Changes in the Nucleotide Pool of *Bacillus licheniformis* During Sporulation

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ABSTRACT

Leitzmann, Claus (University of Minnesota, Minneapolis), and Robert W. Bernlohr. Changes in the nucleotide pool of *Bacillus licheniformis* during sporulation. J. Bacteriol. 89:1506–1510. 1965.—An analysis of the amount of acid-soluble nucleotides in *Bacillus licheniformis* cells showed a 75% increase during presporulation over that in log-phase cells. Cultures in which presporulation was inhibited by actinomycin D showed a decrease in acid-soluble nucleotides during the same time interval. A separation and quantitative determination of the nucleotides in the pool revealed that the relative proportion of each nucleotide remained fairly constant during presporulation. The detection of an intracellular ribonuclease activity and a decrease of the total nucleic acid concentration in the cells suggests that the increased pool arises from polymer breakdown. The effect of actinomycin D on sporulation was examined on both a quantitative and a temporal basis. The data indicate that messenger ribonucleic acid is essential for the completion of the sporulation process and must be resynthesized constantly.

The conversion of the vegetative cell into a mature spore is accompanied by changes in structure, metabolism, and chemical composition. This conversion may be regarded as a model of cellular development or differentiation. Several reviews and recent papers discuss these aspects of sporogenesis in detail (Robinson, 1960; Halvorson, 1962; Schaeffer, Acta Microbiol. Hung., in press; Szulmajster, 1964; Bernlohr, 1965). The changes taking place during the differentiation have been used to separate sporulation in several stages (Hashimoto, Black, and Gerhardt, 1960).

A quantitative determination of the intracellular nucleotide pool during sporulation in *Bacillus* species has not been reported. It is conceivable that if a unique type of nucleic acid metabolism occurs during presporulation, this metabolism may be reflected in the quantities of individual nucleotides in the intracellular pool. Schmitz (1961) has divided yeasts and mammalian tissues into energy types and metabolic types on the basis of free nucleotides present.

The finding that actinomycin D (Act-D) inhibits sporulation (del Valle and Aronson, 1962) if added before commitment to sporulation, i.e., before the end of log-phase growth, allows an analysis of the culture under conditions when the changes leading to sporogenesis are suppressed. Act-D is a chromopeptide antibiotic that exerts its bacteriostatic effect by inhibiting the synthesis of deoxyribonucleic acid (DNA)-primed ribonucleic acid (RNA; Kirk, 1960). Recent information on the structure and function of Act-D was presented by Reich and Goldberg (1964).

Initially, an examination of the total quantity of free nucleotides in the pools of the cells was undertaken with normal and Act-D-inhibited cultures. These analyses showed that the concentration of intracellular nucleotides in normal cultures rose 75% during presporulation when compared with log-phase cells. In Act-D-treated cells, the level of free intracellular nucleotides decreased slightly.

MATERIALS AND METHODS

*Cultivation of the organisms.* *B. licheniformis* strain A-5 was used exclusively for the experiments. The medium for spore production and for the growth of cells was described by Bernlohr and Novelli (1963). This simple salts medium was supplemented with 20 mM glucose and 50 mM ammonium lactate.

Spore stocks were prepared by 72 hr of growth at 37°C. The free spores were separated from lysed cell-wall material and repeatedly washed by centrifugation at 3,000 × g for 20 min at 3°C, suspended in water, and pasteurized at 60°C for 90 min in small vials containing 10⁶ spores.

Cultures were started with vegetative cells

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(always 8% inoculum) obtained from 10^9 spores in 23 ml of growth medium after incubation at 37 C for 17 hr. The 25-ml cultures were grown in 125-ml Erlenmeyer flasks in an Eberbach water-bath shaker having 120 strokes (4 cm) per min. The 15-liter cultures were grown in 20-liter carboys placed in a water bath (40 C) in which vigorous aeration with warmed and sterile water-saturated air maintained the cultures at 37 C. The stage of growth in these cultures was determined at hourly intervals by measuring the pH with a Beckman Zeromatic pH meter and the optical density in a Klett-Summerson photoelectric colorimeter with a no. 54 filter. Microscopic observations were made with a Leitz phase-contrast microscope, and counts of the sporangia were made with a Petroff-Hausser chamber. The efficiency of sporulation is always expressed as total number of spores or sporangia over the total number of cells at the end of log phase.

In experiments designed to determine the effects of Act-D on sporulation and on the composition of the acid-soluble pool, this antibiotic, at 0.5 µg/ml, was added to different 25-ml cultures at hourly intervals and to the 15-liter cultures before commitment to sporulation, i.e., at 10 hr. Higher concentrations of Act-D lyzed the cells under our conditions.

Isolation of acid-soluble nucleotides. Samples (2.5 liters) were removed from 15-liter cultures and immediately harvested at 0 C by centrifugation at 10,000 X g for 10 min in a refrigerated Servall RC-2 centrifuge. The cells were washed with cold salt medium containing 0.5% glucose and weighed. The ratio of wet weight to dry weight during growth and sporulation was found to be constant. The acid-soluble nucleotides of 10 g (wet weight) of cells were extracted with 150 ml of 5% trichloroacetic acid by suspending and manually shaking the cells for 5 min. The suspension was further shaken for 10 min at 0 C with the 1.0-cm (diameter) probe of a 20-ke MSE sonic oscillator and centrifuged as before; the pH of the supernatant solution was then adjusted to 5.5 with saturated KOH. The extracted cells were discarded. To the solution, 5 g of the acid-washed Norit were added, and the mixture was stirred for 2 hr at 1 C to adsorb all nucleotide material. The Norit was separated by centrifugation, washed twice with 50 ml of 0.05 M sodium acetate buffer (pH 4.5) and once with distilled water, and then eluted with cold 50% ethyl alcohol containing 1% NH₄OH (Klouwen, 1962). Five 20-ml elutions were sufficient to remove 90 to 95% of the bound nucleo-
tides. The eluates were combined, filtered, and reduced to 10 ml by vacuum evaporation with a Rinco flash evaporator.

Determination of total nucleic acid. The acid-
soluble nucleotides of cells harvested from a 250-
ml sample of the 15-liter cultures were extracted with 20 ml of 10% perchloric acid by manually shaking for 5 min and centrifuging at 10,000 X g. The supernatant liquid was discarded; the pellet was washed twice with 20 ml of 10% perchloric acid, and then was suspended in 10 ml of 10% perchloric acid and heated at 90 C for 30 min. The suspension was centrifuged as above, and the absorbance of the supernatant solution was determined at 260 mµ with 0.01 N HCl. The amount of nucleic acids was calculated on a dry weight basis by use of the value of E₁₆₅ = 31 per microgram of nucleotide per milliliter.

Chromatography. Ion-exchange columns (25 by 1.1 cm) were prepared by the method of Cohn (1951) with Dowex 1, X-8, 200 to 400 mesh, chloride form. The 10-ml samples were put on the column at a flow rate of 0.3 ml/min. The nucleotides were eluted with a gradient elution system as described by Hurlbert et al. (1954) at about 0.4 ml/min. An LKB (Stockholm) fraction collector fitted with a LKB Uvicord ultraviolet (UV) adsorption meter and recorder monitored the effluent. The 5-ml fractions containing UV-absorbing material were pooled, reduced in volume, and freeze-dried. The residues were dissolved in distilled water and characterized.

Descending paper chromatography with the use of Whatman 3MM paper and the solvent system described by Strominger (1959) were employed.

Spectrophotometry. Beckman DU and Beckman DK spectrophotometers were employed to measure the absorption spectra and the absorbance of the individual nucleotides under acid (0.01 N HCl), neutral (pH 7.0 with KOH), and alkaline (0.01 N KOH) conditions.

Chemical analysis. The acid-labile phosphate was determined by the method of Fiske and Sub-
baRow (1925) after hydrolysis of the samples in 1.0 N HCl at 100 C for 10 min. The pentose de-
termination was performed as described by Schneider (1957).

Chemicals. Nucleoside-5'-phosphate samples were purchased from Schwarz BioResearch, Inc., Mount Vernon, N.Y., and from Sigma Chemical Co., St. Louis, Mo. Dowex-1 was obtained from Calbiochem. The Act-D was a gift from H. B. Woodruff, Merck, Sharpe and Dohme Research Laboratories, West Point, Pa. All other reagents and chemicals were of reagent-grade purity.

Results and Discussion

The typical growth curve, pH changes, and the appearance of sporangia of a 15-liter culture with and without Act-D addition are shown in Fig. 1. It can be seen that the pH and the optical density stayed about constant after Act-D addition. In the normal cultures, the first sporangia appeared in the culture after 15 hr, and the culture completed sporulation in 48 hr, with an 80 to 90% efficiency. The development of 25-ml cul-
tures was the same in all respects, except that these finished the life cycle somewhat faster.

To different 25-ml cultures, Act-D was added at hourly intervals. At the time of addition of Act-D, and at the end of 26 hr, a sample was removed for microscopic observation (Table 1). Sporulation was inhibited when Act-D was added
before a certain developmental stage had been attained (in this experiment, before 11 hr). After this stage was reached, sporangia were resistant to Act-D inhibition and completed their development. The data indicate that about 10% of the total population attained this insensitive stage per hour. This insensitive stage was reached about 1 hr before sporangia could be seen under phase contrast, because the total increase in the number of sporangia after the time of addition of the antibiotic was equivalent to the number of sporangia normally found 1 hr later in the control culture. Our data indicate that messenger RNA (m-RNA), the synthesis of which is presumed to be inhibited by Act-D, is essential for the completion of the sporulation process, and that this m-RNA has to be synthesized constantly. This observation was also made by Szulmajster (1964). However, del Valle and Aromson (1962) suggested that a stable m-RNA for bacterial spore formation in B. cereus is formed when active growth has stopped, and this m-RNA can function over an extended period in the absence of any further RNA synthesis.

We proceeded to investigate the quantities of nucleotides in the intracellular pool of 15-liter cultures grown in the absence of Act-D and of cultures to which Act-D was added at 10 hr. [Samples (2.5-liter) were taken before and during presporulation, and the acid-soluble nucleotides from 10 g (wet weight) of these cells were extracted and put on the ion-exchange columns described in Materials and Methods.] The total free intracellular nucleotides in cultures grown with and without Act-D are plotted in Fig. 2. The 75% increase of soluble nucleotides in normal cultures and the decrease of soluble nucleotides in Act-D-treated cultures during presporulation are apparent.

![Fig. 1. Growth, pH change, and sporangia formation of normal and actinomycin D-inhibited 15-liter cultures. Open symbols represent normal cultures; closed symbols, actinomycin D-treated cultures. Symbols: O, growth; Δ, pH; □, sporangia.](http://jb.asm.org/)

![Fig. 2. Total acid-soluble nucleotide concentrations in the absence and presence of actinomycin D. Open symbols represent normal cultures; closed symbols, actinomycin D-treated cultures. Symbols: O, acid-soluble nucleotides; Δ, nucleic acid concentration.](http://jb.asm.org/)

**Table 1. Effect of actinomycin D on 25-ml cultures**

<table>
<thead>
<tr>
<th>Age (hr)</th>
<th>Control pH</th>
<th>Klett units</th>
<th>Optical density at 540 nm of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>6.5</td>
<td>265</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>7.1</td>
<td>310</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>7.2</td>
<td>320</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>7.2</td>
<td>340</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>7.2</td>
<td>355</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>7.2</td>
<td>370</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>7.3</td>
<td>405</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>7.3</td>
<td>420</td>
<td>8</td>
</tr>
<tr>
<td>16</td>
<td>7.3</td>
<td>445</td>
<td>9</td>
</tr>
<tr>
<td>17</td>
<td>7.4</td>
<td>465</td>
<td>10</td>
</tr>
</tbody>
</table>

*ADD indicates the time of actinomycin D addition. The control culture contained 35% sporangia at 26 hr. The per cent sporangia in each culture at ADD time was as follows: in 1, 2, and 3, 0; in 4, 1; in 5, 5; in 6, 10; in 7, 20; and in 8, 25. After 26 hr, the per cent sporangia in each culture was: in 1 and 2, 0; in 3, 5; in 4, 10; in 8, 15; in 6, 20; in 7, 25; and in 8, 35.
TABLE 2. Acid-soluble nucleotide concentration of normal and actinomycin D-treated 15-liter cultures

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Amt (μmoles) per 10 g of cells</th>
<th>Normal cultures</th>
<th>Treated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 hr</td>
<td>12 hr</td>
<td>13 hr</td>
</tr>
<tr>
<td>AMP</td>
<td>1.67</td>
<td>2.46</td>
<td>3.29</td>
</tr>
<tr>
<td>ADP</td>
<td>0.52</td>
<td>0.80</td>
<td>1.90</td>
</tr>
<tr>
<td>ATP</td>
<td>0.84</td>
<td>0.65</td>
<td>0.74</td>
</tr>
<tr>
<td>GMP</td>
<td>0.54</td>
<td>1.20</td>
<td>1.33</td>
</tr>
<tr>
<td>GDP</td>
<td>0.18</td>
<td>0.28</td>
<td>0.33</td>
</tr>
<tr>
<td>GTP</td>
<td>0</td>
<td>0</td>
<td>0.14</td>
</tr>
<tr>
<td>CMP</td>
<td>1.34</td>
<td>1.02</td>
<td>1.79</td>
</tr>
<tr>
<td>CDP</td>
<td>0</td>
<td>0.20</td>
<td>0.31</td>
</tr>
<tr>
<td>CTP</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UMP</td>
<td>0.54</td>
<td>0.34</td>
<td>0.48</td>
</tr>
<tr>
<td>UDP</td>
<td>0.72</td>
<td>1.14</td>
<td>0.81</td>
</tr>
<tr>
<td>UTP</td>
<td>0</td>
<td>0.24</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>6.35</td>
<td>8.33</td>
<td>11.12</td>
</tr>
</tbody>
</table>

The quantities of the individual free nucleotides are listed in Table 2 for normal cultures and for Act-D-inhibited cultures. The concentration changes of all nucleotides do not follow a common pattern. The concentration of the nucleoside monophosphates is higher than for nucleoside di- and triphosphates, except for the uridine nucleotides, where uridine diphosphate occurs at higher concentrations than does uridine monophosphate. The nucleoside triphosphates were detected only during early presporulation; cytosine triphosphate was not detected at all. The mole per cent guanine plus cytosine in DNA (GC content) for B. licheniformis is 46 to 48% (Marmur, Falkow, and Mandel, 1963). The GC content of the mononucleotides of the pool was constant at about 43% in our experiments.

The identity of the nucleotides from the pool was established by comparing literature values (Pabst Laboratories, 1956) with the physical constants of the nucleotides in a system standardized with authentic nucleotides. The authentic nucleotides were treated in the same manner as the experimental samples to allow a valid comparison under our conditions. This included acid treatment, sonic treatment, adsorption to charcoal, elution, freeze-drying, and chemical analysis. The elution patterns, adsorption characteristics, per cent recoveries, RF values, and the ratios of base-ribose-phosphate of the isolated nucleotides agreed closely with the same data obtained for authentic samples, and allowed a positive identification.

If the observed increase of the pool of acid-soluble nucleotide is a result of polymer degradation rather than de novo synthesis, then the quantity of nucleic acid should decrease during sporulation. This has been shown, and the data from a typical experiment are plotted in Fig. 2. The decrease of nucleic acids is about 40 μmoles per 10 g from 10 to 13 hr. During this same interval, the intracellular acid-soluble nucleotide concentration increased 5 μmoles per 10 g to a value of 11 μmoles per 10 g. This latter concentration may reflect the total capacity of the pool, and the additional nucleotides would be secreted into the medium. Measurements of the extracellular acid-soluble nucleotides in normal and in Act-D-inhibited cultures show that the concentration in both cultures is about the same and rises to 60 to 80 μmoles per 10 g of cells.

The occurrence of a weak ribonuclease activity in extracts of well-washed cells of B. licheniformis was detected recently in our laboratory. This activity increases during presporulation, but we have been unsuccessful in attempts to purify the enzyme. It is possible that a specific inhibitor is present in the extracts, and the system may be similar to that reported by Smeaton, Elliott, and Coleman (1965). It is possible that a ribonuclease functions in normal cultures during presporulation as one of the early genetic expressions of sporogenesis. An uncontrolled biosynthesis would result in the secretion of the enzyme into the extracellular medium. However, it is logical to assume that a ribonuclease activity would appear in postlogarithmic cells of many bacterial species, and may be unrelated to sporulation.

Although extracellular ribonucleases have been detected in cultures of a variety of microorganisms (Nishimura and Nomura, 1958; Rushizky et al., 1964; Coleman and Elliott, 1964), the function of these enzymes is not clear. We are not aware of a previously reported intracellular
ribonuclease activity in any Bacillus species. It is clear that the concentration of intracellular acid-soluble nucleotides transiently increases during presporulation in normal cultures, but not in inhibited cultures. The relationship between this increase and other activities required for sporulation must await further investigation.

ACKNOWLEDGMENT

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LITERATURE CITED


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