Isolation and Characterization of Ribosomes from *Bacillus subtilis* Spores

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ABSTRACT

BISHOP, HELEN L. (Syracuse University, Syracuse, N.Y.), AND ROY H. DOI. Isolation and characterization of ribosomes from *Bacillus subtilis* spores. J. Bacteriol. 91:695–701. 1966.—The isolation of ribosomes from *Bacillus subtilis* spores was accomplished by freezing the spores in liquid nitrogen and grinding the spore pellet with an equal weight of levigated alumina. The ribosomes, which were adsorbed to the alumina, were freed by the addition of vegetative-cell ribosomes or bulk ribonucleic acid (RNA) to the crude alumina-ground extract. The spore ribosomes had sedimentation properties and RNA and protein compositions similar to those of vegetative-cell ribosomes. The difficulty encountered in obtaining spore ribosomes by ordinary extraction methods may be the result of nuclease and protease activities which were demonstrated in spore extracts.

Bacterial spores represent an extreme case of dormancy among biological systems. Studies to date have indicated that bacterial spores have little or no metabolism (8). Thus, an investigation of some of the properties of the dormant spore contributes to an understanding of the mechanism of dormancy. Since the protein-synthesizing mechanism is one of the basic functions of the cell, it would be of interest to examine each of the components of the system to see whether it has been altered or is in any way deficient for the process of protein synthesis. Woese, Landridge, and Morowitz (17) demonstrated that spores contained particles with sedimentation coefficients of 50S and 68S. They were unable to identify or find particles which had a 30S sedimentation coefficient. Since their studies were primarily based on the sedimentation properties of ribosomes and no chemical analyses had been made of these particles, a further analysis of these particles may be revealing in terms of their possible function in dormancy. To perform the biochemical analyses, a method has been developed for the isolation and characterization of the particles from dormant spores. This was necessary because spore ribosomes were unstable during the isolation process. The presence of an active ribonuclease and a protease in spores has been demonstrated, a fact which may account for the instability of ribosomes under ordinary isolation procedures. A comparison of spore and vegetative-cell ribosomes has revealed that they have essentially the same composition.

MATERIALS AND METHODS

Preparation of vegetative cells and spores. *Bacillus subtilis* W23 cells and labeled spores were grown at 37 C in the synthetic medium (SCM) of Doi and Igarashi (6), supplemented with 0.3% casein hydrolysate and 0.1% glucose minus sodium citrate. Unlabeled spores were obtained from cells grown in Penassay medium.

Vegetative cells were grown in SCM to an absorption at 660 mu of 0.5 as measured in a Beckman DU spectrophotometer. The cells were pooled by centrifugation, washed twice with 0.01 M tris(hydroxy-methyl)aminomethane (Tris) containing 5 X 10^-5 M MgCl2, and the pellet was frozen at -15 C in 0.01 M Tris containing 0.01 M MgCl2. All buffers were adjusted to pH 7.2.

For P32-labeled spores, 5 mc of P32O4^- was added to 100 ml of culture after the 2nd hr of the stationary phase. The SCM medium contained only 0.001 M inorganic phosphate. The cells were agitated for an additional 15 to 20 hr after the addition of P32O4^- for complete sporulation. Clean spore preparations were obtained by treating the cultures with 100 mcg/ml of lysozyme and 10 mg/ml of crude pancreatic deoxyribonuclease and with a final concentration of 0.005 M MgCl2 to remove the vegetative cells and debris. The two-phase "Y" system of Sacks and Alderton (15) was used to produce microscopically clean spores.

Extraction of ribosomes. Vegetative-cell ribosomes

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were extracted by grinding the pellets in precooled mortars at 4 C until a paste was obtained. The paste was extracted with 0.01 M Tris-0.01 M MgCl₂ buffer and was centrifuged at 17,000 × g for 15 min. The supernatant fluid was designated as the crude ribosomal extract. Partially purified ribosomes were obtained by centrifuging the crude extract for 3.5 hr at 100,000 × g in a Spinco model L preparative ultracentrifuge. The complete method for extraction and characterization of P³²-labeled spore ribosomes is shown in Fig. 1. Spores were alternately frozen in liquid nitrogen and thawed twice at room temperature, and were then immediately ground in a precooled mortar in the cold with 1 volume of alumina, which was used as a suspended ribosomes-P³², was centrifuged for 3.5 hr at 100,000 × g. The supernatant fluid was decanted, and the pellet containing the partially purified ribosomes was suspended in 2.0 ml of 1.5% sucrose in 0.01 M Tris-0.01 M MgCl₂ buffer (pH 7.2).

**Determination of ribosome sedimentation pattern.** The ribosome pattern was obtained by layering 2 ml of the crude extract containing vegetative-cell carrier ribosomes and P³²-labeled spore ribosomes equivalent to an absorbancy of 20 at 260 μm on a 3 to 20% linear sucrose density gradient. The gradient was centrifuged in the SW 25.1 rotor of a Spinco model L ultracentrifuge at 25,000 rev/min for 5 hr at 4 C. At the end of the run, equal fractions were collected by piercing the bottom of the tubes. The optical density at 260 μm of each fraction was determined, as well as its content of radioactivity. Radioactivity was measured according to the method of Doi and Igarashi (6).

**Column chromatography of purified RNA.** RNA from pooled 70S and 50S ribosomes was purified by the phenol method of Gierer and Schramm (9).

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**Spore-P³² pellet (100 mg)**

- Freeze in liquid nitrogen and grind at 4 C with 1 volume of alumina and vegetative ribosomes equivalent to an optical density at 260 μm of 75. Resuspend in 2 ml of 0.01 M Tris (pH 7.2), 0.01 M MgCl₂, and 2% deoxycholate. Incubate in cold for 30 min. Centrifuge at 17,000 × g for 15 min

**17,000 × g pellet**

- (cellular debris + alumina)

- Resuspend in 2 ml of 0.01 M Tris, pH 7.2, containing 0.01 M MgCl₂ and 1.5% sucrose. Centrifuge at 17,000 × g for 15 min

**17,000 × g supernatant fluid**

- (crude ribosomal extract)

**100,000 × g pellet**

- (discard)

**17,000 × g supernatant fluid**

- (crude ribosomes)

**Centrifuge at 100,000 × g for 3.5 hr**

- 100,000 × g supernatant fluid (discard)

- Differential sedimentation through linear sucrose gradient with carrier vegetative-cell ribosomes

**Pooled 70S ribosomes-P³²**

- (pure)

**Determination of protein and RNA content**

**Purify RNA**

**Fractionation by MAK column**

**Base ratio analyses**

**FIG. 1. Procedure for the extraction of spore ribosomes-P³².**
Fractionation of the preparations was accomplished by use of the methylated albumin kieselguhr (MAK) column of Mandell and Hershey (14). A linear gradient of NaCl was used to elute RNA. Base ratio analyses were performed by the method of Hayashi and Spiegelman (10).

Preparation of H₁-ribosomes from B. subtilis vegetative cells. For preparation of H₁-ribosomes, 100 ml of SCM medium was inoculated with cells having an optical density at 660 μm of 0.120 and the cells were grown to an optical density of 0.4 in the presence of 50 μg and 50 μc of H₁-uridine (4.4 c/mole). The cells were then collected by centrifugation, washed with SCM plus 15% sucrose, and resuspended in SCM supplemented with 10 μg/ml of unlabeled uridine. At an optical density (660 μm) of 0.8, the cells were collected, and the 70S ribosomes were purified as previously described.

Assay for nuclease and protease activity. Assays for nuclease activity were performed with crude extracts from log-phase cells (optical density at 660 μm of 0.3), 2-hr stationary-phase cells, and spores ground with and without alumina. Duplicate reaction mixtures contained 1.5 mg of protein from the individual extracts and H₁-ribosomes (9 × 10⁸ count/min) or RNA-H₁ (1.6 × 10⁶ count/min) in a total volume of 2 ml. Potential enzyme inhibitors (bentonite, polyvinylsulfate, and Macaloid) when tested were added at a concentration of 50 μg/ml. The reaction mixtures were incubated at 32 C with 0.1-ml samples removed every 15 min for assay of radioactivity.

Protease activity was determined by the method of Charney and Tomarelli (3). Each reaction mixture contained 100 mg of ground or whole spores, 25 mg of azocasein, and 1% NaH₂CO₃ in a total volume of 2 ml. The mixtures were incubated at 37 C for 45 min.

The protein content of ribosome extracts was determined by the Folin method of Lowry et al. (13). RNA content was determined by the orcinol method of Dische and Schwartz (4) on a sample treated with 1 M perchloric acid at 100 C for 15 min.

Materials. The following chemicals were used: uridine-H₁ (4.4 c/m mole), Schwartz Bio Research, Inc., Orangeburg, N.Y.; P₃₂-phosphoric acid, Oak Ridge National Laboratory; lysozyme and pancreatic deoxyribonuclease, Worthington Biochemical Corp., Freehold, N.J.; azoprotein, Calbiochem; levigated alumina, Norton Co., Worcester, Mass.

RESULTS

Isolation of spore ribosomes. In a previous investigation of the RNA of bacterial spores (6), it was found that ribosomal RNA was degraded rapidly unless spore extracts were prepared at a low temperature (−20 C) and under conditions in which ribonuclease activity was inhibited quickly. Therefore, some difficulty was encountered in the initial phases of this study in the isolation of intact ribosomes. Since purification of ribosomes required several hours, a method of isolation was required which would allow the ribosomes to remain intact for this period. Several methods of ribosomal extraction were tested to obtain ribosomes whose properties could be examined and compared with ribosomes from vegetative cells. When spores frozen in liquid nitrogen were ground in prechilled (−20 C) mortars, it was found that intact ribosomes could not be extracted. Since spores contain dipicolinic acid, a chelating agent, the Mg ion concentration was raised to 0.05 M; however, even at this concentration of Mg, no intact ribosomes could be isolated. Microscopic examination of the spore pellets which had been ground revealed that over 90% of the spores were broken by the freezing and grinding treatments.

Since Woese et al. (17) obtained spore ribosomes from B. subtilis by grinding with alumina, P₃₂-labeled spores were prepared and ground with alumina. The P₃₂-labeled spore extract was then combined with vegetative cell ribosomes as an optical-density marker, and was layered on a linear sucrose density gradient. The vegetative-cell ribosome pattern was clearly discernible, but a pattern attributable to spores was absent (Fig. 2). In addition, when spores were ground with alumina, the recovery of ribosomes as judged by the recovery of radioactivity was extremely low. In most experiments, the recovery was less than 1% of the total radioactivity of the spore. Furthermore, the radioactivity of the spore pellet after grinding was extremely high, and therefore it seemed possible that the ribosome...
somes were not being released into the extract but were bound to the alumina or to the spore cell wall-membrane complex. It was found that the radioactive spore ribosomes could be displaced from the alumina-ground pellet by adding vegetative-cell ribosomes to a suspension of the alumina-ground pellet. The recovery of P32 in the crude extract was increased to 12%. It appeared that the vegetative-cell ribosomes were displacing the P32-labeled spore ribosomes from the alumina. This is illustrated in Fig. 3 which shows a comparative ribosomal pattern of vegetative cells and P32-labeled spore. The vegetative-cell and spore ribosomal patterns coincided with clearly recognizable 70S, 50S, and 30S ribosomal peaks. In a control experiment, the ribosomes obtained from vegetative cells ground with and without alumina were analyzed for their sedimentation coefficients. The sedimentation coefficients of 69S, 52S, and 33S were essentially identical for both ribosome preparations; this signified that the alumina was not affecting the sedimentation pattern. Furthermore, it was shown that ribosomal RNA could also displace ribosomes from the alumina-ground pellet. This latter observation allowed us to isolate and characterize the bacterial spore ribosomes and compare them to the ribosomes of the vegetative cell.

The chemical composition of the 70S and 50S spore ribosomes is shown in Table 1. The spore ribosomes were obtained by mixing the alumina-ground spore pellets with bulk vegetative-cell RNA. The released spore ribosomes were then separated from the bulk vegetative-cell RNA by sedimenting the spore ribosomes. Further purification was obtained by differential sedimentation through a linear sucrose gradient. The spore ribosomes were then analyzed for their RNA and protein composition. The protein and RNA composition was 39.9 and 60.1, respectively. This compares favorably with the values of 40.1 and 59.9 found for vegetative-cell ribosomes. The RNA was isolated from the purified spore ribosomes and fractionated by MAK column chromatography. The RNA-P32 from the 70S ribosomes showed the presence of 16S and 23S ribosomal components (see Fig. 4). Both components have been shown to be prominent in the composi-

### Table 1. Chemical composition of 70S ribosomes from vegetative cells and spores

<table>
<thead>
<tr>
<th>Source of ribosomes</th>
<th>Protein*</th>
<th>RNA*</th>
<th>Protein</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cells...</td>
<td>0.251</td>
<td>0.375</td>
<td>40.1</td>
<td>59.9</td>
</tr>
<tr>
<td>Spores..............</td>
<td>0.069</td>
<td>0.173</td>
<td>39.9</td>
<td>60.1</td>
</tr>
</tbody>
</table>

* Average of three experiments.

![Fig. 3. Sucrose gradient sedimentation pattern of P32-labeled spore ribosomes after displacement from the alumina-ground pellet by vegetative-cell ribosomes. The solid line represents the optical density at 260 mg/ml of the vegetative-cell ribosomes; the dashed line, the P32-labeled spore ribosomes. The arrows represent the fractions pooled for MAK column fractionation and base ratio analysis (see Fig. 4 and Table 2).](image1)

![Fig. 4. MAK column chromatography of RNA-P32 extracted from 70S spore ribosomes. The solid line represents the optical density at 260 mg/ml of the carrier vegetative-cell RNA, and the dashed line represents the spore ribosomal RNA-P32. The arrows indicate the fractions pooled for base ratio analyses (see Table 2).](image2)
tion of the 50S and 30S monomers by Kurland (12). The base ratio of the spore ribosomal RNA is shown in Table 2. The base composition of the ribosomal RNA is essentially that reported previously by Doi (5). Therefore, the protein and RNA composition and the base composition of the RNA have been shown to be similar to those obtained from vegetative cells.

Examination of spore extracts for nuclease and protease activities. The absence of intact spore ribosomes upon extraction by the usual method suggested the presence of degradative enzymes in the spore extracts. An investigation was therefore undertaken to determine whether nucleases or proteases capable of digesting ribosomes or purified RNA were present in the crude spore extract. Table 3 shows the effects of several extracts upon 70S tritiated ribosomes. Extracts from log-phase cells, stationary cells, and spores ground with and without alumina were examined for their ability to degrade the tritiated RNA of ribosomes. The tritium was present in the uracil of the ribosomal RNA. The degradation of the ribosomal RNA was followed by the decrease in acid-precipitable counts. Only with the crude

Table 2. Base composition of RNA from spore ribosomes

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>Moles per cent</th>
<th>Per cent GC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytidine (C)</td>
<td>Ade-</td>
</tr>
<tr>
<td>Ribosomal I</td>
<td>24.8</td>
<td>26.5</td>
</tr>
<tr>
<td>Ribosomal II</td>
<td>25.0</td>
<td>23.4</td>
</tr>
</tbody>
</table>

* Ribosomal I and II RNA were extracted from purified ribosomes; ribosomal I RNA was further purified by MAK column chromatography.

Table 3. Decrease in radioactivity of tritiated ribosomes and RNA after incubation with various extracts

<table>
<thead>
<tr>
<th>Source of crude extract</th>
<th>Percent decrease with H1-ribosomes*</th>
<th>Percent decrease with H1-RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log-phase cells</td>
<td>0</td>
<td>23.0</td>
</tr>
<tr>
<td>Stationary-phase cells</td>
<td>0</td>
<td>26.9</td>
</tr>
<tr>
<td>Spore†</td>
<td>0</td>
<td>17.7</td>
</tr>
<tr>
<td>Spore‡</td>
<td>13.8</td>
<td>29.9</td>
</tr>
<tr>
<td>Buffer (0.01 M Tris-0.01 M MgCl2)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* After 2 hr of incubation; rate was linear with H1-RNA.
† Ground with alumina.
‡ Ground without alumina.

Table 4. Protease activity of spore extracts and whole spores

<table>
<thead>
<tr>
<th>Source of protease activity</th>
<th>Increase in optical density at 440 nm/45 min*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spores ground without alumina</td>
<td>0.282</td>
</tr>
<tr>
<td>Spores ground with 150 mg of alumina</td>
<td>0.126</td>
</tr>
<tr>
<td>Spores ground with 300 mg of alumina</td>
<td>0.062</td>
</tr>
<tr>
<td>Spores ground with 450 mg of alumina</td>
<td>0.059</td>
</tr>
<tr>
<td>Spores, unground</td>
<td>0.091</td>
</tr>
<tr>
<td>Spores, heat shocked (60 C, 15 min), unground</td>
<td>0.144</td>
</tr>
</tbody>
</table>

* Protease activity was assayed according to the method of Charney and Tomarelli (3).

extracts from the spores ground without alumina was there any decrease in the radioactivity of the tritiated ribosomes. A loss of roughly 14% was recorded. Addition of any of the other crude extracts did not result in the degradation of the ribosomes. Addition of potential nuclease inhibitors (bentonite, polyvinylsulfate, Macaloid) served only to stimulate breakdown with the extracts which had previously been without activity. The presence of nuclease activity was shown more decisively when tritiated RNA was used as the substrate. This is shown in Table 3. Ribonuclease activity was present in all the extracts. Again the spore extracts ground without alumina showed the greatest effect upon tritiated RNA, as shown by the loss of acid-precipitable counts.

Table 4 shows the results of the assay for protease activity in various spore extracts. When spores were ground in the absence of alumina, the highest activity of protease was observed. As an increasing amount of alumina was added to the spores during grinding, the amount of protease activity in the extract decreased. This suggested that the protease was adsorbing to the added alumina. The other interesting observation was the presence of proteolytic activity of whole spores; furthermore, the protease activity of unground spores was increased by heating the spores at 60 C for 15 min.

Discussion

These results, in addition to previous results of Woese et al. (17) and Doi and Igarashi (6), have demonstrated that bacterial spore ribosomes are essentially identical to those found in the vegetative form. Furthermore, Kobayashi and co-workers (11) recently found that B. cereus spore ribosomes are active in the polyuridylate stimula-
tion of phenylalanine incorporation. Therefore, from both the physicochemical and functional aspects the bacterial spore ribosome appears to be similar to that found in vegetative cells. The major difference between these results and those of Woese et al. (17) is the presence of 30S ribosomes in the spore extracts. Whether this is due to adsorption and displacement from alumina is not known.

The instability of bacterial spore ribosomes in the spore extracts presented a major problem in terms of isolation and characterization. The presence of ribonuclease and protease in the spore may account for this instability. It is not surprising that these enzymatic activities are present in spores. Several workers have demonstrated active turnover of RNA (1) and protein (2) during the sporulation phases of bacilli. From these results, it appears that these enzymes are incorporated or trapped during spore formation. Since protease activity is observed with whole spores, there must be protease molecules adsorbed to the outer wall of the spore or present just under the outer spore coat. Of interest is the fact that heat-shocked whole spores exhibited greater protease activity. Since greater protease activity is observed when spores are broken, this suggests that there is also an intracellular protease which is released upon breakage of the spore. Since ribosomes appear to be relatively stable to ribonuclease activity under most isolation conditions, the instability of spore ribosomes is probably due to the presence of both a protease and a ribonuclease. The action of both these enzymes may be necessary and sufficient to degrade the ribosome structure.

The similarity of the spore ribosome to the ribosome found in vegetative cells suggests that the ribonuclease protein particles have not been altered during the sporulation process and are not the cause of dormancy in spores. The major RNA fractions ordinarily found in living cells have been demonstrated in bacterial spores. The only fraction that has not been demonstrated in dormant spores is the messenger RNA fraction (6, 11). However, during germination there is a rapid and extensive synthesis of messenger RNA (7). Presumably these messenger RNA molecules are used to form the enzymes necessary for the germination process. It appears, therefore, that the transcription process is controlled during sporulation, dormancy, and germination. The presence of the major components of the protein-synthesizing system, except for messenger RNA, suggests that dormancy may be caused by a factor which controls the transcription process. There is insufficient evidence at the moment to decide whether dormancy is controlled primarily by regulating the transcription process or whether the regulation of the transcription process is a secondary factor in the overall process of converting a vegetative cell to a dormant spore. Further analyses on the sporulating cells should be fruitful since several investigators have found that regulation occurs during various steps of sporulation (16).

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