# STUDIES ON SPORE GERMINATION: ITS INDEPENDENCE FROM ALANINE RACEMASE ACTIVITY

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The demonstration in the past few years that simple nutrients are sufficient to enable a rapid germination of spores from aerobic bacteria has stimulated an interest in the biochemical reactions involved. Glucose, adenosine, and certain amino acids, added singly or in combination, were found by a number of workers to be sufficient to lead to over 90 per cent germination of spores from a number of aerobic strains within a 30 minute period (Knaysi, 1945; Hills, 1949*a*, *b*, 1950; Pulvertaft and Haynes, 1951; Powell, 1950). Among the bacillus strains, Hills and co-workers found that of these nutrients L-alanine played the most prominent and general role. Its activity could be inhibited by D-alanine but not by a series of other structural analogues (Hills, 1949*b*). A similar observation was made recently by Stewart and Halvorson (1953). They found that approximately 90 per cent of the spores of *Bacillus terminalis* would germinate in the presence of adenosine and L-alanine, but if the cells were removed then by centrifuging, the resulting supernatant was unable to bring about the germination of freshly added spores. Subsequent studies made to explain this revealed the presence of an active enzyme that would convert the L-alanine to D-alanine, and the latter would inhibit the germination initiated by the L-alanine. Nine other aerobic spore formers were studied, and the racemase was found in all of them.

The properties of this enzyme which have been studied thus far, pH optimum, affinity for the substrate, and the vitamin $B_6$ requirement, suggest that it is identical with the alanine racemase reported by Wood and Gunsalus (1951). Although all racemase enzymes studied to date can convert either the D- or L-amino acid to the racemase mixture, the observed inhibition of the germination by D-alanine warranted further study of the spore enzyme to see if it also would do this.

If the enzyme is a true racemase, then the D-alanine inhibition would suggest that the racemase enzyme plays a secondary role in the germination and that the L-alanine is serving as a substrate for some other enzyme.

It is the purpose of this paper to characterize this enzyme more precisely and determine whether or not it plays a role in the germination process. If its activity is essential for germination, then one would expect the following correlations:

(1) Response to L-alanine should parallel the content of the enzyme in different strains of the organism.

(2) Inactivation of the enzyme should preclude germination of the spore.

## MATERIALS AND METHODS

*Production and purification of spores.* In order to study the activity of enzymes associated with spores, it is essential that the spores be obtained free from contamination with materials from the vegetative cells. This is particularly important in studying an enzyme which is common to both the vegetative cell and its spore (Stewart and Halvorson, 1953). Consequently, purity gauged only by microscopic observation is insufficient. It would be more accurate to depend upon the elimination of heat-sensitive enzymes which are associated with the vegetative cell. For aerobic spore formers, catalase activity is a convenient choice because of its wide distribution and high turnover number. However, since some of the spores contain a heat-stable catalase (Lawrence and Halvorson, 1954) as an integral part of the cell, only the heat labile enzyme can be used as an index of cleanliness.

The problem of purification was simplified by the selection of a liquid medium which was free from particulate matter and at the same time favored the rapid formation of spores, thus giving both high spore yields and a favorable ratio of spores to contaminating materials. It has been our experience that one cannot rely upon a single medium for the formation of spores in a

TABLE 1

*The source and media used to sporulate strains of bacilli*

| ORGANISM SPORULATED | SOURCE | SPORULATING MEDIA |
|---|---|---|
| *B. terminalis* | University of Illinois | G* |
| *B. cereus* | NRRL B-569, American Type Culture | G* |
| *B. globigii* | Camp Detrick, Frederick, Maryland | G* |
| *B. polymyxa* | 297 American Type Culture | BHI† |
| *B. megaterium* | American Type Culture | BHI† |
| *B. subtilis* | University of Michigan | BHI†, 0.01 per cent $MnSO_4$ |

* "G" medium was prepared by adding the following to one liter of water: 1 g $K_2HPO_4$, 5 g $(NH_4)_2SO_4$, 2 g yeast extract (Difco), 4 g glucose, 0.1 g $MnSO_4 \cdot H_2O$, 0.8 g $MgSO_4$, 10 mg $ZnSO_4$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $CaCl_2$, and 1 mg $FeSO_4$.

† Difco brain heart infusion broth (BHI).

variety of bacillus species. The media found adequate for spore formation for the various species studied in this work are recorded in table 1. The organisms were grown for 24 to 48 hours at 30 C in four liter flasks which were aerated vigorously by bubbling compressed air through the medium. Dow Corning antifoam C was added to prevent excessive foaming.

For the elimination of vegetative debris, a variety of methods are possible. Frequently, following the completion of spore formation, the vegetative cells will autolyze, freeing the spore from the vegetative cell. This process can be hastened by incubating the suspension at temperatures above the growth temperature. It is important prior to such incubation to centrifuge the spore mass out of the growth medium and resuspend it in distilled water or buffer to remove nutrients that might stimulate germination (Powell and Hunter, 1953).

Following autolysis, the spores were removed by differential centrifugation and washed with distilled water until they were microscopically clean and free from heat labile catalase. In the case of *Bacillus cereus*, 12 washings were sufficient. For the remaining species studied, this procedure failed to yield pure spores. In these cases, by suspending and incubating the spores in dilute HCl solution at pH 2, subsequent differential centrifugation yielded clean spores.

*Determination of viability.* The viability of the spores obtained by the above method was determined by a comparison of a direct count with a viable count. The latter was performed by plating aliquots in duplicate on a medium containing one per cent peptone (Difco), one per cent yeast extract, 0.5 per cent sodium chloride, and two per cent agar. The plates were incubated at 30 C and counted after 24 and 48 hours.

*Measurement of germination.* The following criteria were used routinely as an index of spore germination (Powell, 1950): (1) the change in the refractive index of a germinating suspension as measured in a Klett-Summerson photoelectric colorimeter using filter no. 420; (2) the ability of the spore to take up one per cent aqueous methylene blue stain; and (3) the loss in heat resistance. With each of the spores studied, it was found necessary to heat shock the suspension before optimal germination could be obtained. This was achieved by placing a suspension of 0.2 mg of dry spores per ml of buffer at pH 7.3 in a 65 C water bath for 15 minutes. Following equilibration of the spore suspension at 30 C, various agents were added and the kinetics of germination measured by the methods described above.

*Determination of alanine racemase.* The alanine racemase activity of intact spores or soluble enzymes was determined as reported elsewhere (Stewart and Halvorson, 1953). The activity of the enzyme at 30 C was stopped at appropriate intervals either by chilling and centrifuging the spores out of the medium or by heat inactivation of the soluble enzyme. The concentration of D-alanine was measured manometrically (Wood and Gunsalus, 1951) using D-amino acid oxidase prepared from acetone dried pig kidney cortex.

*Materials.* D-Alanine and L-alanine were purchased from Nutritional Biochemical Corporation and used without further purification. The amount of L- or D-contamination present in the enantiomorph was determined manometrically with the D-amino oxidase assay.

Pyridoxal phosphate ($B_6$ al P) was obtained from the California Foundation for Biochemical Research.

All of the water used in these studies was double distilled and passed through a Barnstead Bantam demineralizer.

## RESULTS

*Reversibility of the enzyme.* Some of the properties of the enzyme converting L-alanine to D-alanine have been determined (Stewart and Halvorson, 1953, 1954). The enzyme from either intact spores or spore extracts has a pH optimum of 8.5, is specific for alanine, and has a $K_s$ value of $5.8 \times 10^{-3}$ M. A threefold increase in enzyme activity was observed upon the addition of pyridoxal phosphate to dialyzed extracts of spores. Such an addition to the intact spore, however, does not influence the enzyme activity. Although these properties are in agreement with those in the alanine racemase enzyme reported by Wood and Gunsalus (1951), the response of germination of L- and D-alanine warrants an investigation of the reversibility of the reaction.

The reversibility of the enzymatic reaction was determined using both intact spores of *B. terminalis* and extracts of the same spores (Stewart and Halvorson, 1954). Ten mg of intact dry spores were placed in each of two tubes containing either 16 mg of D-alanine or 16 mg of L-alanine. These tubes were incubated for various intervals of time at 30 C, after which the enzymatic reaction was stopped by rapid chilling in crushed ice and the supernatants removed by centrifugation. The supernatants were assayed then for their content of D-alanine. Parallel tubes were incubated in the presence of spore extracts. The activity of the soluble enzyme was stopped by quick freezing the samples and inactivating the enzyme by autoclaving the solution at 120 C. These supernatants also were assayed as previously described. The results obtained are shown in figure 1. The reversibility of the enzyme both in intact spores and extracts further characterizes the enzyme as a typical alanine racemase.

*Germination requirements and alanine racemase distribution.* A survey of the germination requirements for a number of aerobic spore forming bacilli was undertaken in order to study the relationship of L-alanine to the germination process and its possible correlation with the presence of the alanine racemase enzyme. The following strains of Bacillus were sporulated and their spores purified as described under methods: *B. cereus*, *B. terminalis*, *B. polymyxa*, *B. subtilis*, *B. megaterium*, and *B. globigii*. In all cases the spores were 100 per cent viable.

In each case an attempt was made to determine the minimal requirements for optimal germination. The three methods of measuring germination described earlier gave close agreement for each strain. Table 2 summarizes the results of experiments carried out with *B. globigii* spores. The effect of substrate concentrations of L-alanine and glucose on the degree of germination is shown in figure 2. As can be seen from inspection of table 2 and figure 2, 12 μM L-alanine, 10 μM glucose, and 30 mM phosphate at pH 7.3 represent the requirements for optimal germination.

The results of similar studies on the other purified spores are summarized in table 3. These conditions are in agreement with those reported by other workers for *B. terminalis* (Stewart and Halvorson, 1953), *B. subtilis*, and *B. megaterium* (Powell, 1950, 1951). In the case of *B. megaterium*, however, it was observed that 70 per cent of the spores could be germinated in 12 μM L-alanine. Although incomplete germination was obtained with *B. cereus*, *B. polymyxa*, and *B. subtilis*, under the conditions of our test, 100 per cent germination was obtained on peptone yeast extract agar plates. Thus far, attempts to recognize further requirements for germination in these cases have been negative.

In addition, the alanine racemase activity (μM alanine racemized per mg dry spores per hr) of each purified spore strain is recorded in table 3. The negative enzyme activity of *B. globigii* spores was confirmed employing both high levels of spores (50 mg) in the test and also in spore extracts.

Extracts were prepared in two manners. In the first instance, spores of both *B. globigii* and *B. terminalis* were treated in the Mickle disintegrator for 30 minutes in the presence of alumina A301 as an abrasive. This treatment led to over 99.99

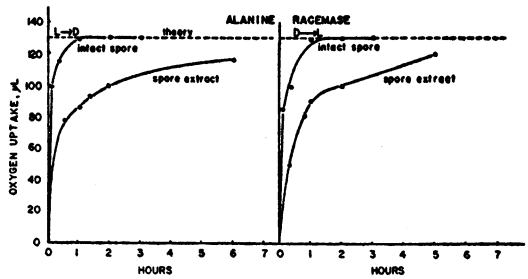


*Figure 1.* Racemization of D (−) and L (+) alanine by extracts and intact spores of *Bacillus terminalis*. D-Alanine determined manometrically employing D-amino acid oxidase. (See text for conditions.)

TABLE 2

*Requirements for germination of spores of Bacillus globigii*

| SUPPLEMENTS | GERMINATION |
|---|---|
| | % |
| Control | 0 |
| Bacto-casitone (Difco), 0.01 per cent | 0 |
| Yeast extract (Difco), 0.01 per cent | 0 |
| L-Alanine, 6 μM | 50 |
| L-Alanine, 18 μM | 70 |
| L-Alanine, 36 μM | 70 |
| Adenosine, 12 mμM | 0 |
| Adenosine, 24 mμM | 8 |
| Adenosine, 48 mμM | 8 |
| Glucose, 5 μM | 40 |
| Glucose, 10 μM | 50 |
| Glucose, 20 μM | 50 |
| L-Alanine, 6 μM + glucose, 5 μM | 70 |
| L-Alanine, 12 μM + glucose, 5 μM | 80 |
| L-Alanine, 20 μM + glucose, 5 μM | 90 |
| L-Alanine, 6 μM + adenosine, 24 mμM | 50 |
| L-Alanine, 18 μM + adenosine, 24 mμM | 70 |
| L-Alanine, 12 μM + glucose, 10 μM | 86 |
| L-Alanine, 24 μM + glucose, 10 μM | 97 |
| L-Alanine, 30 μM + glucose, 10 μM | 100 |

A heat-shocked suspension of *B. globigii* spores containing 1 mg of spores in 5 ml of 0.067 M phosphate at pH 7.3 was incubated with a series of supplements at 30 C. After 30 minutes the per cent germinated spores was determined by the uptake of 1 per cent aqueous methylene blue and by the change in light transmission of the suspension.

per cent decrease in viable count of *B. terminalis* spores without the loss of enzyme activity. Although more than 40 per cent loss of viability of *B. globigii* occurred, there was no observable enzyme activity in the extracts. A more complete rupture (93 per cent) of the latter was obtained by grinding the spores in a mortar and pestle for four hours, using dry ice as an abrasive. Again the extracts were negative for alanine racemase activity. Addition of pyridoxal phosphate was without effect, indicating the absence of the apoenzyme in the spore. Although spores of *B. globigii*

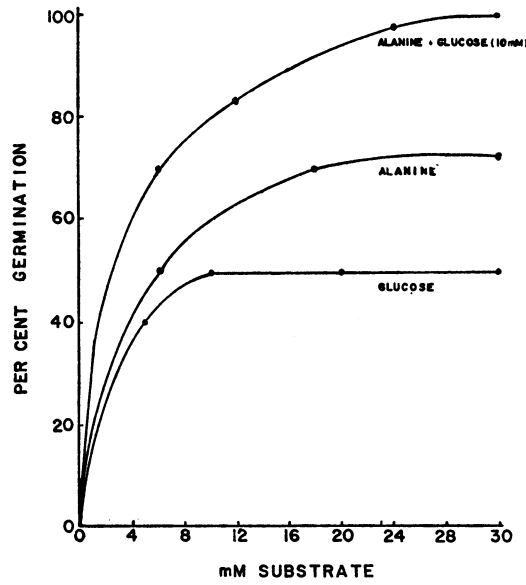


*Figure 2.* The effect of glucose and L-alanine on the germination of spores of *Bacillus globigii*. One mg of heat-shocked spores was suspended in 5 ml 0.067 M phosphate, pH 7.3, at 30 C with various supplements. Suspension was incubated until maximal germination occurred.

lack the racemase enzyme, their germination by L-alanine is inhibited by D-alanine as are the spores of *B. terminalis*. The germination of *B. globigii* spores in the presence of glucose is not inhibited by D-alanine but rather is slightly stimulated. Preliminary attempts to demonstrate any appreciable disappearance of L-alanine in the presence of *B. globigii* spores have been unsuccessful thus far.

*Separation of alanine racemase activity from germination in spores of B. terminalis.* Although the previous results suggest that germination does not require alanine racemase activity, a conclusive separation can be established only in a system where both are initially operative. Attempts to achieve such a separation in *B. terminalis* were undertaken by varying the pH. The buffers that were found to be noninhibitory for either germination or the racemase enzyme and their effective buffering range are listed in table 4. The activities of both systems were checked with two different buffers for each pH determined.

The rate of alanine racemase activity for the intact spores was measured at various pH's by the static assay method previously described. In each case the final pH was adjusted to 8.3 for the

TABLE 3

*A survey of the optimal conditions for germination and the alanine racemase activity of clean bacillus spores*

| SPORES | CONDITIONS FOR GERMINATION | | | | GERMINATION | RACEMASE ACTIVITY $Q_n$* |
|---|---|---|---|---|---|---|
| | Phosphate | Adenosine | L-Alanine | Glucose | | |
| | | | | | % | |
| *B. terminalis* | 67 mM pyro | 12 mμM | 6 μM | | 100 | 84 |
| *B. cereus* | 30 mM $PO_4$ | 12 mμM | 12 μM | | 55 | 45 |
| *B. globigii* | 30 mM $PO_4$ | | 12 μM | 10 μM | 100 | 0 |
| *B. polymyxa* | 30 mM $PO_4$ or 67 mM pyro | 12 mμM | 12 μM | | 35 | 15 |
| *B. subtilis* | 30 mM $PO_4$ | | 2 μM | 5 μM | 35 | 31 |
| *B. megaterium* | 30 mM | | | 5 μM | 90 | 24 |
| | | | 12 μM | | 70 | |

* μM alanine/mg/hr.

Heat-shocked spores were incubated in phosphate medium, pH 7.3, at 30 C for 60 minutes in the presence of various supplements. The per cent germinated spores was determined by the change in light transmission of the suspension and microscopically by the uptake of 1 per cent aqueous methylene blue. Alanine racemase activity was measured manometrically employing the D-amino acid oxidase assay.

TABLE 4

*Composition of noninhibitory buffers used in germination studies and in the assay of racemase*

| BUFFER* | pH RANGE |
|---|---|
| M/5 acetate<br>0.02 N veronal | 3.4–4.5 |
| +M/5 acetate | 4.0–9.0 |
| M/15 pyrophosphate | 6.1–9.0 |
| M/10 arginine | 7.0–11.8 |
| M/10 creatine | 7.0–12.4 |

* 0.067 M phosphate included in each buffer.

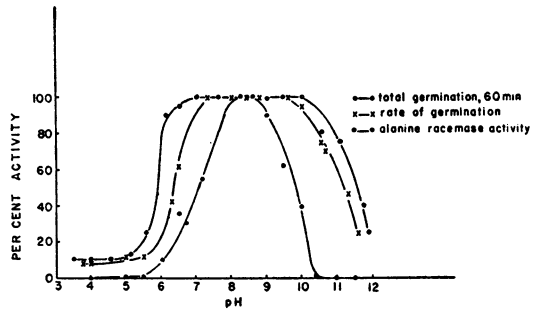


*Figure 3.* The effect of pH on the germination and alanine racemase activity of spores of *Bacillus terminalis*. (See text for details.)

TABLE 5

*Effect of pH on the alanine racemase activity and germination of spores of Bacillus terminalis*

| pH | GERMINATION | RATE OF GERMINATION* | ALANINE-RACEMASE ACTIVITY $Q_n$† |
|---|---|---|---|
| | % | | |
| 8.5 | 100 | 100 | 130 |
| 10.5 | 90 | 73 | 1.1 |
| 11.3 | 73 | 52 | 0 |

* Calculated from the refractive index change during first five minutes. Control rate set at 100.

† μM alanine/mg/hr.

Heat-shocked spores were suspended in buffer, 12 mμM adenosine and 6 μM L-alanine at 30 C. The per cent germination after one hour was determined microscopically and by the change in refractive index of the suspension. Alanine racemase activity was determined by measuring the D-alanine concentration with D-amino acid oxidase.

measurement of D-alanine. Two criteria were chosen to determine the relationship between germination and pH: the percentage of spores which germinate after one hour and also the rate of germination, as measured by the change in optical transmission, during the first 5 minute period. The results obtained are shown in figure 3. Inspection of this curve indicates that the activity of the racemase enzyme is more sensitive to the higher pH's than is germination. In order to establish germination more adequately in the absence of racemase activity, the experiments at the higher pH ranges were repeated using a 100-fold increase in the spore concentration (10 mg per ml). The results shown in table 5 clearly demonstrate the germination of spores in

L-alanine at pH 11.3 in the absence of alanine racemase activity.

## DISCUSSION

The results reported here and elsewhere have demonstrated that spores of aerobic bacteria can be germinated rapidly in the presence of various combinations of L-alanine, glucose, and adenosine. The mechanism whereby these compounds initiate germination is obscure, however, largely due to our ignorance of the biochemical activities of spores.

Hardwick and Foster (1952) have shown that 17 enzymes which are present in the vegetative cell are absent from the homologous spores. It would seem reasonable to assume that this apparent inertness represents a lack of sufficiently sensitive test systems rather than a paucity of enzymes in spores.

Although numerous claims have been made for the presence of enzymes in spores, only three enzymes have been reported in spores that are sufficiently clean to preclude possible contamination from vegetative debris. These are a diaphorase-like enzyme (Spencer and Powell, 1952), alanine racemase (Stewart and Halvorson, 1953), and catalase (Lawrence and Halvorson, 1954). Of these enzymes, only alanine racemase acts directly on one of the compounds which stimulates germination. It seemed necessary, therefore, to determine whether or not the activity of this enzyme was essential for the germination process. The results presented in this paper indicate that the germination of spores from aerobic bacteria in the presence of L-alanine is independent of the activity of the alanine racemase enzyme. The often-observed inhibition by D-alanine was shown not to be due to an inhibition of the racemase enzyme. This enzyme obtained either from extracts or intact spores of *B. terminalis* produced racemic mixtures starting with either D- or L-alanine. A survey of the requirements for germination and the distribution of the alanine racemase enzyme showed that spores of *B. megaterium*, although they contain the racemase enzyme, germinate in the absence of alanine. On the other hand, *B. globigii* spores, which lack this enzyme, are stimulated to germinate by L-alanine. A more significant separation was observed in spores of *B. terminalis* which require alanine for germination and also contain the racemase enzyme. At a pH of 11.3, 70 per cent of the spores germinated in one hour under conditions in which the enzyme was completely inactive.

Although these results tend to eliminate the need of alanine racemase for germination, they do not explain the role of L-alanine in the process. The ability of glucose to replace L-alanine and the small but significant activity of pyruvate, lactate, glycerophosphate, and ribose (Powell, 1951) suggested the possibility that the active agent may be a triose which is derived from both alanine and glucose. That alanine itself might be the intermediate formed from glucose is made unlikely not only by the failure to detect alanine in the medium during glucose stimulated germination but also by the failure of D-alanine to inhibit this germination.

Some recent observations by Harrell and Halvorson (1954) suggested that L-alanine may directly or indirectly play a catalytic role in the germination. Following a 45 second exposure to alanine at pH 8.5 and subsequent washing, over 40 per cent germination was observed upon continual incubation in the absence of alanine. Experiments are in progress employing $C^{14}$ labeled alanine in an attempt to reveal the role of alanine during this period.

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## SUMMARY

A method for the preparation of reasonably clean spores from 6 species of aerobic spore forms is described.

The enzyme, widely distributed in spores, which converts L-alanine to D-alanine was shown to be reversible, giving racemic mixtures from either L- or D-alanine.

The germination requirements and the distribution of the alanine racemase enzyme were determined. Four strains were found to possess the enzyme and also require L-alanine for germination. *Bacillus megaterium* has an active racemase enzyme but will germinate in the presence of glucose, whereas *Bacillus globigii* requires L-alanine for germination but lacks the enzyme.

The pH curve for alanine racemase activity and the germination of *Bacillus terminalis* spores were determined. At pH 11.3 the enzyme was inactive but 70 per cent of the spores germinated in one hour.

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