JOURNAL OF BACTERIOLOGY, Apr. 1973, p. 455-456 Vol. 114, No. 1
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Dipicolinic Acid Location in Intact Spores of *Bacillus megaterium*

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Received for publication 19 December 1972

Beta-attenuation analysis of intact spores of *Bacillus megaterium* containing tritium-labeled dipicolinic acid has shown that dipicolinic acid is located in the spore protoplast and not in the cortex.

Since its discovery as a unique and major component of bacterial spores, dipicolinic acid (DPA) has been implicated in the remarkable resistance of spores to environmental hazards (13). To understand the physiological role of DPA, much effort has been expended to determine its location within the spore. Some experiments have suggested a core location for DPA (4, 7, 10, 15, 17); others favor a cortical location (6, 8, 9, 12, 14); some additional work tentatively excludes the coats (1) and the exosporium (3) as sites of DPA. This great ambiguity stems from the fact that in none of these experiments was DPA location directly determined using intact spores. The spores were physiologically, chemically, or mechanically altered, and DPA location was assigned by inference.

To resolve the DPA controversy, we have developed the method of beta-attenuation analysis, which should permit the in vivo localization of DPA (11). This method depends on the fact that a certain fraction of beta emissions from intracellular tritium-labeled compounds is completely absorbed by spore tissue before they can escape and be detected. The closer the isotope is to the center of the spore, the greater the fraction of radiation which will be absorbed.

To determine the dependence of the attenuation on the tritium location, spores of strain M-46, a double auxotroph of *Bacillus megaterium* (Lys^-, DAP^-) (5) were prepared with a tritium-labeled marker for each structural region: ^3H-uracil for core nucleic acids; ^3H-α,ϵ-diaminopimelic acid for cortical mucopeptide; and ^3H-L-lysine added late in sporulation for outer protein coats. Counting rates for the intact spores were compared with the total incorporated radioactivity (determined by counting digested spores). The reduction in counting rate in the intact spores was 26, 18, and 3% for uracil, diaminopimelic acid, and lysine, respectively (11). The location of ^3H-DPA within suitably labeled spores could then be determined by comparing its attenuation value with the standards.

Tritium-labeled dipicolinic acid was prepared by the spontaneous conversion of α,ϵ-diketopimelic acid to DPA in the presence of ammonia (16). The ^3H-α,ϵ-diketopimelic acid was generated in situ by the action by L-amino acid oxidase from *Neurospora crassa* (2) on uniformly labeled ^3H-α,ϵ-diaminopimelic acid. The reaction mixture contained 0.29 mCi of ^3H-α,ϵ-diaminopimelic acid per ml, 0.043 M ammonium acetate, 0.2 mg of catalase per ml, and 0.029 M imidazole buffer (pH 7). The reaction was allowed to proceed for 24 h in an O_2 atmosphere, with gentle swirling in a 37 C water bath. After the addition of carrier potassium dipicolinate and acidification to 3 N in H_2SO_4, the DPA was extracted into ether and subsequently recrystallized from hot water. A minor radioactive contaminant was removed by anion exchange chromatography in Bio-Rad Ag-1X2 resin (acetate form); after this final purification, the radioactivity profile of column fractions coincided with absorbance at 270 nm. Nuclear magnetic resonance studies of solutions of DPA in heavy water showed that the aromatic protons, which would be labeled with tritium, are stable with respect to deuterium exchange at physiological pH and temperature over a period of weeks.

Strain M-46 was grown on minimal FCG medium (5) containing α,ϵ-diaminopimelic acid and lysine; ^3H-DPA and calcium chloride were added 1 h after vegetative growth ceased. Spores were harvested, purified on a Renogra-

fin gradient, washed, and disaggregated by brief sonic treatment as described previously (11). The counting rates for the intact spores and samples that were digested by lysozyme are presented in Table 1. In a separate experiment it was confirmed that the DPA is quantitatively released as a result of lysozyme treatment.

A value of 33% was obtained for the diminution of the counting rate for the ^{3}H-DPA-labeled spores. This high value is to be contrasted with the 18% beta attenuation noted for the cortical marker, diaminopimelate. Clearly, DPA cannot have a cortical location. It is of some interest that the attentuation noted for the ^{3}H-DPA is even greater than that observed for the uracil which was selected to be the core marker. This may reflect on the suitability of uracil as a core marker. There have been reports of mother-cell cytoplasm being trapped between the outer membrane of the forespore and the coat proper (13). Alternatively, it may indicate that the core nucleic acids have a more peripheral location than the core DPA.

It is important to reiterate that the *B. megaterium* spores remained viable in the Bray's solution that was used for determining the counting rate (11). This means that the assignment of localization by the beta-attenuation method has been made without perturbing the ability of the spores to germinate and form colonies.

These data do not generate any hypothesis bearing on the physiological role of DPA. However, one can at least eliminate from consideration speculations that would place the bulk of the DPA in the cortical, coat, or exosporial regions of the spore.

TABLE 1. *Beta-attenuation values for dipicolinic acid-labeled spores*[a]

Determination	Spore counting rate (counts/min)				Beta attenuation (%)
	Intact		Disrupted		
	Sample 1	Sample 2	Sample 3	Sample 4	
1	479	461	698	700	32.8
2	397		595	596	33.4

[a] The specific activity of the ^{3}H-DPA used was 4.3 $\times$ 10^{4} counts per min per μmol. About 10^{7} spores were used in each determination. Dispersal of the clumped spores was verified by quantitative microscope evaluation in a Petroff-Hauser counter.

This investigation was supported by Public Health Service grant AM-09058 from the National Institute of Arthritis and Metabolic Diseases.

We thank Renee Gruber for her technical assistance.

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