JOURNAL OF BACTERIOLOGY, July 1978, p. 133-137
0021-9193/78/0135-0133$02.00/0

Vol. 135, No. 1
Printed in U.S.A.

Study of Calcium Dipicolinate Release During Bacterial Spore Germination by Using a New, Sensitive Assay for Dipicolinate

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Received for publication 21 December 1977

The release of calcium and dipicolinic acid from spores of *Bacillus megaterium* KM during L-alanine-induced triggering of germination has been studied using a new, simple, and rapid assay for dipicolinic acid capable of detecting a concentration of 0.5 μM. The release of both calcium and dipicolinate started within seconds of exposure of the spores to L-alanine, thus preceding other measurable changes associated with germination. From the earliest times, the two substances were released in equimolar quantities, although later in germination calcium predominated.

Dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) is a unique constituent of bacterial spores that has been suggested to be involved in their dormancy, heat resistance, and germination. It is normally present in equimolar amounts with calcium or other metals (11), but deviations from this ratio have been observed (9). The time of release of calcium DPA during spore germination has been measured in the past (1, 13, 15) and has been shown to parallel other germination changes such as loss of phase whiteness and resistance to stains, but to be slower than loss of heat resistance (4). Thus, the release of calcium DPA appeared to occur too late to be a primary event in the triggering of germination. There is, however, considerable evidence that calcium DPA is involved in this triggering, including the facts that DPA-deficient spores are reluctant to germinate unless exogenous DPA is added (16) and that a 1:1 chelate of calcium and DPA is a universal germinant (10). One possible explanation for this inconsistency is that the release of the bulk of the calcium DPA is a post-triggering event, but that a small amount of calcium DPA is involved in the triggering.

Two questions relating to this possibility have not yet been answered. The first is whether release of calcium or DPA starts sufficiently early to be associated with the triggering of germination. The second is whether calcium and DPA are released as a 1:1 chelate initially, as well as later in germination when the bulk of the calcium DPA is released. It has been suggested that germination involves displacement of calcium from the spore membrane (2), in which case an excess of calcium over DPA would be expected to be released initially. Conversely, it has been suggested that calcium binding to the spore cortex triggers germination (5), in which case an excess of DPA over calcium might be expected at this early time.

Studies on the initial release of DPA have been hampered by the lack of a simple and sufficiently sensitive assay for this compound. The commonly used method of Janssen et al. (6) is quite insensitive, and the UV absorbance method described by Lewis (7) is limited in its sensitivity to five times that of Janssen's method by residual blank UV absorbance. The gas-liquid chromatography method described by Tabor et al. (12) is capable of detecting 1 nmol of DPA but is quite lengthy, involving two extraction steps in the purification procedure.

In this paper we describe a simple, very sensitive, and rapid technique for estimation of DPA in aqueous solutions and the use of this technique to investigate the precise timing and stoichiometry of calcium and DPA release during germination.

MATERIALS AND METHODS

Growth and preparation of spores. The organism used was the sporogenic strain of *Bacillus megaterium* KM described by Ellar and Posgate (3). Spores were grown in modified CCY medium supplemented with 10 μCi of ^{45}Ca per liter and harvested and cleaned as previously described (14). Before use, the spores were washed twice in 50 mM KCl (4°C), then three times in deionized water (4°C), to remove readily exchangeable calcium from the spores.

Germination of spores. Spores were germinated at 2 mg (dry weight)/ml in 50 mM KCl and 1 mM L-alanine at 37°C. Where appropriate, they were first

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heat activated by heating a suspension of 20 mg/ml in deionized water at 70°C for 30 min, then cooling to 37°C, and immediately adding to the germinants. Samples of heat-activation exudate were prepared by taking 0.5-ml samples of the spores during heat shock, rapidly mixing them with 4.5 ml of ice-cold deionized water, and filtering the sample through a 0.45-μm membrane filter using a Swinnex 2.5-cm syringe filter (Millipore Corp., Bedford, Mass.). Samples of germination exudate were taken by rapidly filtering the germination spore suspension in the same way. The filtration process took approximately 5 s.

DPA assay. The principle of the method was to measure the characteristic change in the spectrum of DPA on addition of calcium. Figure 1 is the spectra of Na_2DPA and calcium DPA showing the peak at 277.5 nm given by the calcium chelate. Figure 2 shows a difference spectrum of DPA in excess $CaCl_2$ against DPA in EGTA (1,2-bis-2-aminoethoxyethane-*N,N,N',N'*-tetraacetic acid). The combination of alkaline pH and the presence of the powerful divalent ion chelator EGTA ensured that the reference gave a pure DPA^{2-} spectrum, while the excess calcium in the sample ensured a pure calcium DPA spectrum. The assay was based on the linear relationship between the concentration of DPA and the peak-to-trough differences A and B shown in Fig. 2.

Because this difference spectrum is only influenced by substances whose spectra between 270 nm and 290 nm change in the presence of calcium, most UV-absorbing impurities do not affect the assay. Because absolute extinction values need not be measured, the sensitivity of the assay is not affected by moderate blank absorbances, and it can therefore detect a concentration of 0.5 μM DPA (change in optical density [ΔOD] = 0.0002).

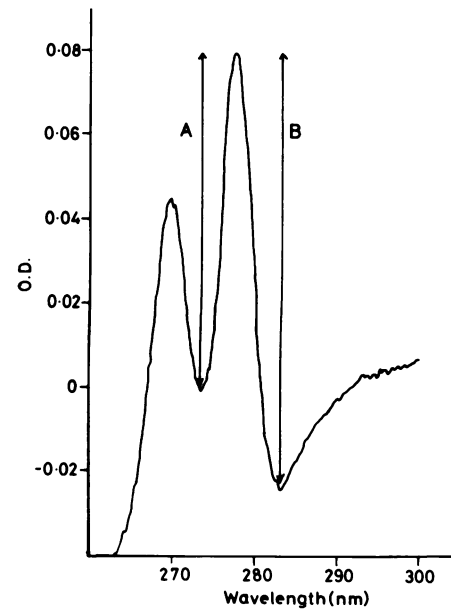

FIG. 2. *Difference spectrum of calcium DPA and DPA^{2-}. Sample cuvette contained 41 μM DPA, 10 mM NaOH, and 5 mM $CaCl_2$ and the reference cuvette contained 41 μM DPA, 10 mM NaOH, and 2.5 mM EGTA. For definition of functions A and B (arrows), see legend to Fig. 3.*

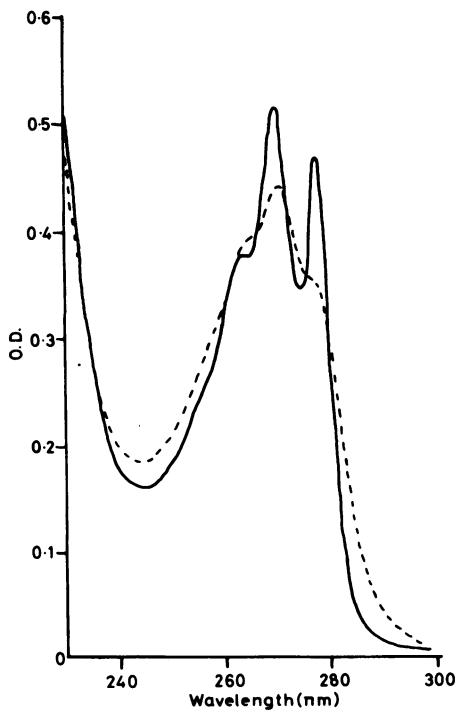

FIG. 1. *Spectra of DPA^{2-} and calcium DPA.* (——) *0.1 mM DPA, 10 mM NaOH, and 5 mM $CaCl_2$.* (-----) *0.1 mM DPA and 10 mM NaOH.*

The assay procedure was as follows: 1-ml samples of the DPA solution were added to two 1-ml 1.000-cm light path cuvettes. A 10-μl volume of 1 M NaOH was added to each, followed by 50 μl of 50 mM EGTA (neutralized with NaOH) in the reference cuvette and 50 μl of 100 mM $CaCl_2$ in the sample cuvette. The difference spectrum was recorded at 30°C using a Unicam SP1750 UV spectrophotometer fitted with an SP1805 program controller and an AR55 linear recorder (Pye Unicam Ltd., Cambridge, U.K.). The band width was 0.8 nm, and the scan speed was 0.2 nm/s. A full scale of 0.2 or 0.5 OD units was used, depending on the concentration of DPA. Standard curves were constructed using the functions A and B shown in Fig. 2 and are shown in Fig. 3.

The identity of the results obtained using these two functions was used as a routine check for interference by other substances. Selected samples were also checked by recording the difference spectrum of the sample in excess calcium versus the calculated amount of DPA, also in excess calcium. A peak or trough at 277.5 nm indicated that the concentration of DPA in the sample was, respectively, higher or lower than in the reference. No such interference was found with any of the samples used in this study.

Determination of calcium. A minor modification of the assay described above made it possible to determine calcium concentration. After the DPA assay

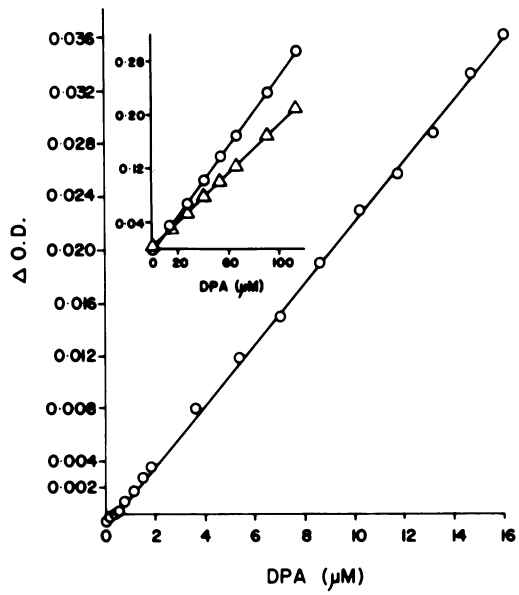

FIG. 3. *Standard curves for the DPA assay.* (○) $OD_{277.5} - OD_{283}$ *(function B);* (△) $OD_{277.5} - OD_{273}$ *(function A). For the standard curve (A), the final concentrations of EGTA and* $CaCl_2$ *in the assays were 0.25 mM and 0.5 mM, respectively, and OD was measured with a Cary 118 spectrophometer. For the inset standard curve (B), the assay conditions were as described in the text.*

was completed, the sample cuvette was removed, washed with EGTA to remove traces of calcium and then washed extensively with deionized water. A 1.0-ml sample of the test solution was then added, followed by 10 μl of 1 M NaOH and 50 μl of deionized water. Sufficient 4 mM DPA was added to both sample and reference cuvettes to make the total DPA concentration 180 μM. The difference spectrum was then recorded as above, and the function B was measured. A standard curve of function B versus calcium concentration is shown in Fig. 4. The curvature of this standard curve was due to the partial dissociation of the calcium DPA complex and was constant provided the total DPA concentration, ionic strength, and temperature of the assay were constant. This assay is not specific for calcium but, provided calcium is the only polyvalent ion present, is simple and sensitive.

The release of ^{45}Ca from spores was measured by counting 1-ml samples of the germination exudate in 15 ml of scintillant fluid containing 7 g of PPO (2,5-diphenyloxazole) and 0.35 g of POPOP [1,4-bis-[2]-(5-phenyloxazolyl)benzene] per liter in 66% toluene-33% Triton X-100 using a Packard Tri-Carb refrigerated scintillation counter. ^{45}Ca was used to measure release of calcium, since contamination of solutions and glassware with calcium made analytical detection of low levels of calcium difficult.

The specific activity of the calcium present in the spores was determined by measuring the concentration of calcium in the exudate from spores germinated for 20 min, using the spectrophotometric calcium assay described above. At this time in germination, calcium is by far the major divalent ion present in the germination exudate.

RESULTS

Figures 5 and 6 show the release of calcium and DPA during the germination of heat-activated and dormant spores, respectively. It is clear that release of both compounds started within seconds of the addition of germinants, although at a low rate. Furthermore, the substances were released in equimolar quantities initially, even though later in germination an excess of calcium was released. Heat activation had little effect on the early, lag period of germination but improved the later synchrony of germination considerably.

Table 1 shows the release of calcium and DPA during heat activation at 70°C. DPA was released progressively throughout the period of heat activation, while at no stage could release of calcium be detected.

DISCUSSION

The fact that release of calcium and DPA started immediately on addition of germinants suggests that this release may be associated with a very early event in triggering germination. Studies on the time of commencement of other biochemical changes during germination of this strain of *B. megaterium* under similar conditions (I. R. Scott and D. J. Ellar, Biochem. J., in press) have shown that the activation of metabolism associated with germination does not commence until 2 to 3 min after the addition of germinants. Unfortunately, the sensitivity of the methods used to detect metabolism was insufficient to

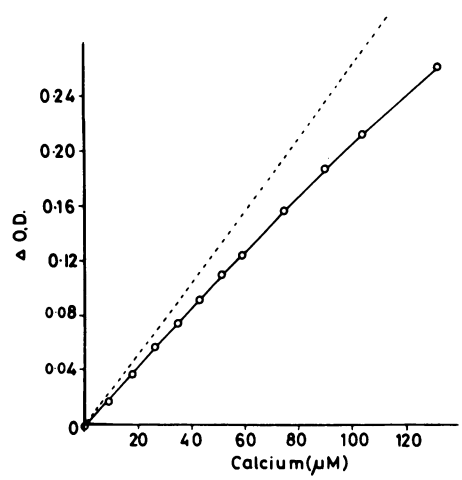

FIG. 4. *Standard curve for calcium assay.* (○) $OD_{277.5} - OD_{283}$ *(function B).* (-----) *Theoretical curve for undissociated calcium DPA.*

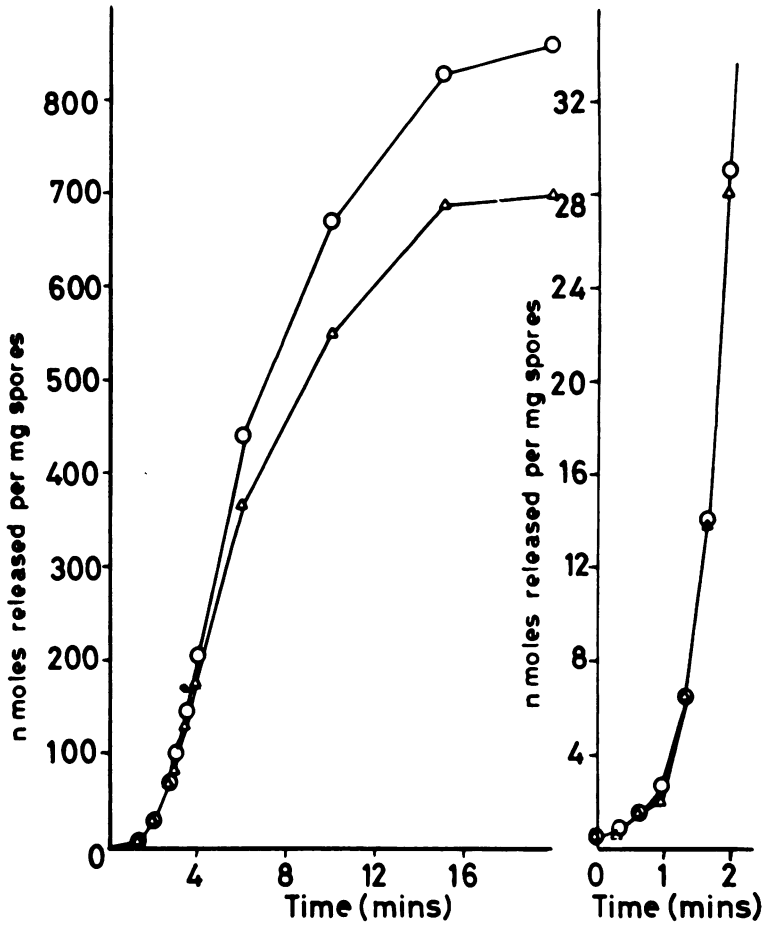

FIG. 5. *Release of ^{45}Ca and DPA during germination of heat-activated spores.* (○) *Calcium;* (△) *DPA. The specific activity of the ^{45}Ca was determined from the 20-min sample using the spectrophotometric calcium assay described in the text.*

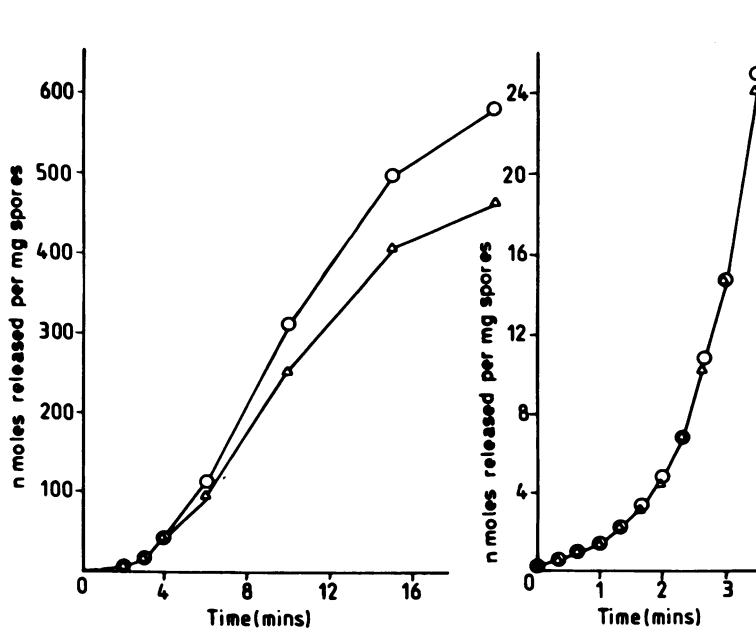

FIG. 6. *Release of ^{45}Ca and DPA during germination of non-heat-activated spores.* (○) *Calcium;* (△) *DPA. The specific activity of the ^{45}Ca was determined from the 20-min sample using the spectrophotometric calcium assay described in the text.*

TABLE 1. *Release of ^{45}Ca and DPA during heat activation at 70°C*

Heat activation (min)	nmol released/mg of spores	
	Calcium	DPA
0	<0.02	<0.2
5	<0.02	<0.2
10	<0.02	1.5
20	<0.02	2.5
30	<0.02	3.0

determine whether this very early calcium DPA release was due to the rapid germination of a small fraction (0.2%) of the spores with complete release of their calcium DPA, or whether the calcium DPA represented a small release from the whole population of spores. Only in the latter case would the early release be significant as an early germination event. Some evidence that the latter explanation is the correct one came from the observation that throughout the early minutes of germination, calcium and DPA were released in equimolar amounts, whereas later, calcium predominated. If the early release of calcium DPA represented the complete germination of a few spores, this excess release of calcium might be expected to be seen from the earliest times.

The fact that, initially at least, calcium DPA was released as the 1:1 chelate indicates that if germination is triggered by the displacement of calcium from a spore structure (2, 8), then the agent causing the displacement must be DPA present in the spore in some other form than the calcium chelate. The DPA could exist either in a completely dissociated form (e.g., Na_2DPA) or as a weak complex (e.g., MgDPA). The possible presence of such a pool of DPA in the spore is suggested by the observation that during heat shock, DPA but no Ca^{2+} was released. Conversely, if calcium binding to the spore cortex is responsible for the triggering of germination (5), the source of the calcium is unlikely to be the calcium DPA pool, since, if this were the case, excess DPA over calcium would probably be excreted early in germination.

ACKNOWLEDGMENTS

These investigations were carried out with the financial assistance of the Medical Research Council.

We thank K. Johnstone, G. S. A. B. Stewart, and M. Ross for assistance in preparing this manuscript.

LITERATURE CITED

1. **Dring, G. J., and G. W. Gould.** 1971. Sequence of events during rapid germination of spores of *Bacillus megaterium*. J. Gen. Microbiol. **65**:101-104.
2. **Ellar, D. J., M. W. Eaton, and J. Posgate.** 1974. Calcium release and the germination of bacterial spores. Biochem. Soc. Trans. **2**:947-948.
3. **Ellar, D. J., and J. A. Posgate.** 1974. Characterisation of forespores isolated from *Bacillus megaterium* at different stages of development into mature spores, p. 21-40. *In* A. N. Barker, G. W. Gould, and J. Wolf (ed.), Spore research 1973. Academic Press, London.
4. **Gould, G. W., and G. J. Dring.** 1972. Biochemical mechanisms of spore germination, p. 401-408. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
5. **Gould, G. W., and G. J. Dring.** 1975. Heat resistance of bacterial endospores and the concept of an expanded osmoregulatory cortex. Nature (London) **258**:402-405.
6. **Janssen, F. W., A. J. Lund, and L. E. Anderson.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26-27.
7. **Lewis, J. C.** 1967. Determination of dipicolinic acid in bacterial spores by ultraviolet spectrometry of the calcium chelate. Anal. Biochem. **19**:327-337.
8. **Lewis, J. C., N. S. Snell, and H. K. Burr.** 1960. Water permeability of bacterial spores and the concept of a contractile cortex. Science **132**:544-545.
9. **Murrell, W. G.** 1969. Chemical composition of spores and spore structures, p. 215-273. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press, London.
10. **Riemann, H., and Z. J. Ordal.** 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science **133**:1703-1704.
11. **Slepecky, R. A., and J. W. Foster.** 1959. Alterations in metal content of spores of *Bacillus megaterium* and the effects on some spore properties. J. Bacteriol. **78**: 117-123.
12. **Tabor, M. W., J. MacGee, and J. W. Holland.** 1976. Rapid determination of dipicolinic acid in the spores of *Clostridium* species by gas-liquid chromatography. Appl. Environ. Microbiol. **31**:25-28.
13. **Uehara, M., and H. A. Frank.** 1967. Sequence of events during germination of putrefactive anaerobe 3679 spores. J. Bacteriol. **94**:506-511.
14. **Wilkinson, B. J., D. J. Ellar, I. R. Scott, and M. A. Koncewicz.** 1977. Rapid, chloramphenicol-resistant, activation of membrane electron transport on germination of *Bacillus* spores. Nature (London) **266**: 174-175.
15. **Woese, C., and H. J. Morowitz.** 1958. Kinetics of the release of dipicolinic acid from spores of *Bacillus subtilis*. J. Bacteriol. **76**:81-83.
16. **Zytkovicz, T. H., and H. O. Halvorson.** 1972. Some characteristics of dipicolinic acid-less mutant spores of *Bacillus cereus, Bacillus megaterium* and *Bacillus subtilis*, p. 49-52. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.