PHOSPHORUS METABOLISM IN *BACILLUS MEGATERIUM* DURING SPORULATION

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Comparison of cellular components of vegetative cells and spores has been extensively studied by many workers, but only a little is known of the general phosphorus metabolism during sporulation in bacteria (1).

The present work was undertaken in an attempt to clarify the phosphorus metabolism in *Bacillus megaterium* during the course of sporulation with special attention on the intracellular flow of nucleic acids. When the sporulation process was defined as a series of successive phases of pre-sporulation, forespore formation, endospore formation, and free spore liberation, the most conspicuous change in phosphorus metabolism occurred during pre-sporulation to the endospore formation.

MATERIALS AND METHODS

Microorganism. *Bacillus megaterium* IAM 1166 (NRRL B-939) maintained on nutrient agar was used throughout this work.

Sporulation medium. The medium used by MARTIN and FOSTER (2) was modified slightly and denoted as the Y medium. It consists of two parts, phosphate buffer of pH 6.8 (KH_2PO_4 , 5.0 g; $(\text{NH}_4)_2\text{HPO}_4$, 1.0 g; and distilled water to 100 ml) and the basal medium of the following composition: Glucose, 1.0 g; L-glutamic acid, 1.0 g; yeast extract (Difco), 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; NaCl, 1.0 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.8 mg; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 7 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.064 mg, and distilled water to 900 ml. These two parts were sterilized separately and combined in a ratio of 1 : 9 by volume before use.

Culture method. Cells grown on a nutrient agar slant at 30° for 12-14 hr were suspended in 1.0 ml distilled water and 0.3 ml of the suspension was inoculated into 100 ml of the Y medium contained in a 500-ml Erlenmeyer flask. After 6 hr of incubation, when the culture was at the end of the log phase, 5 ml of this seed culture was inoculated into 100 ml of the Y medium and shake cultured at 30° on a rotary shaker. Under the present cultural conditions, a fairly good synchrony in sporulation was observed after 10 hr of cultivation.

Fig. 1. The fractionation procedures for cellular phosphorus components.

^a Successive fractionations of phosphorus components in the acid-soluble fraction.

^b Abbreviations for reagents (for details see reference 4)

TFA: trifluoroacetic acid solution

AM: ammonium molybdate solution

IB: isobutanol-benzene (1:1) mixture

^c Radioactivity of this fraction was obtained by the subtraction of those of the orthophosphate and labile-sugar phosphate from that of the TFA extract.

Measurement of growth and sporulation. The bacterial growth was followed by the plate counting and by the measurements of optical density at 650 m μ and dry cell weight. Morphological changes during sporulation were observed under a phase-contrast microscope and by WIRTZ's spore staining method.

Labeling experiment with ^{32}P . Orthophosphate- ^{32}P was used as a tracer. To remove a possible contaminant (phosphate polymer), ^{32}P -sample contained in a vial was heated on a boiling water bath before use.

Orthophosphate- ^{32}P (150 μc) was added to 100 ml of the Y medium and cells were cultured in the medium as usual. Samples were taken at intervals, chilled quickly by pouring them over a frozen phosphate buffer, and NaCN was added to a final concentration of 10^{-3} M. The labeled cells were washed several times with phosphate buffer until no radioactivity was detected in the supernatant. The measurement of radioactivity was carried out by a Geiger-Müller counter.

Fractionation of phosphorus compounds. SCHMIDT-THANNHAUSER-SCHNEIDER method modified by NISHI (3) was used for the fractionation of intracellular phosphorus compounds, and, when necessary, YANAGITA's method (4) was also employed for the separation of orthophosphate in the acid-soluble fraction. The flow-sheet of the whole procedure is shown in Fig. 1.

Chemical analyses. The amounts of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were estimated by the orcinol (5) and diphenylamine reactions (6), respectively, employing yeast RNA (Nutritional Biochemicals Co.) and herring sperm DNA (Nutritional Biochemicals Co.) as reference standards. Changes in glucose and glutamate contents in culture medium were followed by the modified method of SOMOGYI (7) and the technique of microbioassay (8), respectively.

RESULTS AND DISCUSSION

Courses of growth and sporulation

As shown in Fig. 2, courses of growth and sporulation of *B. megaterium* were followed in relation to environmental changes. As seen from the curves of viable cell number and dry cell weight, the maximum growth was attained after 7 hr. As already known, the course of change in the optical density was characteristic showing a stepwise increase after the maximum growth was [reached]. The number of cells bearing forespores (sporulating cells) increased in parallel with the stepwise increase in the optical density. Prior to the formations of the forespores, granular materials appeared in cells as a primary event at the period of 8 to 9 hr of cultivation (pre-sporulation phase), and the forespore formation followed at around 10 to 11 hr. Following the forespore formation, maturation of endospores proceeded in cells from 11 to 13 hr and finally the liberation of the mature free spores from cells followed (17-24 hr). It should be noted that the pre-sporulation phase was preceded by

Fig. 2. Growth and sporulation curves of *Bacillus megaterium* in the Y medium.

the consumption of glucose and glutamate, and by a slight increase in pH of the medium (from pH 6.7 to pH 7.0). The change in dry cell weight during the course of sporulation is also worthy of note. The dry cell weight decreased gradually by 3% from the stationary phase to the phase of maturation of endospore and then it decreased markedly by 33% during the course of free spore liberation. Thus, the final recovery of the dry cell weight of spores was about two-thirds that of the original vegetative cells in the stationary phase.

Changes in DNA and RNA contents

In Table 1 are listed the DNA and RNA contents in cells of different sporulation phases. The DNA content was the highest in the late log phase and decreased gradually during sporulation, followed by an abrupt decrease during the free spore liberation. In free spores, the DNA content was only one-third of that in the stationary phase cells on a unit volume of culture.

It is of interest that the change in RNA content was almost similar to that of DNA; the content of RNA in the free spore was also one-third that of the stationary phase cells.

Table 1. Amount of DNA and RNA during spore formation

Time of cultivation (hr)	Phase	Based on unit volume of culture			Based on dried cell weight	
		DNA	RNA	RNA/DNA	DNA	RNA
		(mg/100 ml culture)			(%)	
5	Late log	1.93	9.37	4.85	1.93	9.46
7	Stationary	1.93	8.25	4.27	1.54	6.60
9	Pre-sporulation	1.77	7.67	4.33	1.40	6.07
11	Forespore formation	1.72	7.50	4.36	1.39	6.05
13	Endospore formation	1.55	6.56	4.23	1.28	5.42
26	Free spore	0.61	2.47	4.05	0.73	2.94

Contents of DNA and RNA of cells in 100 ml culture in various phases were determined by the diphenylamine- and orcinol- method, respectively.

Changes in radioactivities of phosphorus fractions of cells during sporulation

Cells grown in the presence of orthophosphate-³²P were taken at intervals and fractionated by the method shown in Fig. 1. By this procedure, changes in the amount of various phosphorus fractions of cells could be followed simply by the measurement of their radioactivities.

As seen in Fig. 3 and Table 2, low molecular weight phosphorus compounds in cells increased, in general, during the course of sporulation and decreased rather markedly during the liberation of free spores, whereas high molecular weight phosphorus compounds, such as polyphosphate and nucleic acids, decreased during the whole of spore formation.

In Fig. 3A is shown the change in radioactivity of the acid-soluble fraction. Prior to the formation of forespores (pre-sporulation phase), conspicuous drop in the radioactivity was noted. The orthophosphate and the sugar phosphate fractions, including acid-labile and -stable sugar phosphates in the acid-soluble fraction, decreased similarly in the presporulation phase,

- A: Change in radioactivity of the acid-soluble fraction. Trifluoroacetic acid extract was fractionated successively into the orthophosphate, labile-sugar-phosphate, and stable sugar-phosphate fractions by the method shown in Fig. 1.
- B: Change in radioactivities of the phospholipid and polyphosphate fractions.
- C: Change in radioactivities of the DNA and RNA fractions.
- D: Change in radioactivities of the nucleotide and residual phosphorus fractions.

Fig. 3. Changing patterns in radioactivities of various phosphorus fractions. Shaded portions, 9-13 hr and 17-24 hr, represent phases of endospore formation and free spore liberation, respectively.

Table 2. Ratios of the phosphorus component on a cell basis at various stages of sporulation process.

Fractions	Sporulation phase				
	Stationary ^a	Pre-sporulation	Forespore	Endospore	Free spore
Acid soluble	1.00	1.13	1.25	1.53	0.67
Ortho-P ^b	1.00	1.01	1.14	1.26	0.59
Labile sugar-P	1.00	0.56	0.77	0.67	0.03
Stable sugar-P	1.00	1.70	1.75	2.60	1.17
Nucleotide	1.00	0.97	1.22	1.19	0.17
Phospholipid	1.00	0.95	0.69	0.61	0.31
Polyphosphate	1.00	0.76	0.15	0.19	0.12
DNA	1.00	0.95	0.89	0.81	0.34
RNA	1.00	0.92	0.89	0.85	0.35
Residual-P	1.00	0.91	1.46	2.77	4.17
Total-P	1.00	1.00	0.94	1.04	0.49

The relative amount of each component was calculated from the results shown in Fig. 3.

^a The values of each cell component at the stationary phase (7 hr) were taken as the standard.

^b P: phosphate

then they increased during the period of endospore formation. These observations suggest that some significant dynamic change in phosphorus metabolism must be occurring in the pre-sporulation phase in parallel with the conspicuous change in nitrogen metabolism as pointed out earlier by HARDWICK and FOSTER (9).

In contrast to the change in radioactivity of the acid-soluble fraction, those of the polyphosphate and phospholipid fractions, known as the phosphorus reserves, are worthy of notice (Fig. 3B). As already known in various bacteria, a high content of polyphosphate was found in cells in the stationary phase of growth, and prior to and during the sporulation phase it decreased conspicuously and the low value maintained during the course of the liberation of free spores. A similar course of change, but not so marked, was also observed in the phospholipid fraction. From these observations, one is tempted to speculate that these phosphorus reserves were utilized as phosphorus or energy sources in the course of the sporulation but not in that of free spore liberation.

The courses of changes in the radioactivities of nucleic acids and related substances are shown in Fig. 3C and D. In the interpretation of these data, however, it should be borne in mind that the efficiencies of extraction of RNA and, in particular, DNA by SCHMIDT-THANNHAUSER-SCHNEIDER's method are apt to change during the course of cell differentiation, especially in the case of sporulation (1). Therefore, gradual decrease of nucleic acids during

the course of forespore formation does not necessarily represent the true situation (see also Table 1). A slight increase in the radioactivity of the nucleotide fraction in the course of forespore formation did not balance the decrease in those of nucleic acids. It should, however, be stressed that the marked decreases in nucleic acids during the courses of endospore formation and liberation may reflect a partial loss of these substances, since after the sporulation had been completed the efficiency of the extraction of these nucleic acids might not change independent of the localization in cells of such spores. It was indeed observed under a microscope that endospores were liberated from the host cells as a result of the lysis of the latter.

A change in radioactivity of the residual fraction seemed to be characteristic. This fraction contained various substances left unextracted by the preceding procedures. Therefore, the biological interpretation of such a characteristic change of this fraction may be less significant than it deserves. It should, however, be pointed out that ^{32}P -phosphate in the medium was incorporated into the spore coat fraction in a bound form, such as phosphoprotein or phosphopolysaccharide (10). If all the radioactivity of the residual fraction can be assumed as that of the bound phosphorus, the marked increase in the radioactivity during the course of free spore liberation may be related to some process by which spores become matured by the formation of phosphorylated biopolymers.

SUMMARY

Phosphorus metabolism in sporulating *Bacillus megaterium* was examined by the fractionation of phosphorus compounds in cells.

Marked changes in the amount of various phosphorus fractions prior to and during the endospore formation suggested that the intracellular reconstruction was occurring in these phases of spore formation. The contribution of intracellular phosphorus reserves, especially of polyphosphate, to the sporulation process was also suggested. Conspicuous decreases (up to one-third) in the amount of RNA and DNA during the course of liberation of free spores was observed. This was discussed in relation to the mode of free spore liberation.

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