GERMINATION PROPERTIES OF SPORES WITH LOW DIPICOLINIC ACID CONTENT

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ABSTRACT

KEYNAN, A. (University of Wisconsin, Madison), W. G. MURRELL, AND H. O. HALVORSON. Germination properties of spores with low dipicolinic acid content. J. Bacteriol. 83:395–399. 1962.—When the dipicolinic acid content of spores of Bacillus cereus strain T is reduced from 7.5 to 2 or 3%, the spores germinate spontaneously after heat activation and are sluggish in their response to L-alanine and other germination agents. Only germination initiated by calcium dipicolinic acid is unaffected. L-Alanine-induced germination is stimulated by exogenous dipicolinic acid. These results support the hypothesis that endogenous dipicolinic acid regulates the L-alanine-triggered germination.

Dipicolinic acid (DPA) is associated in bacteria exclusively with the dormant spore. This compound is absent in vegetative cells, is synthesized during sporulation, and is excreted from the spore during germination. These correlations, as well as its high content in spores, have led investigators to assign various roles to DPA in the physiology of the spore. Of particular interest was the recent finding by Riemann and Ordal (1960) and Riemann (1961) that exogenous DPA in the presence of calcium can serve as a germination stimulant. They proposed that endogenous DPA and calcium participated in this process, although no direct evidence was available. A possible involvement of endogenous DPA in L-alanine-induced germination was suggested by the finding that L-alanine dehydrogenase was driven by a DPA-stimulated reduced diphenoylpyridine nucleotide (DPN) oxidase (O’Connor and Halvorson, 1961b). L-Alanine-induced germination was found to be dependent upon the activity of the dehydrogenase (O’Connor and Halvorson, 1961a).

If DPA plays an integral role in the germination process, then one would expect that this process might be altered in spores with a low DPA content (hereafter referred to as “low DPA spores”). The demonstration in recent years that the DPA content can be varied by controlling the conditions of sporulation has provided a method for further examination of this relationship. The present paper is an analysis of the germination properties of low DPA spores of Bacillus cereus strain T.

MATERIALS AND METHODS

Production of low DPA spores. Two methods of producing low DPA spores were employed. First, spores were grown endotrophically in the presence of L-phenylalanine (Church and Halvorson, 1950); second, regular G medium, in which calcium was withheld, was used (Slepecky and Foster, 1959; Black, Hashimoto, and Gerhardt, 1960). No difference was observed in the properties or germination behavior of spore suspensions prepared by the two methods.

1) P spores:—B. cereus strain T was grown in G medium (Church, Halvorson, and Halvorson, 1954) until the appearance of granulated forms. The cells were removed aseptically by centrifugation, washed twice with water, and then resuspended in one-tenth their original volume in 0.006 M phosphate buffer (pH 7.3) containing 1 mg/ml L-phenylalanine. Free spores were produced after incubation for 30 hr on a shaker at 30 C.

2) A spores:—These spores were grown on regular G medium from which calcium was omitted. The spores were harvested after 30 hr growth on a shaker at 30 C.

Both suspensions were washed six times with distilled water in the cold. During this procedure both preparations showed some tendency to
germinate spontaneously. In the suspensions employed in the subsequent experiments, microscopic examination showed 5% germinated forms in the P spores and 10% germinated forms in the A spores. These suspensions, however, were stable, since no further germination was observed during subsequent storage at 2 C for several months. The P spores contained 2.3% dry wt of DPA; the A spores contained 3% dry wt of DPA. The normal DPA content for this strain, grown on regular G medium, is about 7%.

Microscopic determination of germination. Heat-fixed films of spores were stained with an 0.1% aqueous solution of crystal violet for 5 min, rinsed with water, wet mounted, and observed under oil immersion with a phase contrast microscope. With this procedure, the deeply stained, nonrefractile, germinated forms could easily be distinguished from the unstained, refractile spores. For estimating percentage germination, 500 to 1,000 cells were counted.

Measurement of germination by changes in optical density. Germination was also followed by measuring the changes in optical density at 625 mµ, either with a Spectronic 20 colorimeter, or with a Beckman DK-2 recording spectrophotometer.

Incubation conditions. Spore suspensions were heat activated as indicated, centrifuged, and resuspended in 5 ml of 0.02 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 8). DPA-induced germination was carried out at 20 C; all other experiments were incubated at 30 C. The germinating agents were added after a 15-min incubation period. In all cases, a control suspension of spores in buffer was included to measure spontaneous germination.

Chemicals. DPA was obtained from the Aldrich Chemical Co., Milwaukee, Wis. All other chemicals were obtained from standard commercial sources.

RESULTS

Decrease in optical density of low DPA spores during germination. Preliminary experiments with low DPA spores indicated that the correlation usually observed between percentage germination, as measured microscopically, and the decrease in optical density did not hold for these spores. This is illustrated in Fig. 1; both normal and low DPA spores were germinated in the presence of CaDPA. Under such conditions, CaDPA induced a final decrease in optical density of 25% with low DPA spores, whereas a 55% decrease occurred with normal spores. When both preparations were examined microscopically at the end of the experiment, total germination was found in both cases. Possibly, low DPA spores are less refractile than spores with normal DPA levels. Similar results were observed when other germinating stimulants were employed. In all subsequent experiments, therefore, germination of low DPA spores was measured microscopically as described in Materials and Methods.

Germination requirements of low DPA spores. Heat-shocked spores of B. cereus strain T normally germinate completely in the presence of trace quantities of L-alanine. Under similar conditions, only 25 to 30% germination of low DPA spores was observed. Elevated concentrations of L-alanine or the addition of adenosine, glucose, or manganese did not increase the extent of germination. A summary of the effect of a variety of substances, known to induce the germination of normal spores, on the germination of low DPA spores is shown in Table 1. Complete germination is observed only in the presence of CaDPA. As described by Riemann and Ordal

FIG. 1. Decrease in optical density in low and normal DPA spores during CaDPA-induced germination. Changes in optical density of suspensions of normal and P spores followed in a Beckman DK-2 recording spectrophotometer at 625 mµ. The sample cuvette contained: 1 ml of a spore suspension in 0.02 M tris buffer (pH 8), 0.4% Difco gelatin, 0.3 ml of an 0.4 M CaCl₂ solution, and 0.5 ml of an 0.2 M DPA solution. The reference cuvette contained 0.02 M tris buffer. Temperature of incubation, 20 C.
(1960) and Riemann (1961), only equimolar concentrations are active; altering the ratio of Ca++ to DPA retards the rate of germination. L-Cysteine was a more effective germinating agent than L-alanine.

Reduction in the DPA content of spores may have a specific effect on the mechanism of germination. This is suggested by the finding that nearly complete germination is observed when low DPA spores are incubated in complex medium or in the presence of five germinating stimulants.

Heat activation. Heat activation has long been recognized to increase the rate of germination of aerobic spores and to simplify the germination requirements. In freshly prepared spores, often several hours of heat activation at 65 C were found necessary to achieve optimal germination. It was, therefore, of interest to examine the effect of heat activation on low DPA spores. In the control suspensions of low DPA spores, only 17% germination was observed after 1 hr incubation in the presence of L-alanine. After a 15-min heat shock at 65 C, 3% of the spores germinated in the same period of time; this rate could not be increased by further heat treatment.

When spores were heated for periods of 30 and 45 min, they displayed the phenomenon of spontaneous germination. An example of this is shown in Fig. 2. Spontaneous germination in B. megaterium has been previously described by Powell and Hunter (1955), but has not thus far been observed in B. cereus. Spores with normal levels of DPA did not spontaneously germinate, even after heat shock for several hours at 65 C; however, some spontaneous germination was observed after the spore suspensions had been stored for 2 to 3 weeks at 2 C.

Low DPA spores have been observed to be more sensitive to heat inactivation than normal spores (Church and Halvorson, 1959; Black et al.,

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The table below lists the germination requirements of low DPA spores:

<table>
<thead>
<tr>
<th>Simple substances</th>
<th>Germination</th>
<th>Complex media</th>
<th>Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine, 1 mg/ml</td>
<td>30</td>
<td>Hyatt and Levinson (1957) germination medium</td>
<td>30</td>
</tr>
<tr>
<td>L-Alanine, 5 mg/ml</td>
<td>26</td>
<td>Same + 1 mg/ml adenosine, 1 mg/ml cysteine, and 1 mg/ml tyrosine</td>
<td>80</td>
</tr>
<tr>
<td>L-Alanine, 1 mg/ml + adenosine, 1 mg/ml</td>
<td>29</td>
<td>Same + spore extract</td>
<td>85</td>
</tr>
<tr>
<td>Cysteine, 4 mg/ml</td>
<td>65</td>
<td>Tryptose, 0.5% (Difco)</td>
<td>88</td>
</tr>
<tr>
<td>CaDPA, 0.04 u</td>
<td>100</td>
<td>Brain-heart infusion, 0.5% (Difco)</td>
<td>89</td>
</tr>
</tbody>
</table>

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* P spores were heat shocked for 15 min at 65 C, centrifuged, and resuspended in 0.02 M tris buffer (pH 8) to an optical density of 0.60. The final volume was 5 ml. Germination was measured microscopically after 45 min incubation at 30 C and corrected for germinated cells present before addition of the germinating agent. No spontaneous germination was observed in the control suspensions.

* Medium contained (g/liter): NaH2PO4, 6; K2HPO4, 6; NaCl, 1; MgCl2, 0.1; glucose, 10; L-alanine, 0.4; MnCl2, 0.08; water to 1 liter.

* Sonic-treated spore extract from 1 g of spores in 10 ml of phosphate buffer; diluted 1:100 with water.

* Germination at 20 C.

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**Fig. 2. Heat-induced germination in low DPA spores. A suspension of P spores, 0.85 optical density, was heat activated in water at 65 C for the times indicated. The suspensions were centrifuged, resuspended in 0.02 M tris buffer (pH 8.5), and incubated at 80 C. Germination was followed microscopically as described in Materials and Methods.**
FIG. 3. Kinetics of DPA stimulation of L-alanine-induced germination. "A" spores were heat shocked for 15 min at 65 C and resuspended in 0.02 M tris buffer (pH 8) containing the following additions: (A) 10 mg/ml L-alanine and 4 × 10^{-3} M DPA; (B) 10 mg/ml L-alanine; (C) 4 × 10^{-3} M DPA; and (D) 10 mg/ml L-alanine, 15 mg/ml d-alanine, and 4 × 10^{-3} M DPA. Incubation temperature, 30 C. Germination was measured microscopically as described in Materials and Methods.

1960). The phenomenon of spontaneous germination could not be attributed to the death of a few cells and the liberation of germinating stimulants, since, under conditions of heat treatment at 65 C reported here, there was no detectable decrease in the viable count of either normal or low DPA spores.

Stimulation of L-alanine-induced germination by DPA. L-Alanine-induced germination has been shown to be dependent upon the activity of L-alanine dehydrogenase (O'Connor and Halvorson, 1961a). Since deamination is driven by a DPA-stimulated DPNH oxidase (O'Connor and Halvorson, 1961b), it seemed reasonable that the poor germination in the presence of L-alanine was due to reduced recycling of DPN required for deamination. If a reduced DPNH oxidation is limited by the low content of DPA, then one would expect that exogenous DPA should stimulate the rate of L-alanine-induced germina-

<table>
<thead>
<tr>
<th>Germination agent</th>
<th>Germination of low DPA spores</th>
<th>Germination of normal spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine, 1 mg/ml</td>
<td>10%</td>
<td>98%</td>
</tr>
<tr>
<td>DPA, 0.04 μM</td>
<td>9%</td>
<td>0%</td>
</tr>
<tr>
<td>L-Alanine, 1 mg/ml + DPA, 0.04 μM</td>
<td>37%</td>
<td>98%</td>
</tr>
<tr>
<td>L-Alanine, 1 mg/ml + DPA, 0.04 μM + d-Alanine, 2 mg/ml</td>
<td>7%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Spores were heat activated for 15 min, resuspended in 0.02 M tris buffer (pH 8). Low DPA spores are P spores (see Materials and Methods), and normal spores have a 7.5% dry wt intrasporal DPA concentration. Germination was measured microscopically after 15 min incubation with germination agents at 30 C, and corrected for initial germination.

This hypothesis is supported by the results shown in Fig. 3. DPA at concentrations of 4 × 10^{-3} M dramatically stimulated the rate and amount of L-alanine-induced germination, whereas DPA alone had no effect after incubation for 1 hr at 30 C.

That the DPA stimulation is operating at the level of the L-alanine trigger mechanism is indicated by the results shown in Fig. 3 (curve D) and in Table 2. Germination in the presence of DPA and L-alanine in low DPA spores is inhibited by d-alanine. In spores with normal DPA content, d-alanine is a competitive inhibitor of L-alanine-induced germination but not of CaDPA-induced germination (Keynan and Halvorson, 1962).

DISCUSSION

These results further illustrate the significance of DPA in the dormancy (Keynan, Murrell, and Halvorson, in press) and germination of bacterial endospores. When the DPA content of the spores is reduced by 60 to 70%, the spores are more heat sensitive (Church and Halvorson, 1959), sluggish in their response to L-alanine, and have a tendency to germinate spontaneously. On the other hand, germination in the presence of CaDPA is unaffected, suggesting that exogenous DPA is replacing a normal function of endogenous DPA.

The present findings pose some problems on the
concept of dormancy in bacterial spores. The term dormancy is usually employed to describe both the failure of spore preparations to respond to germinating stimulants and the physiological state in which the mechanism responsible for germination is inhibited. Based on the experiments described in the present paper, this generalization is not justified, since the degree of dormancy observed in a suspension of spores differs depending upon the germinating agent employed. Compared to spores with normal DPA levels, low DPA spores are less dormant when measured by the criterion of heat-induced germination, are more dormant by the criterion of L-alanine-induced germination, and are equally dormant when CaDPA is used as the test system. These and other findings (Keynan et al., in press) indicate that the concept of dormancy, as used to describe a general property of a suspension of spores, is not valid and must be restricted to be a specific germination condition. These observations could result from at least two different mechanisms. First, there may be more than one enzymic mechanism responsible for germination, some of which are more dormant in the intact spore than others. Second, germination may be initiated by a single mechanism. Various germinating agents activate different steps of the metabolic chain responsible for germination; only the first step in this sequence is subject to the control of dormancy.

Although little is known concerning the biochemical nature of CaDPA-induced germination, the present experiments support the suggested role of DPA in L-alanine-induced germination acting via the L-alanine dehydrogenase (O’Connor and Halvorson, 1961a). Internal DPA appears to be responsible for several functions in the physiology of the spore. At high concentrations it retains the dormancy to spontaneous or heat-induced germination, but supports L-alanine-triggered germination. A DPA content of 2.3% is apparently insufficient to maintain the dormant state.

ACKNOWLEDGMENTS

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LITERATURE CITED


