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Preliminary Report

Possible Involvement of Sporangial Cytoplasm as a Biosynthetic Site in Dipicolinic Acid Formation by *Bacillus subtilis*

It is known that dipicolinic acid (DPA) is a unique constituent of bacterial spores and the formation of this compound is one of the most dramatic chemical changes occurring during sporulation. Although much attention has been directed towards the state and formation of DPA, only a very little information is available about the intracellular site for DPA biosynthesis, whose knowledge would facilitate the approach to the problems of the physiological function of this compound. Since DPA appears only after the formation of forespores and is not found associated with the vegetative fractions during the lysis of the sporulating cells (Halvorson, 1957), it has been assumed that DPA may be synthesized within the forespore (Halvorson and Howitt, 1961). However, while studying the intracellular distribution and the biosynthesis of DPA in *Bacillus subtilis* particularly in the initial stage of sporulation, we have obtained evidence suggesting a close association of DPA biosynthesis with the cytoplasm surrounding the developing spore.

One loopful of cells of *Bacillus subtilis* NRRL 558 (lysozyme sensitive strain) was inoculated into 100 ml of peptone broth medium and incubated at 35°C on a reciprocal shaker for 4 hours. Five ml of this culture was inoculated into 100 ml of fresh peptone medium and incubated with shaking at 35°C for one hour. The latter treatment was repeated three times and finally, one ml of the culture was transferred into new medium. Under these conditions, sporulation was initiated after approximately 16 hours incubation. Cells at various stages of sporulation of the culture were then collected by centrifugation at 8,000 rpm for 20 minutes in the cold after different time intervals and washed twice with chilled distilled water. The cell suspension in M/75 phosphate buffer (pH 7.0) containing 10 μgs/ml of $MgCl_2$ and 50 μgs/ml of lysozyme ($\times$ 3 crystallized, Sigma Co.) was incubated at 37°C for 15 minutes and the lysates were centrifuged at 12,000 rpm for 30 minutes in the cold. The supernatant (S_1) and residue (P_1) fractions thus obtained were used for measuring both the DPA content and the oxidative activity for α, ε-diketopimelic acid (a possible intermediate in DPA biosynthesis, as reported by Powell and Strange (1959)). The content of DPA was determined by the method of Janssen *et al.* (1958) and the diketopimelic acid oxidative activity was measured by the method of Powell and Strange (1959).

Figure 1 shows the intracellular distribution of DPA in this strain of *Bacillus subtilis* during sporulation. It will be seen that, during the initial stage of sporulation (after 16 to 18 hours incubation), DPA is detectable only in the soluble fraction (S_1) and the content rapidly decreases with increase in the content of DPA in the residue fraction (P_1). In contrast, no DPA is detectable in the supernatant derived from the cells after 22 hours incubation, when almost maximal sporula-

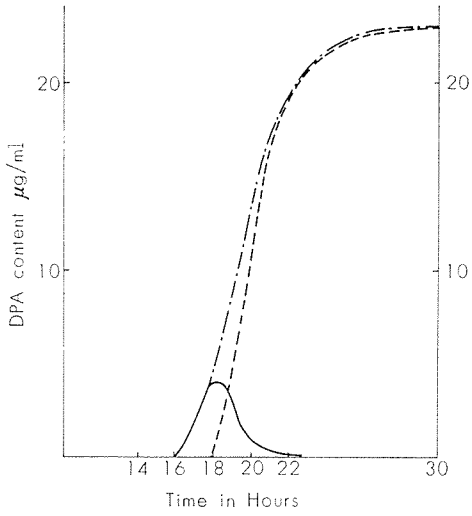

Fig. 1. Intracellular Distribution of DPA in Cells during Sporulation

tion takes place. Similar results were also obtained when the cells were incubated with lysozyme in a hypertonic solution of Carbowax 5,000 (Polyethylene glycol) and the protoplasts obtained were lysed gently by lowering the tonicity. Incidentally, a recent experiment (Kondo, 1963) has shown that the P_1 fraction, which was obtained during the initial stage of sporulation and contained no detectable DPA, is mainly composed of spore-like bodies which presumably correspond to forespores and that the spore-like bodies remain viable after lysozyme treatment

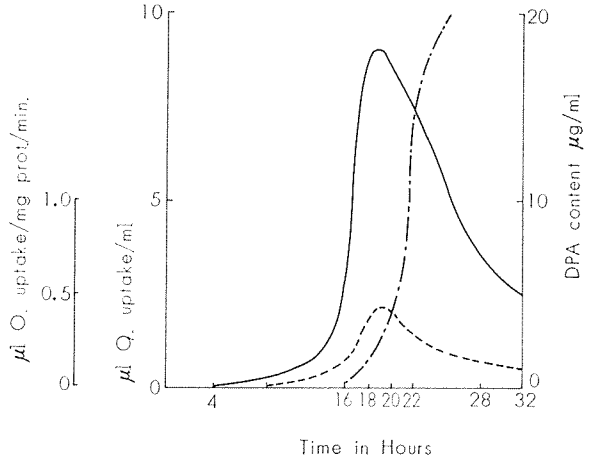

Fig. 2. Kinetics of the Distribution of α, ε-diketopimelic Acid Oxidative Activity in Cells during Sporulation

in a medium of low tonicity. This indicates the lysozyme insensitive nature of the bodies and further suggests that no serious damage of their surface structure occurs by the action of this enzyme. If such damage occurred, it would subsequently cause the release of DPA from intact spore-like bodies into the soluble fraction (S_1), assuming that DPA is present inside these bodies.

The changes in the distribution of the α, ε-diketopimelic acid oxidation system in cells are illustrated in Figure 2. As is seen in this figure, the oxidative activity is found in the soluble fraction (S_1) and not in the residue fraction (P_1) during all stages of sporulation. In addition, all attempts have so far failed to detect diketopimelic acid oxidative activity in mechanical disintegrates of the P_1 fraction, suggesting the absence of the oxidation system in the developing spore. Of particular interest was the observation that lysozyme lysates of the vegetative cells, particularly in the exponential growth phase did not show any measurable activity and the activity suddenly emerged just before the onset of sporulation.

Assuming that the site for DPA biosynthesis is located outside the developing spore, as suggested from the results described above, one would naturally expect that the degradation of the cell structure could profoundly affect DPA biosynthesis by the sporangium. Therefore, an experiment was done to see the effect of lysozyme on DPA formation of a sporulating culture of this organism. It was found that, when lysozyme was added to sporulating cultures at various stages, the further synthesis of DPA was immediately and almost completely inhibited. The result is shown in Figure 3.

Although it is still uncertain whether the surface structure of the sporangium

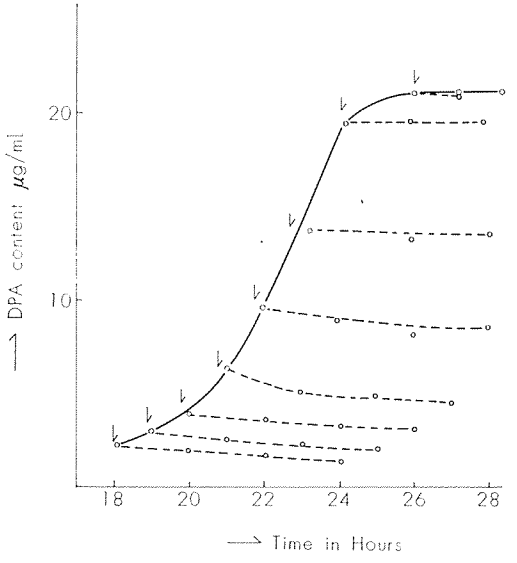

Fig. 3. Inhibition of DPA Synthesis by the Addition of Lysozyme
Arrows show the times of addition of lysozyme to the sporulating culture

is directly involved as a site for DPA biosynthesis in this organism, these results clearly suggest that, in the initial stage of sporulation, DPA may be mainly synthesized outside the developing spore (presumably forespore) and then incorporated into the spore or at least that the cytoplasm surrounding the developing spore may be required for DPA biosynthesis. The details of this study will be published elsewhere.

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Masaomi Kondo*
Yoshifumi Takeda*
Masahiko Yoneda**

* *Department of Bacteriology and Serology,*
** *Department of Tuberculosis Research I.*
The Research Institute for Microbial Diseases,
Osaka University, Osaka, Japan.
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