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Synthesis of Ribosomal Ribonucleic Acid During Sporulation of *Bacillus subtilis*

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The incorporation of radioactive uracil into 50*s* and 30*s* ribosomal subunits and ribosomal ribonucleic acid (rRNA) was studied during the growth cycle of different sporogenic and asporogenic strains of *Bacillus subtilis*. It was found that partially synchronized cultures of the strains examined incorporated labeled uracil into the two ribosomal subunit species and rRNA during sporulation and during the stationary phase of the asporogenic strains. Kinetic studies have shown that, compared to vegetative cells, the percentage of uracil incorporated into the ribosomal subunits of cells taken 30 min after the end of exponential growth was decreased by about 25 to 35%. This decrease, however, appeared to be a general characteristic of stationary-phase cells and seems to depend on the nature of the sporulation medium and to some extent on the nature of the strain but not on the sp^+ or sp^- phenotype of the strain. Moreover, by use of actinomycin D it was shown that the labeled uracil incorporated, in the presence of the drug, during the sporulation period was located in the ribosomal subunits (stable RNA). Based on these results, we concluded that during sporulation ribosomal genes are transcribed and consequently rRNA continues to be synthesized, although to a lesser extent than during vegetative growth. These results are discussed in the light of those obtained by Hussey et al.

Although the net synthesis of ribonucleic acid (RNA) in *Bacillus subtilis* stops at the end of the exponential growth phase (T_0), studies on the incorporation of radioactive precursors have indicated that active turnover of RNA takes place throughout the sporulation period (1, 21, 27). It was also shown that all three types of RNA (messenger [mRNA], ribosomal [rRNA], and transfer [tRNA]) are synthesized during sporulation in the same proportions as during growth (2, 3).

More recently, Hussey, Losick, and Sonenshein (8) reported that the incorporation of radioactive uracil into 50*s* and 30*s* ribosomal subunits stops very early during sporulation. They also found that there was no incorporation of radioactivity into 23*s* rRNA and very little into the 16*s* RNA, even though other species of RNA were made during sporulation. These authors suggested that the rRNA genes are not expressed in sporulating cells. This was further substantiated by the observation that a rifampin-resistant mutant of *B. subtilis* (Rfr 10) which is also defective in sporulation continued to synthesize rRNA during the stationary phase.

Hussey et al. (9) have further observed that RNA polymerase purified from wild-type sporulating cells synthesized little rRNA in vitro, whereas such synthesis took place in the presence of the homologous enzyme isolated from stationary cells of the sporulation-defective rifampin-resistant mutant. Since "sporulation RNA polymerase" is lacking the sigma factor and contains an altered β subunit (13), these authors suggested that the turn-off of the rRNA genes might be due to the alteration of the vegetative RNA polymerase.

In our studies on rRNA synthesis by a temperature-sensitive sporulation mutant (ts-4), we have observed that the percentage of incorporation of ^{3}H-uracil into ribosomal subunits during the stationary phase is the same whether the cells were grown at the restrictive or at the permissive temperature. At the restrictive temperature the sporulation process is blocked at stage zero, wheras under permissive conditions spores are formed with the same

efficiency as in the parental strain. These observations are at variance with the results and conclusions of Hussey et al. (8), and, because of the importance of this problem for comprehension of the sporulation process in general, we have undertaken a more detailed examination of rRNA synthesis during the growth and stationary phase of a number of different sporogenic and asporogenic strains of *B. subtilis*.

We report here that in partially synchronized cells of *B. subtilis* incorporation of radioactive uracil into ribosomal subunits takes place in sporulating cells as in the stationary-phase cells of asporogenic mutants. Similar conclusions were drawn from experiments with actinomycin D. We further show that, during sporulation, ^{3}H-uracil is incorporated into 16*s* and 23*s* rRNA at the same efficiency as in stationary-phase cells of an Rfr, sp$^-$ mutant. The decrease in the incorporation of radioactive RNA precursors into ribosomal RNA in the early stationary phase is independent of the spore phenotype but seems to vary with the nature of the sporulation medium and, to some extent, with the nature of the strain.

MATERIALS AND METHODS

Strains. The strains used in this work are described in Table 1.

Media. The carrier cells were grown in nutrient broth (Difco) supplemented with metals as described previously (4). In the incorporation studies, either the resuspension medium of Sterlini and Mandelstam (22) or the double strength nutrient broth medium of Leighton et al. (11) was used.

Sporulation. Sporulation was induced in partially sychronized cultures in the two media mentioned above (11, 22). Synchronization with respect to sporulation was achieved in Difco medium as follows (J. Szulmajster, *in press*). An overnight preculture, inoculated from a single-cell colony and grown at 25 C, was centrifuged, suspended in fresh medium at a 100-fold dilution, and allowed to grow until the optical density at 650 mm (OD_{650}) reached about 1.0. This culture was then centrifuged and was washed with prewarmed fresh medium; the cells were suspended in this medium (OD_{650} of 0.1 to 0.2) and growth was allowed until no more increase in the optical density was observed. The cells were then rapidly collected by centrifugation at the growth temperature, washed as before, and suspended to an OD_{650} of about 1.0, which then permitted the cells to accomplish two to three generations before they reach the stage T_0 (end of exponential growth). Synchronization of the sporulating cultures was followed by examination in an electron microscope of the different morphological stages as a function of time after T_0. We observed that, under these conditions of synchronization, at T_0, 85 to 95% of the bacterial population were at stage 0-I, recognizable by the formation of the axial filament from the two nuclei of the vegetative cell. It was also observed that it took about 90 to 100 min for the great majority (60 to 70%) of the cells to reach stage II (formation of spore septum). As sporulation proceeded, there seemed to be a scattering of all stages. However, at T_5 to T_6, there was a rapid increase in the frequency of the last stages (V–VI). From these observations, we concluded that under our conditions of sporulation the cells are synchronized in the sense that at the end of exponential growth almost all of the bacterial population enters the sporulation process.

Labeling of cultures. Cultures (10 ml) were labeled at various times during the growth cycle with 25 μg of [5-^3H]uracil containing 2.5 μCi (final specific activity, 0.1 μCi/μg of uracil). The cultures were labeled for 20 min and labeling was stopped by diluting the radioactive cells into 100 ml of a nonradioactive exponential culture, grown in nutrient broth (carrier cells). The combined cultures were centrifuged, washed with Tris-Mg buffer (10 mM tris[hydroxymethyl]aminomethane-1 mM magne-

TABLE 1. *Bacillus subtilis strains used*

Strain	Relevent genotype or phenotype[a]	Spore formation	Origin
168, Marburg	Wild type	+	
168, TT	*trp*$^-$, *thy*$^-$	+	Anagnostopoulos
ts-4[b]	*trp*$^-$, *thy*$^-$	+ at 30 C – at 42 C	168 TT
12 A	*trp*$^-$, *prot*$^-$	–	Spizizen (19)
110-NA	ab$^-$, prot$^-$, est$^-$	–	Michel and Cami (15)
BS-15[c]	*trp*$^-$, Rfr	–	12 A
BS-22[c]	*trp*$^-$, Rfr	–	168 M
BS-B-31[d]	*trp*$^-$, *lys*$^-$, Rfr	+	GSY-1540, Anagnostopoulos

[a] Abbreviations: prot, protease; ab, antibiotic; est, esterase; Rfr: rifampin resistance.
[b] Isolated in the Laboratoire d'Enzymologie by mutagenesis of 168 TT.
[c] Isolated in the Laboratoire d'Enzymologie by spontaneous mutation on rifampin.
[d] Isolated in the Laboratoire d'Enzymologie by mutagenesis of GSY-1540.

sium acetate) at pH 7.2, and stored at −20 C. For the extraction of RNA, the cultures were labeled with ^{3}H-uracil, 2.5 μg/ml containing 2.0 μCi/ml (final specific activity, 0.8 μCi/μg).

The labeling of the culture with ^{14}C-uracil and the treatment with actinomycin D are described in the corresponding figure legends.

Preparation of ribosomal subunits. Frozen cells were suspended in 1 ml of low-magnesium buffer (10 mM Tris-0.02 mM magnesium acetate-5 mM β-mercaptoethanol), pH 7.2, and 1 mg of lysozyme/ml. The mixture was incubated for 20 min at 30 C. Cell debris was removed from the extract by centrifugation for 10 min at 10,000 rpm. The supernatant liquid was dialyzed for 3 h against 1 liter of the same buffer with three changes of buffer. To complete the dissociation into ribosomal subunits, the dialyzed supernatant fluid was stirred overnight at 4 C in the low-magnesium buffer. A 1-ml amount of the extract was then layered onto a 25-ml linear sucrose gradient (5 to 20%) in Tris-Mg buffer and centrifuged for 6 h at 25,000 rpm (Spinco SW 25.1 rotor). If not otherwise stated, fractions of 0.7 ml were collected dropwise directly into scintillation vials; 0.1 ml of each fraction was diluted 10 times and the absorbancy at 260 nm was measured on a Zeiss spectrophotometer. To the remainder of each fraction, Bray scintillation liquid was added, and the radioactivity was determined in a scintillation counter. The labeling of cultures and the preparations of ribosomal subunits from cells taken at different times after the end of exponential growth were done in a similar manner.

Extraction of RNA. RNA was extracted according to the method of Dubnau, Davidoff, and Smith (6). Frozen cells (labeled at different times, mixed with unlabeled vegetative carrier cells) were suspended in 5 ml of acetate buffer (0.1 M), pH 5.0, in the presence of 50 μg of deoxyribonuclease/ml and 1 mg of macaloid/ml. The suspension was subjected to sonic disintegration for 3 min under cooling in acetone-dry ice. Cell debris was removed by centrifugation at 10,000 rpm for 15 min, and the RNA was extracted by shaking (5 min) the supernatant fluid with an equal volume of cold buffer-saturated redistilled phenol containing 1% sodium dodecyl sulfate. The aqueous phase was removed and treated twice again with buffer-saturated phenol. The RNA was precipitated at −20 C from the aqueous phase by two volumes of ethanol (left in ethanol for at least 4 h). The RNA was collected by centrifugation and dissolved in 1 ml of acetate buffer. A sample was taken for measurement of optical density and radioactivity. The RNA preparation was then layered on a sucrose gradient (5 to 20% in acetate buffer) and centrifuged for 16 h at 22,500 rpm in the SW 25.1 rotor of a Spinco centrifuge.

RESULTS

Incorporation of ^{3}H-uracil into 50s and 30s ribosomes. The labeling of the cultures and the preparation of ribosomal subunits were as described in Materials and Methods. The percentage of RNA in ribosomal subunits is the percentage of total radioactivity in the gradient located in the 50*s* and 30*s* peaks.

It has to be emphasized that, in the stationary phase, *B. subtilis* cells (sp$^+$ or sp$^-$ strains) incorporated only about 10% as much ^{3}H-uracil as the vegetative cells. A typical example of the quantitative aspect of incorporation of ^{3}H-uracil at various times during the growth cycle and of the recovery of the label in the ribosomal subunits is described in the legend of Fig. 1. A similar example for the rRNA is shown in Table 2.

Figure 1 shows the incorporation of ^{3}H-uracil into ribosomal subunits prepared from exponential (A) and sporulating (B, C) cultures of *B. subtilis* strain 168wt. It was calculated that at 30 ($T_{0.5}$), 120 (T_2), and 180 min (T_3, not shown on the figure) after the end of exponential growth (T_0), respectively, 59, 63, and 50% of the total radioactivity incorporated and layered onto the sucrose gradient still entered into the ribosomal subunits of the sporulating cells. The percentage of radioactivity incorporated into the ribosomal subunits of the exponential cells was 79%. The drop of uracil incorporation into the ribosomal subunits in postexponential cells is not the result of the sporulating state of the cells, since an even more pronounced decrease was observed with

TABLE 2. *Extraction of ^{3}H-uracil-RNA at different times during the growth cycle*[a]

Time of incorporation	Total ^{3}H-uracil incorporated in 20 min (counts/min)[b]	Recovery after phenol extraction (counts/min)	Recovery in ethanol precipitate (counts/min)	Percentage of incorporation into RNA[c]
Exponential	3.73×10^6	3.47×10^6	2.9×10^6	72
$T_{0.5}$	8.65×10^5	8.24×10^5	4.62×10^5	51
T_2	4.91×10^5	4.24×10^5	2.31×10^5	50

[a] The labeling of cultures, treatment of the cells, and the extraction of RNA were as described in Materials and Methods and the legend of Fig. 7.

[b] Amount of ^{3}H-uracil present in the crude extracts.

[c] Amount of label located in the 16*s* and 23*s* peaks recovered from the sucrose gradient relative to the total amount of RNA (ethanol precipitable) layered on this gradient.

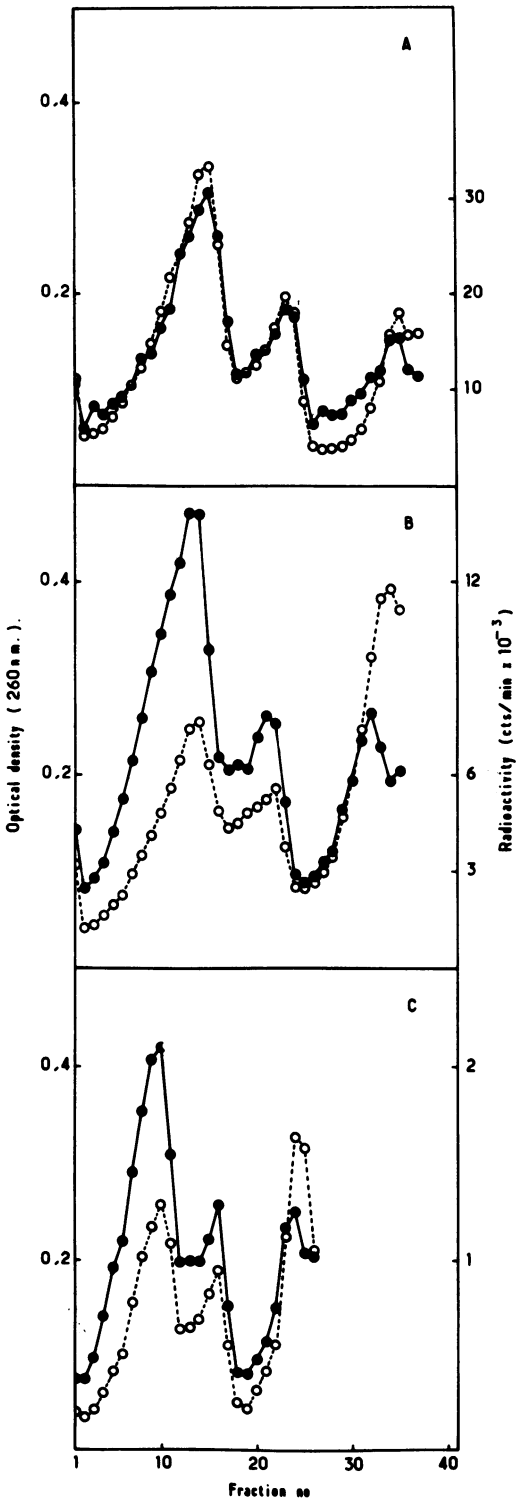
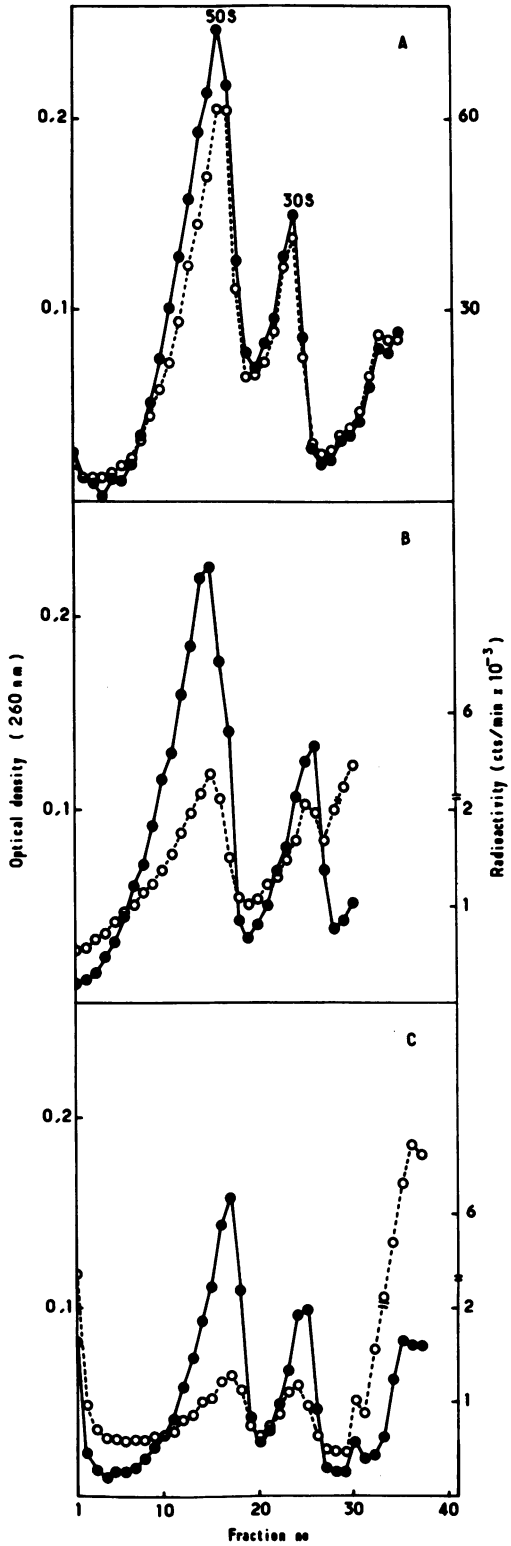


FIG. 1. *Sucrose density-gradient sedimentation of ^{3}H-labeled ribosomal 50s and 30s subunits of B. subtilis 168 wt during growth and sporulation. Cells were grown in nutrient broth (Difco) medium and labeled for 20 min with 10 μg of [5-^{3}H]uracil/ml (final specific activity, 0.1 μCi/μg): (A) during exponential growth, and (B and C) 30 min ($T_{0.5}$) and 120 min (T_2), respectively, after the end of exponential growth. The labeled cells were mixed with unlabeled vegetative cells grown under the same conditions and extracted with Tris-Mg buffer as described in Materials and Methods. The dialyzed extracts were layered onto a 25-ml linear sucrose gradient (5 to 20%) in Tris-Mg buffer and centrifuged at 4 C for 6 h at 25,000 rpm (Spinco SW-25 rotor). In this experiment, the total incorporation of ^{3}H-uracil in the crude extracts was 1.41 × 10^6, 4.32 × 10^5, 1.24 × 10^5, and 5.15 × 10^4 counts/min for exponential, $T_{0.5}$, T_2, and T_3 cultures, respectively. After dialysis, the corresponding figures were 1.36 × 10^6, 3.49 × 10^5, 5.64 × 10^4, and 1.31 × 10^4 counts/min. These quantities (taken as 100%) were layered onto the sucrose gradients, and the recovery of radioactivity located in the ribosomal peaks was 1.07 × 10^6 (79%), 2.06 × 10^5 (59%), 3.56 × 10^4 (63%), and 6.55 × 10^3 counts/min (50%), respectively, for the exponential, $T_{0.5}$, T_2, and T_3 extracts. Symbols: ●, optical density at 260 nm; ○, radioactivity/fraction.*

the asporogenic *B. subtilis* strain 12A (Fig. 2): here, the percentage of incorporation of ^{3}H-uracil into the ribosomal subunits was 78, 44, and 37, respectively, for exponential cells and for cells taken at $T_{0.5}$ and T_3. It is important to note that the 12A strain is a mutant blocked at stage zero of the sporulation process.

Since these results are in contradiction to those obtained by Hussey et al. (8), it was essential to test several other sporogenic and asporogenic strains of *B. subtilis* with respect to their ability to synthesize rRNA during the stationary growth phase or during sporulation (Table 1). The results obtained with five different strains and in two different sporulation media are summarized in Fig. 3. Each curve represents the kinetics of the incorporation of ^{3}H-uracil into the 50*s* and 30*s* ribosomal subunits as a function of the growth cycle. The following conclusions can be drawn from these data. (i) The incorporation of uracil into rRNA continues during stationary growth in all strains tested. (ii) In most cases, the decrease in the ratio of ribosomal to total incorporation of uracil is greater in the nutrient broth resuspension medium than in the synthetic one: the decrease at T_2 varies from 21 to 43% in the first medium and only from 0 to 25% in the second. (iii) Whatever the extent of the decrease might be, it is apparently independent of the sp$^+$ or sp$^-$ phenotype of the strain.

Relative to these experiments, it is worthwhile to mention an observation made by



Spotts and Szulmajster in 1962 (*unpublished data*) in the course of their investigations on the synthesis of unstable and stable RNA during sporulation: when the cells of the wild-type strain of *B. subtilis* (SMY) or the cells from an asporogenic strain (01003) were labeled at T_3 for 20 min with ^{14}C-uracil, the ratio of the specific activities (counts per minute per OD_{260} unit) of sp^+ to sp^- was 1.5 for the 50*s* ribosomal subunits and 1.0 for the 30*s* subunits. This would suggest that during sporulation not only does rRNA continue to be made but also the 50*s* subunits are synthesized more rapidly than the 30*s* subunits.

Incorporation of radioactive uracil into ribosomal subunits in the presence of actinomycin D. Actinomycin D is known to prevent the deoxyribonucleic acid-dependent RNA formation. In the presence of this drug, mRNA decays rapidly (unstable RNA) whereas rRNA and tRNA remain unbroken (stable RNA). We therefore used actinomycin D as another criterion to study the formation of stable RNA during sporulation.

Figure 4 shows the incorporation of ^{14}C-uracil (20-min labeling) into a culture of *B. subtilis* wild type at T_1 and $T_{2.5}$ as a function of time after addition of actinomycin D. It can be seen that in both cases 35% of label was incorporated into acid-precipitable material. Under the same conditions, exponential cells incorporated 73% of uracil after addition of the drug. These values are similar to those observed by measuring directly the incorporation of ^{3}H-uracil into ribosomal subunits (Fig. 3A, B, C) isolated from cultures grown in nutrient broth medium. Figure 5 shows the incorporation (in the presence of actinomycin D) at T_1 and $T_{2.5}$ of ^{14}C-uracil into stable, acid-precipitable material by two different asporogenic strains, 110NA (blocked at zero stage of the sporulation process) and Rfr BS-15 compared to the *B. subtilis* wild-type strain. These results indicate that the percentage of decrease of uracil incorporation in the 110NA, sp^- strain is almost identical to that of the wild type tested. Similar results were obtained with the ts-4 mutant at the restrictive temperature, when sporulation is arrested, and with the 168TT parental strain at the same temperature, where sporulation occurs normally. To attribute more significance to the actinomycin experiments, it was necessary to demonstrate that the stable frac-

FIG. 2. *Sucrose density gradient sedimentation of ^{3}H-labeled ribosomal subunits of B. subtilis 12A (sp^-) during growth and stationary phase. Conditions as described in the legend of Fig. 1.*

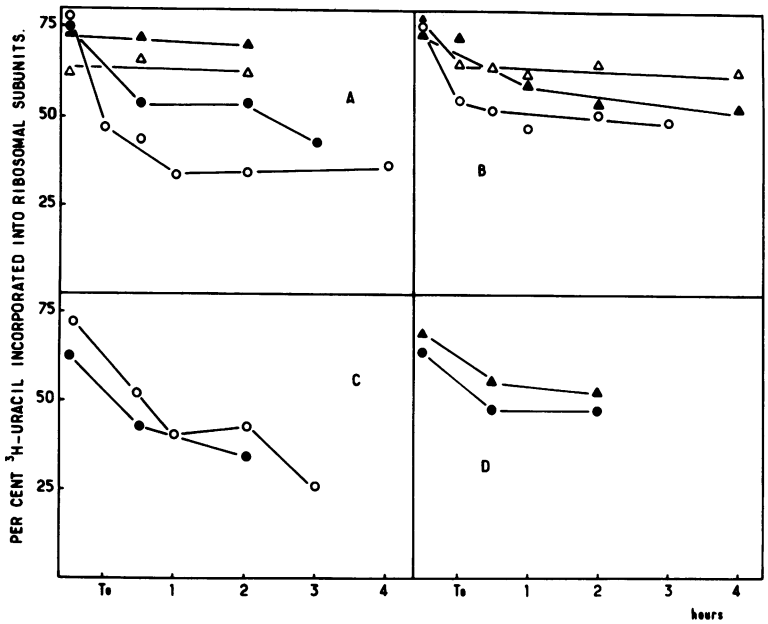


FIG. 3. *Incorporation of ³H-uracil into ribosomal subunits of various sporogenic and asporogenic strains of Bacillus subtilis. Labeling of cells, preparation of extracts, and sedimentation of ribosomal subunits as described in legend of Fig. 1. Symbols:* ▲, △, *resuspension medium of Sterlini and Mandelstam (22);* ●, ○, *double-strength nutrient broth medium (11); (A)* ▲, ●, *B. subtilis 168 TT, sp⁺;* △, ○, *B. subtilis 12 A, sp⁻; (B)* ▲, *ts-4 (30 C), sp⁺;* △, ○, *ts-4 (42 C), sp⁻; (C)* ●, *Rfr, sp⁺,* ○, *Rfr, sp⁻; (D)* ▲, ●, *168 wt, sp⁺.*

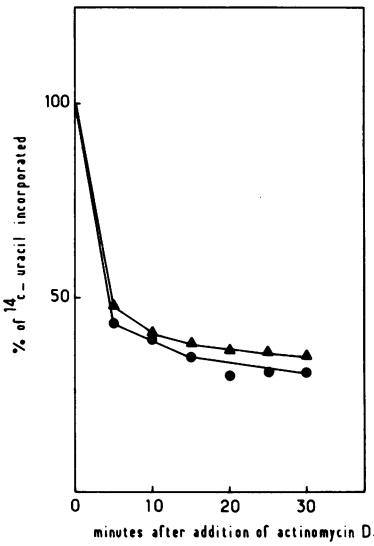


FIG. 4. *Incorporation of ¹⁴C-uracil in the presence of actinomycin D. To 10 ml of B. subtilis 168 culture taken at* T_1 *and* $T_{2.5}$, *7 μg of ¹⁴C-uracil/ml (58 mCi/mmol; final specific activity, 0.1 μCi/μg) was added. After 20 min of labeling, 10 μg of actinomycin D/ml was added, and samples of 1 ml were taken at 5-min intervals, precipitated, washed by 5% trichloroacetic acid containing* 10^{-2} *M cold uracil, and counted in a scintillation counter. Cultures of* T_1 (▲) *and* T_2 (●).

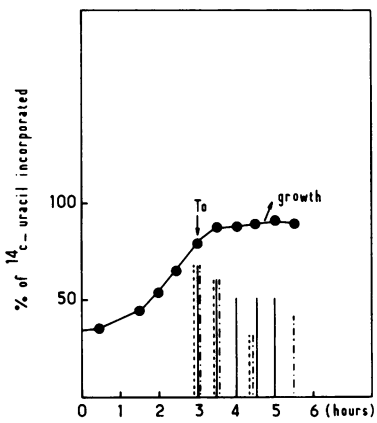


FIG. 5. *Incorporation of ¹⁴C-uracil in the presence of actinomycin D by sp⁺ and sp⁻ B. subtilis strains. Samples of 10 ml from cultures taken at the time indicated on the figure were labeled and treated with actinomycin D as described in the legend of Fig. 4. After 30 min of incubation in the presence of actinomycin D, 1-ml samples were precipitated and counted as described in the legend of Fig. 4. Symbols: ---, wt; -·-·-, 110 NA, sp⁻; ——, BS-15, Rfr, sp⁻.*

tion of RNA synthesized in the actinomycin experiments is present in the ribosomal subunits. This is clearly shown in Fig. 6. Altogether, the actinomycin experiments also confirm that labeled uracil is indeed incorporated into rRNA

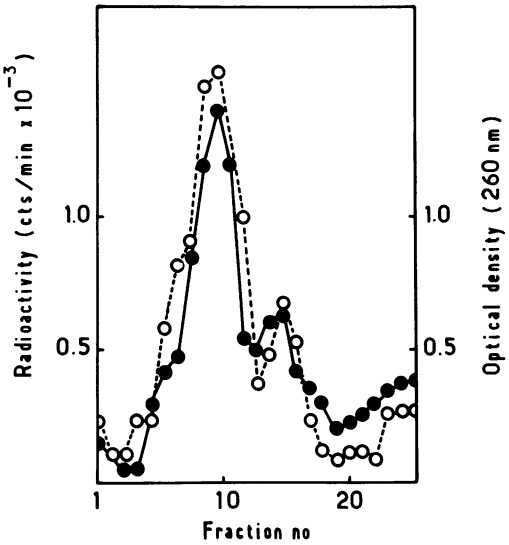


FIG. 6. *Sucrose density gradient sedimentation of ^{3}H-labeled ribosomal subunits after actinomycin D treatment of sporulating cells. A 20-ml amount of a B. subtilis 168 M culture taken at T_1 was labeled for 20 min with 10 μg of [5-^{3}H]uracil/ml (final specific activity, 0.2 μCi/μg) followed by a 30-min incubation in the presence of actinomycin D. The cells were then diluted with a 100-ml unlabeled culture of vegetative cells grown under the same conditions, centrifuged, and washed three times in Tris (10 mM)-magnesium acetate (10 mM) buffer, pH 7.5, containing 5 mM β-mercaptoethanol and 1 mg of macaloid/ml. The cells were then suspended in the same buffer and disrupted by a 3-min sonic treatment in a Branson Sonifier under cooling. Cell debris was removed by low-speed centrifugation, and the supernatant fluid was centrifuged for 1 h at 65,000 rpm. The ribosomal pellet was suspended in Tris-Mg buffer at low Mg^{2+} concentration (0.1 mM magnesium acetate) and dialyzed for 16 h against the same buffer. A sample of the dialyzed ribosomes was layered onto a sucrose gradient and treated as indicated in Materials and Methods. Symbols: ●, optical density; ○, radioactivity.*

during the stationary phase of sporulating (sp^+) and nonsporulating (sp^-) cells of *B. subtilis*.

Synthesis of 16s and 23s rRNA. It was shown by Spotts and Szulmajster (21) that *B. subtilis* cells, when labeled with ^{3}H-uracil during sporulation (T_3) for 20 min, incorporated most of the label into rRNA species (stable RNA). However, when the cells were given a 3-min pulse label, the radioactivity was found in the mRNA fractions (unstable RNA). Using actinomycin D, Szulmajster et al. (24) further showed that in *B. subtilis* the half-life of sporulation mRNA is of the same order of magnitude (2 to 3 min) as that in exponentially growing cells (12). On the other hand, the unstable RNA fraction represents only 3% (17) or a maximum of 9% (18) of the total RNA in *B. subtilis*.

To examine the synthesis of rRNA during the growth cycle of *B. subtilis*, we labeled the cells for 20 min and then extracted the RNA as described in Materials and Methods. We observed that in the extraction of RNA from exponential cells of *B. subtilis* by the method of Scherrer and Darnell (19), using hot phenol, there was a dramatic loss of the 23*s* RNA and only part of the 16*s* species was recovered after precipitation with ethanol. This difficulty was overcome by using the extraction procedure of Dubnau (6). It is shown in Fig. 7 that in cells of *B. subtilis* 168TT radioactive uracil was incorporated into their 16*s* and 23*s* RNA for at least 2 h after exponential growth was completed. In this experiment, the relative amounts of label incorporated in these two species of RNA were 84% of total ^{3}H-uracil incorporated for exponential cells (Fig. 7a) and were 56 and 58% when the RNA was extracted from cells labeled at $T_{0.5}$ and T_2, respectively (Fig. 7B, C). The remainder of the label at $T_{0.5}$ and T_2 was found in the interphase after phenol extraction and could be partially recovered after extraction with 1 M sodium chloride. Using the BS-22 nonsporulating Rfr mutant (obtained by a single mutational event from the 168M strain), we found that 72% of the total radioactive uracil incorporated into exponential cells was in the 16*s* and 23*s* RNA (Fig. 8A); 51 and 40% were found in these two RNA species when extracted from stationary-phase cells labeled, respectively, at $T_{0.5}$ and T_2 (Fig. 8B, C).

When the RNA was extracted from the 12A strain (sp^-, $prot^-$), 74% of the total ^{3}H-uracil incorporated was found in the 16*s* and 23*s* RNA from exponential cells, and only small fragments of degraded RNA were found when it was extracted from cells labeled at $T_{0.5}$ or T_2. In all cases, whatever the extent of the degradation of the stationary-phase rRNA, the rRNA from the vegetative carrier cells remained unbroken.

The rRNA recovered from the gradient of the experiment shown in Fig. 7 was completely degraded by alkali treatment (0.3 M KOH, left overnight at 37 C), the mononucleotides were separated by paper electrophoresis, and the radioactivity on each spot (localized by ultraviolet light) was counted in a scintillation counter. The label was found in uridine monophosphate and cytidine monophosphate in almost

equal quantities.

DISCUSSION

Our results clearly show that during sporulation radioactive uracil is incorporated into 50*s* and 30*s* ribosomal subunits. It is true that the extent of incorporation decreases early in the postlogarithmic cells, but this decrease seems generally to depend on the nature of the medium or on the type of strain, but not on the sporulation capacity of the strain: in nutrient broth complex medium, a decrease of about 25 to 35% of uracil incorporated into ribosomal subunits was observed between T_0 and T_2 to T_4, whereas in the synthetic resuspension medium the decrease was even less and varied only from 0 to 20%. In both cases, however, ribosomal subunits continued to be synthesized. Similar results were obtained in experiments in which actinomycin D was added after a 20-min labeling with radioactive uracil of stationary-phase cells of sporogenic and asporogenic strains. Here again, it was shown that the actinomycin-stable RNA fraction was present in the ribosomal subunits.

In similar experiments, we have shown that ^{3}H-uracil was incorporated into 16*s* and 23*s* rRNA of sporulating cells (Fig. 7) and into stationary-phase cells of the BS-22 Rfr asporogenic mutant (Fig. 8). Here too, we observed that the percentage of incorporation of uracil into rRNA decreases early in the stationary-phase cells in the same proportions as described for the ribosomal subunits. This decrease is also independent of the sporulation phenotype of the strain and appears to be characteristic of stationary-phase cells.

Although earlier experiments from different laboratories (2, 3, 21) led to the assumption that stable RNA was synthesized during sporulation of *B. subtilis*, there was some doubt on the validity of this assumption since no evidence of synchronization of the sporulating cultures was reported in these experiments.

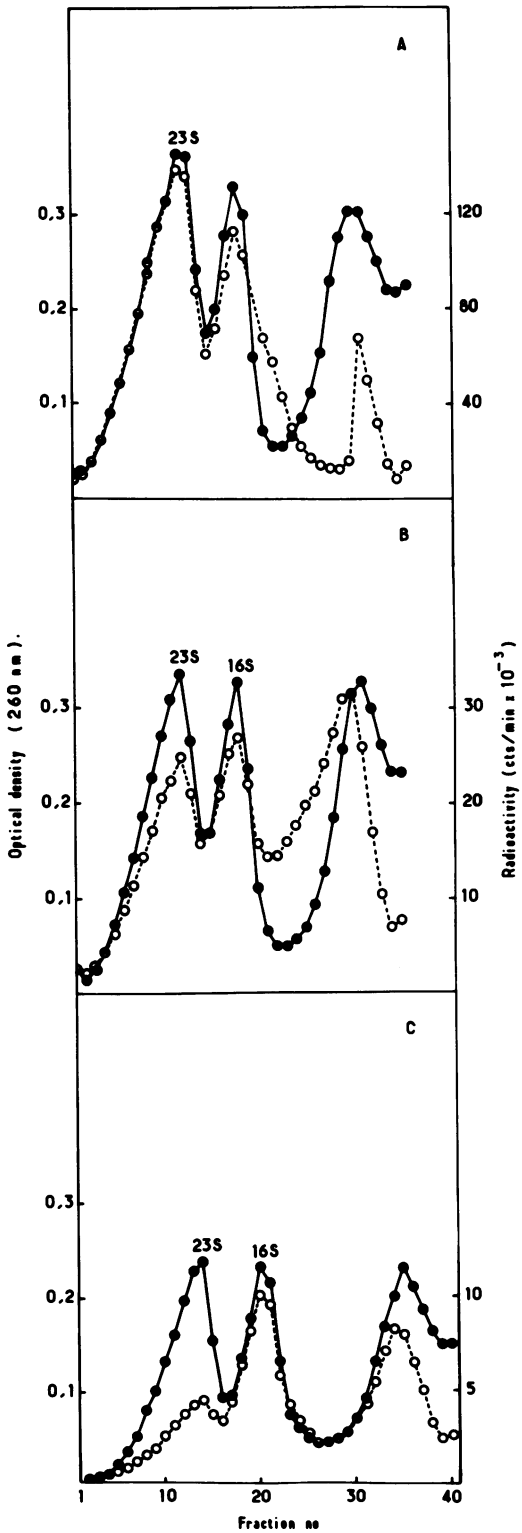


FIG. 7. *Incorporation of ^{3}H-uracil into 16s and 23s ribosomal RNA during growth and sporulation of B. subtilis 168 TT, sp$^+$, ^{3}H-RNA was extracted from cells labeled for 20 min and mixed with unlabeled vegetative carrier cells as described in Materials and Methods. The RNA preparation was layered on a sucrose gradient (5 to 20% in acetate buffer) and centrifuged for 16 h at 22,500 rpm in the SW-25.1 rotor of a Spinco centrifuge. Fractions of 1.2 ml were collected for measurement of optical density and radioactivity. Symbols: ●, optical density; ○, radioactivity. RNA extracted from cells labeled (A) during logarithmic growth; (B) at $T_{0.5}$; (C) at T_2.*

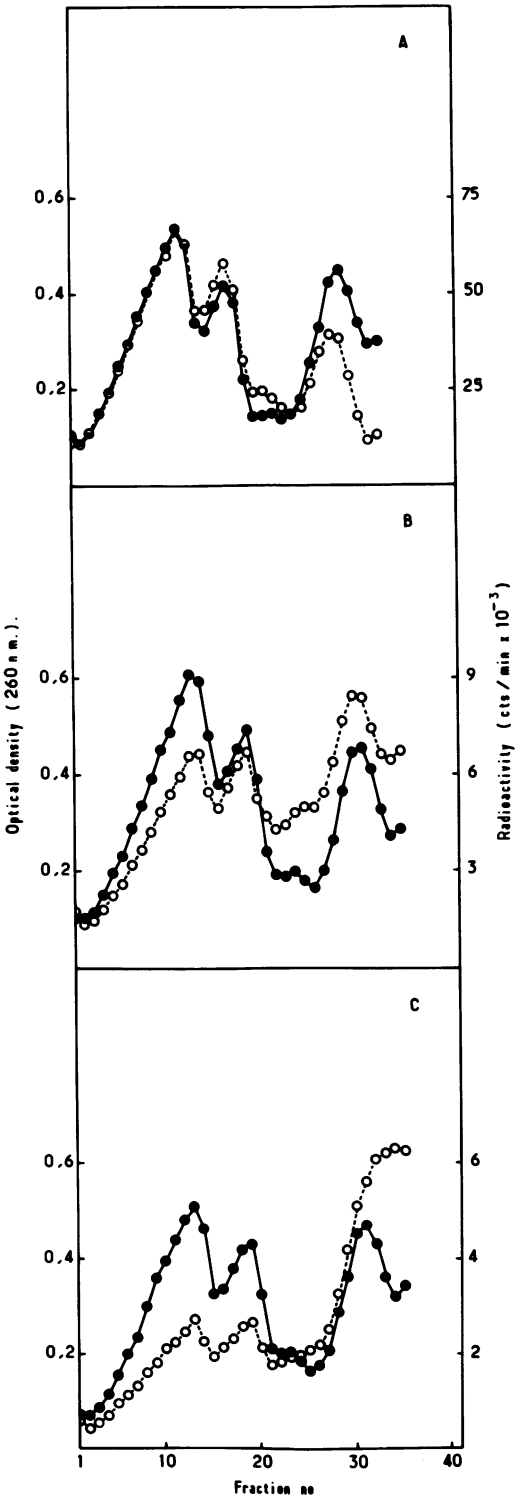


FIG. 8. *Incorporation of ^{3}H-uracil into 16s and 23s ribosomal RNA during growth and stationary phase of B. subtilis BS-22, Rfr, sp$^-$. Conditions and symbols as in legend of Fig. 7.*

Indeed, a lack of synchronization could lead to the impression of rRNA synthesis, which in fact could well be due to the presence of some growing vegetative cells and not to those which had already started to sporulate. This objection was removed in the experiments described here. Electron microscopy has shown that the cultures we were dealing with were synchronized with respect to the first morphological stages of sporulation: at T_0, 85 to 95% of the bacterial population reached stage 0-I, and at $T_{1.5}$ 60 to 70% of the cells entered stage II. Furthermore, the incorporation studies reported here have shown that even at T_3 or T_4 labeled uracil was still incorporated into ribosomal subunits. In no case was the incorporation into the latter observed to stop, irrespective of the strain and sporulation medium used. This was also true, at least in our hands, when the wild-type strain *B. subtilis* 3610 grown on medium 121B of Hussey et al. (8) was used. In this case too, we found that 64% of total uracil was incorporated into the 50*s* and 30*s* ribosomal subunits of vegetative cells, 48, 50, and 37% into these two subunit species of cells taken at $T_{0.5}$, T_1, and T_3, respectively.

As already mentioned, the decrease in the percentage of incorporation of RNA precursors into rRNA appears to be characteristic of stationary-phase cells. Another characteristic of this type of cells is that some of their rRNA is released from the ribosomes during extraction and degraded to fragments nonprecipitable by ethanol or trichloroacetic acid (Table 2). In some cases (strain 12A), the 16*s* and the 23*s* rRNA species were completely degraded into soluble material. The presence in the extracts of stationary-phase cells of an active ribonuclease which could be responsible for this degradation after breakage of the cells is to be discarded, since the carrier RNA derived from exponential cells remained, apparently, intact.

It is known that ribosomal proteins turn over during nitrogen starvation or starvation for Mg^{2+}, which may lead to a net destruction of ribosomes (15). On the other hand, the degradation of rRNA has been indicated to occur in cells starved for phosphate (7) or for uracil (20), or in low concentrations of Mg^{2+} (26). It was also shown that the RNA to protein ratio for purified ribosomes falls sharply from 0.95 in the carbon-limited media to 0.45 in the Mg^{2+}-limited samples (23). This corresponds to a shift in the composition of the ribosomes from 48 and 31% RNA. It appears therefore that the

instability of ribosomes and rRNA in stationary-phase cells is a general characteristic of these cells. In contrast, in exponentially growing cells the ribosomes are completely stable; no breakdown of labeled rRNA into soluble material could be detected (25).

It is conceivable that in *B. subtilis* at the end of exponential growth an activation of some specific nucleases present in the ribosomes (one of the ribosomal proteins may have this property) takes place. This activation may be caused by derepression resulting from lowering of the concentration of Mg^{2+} or by limitation of any other ingredient in the medium. Such an event could lead to a partial degradation of ribosomes and ribosomal RNA in situ. It is possible that in stationary-phase cells some breaks induced by specific nucleases are already present in the RNA on the ribosomes. Part of this RNA would be solubilized during the extraction.

Whatever the reasons for the degradation of the ribosomal subunits and the rRNA in stationary-phase cells, it seems to be unrelated to the sporulation capacity of the strain. Although some decrease in the synthesis of the 50*s* and 30*s* ribosomal subunits and of the corresponding rRNA species was observed during sporulation, a similar decrease was also observed in stationary-phase cells of various zero stage asporogenic mutants. Therefore, the main conclusion to be drawn from the experiments reported in this paper is that during sporulation ribosomal genes are transcribed and consequently rRNA continues to be made.

The results described here are not unique to sporulating bacterial cells. A similar situation was observed during the development of the slime mold *Dictyostelium discoideum* (5). In stationary-phase cells of this organism, about 75% of the ribosomes made during exponential growth disappear and are replaced by new ones synthesized during the sequence of morphogenetic events.

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