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Dipicolinic Acid-less Mutants of *Bacillus cereus*

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When J. Powell (Biochem. J. **54**:210, 1953) established dipicolinic acid (DPA) as an important constituent of bacterial spores, microbiologists became concerned about the function this compound serves and about its biosynthetic pathway. It is now generally considered that DPA is involved in the heat resistance of spores, but the mechanism is unknown. A number of schemes have been proposed for its biosynthesis. J. Powell and R. E. Strange (Nature **184**:878, 1959) proposed α,ϵ-diketopimelic acid as a precursor with dihydrodipicolinic acid (DHDPA) as an intermediate that could be oxidized to DPA. However, when they found that DHDPA could be oxidized to DPA nonenzymatically in air, they had some doubts as to the validity of this pathway.

H. H. Martin and J. W. Foster (J. Bacteriol. **76**:167, 1958) suggested, and Y. Yugari and C. Gilvarg (J. Biol. Chem. **240**:4710, 1965) published supporting evidence for, the involvement of the lysine pathway in the synthesis of DPA. In this pathway, DHDPA is an intermediate that could yield DPA through oxidation.

Srinivasan (*unpublished data*), working in Halvorson's laboratory in Urbana, Ill., found that *Bacillus cereus* growing in a medium containing ethyloxamate produced spores devoid of DPA, and from such cultures he isolated a compound that he thought might be an intermediate in the synthesis of DPA. He believed this compound was formed through condensation of acetoacetyl coenzyme A with alanine and that methylpicolinic acid was then formed by cyclization. This is another possible pathway.

Since we still lack convincing evidence as to the actual pathway, we felt it would be helpful to find mutants of spore-formers that lack the ability to synthesize DPA. The fact that DPA-deficient spores can be produced by use of inhibitors, encouraged us to try to find such mutants.

We first subjected cultures of *B. cereus* to a variety of mutagenic agents and then allowed the cultures to grow until they formed spores. We treated these cultures with octyl alcohol to kill the vegetative cells and made spread plates of the survivors. When an examination of several colonies indicated that spores had been formed, we made replicate plates with velvet pads; first to one plate, and then after exposing the pad to a heat lamp to kill heat-sensitive colonies, another replicate plate was made. After growth, we examined the paired plates and looked for colonies that appeared only on plates made from the unheated pad. We found a number of such colonies, but we were apparently selecting for late spore-formers rather than mutants that formed heat-sensitive spores. After spending considerable effort over a period of several months with negative results, we abandoned this procedure.

H. Tamir and C. Gilvarg (J. Biol. Chem. **241**: 1085, 1966) reported a method for isopycnic gradient centrifugation of spores and showed that *B. megaterium* spores produced in calcium-deficient sporulation media were lighter in density than normal spores. We assumed that mutants which could not synthesize DPA would produce spores which also lack calcium, and therefore, would be less dense than the majority of the spore population.

For these density gradients, Renografin-76 (*N,N'* - diacetyl - 3,5 - diamino - 2,4,6 - triiodobenzoate, an X-ray contrasting agent purchased from Squibb) was obtained in 20-ml sterile bottles as a 76% solution. Linear, 5-ml gradients were formed at room temperature from 50% (1:1 dilution of Renografin with water) to 100% (highest obtainable concentration of Renografin) and were placed at 4 C for 15 to 20 min. Washed spores from cultures irradiated with ultraviolet were suspended in 0.2% Tween 80, and, after adding an equal volume of 100% Renografin, the suspension was kept at 4 C for 15 to 20 min before layering on top of the gradient. Centrifugation was carried out in a Spinco model L ultracentrifuge with a SW-39 swinging bucket rotor at 4 C for 45 min at 15,000 rev/min. After centrifugation, a visible band of spores appeared about two-thirds down the tube. In such gradients, vegetative cells were found to form a layer on the surface, while germinated spores banded several millimeters from the top.

When 10^9 spores were layered on a gradient, the majority of the spores were present in a

distinct band, but 1 to 3% of the colony-forming units were found distributed through the gradient above the spore band. The entire gradient above the spore band was carefully removed with a pipette, diluted 1:10 with distilled water and plated on G medium (B. T. Stewart and H. O. Halvorson, J. Bacteriol **65**:160, 1953) supplemented with 2.0% agar. Plates showed confluent growth, and, after 24 hr, spores were harvested, washed, and the gradient centrifugation repeated. Upon repeating this procedure several times, a very broad, diffuse band appeared in the gradient with some spores layering at normal density but the majority of the spores distributed through a lower density region. These light spores were plated and single colony isolates were found which produced spores that were less heat resistant than normal, were completely devoid of DPA, and which formed a sharp band in the gradient about one-third down the tube.

The mutant spores are stable at 70 C and very little drop in population occurs after 20 min. At 80 C, they lose viability completely within 1 to 2 min. In contrast, the wild type spores show no drop in population at 80 C for 30 min and a very slight drop at 95 C after 5 min. Vegetative cells die off within several min at temperatures of 55 to 60 C.

When DPA is added to sporulating cultures of the mutant, the resulting spores are completely heat resistant at 80 C. Studies using ^{14}C-labeled DPA indicate that the added DPA is incorporated into these spores.

A more detailed report of the characteristics of heat-sensitive DPA-less spores will be presented in a future publication.

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