STUDIES ON GERMINATION OF CALCIUM- AND ZINC-ENRICHED SPORES OF *BACILLUS CEREUS* T

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The calcium-dipicolinic acid (Ca-DPA) complex in the spores of *Bacillus cereus* T is believed to play an important role in the phenomenon of heat resistance. Studies were carried out to determine whether calcium was present in the form of a complex with DPA. Ca and DPA are known to be released into the medium following germination. The presence of Ca-DPA complex in the spores can be established, if Ca and DPA are released in the same ratio in which they are present in the spores. This was studied by preparing spores having different Ca-DPA ratio, and subsequently determining the ratio in which they were released during germination. The present results indicate that the Ca-DPA ratio during the release was the same as in the spores. Attempts were also made by increasing the Zn concentration in the medium to see whether Zn could replace Ca in Ca-DPA complex. The results obtained show that Zn is not taken up by the spores in the presence of Ca.

During the life cycle of spore-forming bacteria there are two important stages, sporulation and germination. During sporulation the bacterial cells are transformed into dormant bodies known as endospores. Endospores are highly refractile and resistant to heat and other deleterious agents. Under favourable conditions they can break their dormancy and become sensitive to heat, leading ultimately to the formation of typical bacterial cells. This process is known as spore germination (1–4). Germination can be measured by release of dipicolinic acid (DPA) (5, 6), and increase in UV resistance (7, 8) or by decrease in optical density. Germination of bacterial spores occurs in two phases. Loss of DPA and heat stability from the spores form the first phase of germination. During the second phase macromolecular synthesis occurs (9).

It is a well-known fact that ions play an important role in spore germination (10). RODE and FOSTER (11) claimed that nonionic compounds such as

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glucose did not cause spore germination in the absence of ions. They proposed a central role for ions in spore germination and a secondary role for other compounds such as glucose, L-alanine, and inosine. It has been well established that several bivalent cations, especially calcium and manganese, are required for germination (12).

The release of Ca^{2+} from germinating spores may be one of the main factors in the opening of cortical structures (13). The unique property of heat resistance of bacterial spores involves the formation of Ca-DPA complex. It is well documented that Ca^{2+} is necessary for DPA synthesis and that it also binds with DPA in the spores. A specific role of Ca^{2+} in DPA synthesis and heat resistance of spores has been suggested (14). However, Ca^{2+} in Ca-DPA complex could be replaced by Zn^{2+} , Sr^{2+} , and Ba^{2+} . Zinc-enriched spores of *B. cereus* T are heat-resistant. It was postulated by SRINIVASAN and SHARMA (16) that Zn^{2+} may be replacing Ca^{2+} and forming Zn-DPA complex in the spores. If this is true then all Zn^{2+} in the spores should be released along with DPA during germination.

Keeping this in view the release of Zn and DPA during germination of Zn-enriched spores was studied. For the sake of comparison, the release of Ca and DPA from Ca-enriched spores was also studied.

MATERIALS AND METHODS

Experimental. The active culture technique proposed by COLLIER (17) with suitable modification by HALVORSON (18) was used to prepare the spores of *Bacillus cereus* T. A synthetic medium containing 5 mg/ml of L-alanine and 1 mg/ml of adenosine in a 10^{-3} M phosphate buffer (pH 7.0) was autoclaved and used for germination. Nutrient agar medium was used for plating. It contained 0.3% beef extract, 0.5% peptone, and 2% agar. The pH was adjusted to 7.0 by adding dilute NaOH solution and then autoclaved. Total viable counts (TVC) were made by plating appropriate dilution of spore solution in nutrient agar. Heat stable counts (HSC) were obtained by heating the appropriately diluted samples at 80° for 30 min prior to plating. They were then plated and incubated at 37° for 24 hr. The colonies were counted on a colony counter (Spencer).

Determination of DPA released during germination. The colorimetric method of DPA determination reported by JANSEN *et al.* (19) was used. The amount of DPA in unknown samples was determined with the help of a standard curve.

Production of Ca- and Zn-enriched spores. The high calcium ($21.9\text{--}219\text{ }\mu\text{g/ml}$, 5.40×10^{-4} to 5.40×10^{-3} M) or zinc ($1.13\text{--}11.30\text{ }\mu\text{g/ml}$, 1.74×10^{-5} to 1.74×10^{-4} M) levels in the medium were obtained by adding appropriate amounts of Ca or Zn stock solutions. Zinc additions were done prior to sterilization and calcium was added under sterile conditions from an autoclaved stock solution of calcium. Before sterilization $0.1\text{ }\mu\text{Ci/ml}$ of ^{65}Zn as $^{65}\text{ZnCl}_2$ was added instead of the normal level of zinc. The specific activity of the radioactive Zn solution added cor-

responded to 1.3 $\mu\text{g/ml}$ in normal G-medium. The higher levels of Zn in the medium were obtained by taking the same amount of ^{65}Zn in each case (0.1 $\mu\text{Ci/ml}$) and adding appropriate amount of cold $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ to the medium.

Since low concentrations of Ca^{2+} in the medium led to loss of heat stability of spores we used normal concentrations of calcium.

Determination of ^{65}Zn activity. Spores having different levels of Zn (approx. 1.0×10^8 cells/ml, heat shocked at 80° for 30 min) were germinated in a medium containing alanine and adenosine as germination stimulants. Samples of 5 ml each were drawn at intervals of 0.5 hr and centrifuged at 5,000 rpm in a refrigerated centrifuge. Spores were washed with 0.04 N HCl ($3 \times$) and subsequently with water, and suspended in 5 ml of distilled water. Radioactivity of samples was determined with a well-type scintillation counter (NaI crystal, Tl activated) to measure the zinc release from spores. Appropriate corrections for isotope dilution were made where higher levels of zinc were used in spores.

Calcium determination. Samples (5 ml) were drawn from the test cultures and centrifuged. The spores thus harvested were washed in 0.4 N HCl, suspended in 5 ml distilled water, and 1.5 ml of conc. HCl was added to this and poured into a beaker. The suspension was heat-dried over a sand bath at 105° . The residue was dissolved in water and the volume was made up to 25 ml. The calcium content of the samples was then determined with a flame photometer (systronic type No. 121) using a calcium filter. Calcium content of supernatant solution from each sample was determined directly, *i.e.*, without subjecting it to acid digestion. The Ca content, corresponding to the reading given by the instrument, was obtained from a standard curve.

All inorganic chemicals and glucose were of Analar-grade supplied by B.D.H. (India), ^{65}Zn was supplied as $^{65}\text{ZnCl}_2$ by Isotope Division, Bhabha Atomic Research Centre, Trombay, India. Agar, peptone, yeast extract, and beef extract were of bacteriological grade.

RESULTS

Effect of zinc and calcium contents of spores on germination

The Zn and Ca contents as well as heat stability of Zn- and Ca-enriched spores were determined and the results are given in Table 1a and b. When Zn concentration of the medium was increased from $1.74 \times 10^{-5} \text{ M}$ to $1.74 \times 10^{-4} \text{ M}$, an increase of Zn content in spores was observed from $9.53 \times 10^{-10} \text{ mol/}10^8 \text{ cells}$ to $3.97 \times 10^{-9} \text{ mol/}10^8 \text{ cells}$. On the other hand, an increase in Ca level from $5.40 \times 10^{-4} \text{ M}$ to $5.4 \times 10^{-3} \text{ M}$ in the medium resulted in an increase of the Ca content in the spores from $1.625 \times 10^{-7} \text{ mol/}10^8 \text{ cells}$ to $3.125 \times 10^{-7} \text{ mol/}10^8 \text{ cells}$.

The spores having various Zn and Ca levels were suspended in the germination medium containing alanine and adenosine. Subsequent loss of heat resistance and absorbance was taken as criteria for germination. It was observed that, within

Table 1. Effect of the levels of Zn and Ca in the medium and the Zn and Ca content of the spores.

(a) Effect of zinc		
Concentration of Zn in the medium (M)	Concentration of Zn in spores (mol/10 ⁸ cells)	Heat stability (%)
1.74×10^{-5}	9.53×10^{-10}	100
8.70×10^{-5}	2.46×10^{-9}	100
1.74×10^{-4}	3.97×10^{-9}	100
(b) Effect of calcium		
Concentration of Ca in the medium (M)	Concentration of Ca in spores (mol/10 ⁸ cells)	Heat stability (%)
5.40×10^{-4}	1.625×10^{-7}	100
2.70×10^{-3}	2.187×10^{-7}	100
5.40×10^{-3}	3.125×10^{-7}	100

Table 2. Cell counts during germination of zinc- and calcium-enriched spores of *B. cereus* T.

Concentration of Zn and Ca in spores (mol/10 ⁸ cells)	Plate counts at 0 hr			Plate counts at 2 hr		
	TVC/ml	HSC/ml	Heat sensitivity (%)	TVC/ml	HSC/ml	Heat sensitivity (%)
Zn concentration						
9.53×10^{-10}	1.14×10^8	1.05×10^8	7.8	1.25×10^8	1.70×10^8	86
2.46×10^{-9}	1.45×10^8	1.19×10^8	11.0	0.93×10^8	1.50×10^8	84
3.97×10^{-9}	1.18×10^8	1.25×10^8	—	0.97×10^8	1.10×10^8	88
Ca concentration						
1.625×10^{-7}	1.60×10^8	1.86×10^8	—	1.08×10^8	1.35×10^8	87
2.187×10^{-7}	1.31×10^8	1.15×10^8	12.0	1.02×10^8	1.40×10^8	86
3.125×10^{-7}	1.27×10^8	1.18×10^8	7.6	1.30×10^8	1.70×10^8	86

Table 3. Release of Zn during germination from Zn-enriched spores of *B. cereus* T.

Time (hr)	Zinc content ($\mu\text{g/ml}$)					
	Spores grown in normal G medium Zn (1.74×10^{-5} M)		Spores grown in 5-fold Zn (8.70×10^{-5} M)		Spores grown in 10-fold Zn (1.74×10^{-4} M)	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	0.304	0.0094	0.773	0.024	1.250	0.0387
0.5	0.288	0.0266	0.735	0.067	1.183	0.1097
1	0.284	0.0274	0.724	0.069	1.170	0.113
1.5	0.281	0.0309	0.717	0.073	1.162	0.127
2	0.280	0.0313	0.714	0.079	1.153	0.128

2 hr, 85% of the spores become heat-sensitive (Table 2). This suggests that the germination of spores is not affected by the initial Zn or Ca content of spores.

Results of the release of DPA from the Zn- and Ca-enriched spores of *B. cereus* T during germination are given in Fig. 1a and b. These data indicate that the amount of DPA released at various Ca and Zn levels was almost the same. High Zn and Ca levels in the spores, therefore, do not influence its DPA content or the release of DPA during germination.

The release of Zn during germination was measured after suspending these spores in a medium containing alanine and adenosine. Approximately 10% of the Zn present in the spores was released within 2 hr of germination. This proportion of release was not affected by the initial Zn content of the spores.

The Ca release during germination was measured. Approximately 75% of Ca present in the spores was released within 2 hr of germination irrespective of the initial content of Ca in the spores.

The results showing the release of Zn, Ca, and DPA have been described previously in Tables 3-6. The changes in molar ratios of Zn-DPA and Ca-DPA

Fig. 1 (a). Release of DPA from Zn-enriched spores of *B. cereus* T during germination.

A, 9.53×10^{-10} mol/ 10^8 cells; B, 2.46×10^{-9} mol/ 10^8 cells; C, 3.97×10^{-9} mol/ 10^8 cells.

○---○, spores; ○—○, supernatant.

Fig. 1 (b). Release of DPA from Ca-enriched spores of *B. cereus* T during germination. A, 1.62×10^{-7} mol/ 10^8 cells; B, 2.18×10^{-7} mol/ 10^8 cells; C, 3.12×10^{-7} mol/ 10^8 cells.

○---○, spores; ○—○, supernatant.

in spores and supernatant solutions during germination were calculated from these results and are presented in Tables 7 and 8, respectively.

Table 4. Release of calcium during germination from calcium-enriched spores of *B. cereus* T.

Time (hr)	Calcium content ($\mu\text{g/ml}$)					
	Spores grown in normal G medium ($5.40 \times 10^{-4}\text{M}$) Ca		Spores grown in ($2.70 \times 10^{-3}\text{M}$) Ca		Spores grown in ($5.40 \times 10^{-3}\text{M}$) Ca	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	6.50	—	8.75	—	12.50	—
0.5	5.50	1.00	6.25	2.25	8.75	3.00
1	4.25	2.00	5.50	3.25	8.00	3.75
1.5	3.50	3.50	5.00	4.50	6.00	6.25
2	1.60	5.00	2.25	6.50	2.50	9.75

Table 5. Release of DPA during germination from zinc-enriched spores of *B. cereus* T.

Time (hr)	DPA content ($\mu\text{g/ml}$)					
	Spores grown in normal G medium ($1.74 \times 10^{-5}\text{M}$) Zn		Spores grown in ($8.70 \times 10^{-5}\text{M}$) Zn		Spores grown in ($1.74 \times 10^{-4}\text{M}$) Zn	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	42.6	6.6	42.6	6.6	40.0	6.6
0.5	33.3	13.3	39.0	13.3	39.0	13.3
1	29.3	16.0	33.3	16.0	26.6	20.0
1.5	26.6	20.0	26.6	20.0	20.0	26.6
2	16.0	26.6	13.3	33.3	13.3	33.3

Table 6. Release of DPA during germination from calcium-enriched spores of *B. cereus* T.

Time (hr)	DPA content ($\mu\text{g/ml}$)					
	Spores grown in normal G medium ($5.4 \times 10^{-4}\text{M}$) Ca		Spores grown in ($2.70 \times 10^{-3}\text{M}$) Ca		Spores grown in ($5.40 \times 10^{-3}\text{M}$) Ca	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	48.0	2.6	48.0	0.0	52.0	0.0
0.5	32.0	16.0	38.6	13.3	38.6	13.3
1	29.3	20.0	34.6	16.0	32.0	20.0
1.5	25.3	25.3	24.0	29.3	25.3	24.0
2	24.0	29.3	20.0	32.0	24.0	29.3

Table 7. Zn-DPA (molar) ratio during germination of zinc-enriched spores of *B. cereus* T.

Time (hr)	Concentration of Zn in spores					
	$(9.53 \times 10^{-10} \text{ mol}/10^8 \text{ cells})$		$(2.46 \times 10^{-9} \text{ mol}/10^8 \text{ cells})$		$(3.97 \times 10^{-9} \text{ mol}/10^8 \text{ cells})$	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	0.0036: 1	0.0013: 1	0.0087: 1	0.0056: 1	0.0149: 1	0.0042: 1
0.5	0.0040: 1	0.0022: 1	0.0123: 1	0.0014: 1	0.0164: 1	0.0046: 1
1	0.0057: 1	0.0008: 1	0.0137: 1	0.0023: 1	0.0197: 1	0.0029: 1
1.5	0.0073: 1	0.0006: 1	0.0177: 1	0.0015: 1	0.0234: 1	0.0023: 1
2	0.0117: 1	0.0004: 1	0.0300: 1	0.0011: 1	0.0539: 1	0.0017: 1

Table 8. Ca-DPA (molar) ratio during germination of calcium-enriched spores of *B. cereus* T.

Time (hr)	Concentration of Ca in spores					
	$(1.625 \times 10^{-7} \text{ mol}/10^8 \text{ cells})$		$(2.187 \times 10^{-7} \text{ mol}/10^8 \text{ cells})$		$(3.125 \times 10^{-7} \text{ mol}/10^8 \text{ cells})$	
	Spores	Supernatant	Spores	Supernatant	Spores	Supernatant
0	0.59: 1	—	0.76: 1	—	1.02: 1	—
0.5	0.60: 1	0.69: 1	0.73: 1	0.77: 1	1.04: 1	1.02: 1
1	0.57: 1	0.57: 1	0.77: 1	0.78: 1	1.02: 1	1.05: 1
1.5	0.59: 1	0.62: 1	0.73: 1	0.76: 1	0.92: 1	1.00: 1
2	0.66: 1	0.56: 1	0.77: 1	0.76: 1	0.95: 1	1.03: 1

DISCUSSION

The life cycle of spore-forming bacilli is usually characterised by two transition stages, namely, sporulation and germination. Many biochemical events operate in the life cycle of a bacillus and lead to the formation of two morphologically and physiologically different forms, spores and vegetative cells, respectively. The spores contain very high concentrations of metal ions and DPA compared to the vegetative cells. The uptake of metal ions and DPA biosynthesis during sporulation have been considered to be closely associated processes (20).

Heat stability of spores has been attributed to a metal and DPA complex, mainly Ca-DPA complex. Further, FOERSTER and FOSTER (15) proposed that the metal ions in spores may be present chiefly as a complex with DPA. During germination, DPA accumulated in spores is known to be released into the medium (21, 22). If metal ions are stored in spores as a DPA-metal complex, then it is logical to assume that these metal ions will be released from the spores along with DPA during germination. In mature heat-resistant spores, Ca and DPA occur in nearly equimolar amounts (23, 24) and are released together with spore peptide during germination.

Present studies on release of calcium and DPA from spores during germination reveal that the molar ratio of Ca-DPA (0.59) is maintained during release. The spores contained 1.62×10^{-7} mol/ 10^8 cells of calcium and 2.85×10^{-7} mol/ 10^8 cells of DPA, out of which 1.25×10^{-7} mol/ 10^8 cells of Ca and 2.2×10^{-7} mol/ 10^8 cells of DPA were released into the medium during germination. It seems likely that Ca in spores is stored as DPA complex and, with the release of DPA, it is also released into the medium. This can further be confirmed by preparing Ca^{+2} -enriched spores. When the medium was enriched 10 times with Ca^{2+} , the spores were found to contain 3.10×10^{-7} mol/ 10^8 cells of Ca^{2+} , although the DPA content remained almost the same (3.20×10^{-7} mol/ 10^8 cells). Hence the molar ratio of Ca: DPA increased to approximately 1. When such spores germinated, 2.43×10^{-7} mol/ 10^8 cells of Ca^{2+} and 2.35×10^{-7} mol/ 10^8 cells of DPA were released in the supernatant. Thus, Ca^{2+} and DPA are released in a ratio of approximately unity, similar to that initially present in spores.

The results obtained with Zn^{2+} were different from those obtained with Ca^{2+} . It was found that only 10% of Zn^{2+} present in spores is released during germination as against 80% of DPA. The molar ratio of Zn: DPA in the spores at zero hour was 0.003 as against 0.0004 in the supernatant after 2 hr. Thus, during germination more DPA is released than Zn^{2+} . This might indicate that most of the endogenous Zn in spores was not present as a Zn-DPA complex. These results are not in agreement with the observation of SLEPECKY and FOSTER (25) who have earlier shown that Zn^{2+} could replace Ca^{2+} in DPA-metal complex. However, it may be pointed out that the conditions in our experiment were different from that of Slepceky and Foster. We have used the normal level of Ca (5.40×10^{-4} M) in the medium used for Zn enrichment. This level of Ca is approximately 30 times

higher than the level of Zn (1.74×10^{-5} M) whereas Slepecky and Foster used a lower level of Ca^{2+} in their medium.

The molar ratio of Ca: Zn in spores is 80. Under such circumstances it is likely that Ca^{2+} is probably the preferred metal ion to form a complex with DPA. Other metal ions probably form complexes with DPA only in the absence or deficiency of Ca^{2+} .

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