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Studies on the ribosomal proteins of *Bacillus subtilis* during sporulation

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Estudios de las proteínas ribosomales de *Bacillus subtilis* durante la esporulación

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Several recent reports indicate that a control at the level of translation might operate during the process of bacterial sporulation and that the ribosome itself may have a regulatory role. On the other hand, dormant spore ribosomes are defective and inactive for protein synthesis.

To find out if modifications of the ribosomal proteins are responsible for the defective ribosomes we have studied the activity and protein composition of the ribosomes during the complete sequence of sporulation. The *in vitro* activity of the ribosomes was measured by their ability to synthesize polyphenylalanine and the ribosomal proteins were analyzed by monodimensional and 2-dimensional polyacrylamide gel electrophoretic systems.

We detected changes of some of the ribosomal proteins early during the sporulation sequence. However, these changes did not seem to affect the *in vitro* activity of the ribosomes. Late in the sporulation sequence and in the dormant spore, where the ribosomes become inactive, we found in addition to the early changes, an alteration of the acidic ribosomal proteins (equivalent to L7-L12 of *E. coli).* Concomitant with this alteration we also observed in polyacrylamide gels the presence of a new less acidic protein band that could be the result of modification of the preexisting acidic ribosomal proteins. The activity of the spore ribosomes was not stimulated by the presence of vegetative state acidic ribosomal proteins.

Our results might explain why dormant *Bacillus* spores show very low levels of protein biosynthesis since the acidic ribosomal proteins are essential for the translation process. The modification of the acidic ribosomal proteins could also constitute a late kind of control in the sporulation or a preparative event for the state of dormancy.

RIBOSOMAL PROTEINS L7-L12 SPORULATION

Bacterial sporulation has been considered as a single model of cell differentiation at the unicellular level (1). The central problem during this process is the control of the synthesis of specific proteins and their organization into a complex biological structure such as the spore. This control may be exerted at different levels i.e. at the translational or transcriptional levels (2).

Several recent experiments have indicated a possible regulatory mechanism of sporulation at the level of translation. Kobayashi (3), Fortnagel and Bergmann (4) and Andreoli *et al.* (5) have suggested that some ribosomal proteins may undergo modification during the sporulation events. Bott *et al.* (6) suggested also that during sporulation the translational apparatus

may be affected by alteration of some structural proteins. Guha ans Szulmajster have recently reported an specific alteration of the 30S ribosomal subunits of *Bacillus subtilis* during sporulation (7). It is also known that ribosomes from dormant spores are defective and inactive for protein synthesis (3, 8, 9).

In additon, mutants resistant to inhibitors of translation such as streptomycin and fusidic acid have been isolated and although these mutants grow normally, they are not able to sporulate (4, 10). In the present report we have used two-dimensional polyacrylamide gel electrophoresis to analyze the ribosomal proteins obtained from ribosomes isolated at different times during the complete sequence of sporulation in an attempt to identify the ribosomal proteins that might undergo modifications and the relationship between these changes and the activity of the ribosomes for protein biosynthesis.

We detected some early and late alterations of some ribosomal proteins during sporulation. Of these proteins, the acidic ribosomal proteins were found to be changed late in the sporulation sequence, in agreement with the previous findings in the dormant spore of *Bacillus subtilis* (11)

MATERIAL AND METHODS

Bacillus subtilis 168 (Marburg) was grown with aeration at 37° in 12 liters of medium G (12). Vegetative cells were harvested at midlog phase (A660 = 1.5). and spores after 36 hours of incubation in the same medium. Dormant spores were freed from contaminant vegetative cells by treatment with lysozyme and SDS (13).

Vegatative cells were initiated to sporulate after harvesting them from 12 liters of medium G and immediately resuspending the cells in 12 liters of the resuspension medium described by Sterlini and Mandelstam (14). After incubation under forced aeration at 37°, samples of 2 liters were taken at different times for the preparation of ribosomes and high-speed supernatants.

Sporulation was followed by the appearence of alkaline phosphatase (in 20 ml aliquots) according to Gleen and Mandelstam (15) and refractile spores were counted in the phase-contrast microscope. After 16-17 hours of resuspension there was 60-70% refractile spores.

Samples (10 ml) were removed from the culture at the indicated times during resuspension and 0.5 μC of [^{14}C]valine (209 μC/mole) was added. A 1 ml portion was removed after 5 min. and placed in 2.5 ml of cold 10% trichloroacetic acid (TCA). After heating for 15 min at 90° the suspension was filtered through glass fiber filters (Watmann GF/C, 25 mm) and washed with cold 10% TCA. The filters were placed in scintillation vials and dried at 110° for 25 min. A 10 ml amount of toluene-based scintillation counting solution was added to each vial and the radioactivity determined.

Methionine incorporation into ribosomal proteins was measured by inicially adding to the resuspension medium (70 ml) 16 μC/ml of methyl-[^{3}H]-methionine (14.6 mC/μmole). At the times indicated, 10 ml aliquots of cells were harvested and after mixing them with carrier cells (300 mg) the ribosomes were obtained and after extracting their total ribosomal proteins, the radioactivity incorporated was determined. Vegetative cells, spores and cells harvested at different times after resuspension (sporulating cells) were all washed with 1 M KCl buffer to reduce proteases (16).

To disrupt the cells, they were suspended in 2.5 vol of buffer A; 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 200mM KCl, 1 mM EDTA, 6 mM 2-mercaptoethanol, 3.5 mM phenylmethylsulfonyfluoride (PMSF) and 5% glycerol and in the presence of 8 mg of makaloid per gram of cells. In the case of vegetative and sporulating cells, the suspensions were incubated in the presence of 1 mg of lysozyme/ml for 20 min at 37° and after adding 10 μg/ml of RNAase-free DNAase sonication during 5 min at 1 min intervals was done. Dormant spores were disrupted in a Bronwill homogenizer (MSK) as previously described (11).

High-speed supernatants and ribosomes were obtained by centrifugation of the cell extracts as previously described (11).

The acidic ribosomal protein fraction from *B. subtilis* (PE proteins) were extracted by the method of Hamel *et al.* (17) and used without further purification. Ribosomes lacking PE will be called extracted ribosomes and the *in vitro* reconstitution of extracted ribosomes by PE proteins was done as in Jerez *et al.* (11).

Total ribosomal proteins were extracted by the acetic acid method (18).

The acetic acid supernatant containing the ribosomal proteins was then dialyzed against 1 liter of 50%, 25%, 12,5% and 2% acetic acid for 2 hours each. The final protein solution was lyophilized and used for polyacrylamide gel electrophoresis (PAGE).

Two-dimensional PAGE of ribosomal proteins was carried out essentially as described by Kaltschmidt and Wittmann (19). The acidic ribosomal proteins were analyzed by PAGE at pH 5 according to Li and Subramanian (20). After staining the gel rods they were scanned at 530 nm in a Canalco Model G densitometer. For determination of radioactivity the gels were sliced into 1 mm slices and after dissolving them in 0.4 ml of 30%H_2O_2 (12 hours at 60°) they were counted in a toluene-triton X-100-based scintillation fluid.

E. coli tRNA was aminoacylated with [^{14}C] phenylalanine (406 μC/ μmole) in the presence of a high-speed supernatant from *E. coli* as the source of activating enzyme (21). *In vitro* polyphenylalanine synthesis was determined by standard procedures (22) and the reaction mixtures were as described in the legends to the figures and tables.

Protein was estimated by the procedure of Lowry *et al.* (23) using bovine serum albumin as standard.

Radioisotopes were obtained from Amersham-Searle. *E. coli* tRNA, PMSF, enzymes and other chemicals were from Sigma Chemical Co [^{3}H]-L7-L12 was a kind gift of Dr. D. Richter.

RESULTS

In vivo and in vitro protein synthesis during sporulation

To trigger the sporulation events we have used the conditions shown in Fig. 1. It can be seen that after 2 hours of resuspension of *Bacillus subtilis* in the sporulation media (14) there is an increase of the specific activity of alkaline phosphatase while there is no growth as indicated by the lack of change in the absorbancy at 650 nm. These results are in agreement with previous reports (14, 24).

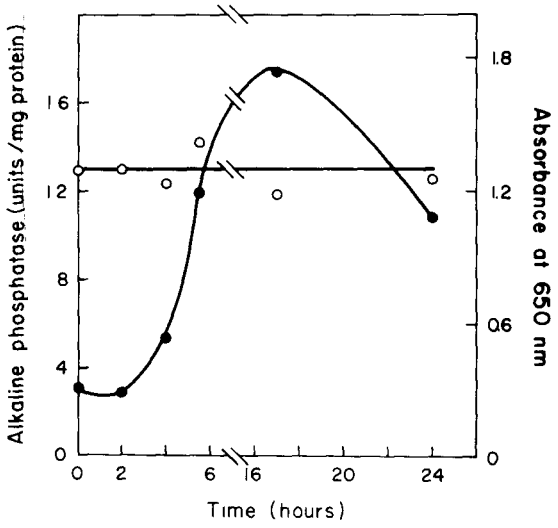


Fig. 1. Appearence of alkaline phosphatase during sporulation. Vegetative cells were resuspended in sporulation medium (14) and at the times indicated, aliquots were removed to determine the absorbance at 650 nm (o____o) and the specific activity of alkaline phosphatase (●____●) as described in Material and Methods.

Using this system we measured the ability of the sporulating cells to incorporate radioactive aminoacids at different time intervals as shown in Fig. 2. Incorporation of [^{14}C]-valine was measured by a 5-min pulse as described in Methods and the incorporation of methionine into ribosomal proteins was determined after resuspending vegetative cells in the resuspension medium containing labeled methionin. It can be seen that there is a continued synthesis of protein during sporulation as it has been previously reported (25) and that this incorporation levels off after 16 hours when most of the cells are sporulated.

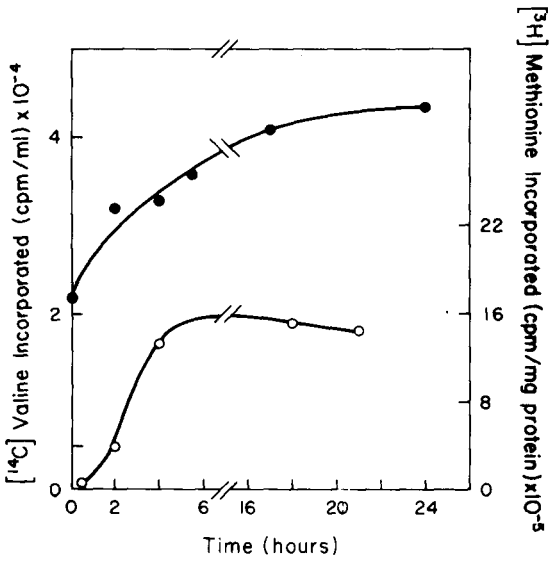


Fig. 2. Incorporation of radioactively labeled aminoacids into proteins during sporulation. [^{14}C] valine and [^{3}H] methionine were added to a culture in resuspension medium and the incorporation of [^{14}C] valine into total proteins (■____■) and the incorporation of [^{3}H] ethionine into ribosomal protein (o____o) were determined as in Materials and Methods.

To compare these results with the *in vitro* protein synthesis activity of the ribosomes we isolated ribosomes at different times after resuspension and analyzed them for their activity to synthetize polyphenylalanine in a polyuridylic acid-directed system as shown in Fig. 3. In Fig. 3A we can see the polyphenylalanine polymerized by ribosomes obtained at the times indicated after resuspension and in the presence of cell-free extracts obtained from the same cells used to prepare the ribosomes. Only at the end of the sporulation sequence it is possible to observe a decrease in the *in vitro* activity of the ribosomes. This decrease occurs by the time the *in vivo* incorporation of aminoacids into proteins has leveled off (Fig. 2).

Fig. 3B shows the activity of the same ribosomes obtained at the times indicated but in the presence of cell-free extracts from the vegetative state. The results are essentially the same, suggesting that the decrease in the ability of the ribosomes to polymerize phenylalanine by the time the cells are mostly sporulated is probably a result of an alteration of the ribosomes rather than an alteration of the soluble factors present in the cell-free extracts from sporulating cells (Fig. 3A).

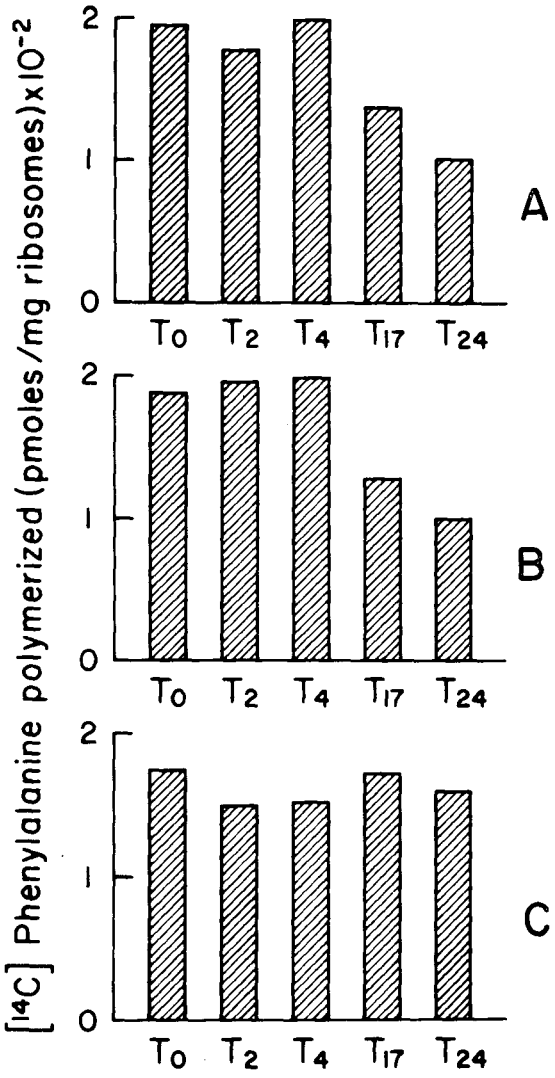


Fig. 3. In vitro polyphenylalanine synthesis during sporulation. At the times indicated after resuspension ribosomes were isolated as described in Materials and Methods and the activity of the ribosomes for polyphenylalanine synthesis was assayed in 125 µl reaction mixtures containing 72 mM Tris-HCl, pH 7.5, 72 mM NH_4Cl, 12 mM magnesium acetate, 2.4 mM DTT, 2 mM ATP, 0.1 mM, GTP, 5 mM creatine phosphate, 0.6 mM EDTA and 5 mM spermidine. Each assay also contained 5 µg creatine phosphokinase, 100 µg poly (U), 50 pmol [^{14}C] phenylalanyl-tRNA (1 pmol- = 132 cpm) and 235 µg of high-speed supernatant protein and 70 µg of ribosomes. After incubation for 30 min at 37° the extent of polyphenylalanine synthesis was determined as described in Materials and Methods. A) high-speed supernatant and ribosomes were from cells harvested at the indicated times, B) high-speed supernatant was from vegetative cells and ribosomes were from cells obtained at the times indicated, C) high-speed supernatants were from cells obtained at the indicated times and ribosomes were from vegetative cells.

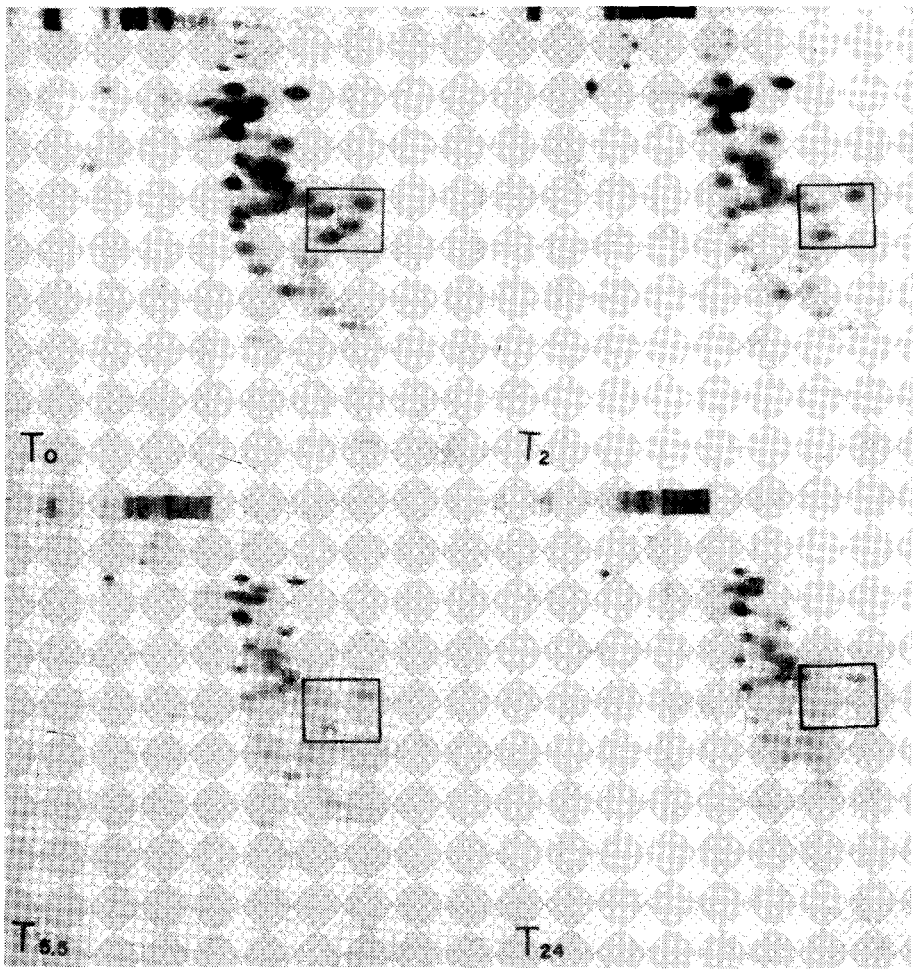
This is supported in part by the results shown in fig. 3C, in which the activity of vegetative state ribosomes was measured in the presence of cell-free extracts obtained from cells harvested at the indicated times after resuspension in sporulation medium. Kobayashi and Halvorson (8) found in addition to an alteration of the ribosomes of sporulating cells a modification of the soluble factors. However, these authors did not use protease inhibitors during the preparation of their ribosomes. We have used cells extensively washed with 1 M KCl and in the presence of nuclease and protease inhibitors (Methods).

Two-dimensional PAGE of ribosomal proteins obtanied at different times of sporulation

To find out if modifications occur at the level of the ribosomal proteins during sporulation, the total ribosomal proteins obtained from ribosomes isolated at different times after resuspension were analyzed by 2-D PAGE according to Kaltschmidt and Wittmann (19). Fig. 4 shows the patterns obtained for the ribosomal proteins from the vegetative state (To) and from 2.5, 5 and 24 hours after resuspension. It can be seen that early in the sequence of sporulation (T2) there is a change in the protein spots enclosed by a square. Using the nomenclature of Geisser *et al.* (26) to name the *B. subtilis* ribosomal proteins, this change corresponds to protein L21, which is apparently absent until late during sporulation (T24). Apparently, the alteration of this protein would not affect the *in vitro* activity of the ribosomes since at T2 or T4 the ribosomes are as active as the vegetative ones (Fig. 3). We believe the changes observed do not correspond to alterations of the ribosomal proteins from the mother-cell ribosomes since the same alterations have been found when ribosomes from dormant spores freed from any other cells were used (11).

Late in sporulation (T24) and in dormant spores (11) there is an aparent change in the intensity of spot L 18.

Although is not readily evident in Fig. 4, we also observed a late alteration of the spot corresponding to the acidic ribosomal proteins. This has been previously observed in the ribosomes from dormant spores of *B. subtilis* (11).



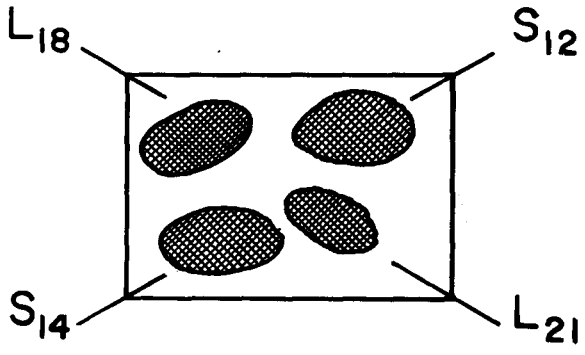


Fig. 4. Two-dimensional gel electrophoresis of total ribosomal proteins obtained at different times of sporulation. Ribosomal proteins were extracted from the ribosomes from cells harvested at 0,2, 5.5 and 24 hours after resuspension in sporulation medium. Electrophoresis was carried out as described in Materials and Methods. The areas enclosed by squares contain the ribosomal protein spots named in the drawing according to Geisser *et al.* (26).

Analysis of the acidic ribosomal proteins during sporulation

The acidic ribosomal proteins (equivalent to L7-L12 of *E. coli)* are essential for protein synthesis (17, 27, 28). It was of interest then to analyze them during the resuspension conditions since it has been reported that the relative levels of these proteins change during the life cycle of *E. coli* (29, 30). We used the electrophoretic procedure of Li and Subramanian (20) to analyze the acidic ribosomal proteins since the 2-D PAGE system is not appropriate.

In the bottom of Fig. 5 we can seen the densitometric tracing (solid line) corresponding to the acidic ribosomal proteins (PE) extracted from vegetative *B. subtilis* ribosomes by the ethanol-NH_4Cl method of Hamel *et al.* (17).

These proteins co-migrate with tritiated L7-L12 from *E. coli* which migrate as a single band under these conditions (open circles). Fig. 5-veg to 5-T17 shows the densitometric tracing of the total ribosomal proteins of which only a few enter the gel. The fastest migrating band (Fig. 5-veg) corresponds to the acidic proteins and up to 5 or 6 hours after resuspension in sporulation medium there is no apparent change of this band (Fig. 5-T3 and 5-T5). However, at 17 hours (Fig. 5-T17) we can see that the acidic ribosomal protein band is absent and there is a new less-acidic band. This coincides with the lower *in vitro* activity of the ribosomes seen in Fig. 3A-B and also wich the leveling off of the *in vitro* protein synthesis (Fig. 2).

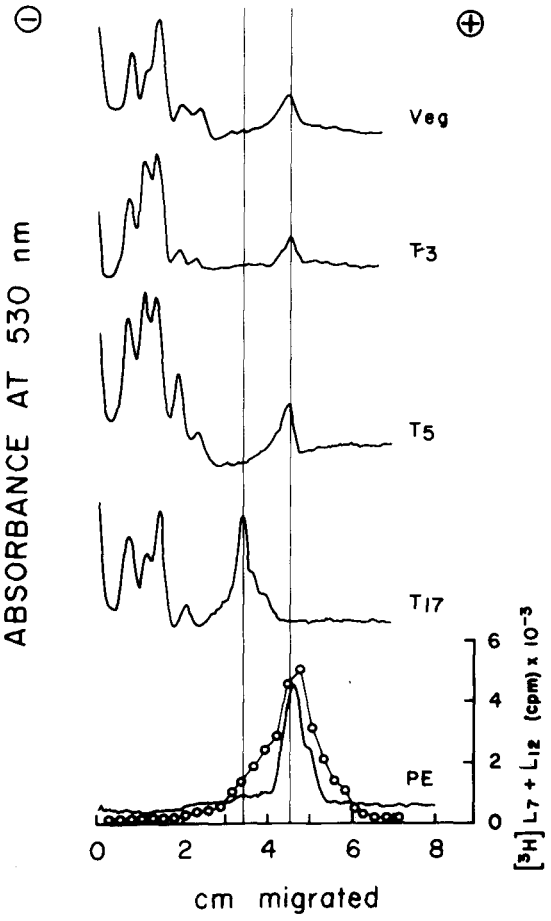


Fig. 5. Analysis of the acidic ribosomal proteins during sporulation. 250 μg of total ribosomal proteins from vegetative cell ribosomes (VEG), from cells harvested at 3 hours (T3), 5.5 hours (T5.5) and 17 hours (T17) after resuspension and 25 μg of acidic proteins (PE) were applied on the indicated gel. In the PE gel the PE proteins were applied together with [^{3}H]-L7 L12 (a total of 65,000 cpm). Electrophoresis at pH 5, densitometric tracings, and counting of the radioactive samples run ot the gels were done as described in Materials and Methods. (———) A 530 nM, (0———0) cpm of tritiated L7 L12.

Effect of vegetative state acidic proteins on the activity of dormant spore ribosomes

Dormant spore ribosomes show very low activity for protein synthesis in almost all kinds of spores that have been analyzed (3, 8, 9). To find out if this reduced activity is the result of the observed change in the acidic proteins, a reconstitution experiment as the one shown in Table 1 was done. When vegetative state ribosomes are extracted with ethanol-NH4Cl the acidic ribosomal pro-

TABLE 1
Effect of PE proteins on the activity of vegetative and dormant state ribosomes for polyphenylalanine synthesis

Ribosomes	*pmoles of phenylalanine polymerized/mg of ribosomes*
Vegetative	302.6
Vegetative + PE	304.3
Extrated vegetative	181.0
Extracted vegetative + PE	354.8
Spore	80.0
Spore + PE	89.4

Polyphenylalanine synthesis was assayed as described in the legend of Fig. 3, except that were indicated, each assay contained 7 μg of PE proteins.

teins (PE proteins) are obtained in the extract and the ribosomes lacking PE have a reduced activity for polyphenulalanine polymerization. However, when PE proteins are added, a total recovery of the activity is observed. The control vegetative ribosomes are not affected in their activity in the presence of the same amounts of PE as seen in Table I. On the other hand, the spore ribosomes, which have only about 25% or less of the activity of vegetative ribosomes, are not stimulated by the presence of vegetative state PE wether they are previously extracted with ethanol-NH_4Cl or not. This would indicate that the low activity of the spore ribosomes is not only due to the apparent lack of PE proteins and that other alterations might also be involved.

DISCUSSION

Our results clearly show modification of some of the ribosomal proteins during sporulation (Figs. 4 and 5) in agreement with several other previous reports (3-6). Guha *et al.* (31) have also used two-dimensional polyacrylamide gel electrophoresis to analyze and compare the ribosomal proteins from vegetative and sporulating cells of *B. subtilis*. However, these authors found no differences in the ribosomal proteins between the two kinds of ribosomes and attributed this discordance to the fact that in the previous reports (3, 4, 6) no protease inhibitors were employed by the authors for the preparation of the ribosomes.

We believe the alterations we describe are not due to proteolysis since we have used cells and spores extensively washed with 1 M KCl to remove extracellular proteases (16) and ribosomes were always prepared in the presence of PMSF (Methods). Also, in some experiments the ribosomal proteins were obtained and processed for polycrylamide gel electrophoresis in the presence of 1-3 mM PMSF.

Early during the sporulation sequence (2 hours), we found a protein spot that is absent (tentatively named L21 according to Geisser *et. al.* (26) in the 2-D electrophoretograms (Fig. 4). Although we do not know what might the function of this protein be in the *B. subtilis* ribosome, the alteration takes place when protein synthesis is essential for the spore formation. However, we believe this change might not be functional since this protein has been described as loosely bound to the ribosomes in several *Bacillus* species (32) and in many of them does not appear when their ribosomal proteins are analyzed by 2-D polyacrilamide gel electrophoresis (26).

Protein spot named L18 apparently changes its intensity late during the sporulation process (24 hours) and in the dormant spore (11). Andreoli *et al.* (5) have also found similar changes to those we find for L21 and L18 and in addition alterations of other ribosomal proteins in the ribosomes from forespores of *B. cereus*.

At present, it is difficult to establish the functional significance of these alterations but the use of mutants blocked at different stages of sporulation might help to correlate these findings with sporulation.

Very late in the sporulation sequence we observed the absence of the protein fraction corresponding to the acidic ribosomal proteins and the formation of a new less acidic band in polyacrylamide gel electrophoresis (Fig. 5). These results, together with the previously reported for the dormant spore ribosome (11) suggest a modification of the acidic ribosomal proteins late in the process of sporulation. It is possible that the formation of the new acidic band is in some way related to sporulation since chloramphenicol which is known to inhibit the appearance of alkaline phosphatase and the formation of spores (14), when added at 2-3 hours after resuspension completely inhibits the presence of this band, suggesting that protein biosynthesis is required for its formation (33).

The lack of stimulation of the *in vitro* activity of spore ribosomes by PE from vegetative state ribosomes (Table I) indicates that sporulating cell ribosomes have not simply lost their acidic ribosomal proteins during their experimental preparation as might be the case with protein L21. It il also possible that in addition to the acidic proteins some other ribosomal proteins (eg. L18) are required to completely restore the activity of the ribosomes.

The new acidic band observed (Fig. 5) may arise by modification of preexisting PE proteins since the former one is also acidic, it is also extractable by ethanol-NH_4Cl and has the same molecular weight of PE proteins, determined by 2-D SDS polyacrylamide gel electropho-

resis (33). However, the use of antibodies specific against vegetative PE proteins will help to confirm that the new acidic protein is the result of modification of PE.

A late modification of PE proteins might be due to a protease but this enzyme would have to be rather specific and insensitive to PMSF. We believe the alteration could be a late change in the degree of methylation of the PE proteins that would alter their mobility in PAGE as it has been observed. In this connection, it is known that L7-L12 are one of the few ribosomal proteins that are methylated in the *E. coli* ribosome (34, 35).

Since the changes in the PE proteins occur by the time the *in vitro* activity of the ribosomes for protein synthesis decreases and considering that the acidic ribosomal proteins are essential for the translation step (17, 27, 28) it is possible that their alteration constitutes a late control at the translational level or else a preparative event for the state of dormancy, where no or very low levels of protein synthesis takes place.

RESUMEN

Una serie de evidencias experimentales recientes indican la posibilidad de que un control a nivel de la traducción opere durante el proceso de esporulación bacteriana y que el ribosoma mismo pudiera tener un papel regulatorio.

Por otro lado, se sabe que los ribosomas provenientes de esporas durmientes son defectuosos e inactivos para la síntesis proteica.

Para establecer si los ribosomas son defectuosos debido a modificaciones de las proteínas ribosomales, hemos estudiado la actividad y la composición proteica de los ribosomas durante la secuencia completa de esporulación.

La actividad *in vitro* de los ribosomas fue medida por su capacidad para sintetizar polifenilalanina, y las proteínas ribosomales fueron analizadas mediante sistemas electroforéticos mono y bidimensionales en geles de poliacrilamida.

Tempranamente durante la secuencia de esporulación encontramos cambios de algunas proteínas ribosomàles. Sin embargo, estos cambios aparentemente no afectan la actividad *in vitro* de los ribosomas. Tardíamente en la secuencia de esporulación, incluyendo la espora durmiente, etapa en la que los ribosomas se hacen inactivos, encontramos además de los cambios tempranos una alteración de las proteínas ribosomales acídicas (equivalentes a L7-L12 de *E. coli).* En forma concomitante con esta alteración detectamos también en geles de poliacrilamida la aparición de una nueva banda proteica menos acídica que podría provenir por modificación de las proteínas ribosomales acídicas preexistentes. La actividad de los ribosomas de esporas no fue estimulada por la presencia de proteínas ribosomales acídicas del estado vegetativo

Nuestros resultados podrían explicar porque las esporas durmientes del género *Bacillus* muestran tan bajos niveles de síntesis proteica, ya que las proteínas ribosomales acídicas son esenciales para el proceso de traducción.

La modificación de estas proteínas acídicas podría también constituir una forma tardía de control de la esporulación o bien un evento preparatorio para el estado durmiente.

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