpreted with caution.

Since the spores were triggered in the presence of chloramphenicol, which blocks protein synthesis in this organism (31), a proline transport system must preexist in the spore and become activated during initiation of germination and outgrowth. Although we have not studied this proline uptake system in detail, it is noteworthy that both D-proline and hydroxyproline competitively inhibited proline uptake (Fig. 5). However, these compounds were not equally effective in triggering germination (Table 2). Hydroxyproline was an effective trigger compound, and D-proline was not. Also, D-proline did not inhibit L-proline-triggered germination at 200 times the concentration of L-proline (data not shown). The apparent K_i for hydroxyproline was 240 μM, whereas that for D-proline was 150 μM, indicating that D-proline had a higher affinity for the proline transport system than hydroxyproline. This competitive inhibition of transport suggests that D-proline can effectively react with the L-proline transport binding protein, but that it cannot trigger initiation of germination, whereas hydroxyproline may effectively interact with both systems. These data indicate that components of the uptake system are not solely responsible for the triggering of germination. Similar data have been used in other systems to differentiate between transport and chemotactic receptor sites (13, 23).

In support of the above hypothesis, the properties of strain JV-137 were investigated. This mutant, isolated as described in Materials and Methods, did not trigger germination when incubated in L-proline, as measured by absorbance, heat-resistance, or dipicolinic acid loss; however, the spores did trigger germination normally in D-glucose (Fig. 6). When these spores were germinated on D-glucose in chloramphenicol and washed and assayed for uptake of L-proline, as described above, the apparent K_m and

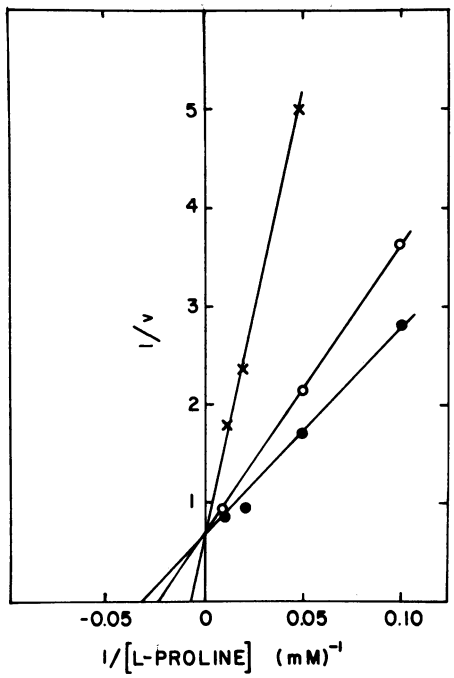

FIG. 5. *Competitive inhibition of* L*-proline uptake. Spores that had completed initiation of germination were obtained as described in the text and diluted into prewarmed 5 mM Tris (pH 8) containing either* L*-[U-*^{14}*C]proline* (●) *(2.2 mCi/mmol) at the indicated concentration,* L*-proline plus 0.6 mM* D*-proline* (×), *or* L*-proline plus 2 mM hydroxyproline* (○). *Samples (100 μl) were removed at intervals, filtered on Millipore HAWP 0.45-μm filters, and washed twice with 5 mM Tris (pH 8) plus 0.1 M NaCl–0.5 mM* $MgCl_2$*; then the filters were dried and counted as described in the text.*

V_{max} for L-proline were identical to those of wild type (data not shown). Thus, the system for L-proline uptake during outgrowth appeared normal in strain JV-137, but the L-proline trigger mechanism was blocked. The simplest interpretation is that the L-proline uptake system is not necessary for triggering germination. The conclusive proof of this would be the ability of a transport mutant blocked in proline transport to trigger germination, but we have been unsuccessful in attempts to isolate a mutant completely blocked in the transport of L-proline.

Collectively, these data indicate that the enzymes necessary for the uptake of L-proline are present in the dormant spore and presumably become activated after triggering of germination. Based on the effect of transport and metabolic inhibitors that did not inhibit the triggering of germination and the properties of strain JV-137, the L-proline uptake system does not play a key role in triggering germination.

In conclusion, we have found that the interaction of L-proline with spores to trigger germination is rapid, stereospecific, and dependent on pH and temperature. Triggering of germination by L-proline is not dependent on detectable metabolism, active transport, generation of a proton motive force, or uptake of oxygen. From our data on triggering of germination by L-proline, it appears that L-proline interacts with the spore and triggers germination, after which the presence of L-proline is no longer required. This conclusion is supported by similar data in other strains of *Bacillus* (12, 24) and for *B. megaterium* QM B1551 with either L-leucine or D-glucose (unpublished data). We are presently trying to identify the site of L-proline interaction to determine the mechanism of triggering germination. One possible model is that L-proline causes a conformational change in some spore receptor site that can then trigger a set of reactions that initiates germination (38, 40). Hopefully, the identification of the L-proline trigger site may allow us to analyze the subsequent reactions.

Preliminary results with protein modification reagents such as acetic anhydride have shown that the system by which L-proline triggers germination may be completely blocked without stopping glucose-triggered germination (Rossignol and Vary, Fed. Proc. **37**:1611, 1978). Furthermore, heat or ethanol activation is required to unmask this site(s) of modification. We are now attempting to isolate the protein to study its interaction with L-proline.

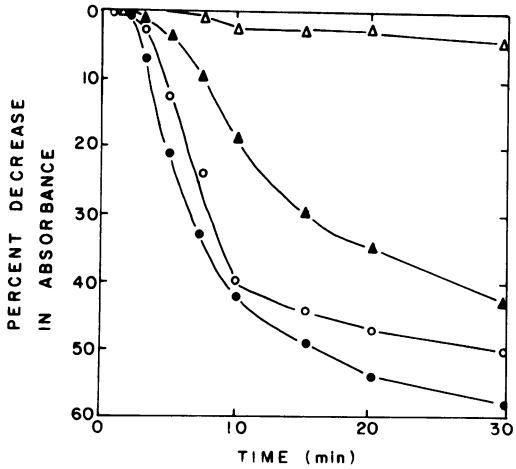

FIG. 6. *Triggering of germination in strain QM B1551 and mutant JV-137. Heat-activated spores of wild type (closed symbols) or JV-137 (open symbols) were diluted into 5 mM Tris (pH 8) plus 0.1 M glucose* (○) *or 10 mM* L*-proline* (△) *at 30°C, and the absorbances at 660 nm were measured.*

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-12678 from the National Institutes of Health.

The technical assistance of W. Jablonski is most appreciated.

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