RIBONUCLEIC ACIDS OF BACILLUS SUBTILIS
SPORES AND SPORULATING CELLS
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ABSTRACT

DOI, Roy H. (Syracuse University, Syracuse N.Y.), and Richard T. Igarashi. Ribonucleic acids of Bacillus subtilis spores and sporulating cells. J. Bacteriol. 87:323-328. 1964.—The ribonucleic acids (RNA) of Bacillus subtilis spores were analyzed for their size and base composition. Soluble and ribosomal RNA identical to those found in vegetative cells were present in spores. A base ratio difference was observed between the 16s and 23s ribosomal RNA. The 16s RNA had a higher cytidylate and lower adenylyl base composition. The ratio of soluble to ribosomal RNA in spores was 30%, in contrast to 15% in vegetative cells, and may be related to their difference in biosynthetic activity. Although active synthesis of informational RNA occurred prior to endospore formation, no detectable level of informational RNA was found in spores. The informational RNA was shown to have the same base composition as that of B. subtilis deoxyribonucleic acid. The compositional difference between spore and vegetative cell RNA appeared to be strictly quantitative.

A characteristic property of bacterial spores is their complete lack of biosynthetic activity. Because the role of ribonucleic acids (RNA) in protein synthesis has been established (Berg, 1961), the nature and properties of spore RNA may give some clue to the dormant condition. Previous investigation revealed that net RNA synthesis stops before the onset of sporulation, and that RNA synthesis and turnover occurs during this period (Young and Fitz-James, 1959). The period of RNA synthesis prior to sporulation appears to be critical, because actinomycin D has no effect on sporulation after a specific amount of RNA synthesis has occurred (Del Valle and Aronson, 1962). The conclusion from these results is that a stable informational RNA is involved in sporulation. The observation that Bacillus subtilis spores contain only 50s and 68s ribosomes, and that unusual ribosomes occur during germination, suggests that the RNA constitution of spores is different from that of vegetative cells (Woese, 1961). Furthermore, a substantial amount of spore RNA turnover is seen during germination, which indicates that a fairly large labile RNA fraction may be present in spores (Woese and Forro, 1960).

The main objective of this study was to characterize the various RNA fractions in spores and sporulating cells and to look for RNA fractions not ordinarily found in vegetative cells. By use of the methylated albumin column (Mandell and Hershey, 1960), one can fractionate and determine the size and relative quantities of various RNA molecules. Base ratio analyses of isolated fractions reveal their compositional relationships. By isotope dilution and chemical methods, results were obtained which reveal that any differences in the RNA fractions of spores and cells are purely quantitative.

MATERIALS AND METHODS

B. subtilis W23 cells and spores were grown at 37 C in a medium (SCM) containing 0.055 M NH4Cl, 0.170 M NaCl, 0.054 M KCl, 0.006 M MgSO4, 0.0025 M PO43-, 0.0006 M FeCl2, 0.050 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.2), 0.0001 M MnCl2, 0.0002 M CaCl2, 0.011 M glucose, 0.0034 M sodium citrate, and 0.2% casein hydrolysate.

Clean spore preparations were obtained by use of the two-phase "Y" system described by Sacks and Alderton (1961). To ensure the removal of vegetative cells and debris, the crude spore suspension was treated with lysozyme (300 µg/ml) and deoxyribonuclease (10 µg/ml) for 3 hr at room temperature prior to purification by three passages through the "Y" system. The Carbowax 4000 and phosphate were removed by extensive washing with water. No sporangia were observed on these cleaned spores.

For growth of P32-labeled spores, the inorganic phosphate was omitted from the SCM medium. The phosphate present in the casein hydrolysate...
allowed the cells to grow to a density of about 3 × 10^6 cells per ml. The P^{32}O_4 was added at 20 μc/ml at the initial stages of the stationary phase. The cells were allowed to shake for an additional 12 to 15 hr for complete sporulation.

For pulse labeling of cells in the stationary phase prior to endospore appearance, a culture was started by heat-shocking a suspension of spores at 80°C for 30 min in SCM medium. An initial density of 2 × 10^6 spores per ml was inoculated into SCM medium minus the inorganic phosphate. The growth of several such identical cultures was followed spectrophotometrically by optical density measurements at 660 μm until the cultures were in the stationary phase. At various times in the stationary phase, P^{32}O_4 was added to one of the cultures at 20 μc/ml, and the culture was shaken for an additional 3 min. The culture was quickly chilled by pouring over frozen buffer [0.01 M tris (pH 7.2) containing 5 × 10^-3 M MgCl_2]. The cells were collected by centrifugation, and their RNA was extracted. The other cultures were treated identically over other periods in the stationary phase.

For preparation of uniformly labeled vegetative-cell RNA, SCM medium minus inorganic phosphate was inoculated with cells at an OD_660 of 0.05, and was grown to a density of 0.30 in the presence of 0.1 μc/ml of P^{32}O_4. At this time, the cells were collected by centrifugation and resuspended in SCM medium plus 0.005 M inorganic phosphate, and were grown to an optical density (at 660 μm) of 0.70. The RNA was then purified by the usual method.

For preparation of spore extracts, the cleaned spores were suspended in 0.01 M tris buffer (pH 7.2) containing 0.05 M MgCl_2, and were then concentrated by centrifugation. The spore pellet was frozen in a methanol-Dry Ice bath and then placed in a prechilled mortar with 2 volumes of Superbrute glass beads (120 μ in diameter; Minnesota Mining and Manufacturing Co., St. Paul, Minn.) and ground vigorously in the cold until a paste was obtained. The paste was quickly suspended in 0.01 M tris-0.05 M MgCl_2 buffer (pH 7.2), and the RNA extraction was performed initially on the crude bead-containing lysate.

The vegetative-cell extracts were prepared by the lysozyme freeze-thaw method described by Hayashi and Spiegelman (1961). RNA was purified by the phenol method of Gierer and Schramm (1956). The methylated albumin-kieselguhr (MAK) column was made according to Mandell and Hershey (1960). A linear gradient of NaCl was used to elute off the RNA. Base ratio analyses were performed by the method of Hayashi and Spiegelman (1961). Radioactivity was measured by precipitation of a suitable portion of the RNA-P^{32} with 10% trichloroacetic acid in the presence of 200 μg of carrier herring-sperm DNA. The precipitate was collected by centrifugation, resuspended with 1.0 ml of 2 N NH_4OH, dried on planchets, and counted with a thin-window gas-flow counter (Tracerlab Inc., Waltham, Mass.).

Results

The synthesis of RNA during the stationary phase preceding sporulation was examined by radioactive pulse labeling. The stationary phase lasted approximately 8 hr before endospore formation became apparent. At arbitrary times, 3-min pulses were made. The pulse-labeled RNA was purified and fractionated concomitantly with bulk RNA of vegetative cells by passage through a MAK column. Active RNA synthesis was observed at all times, except 1 to 2 hr prior to endospore appearance. The amount of incorporation during the pulse fell at least tenfold in the later stages. The pattern of RNA synthesized during a pulse is illustrated in Fig. 1. The results are typical of the pulse RNA patterns found in all the stationary-phase periods tested. The classical pattern of 4s, 16s, and 23s RNA is shown by the optical-density profile. The radioactivity curve represents the pulse RNA. Not only soluble RNA (sRNA) and ribosomal RNA (R-RNA) were synthesized, but also an appreciable amount of informational type RNA (D-RNA) was found. This fact was established by pooling fractions 47 to 58 of Fig. 1, and by determining the base ratio of this pooled fraction by the isotope-dilution method (see Materials and Methods). The base ratio indicated a guanylate-cytidylate (GC) composition of 43%, which is that of B. subtilis deoxyribonucleic acid (Table 1). Several results from different time periods in the stationary phase are listed in Table 1. The D-RNA which eluted from the column after the 23s R-RNA comprised approximately 50% of the pulse RNA.

The heterogeneity of the D-RNA was illustrated more clearly when the D-RNA of Fig. 1 was eluted from the column with a shallow NaCl gradient of 0.7 to 1.2 M. Many additional peaks were evident. The active synthesis of D-RNA during the period before endospore formation
prompted a thorough investigation of the spore RNA content.

Because the detection of a small fraction of spore RNA different from that in vegetative cells would be enhanced by use of $^{32}P$-labeled spore RNA, most of the analyses were performed on $^{32}P$-labeled material from spores. When large batches of spores were used as the source of spore RNA for chemical analyses, similar results were obtained. This critical control will be discussed later. Spore RNA was labeled by adding $^{32}P$ at various phases of growth preceding sporulation.

Identical results were obtained when label was added before or during the stationary phase of growth. Figure 2 illustrates the patterns obtained for spore RNA-$^{32}P$ and for carrier vegetative-cell RNA, represented by the radioactivity and optical-density curves, respectively. Figure 3 demonstrates the pattern for vegetative-cell RNA-$^{32}P$. One can immediately see that the specific activity of the vegetative cell in Fig. 3 is uniform, but that the spore RNA-$^{32}P$ profile is different from the carrier vegetative-cell pattern.

Several features of the spore RNA can be noted.

(i) Spore RNA contained the three classical types

(ii) The base ratios of the spore RNA were identical to those found in vegetative cells (Table 2). The base ratios of fractions marked with arrows in Fig. 2 and 3 were determined for several column runs by the isotope-dilution method. The sRNA base ratio was consistently regular in several determinations, and appeared to be pure 4s RNA uncontaminated by breakdown products. Base ratios of the 23s R-RNA region (Fig. 2) gave results identical to those obtained for vegetative cells (Fig. 3). When the trailing edge of the 23s R-RNA (Fig. 2) was examined, the base ratio was still that of 23s R-RNA. Thus, base ratio analyses also indicated the absence of D-RNA. There was a consistent difference between 16s and 23s R-RNA, in that the 16s RNA had a higher cytidylate and a lower adenylate content than did 23s RNA. This was true for both spore and vegetative-cell RNA. The base compositions of spore and cell RNA were, on the whole, similar to those reported previously (Belozersky and Spirin, 1960).

(iii) The ratio of sRNA to R-RNA was higher in spores than in vegetative cells (Table 3). This can easily be seen by comparing the radioactivity profiles of Fig. 2 and 3. In spores, sRNA comprised 30% of the total RNA, as compared with

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Table 1. Base ratios of pulse RNAs from the stationary phase of growth

| Time of radioactive pulse experiment after stationary phase is reached | Moles per cent |
|---|---|---|---|---|
| hr | Cytidylate | Adenylate | Uridylate | Guanylate |
| 1 | 20.7 | 29.1 | 28.2 | 22.0 | 42.7 |
| 3 | 20.2 | 27.4 | 29.4 | 22.9 | 43.1 |
| 5 | 20.6 | 29.3 | 27.5 | 22.6 | 43.2 |

*GC composition of Bacillus subtilis DNA is 43% (Marmur, Seaman, and Levine, 1963). The fractions indicated by the arrows in Fig. 1 were pooled and analyzed for base composition by the isotope-dilution method. The Fig. 1 experiment corresponds to the 5-hr time in this table; similar experiments were performed at 1 and 3 hr.

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FIG. 1. Column chromatography of pulse RNA-$^{32}P$ from cells in the stationary phase. Pulse RNA-$^{32}P$ containing 50 $\mu$g of RNA and $8 \times 10^8$ counts per min was mixed with 1,200 $\mu$g of bulk vegetative-cell RNA before charging the MAK column. The RNA was eluted with a linear gradient of NaCl from 0.3 to 1.2 M. Fractions of 5 ml were collected. The optical density (solid line) and counts per min (dashed line) represent bulk carrier RNA and pulse RNA, respectively. The arrows indicate the fractions pooled for base ratio analyses (see Table 1).
15% in the vegetative cell. This difference in ratios was found when pure spore RNA was analyzed chemically; also, the optical density profile of spore RNA corresponded to its radioactivity pattern. Because the base ratio of the 4s peak was that of sRNA, the increase in the amount of material in the spore 4s peak was not due to breakdown of larger labile fractions during the extraction and purification procedures.

(iv) The quantity of specific sRNA differed between spores and cells, because the profile of radioactive spore sRNA did not correspond to the optical-density pattern of vegetative-cell sRNA (Fig. 2). When a shallow NaCl gradient (0.3 to 0.7 M NaCl) was used in the analysis of the sRNA region, the discrepancy between the spore and vegetative-cell sRNA profile was more obvious. Thus, a differential incorporation of sRNA into bacterial spores occurred.

**FIG. 2. Column chromatography of spore RNA-P32.** Spore RNA-P32 containing 100 μg of RNA and $2.2 \times 10^4$ counts per min was mixed with 1,200 μg of bulk vegetative-cell RNA before charging the MAK column. The RNA was eluted with an 0.4 to 1.2 M linear gradient of NaCl. The optical density (solid line) and counts per min (dashed line) represent carrier bulk RNA and uniformly labeled vegetative-cell RNA, respectively. The arrows indicate the fractions pooled for base ratio analysis.

**FIG. 3. Column chromatography of vegetative-cell RNA-P32.** Vegetative-cell RNA-P32 containing 50 μg of RNA and $3 \times 10^5$ counts per min was mixed with 1,200 μg of vegetative-cell RNA before charging the MAK column. The material was eluted with a 0.4 to 1.2 M linear NaCl gradient. The optical density (solid line) and counts per min (dashed line) represent carrier bulk RNA and uniformly labeled vegetative-cell RNA, respectively. The arrows indicate the fractions pooled for base ratio analysis.

**TABLE 2. Base ratios of spore and vegetative-cell RNA**

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>Type of RNA</th>
<th>Cytidylate</th>
<th>Adenylate</th>
<th>Uridylate</th>
<th>Guanylate</th>
<th>%GC1</th>
<th>Pu/Pyr</th>
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<tbody>
<tr>
<td>Spore</td>
<td>4s</td>
<td>29.4</td>
<td>18.9</td>
<td>18.2</td>
<td>33.5</td>
<td>63</td>
<td>1.10</td>
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<tr>
<td>Spore</td>
<td>16s</td>
<td>24.0</td>
<td>24.4</td>
<td>20.6</td>
<td>30.9</td>
<td>55</td>
<td>1.24</td>
</tr>
<tr>
<td>Spore</td>
<td>23s</td>
<td>22.6</td>
<td>26.2</td>
<td>19.8</td>
<td>31.4</td>
<td>54</td>
<td>1.36</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>4s</td>
<td>28.5</td>
<td>19.1</td>
<td>19.4</td>
<td>32.9</td>
<td>61</td>
<td>1.09</td>
</tr>
<tr>
<td>Vegetative cells</td>
<td>16s</td>
<td>23.4</td>
<td>24.8</td>
<td>21.1</td>
<td>30.8</td>
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<td>26.4</td>
<td>20.7</td>
<td>30.0</td>
<td>53</td>
<td>1.30</td>
</tr>
</tbody>
</table>

* All base composition analyses are accurate to ±1.0%.
† GC composition of Bacillus subtilis DNA is 43% (Marmur et al., 1963). Base ratios were determined by the isotope-dilution method.
that the sRNA and R-RNA of spores and cells are identical. The ratio of sRNA to R-RNA in spores is, however, higher, and may be related to the absence of growth. Kjeldgaard and Kurland (1963) found an increasing amount of sRNA to R-RNA when the growth rate of *Salmonella typhimurium* was reduced. At the lowest growth rate of 0.2 doublings per hr, they found that 65% of the RNA was sRNA. Because the growth rate is nil in the case of spores, one might expect a higher proportion of sRNA than the 30% that we found. However, the exact relationship between the growth rate and the proportion of sRNA to R-RNA in *B. subtilis* has not been established.

The lack of symmetry between the sRNA profiles indicates that there are quantitative differences in the specific sRNA species. Whether this represents a preferential synthesis, or an incorporation of certain sRNA species during morphogenesis, is open to conjecture.

The absence of D-RNA in spores was surprising, because the results of Del Valle and Aronson (1962) and of Woese and Forro (1960) suggested that this type of RNA would be present in spores in detectable quantities. No D-RNA was ever detected in spores which were labeled by the addition of $^{32}P^{32}O_7$ during various stages preceding sporulation. While this manuscript was in preparation, Balassa (1963) reported the presence of spore RNA of 8 to 10s size. Initially, similar results were obtained by us when $^{32}P$-labeled spores were broken in the presence of 0.01 M MgCl$_2$. A major radioactive peak appeared between the 4s and 16s RNA regions, with very small peaks in the 16s and 23s regions. A base ratio analysis of this peak indicated that it was similar to R-RNA. When the Mg$^{2+}$ level was raised to 0.05 M during rupture of the spores, the peak between the 4s and 16s RNA disappeared. Furthermore, when RNA from large batches of spores was examined under these conditions, only the three classical RNA peaks appeared. Therefore, not only clean spores, but also the method of breakage, is extremely important for spore RNA analysis. The presence of dipicolinic acid (Powell, 1953), a chelating agent in spores, requires a high level of Mg$^{2+}$ to prevent breakdown of ribosomes and the release of ribonuclease. Whether a specific stable D-RNA is involved in the control of protein synthesis in dormant spores is not answered by these results (see Jacob and Monod, 1961).

The base ratio analyses of the various RNA fractions conclusively demonstrate that no difference exists between spore and cell RNA. An interesting observation was the consistent difference in the adenylate and cytidylate base composition between 16s and 23s RNA. Recent results of Yankofsky and Spiegelman (1963) revealed that 16s and 23s RNA have distinct cistrons. Thus, it does not seem unreasonable for this difference in base composition to occur. Midgley (1962) and Aronson (1962) also noted differences in the composition of RNA of 50s and 30s ribosomes. Exact comparison with their results cannot be made because Green and Hall (1961) found that, although 30s particles contain only 16s RNA, 50s particles contain both 16s and 23s RNA.

One can conclude from these results that the dormant biosynthetic state of spores does not entail any gross changes in RNA pattern or composition.

**Acknowledgments**

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**Literature Cited**


**Table 3. Ratio of soluble RNA to ribosomal RNA**

<table>
<thead>
<tr>
<th>Source of RNA</th>
<th>Soluble RNA</th>
<th>Ribosomal RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cells</td>
<td>16.6</td>
<td>83.4</td>
</tr>
<tr>
<td>Spores</td>
<td>30.0</td>
<td>70.0</td>
</tr>
</tbody>
</table>

*The average deviation from the mean of several determinations was ±1% for all the values. The amount of each type of RNA was determined by summing the optical density readings or the radioactivities of the relevant fractions from the chromatographic MAK columns.*


