JOURNAL OF BACTERIOLOGY, Sept. 1977, p. 866-871

Vol. 131, No. 3
Printed in U.S.A.

# Specific Alteration of the 30S Ribosomal Subunits of *Bacillus subtilis* During Sporulation

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Received for publication 24 January 1977

Active 30S ribosomal subunits were isolated from vegetative and sporulating cells of *Bacillus subtilis*. Both subunits were able to function in polyuridylic acid or phage $\phi$e messenger ribonucleic acid-dependent protein synthesis in vitro. The sporulation 30S subunits were highly active in polyuridylic acid-dependent polyphenylalanine synthesis but showed a reduced activity in the presence of natural messenger ribonucleic acid as compared with their vegetative counterparts. The reduced activity was independent of the source of 50S particles and initiation factors (vegetative or sporulation). The alteration of the 30S sporulation subunits appears to be related to the sporulation process, since the same subunits isolated from stationary-phase cells of an asporogenic mutant did not show any impairment in protein synthesis in vitro.

There is now an increasing amount of information that the control of sporulation operates at several different levels of gene expression. Evidence for a sequential control at the transcription level has been reported (2, 4, 16, 17, 25), although the molecular mechanisms of this control still remain unclear.

Other evidence suggests concomitant controls of sporulation at the translational level. As an example, it was found that certain mutants of *Bacillus subtilis* resistant to antibiotics inhibiting ribosomal functions were deficient in sporulation capacity (5, 6, 11). Furthermore, changes in ribosomal proteins have been detected during spore development (5, 6). Changes in specificity were also detected in in vitro protein-synthesizing systems from cells at different stages of spore development. Thus, Chambliss and Legault-Demare (3), working with washed and functionally identical ribosomes from vegetative and sporulating cells, have shown that the initiation factor fraction derived from sporulating cells has a reduced ability to translate exogenously added SPO1 messenger ribonucleic acid (mRNA) compared with the initiation factors isolated from vegetative cells. An asporogenic mutant of *B. subtilis* does not exhibit this discrimination. However, as shown by these authors, this discrimination was only observed with SPO1 RNA and not with other RNA templates such as Q$\beta$, T4, or *B. subtilis* vegetative RNA.

We show here that initiation factors from vegetative or sporulating cells have the same binding capacity for formyl-methionyl transfer RNA (tRNA) by $NH_4Cl$-washed 70S vegetative ribosomes in the presence of either poly(AUG) or phage $\phi$e mRNA. However, the binding activity was considerably reduced when 70S ribosomes from sporulating cells were used, and this effect was seen only in the presence of natural mRNA and not in the presence of poly(AUG).

Recently we reported the isolation of active 30S and 50S ribosomal subunits of *B. subtilis* (9). These subunits were able to perform their individual functions and were also active in functions needing their association, such as protein synthesis (artificial or natural mRNA dependent) or elongation factor G-catalyzed guanosine triphosphatase activity. Thus, it was interesting to investigate whether the ribosomal subunits from sporulating cells were able to function in association with their vegetative counterparts in the polyuridylic acid [poly(U)]-dependent polyphenylalanine synthesis and in the natural mRNA-dependent protein synthesis. The results obtained indicate that in the latter case the 30S ribosomal subunits from sporulating cells had a reduced activity.

## MATERIALS AND METHODS

**Bacterial culture.** *B. subtilis* 168M (*trpC2*) and its Spo$^-$ derivative, Spo12A$^-$, protease negative (23), were used. Cells were cultivated at 37°C with vigorous agitation in nutrient broth medium supplemented with metals (14) and harvested at midlog phase or at 2.5 to 3 h after the end of exponential growth ($t_{2.5-3}$). Washing of the cells to remove extracellular protease activity before breakage was carried out according to Orrego et al. (15). Sporulation was controlled as described (7).

Phage $\phi$e was cultivated and concentrated accord-

ing to Sonenshein and Roscoe (22).

**Materials.** Poly(U) and poly(AUG) were purchased from Boehringer Mannheim Corp., France. [$^{14}$C]leucine (specific activity, 236 mCi/mmol), [$^{14}$C]-phenylalanine (specific activity, 250 mCi/mmol), and [*methyl*-$^{3}$H]methionine (specific activity, 6.2 Ci/mmol) were purchased from Commissariat á l'Energie Atomique, Saclay, France. $\gamma$-[$^{32}$P]guanosine triphosphate (specific activity, 15.2 Ci/mmol) was from the Radiochemical Centre, Amersham, England. Analytical-grade sucrose for density gradients was from Prolabo, France.

The preparation of high-salt-washed ribosomes has been described elsewhere (7). Phenylmethanesulfonyl fluoride (3.5 mM) and diisopropylfluorophosphate (0.1 mM) were added throughout the isolation procedure. The efficiency of high-salt washing of the 70S preparations was checked for the absence of endogenous guanosine triphosphatase activity and formyl-methionyl-tRNA binding capacity, and for the overwhelming dependence on initiation factors of natural mRNA-coded protein synthesis. Only the 70S preparations showing an incorporation of at least 2 nmol of phenylalanine per mg (14.5 absorbancy units at 260 nm [$A_{260}$]) of ribosomes were used for further separation of subunits.

The preparation of ribosomal subunits has been described previously (9).

In some experiments, the subunits were separated by centrifugation through a 5 to 20% sucrose gradient in an SW25.2 Spinco rotor for 8 h at 25,000 rpm.

High-speed supernatant (S-150) from midlog cells and partially purified elongation factor G were prepared as previously described (8) in the presence of phenylmethanesulfonyl fluoride and diisopropylfluorophosphate. The latter was found to be devoid of elongation factor activity. Formyl-[$^{3}$H]methionyl-tRNA and crude initiation factors were prepared as before (9).

Phage $\phi$e mRNA was isolated from wild-type *B. subtilis* infected with phage $\phi$e (multiplicity of infection, ≈5 or 20 [Table 5]) for 4 min ($\phi$e-4 min) or 15 min ($\phi$e-15 min). Total RNA from these cells was isolated and used as mRNA.

Formyl-methionyl-tRNA binding capacity, peptidyl transferase activity, elongation factor G-dependent guanosine triphosphatase activity, and natural mRNA-dependent protein synthesis were assayed as before (9). The amount of subunits (expressed in $A_{260}$ units) used in each experiment is given in the tables. Lower concentrations of $A_{260}$ units were used in the presence of poly(U) as mRNA.

Nuclease activity was assayed according to Miller et al. (14). The incubation mixture contained (volume, 0.5 ml): tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, 10 mM; $NH_4Cl$, 60 mM; magnesium acetate, 10 mM; ethylenediaminetetraacetic acid at pH 7.0, 10 mM; [$^{3}$H]uridine pulse-labeled (4 to 6 min) RNA (about 9,000 cpm), obtained from phage $\phi$e-infected cells; and 2.1 $A_{260}$ units of 30S vegetative or sporulation subunits. After 30 min of incubation at 30°C, 0.1 ml of tRNA (10% solution) (9) dissolved in 10 mM Tris, pH 7.4, containing 0.6 M NaCl and 50 mM $CaCl_2$ followed by 1 ml of absolute alcohol, was added and left for 1 h at −20°C. The precipitate was recovered by centrifugation, dissolved in water, and counted in Bray scintillation mixture.

Protease activity was assayed in the following incubation mixture (100 $\mu$l): Tris-hydrochloride, pH 7.8, 50 mM; $NH_4Cl$, 60 mM; magnesium acetate, 10 mM; calcium chloride, 0.2 mM; dithiothreitol, 5 mM; about 10 $\mu$g of [$^{35}$S]methionine pulse-labeled protein obtained from phage $\phi$e-infected (4 to 6 min) *B. subtilis* cells (1 $\mu$g of protein ≈1,300 cpm), and 1.7 $A_{260}$ units of 30S vegetative or sporulation subunits. After 30 min of incubation at 37°C, 100 $\mu$l of bovine serum albumin solution (5 mg/ml) was added, followed by 1 ml of cold trichloroacetic acid solution (10%). The precipitate was heated at 90°C for 15 min, filtered, washed with 5% trichloroacetic acid, dried, and counted in a toluene-based scintillation mixture.

## RESULTS

We have first determined the optimal conditions for poly(U)-dependent [$^{14}$C]phenylalanine incorporation by vegetative (Fig. 1a) and sporulation (Fig. 1b) ribosomal subunits. It can be seen that, under our experimental conditions, a four- to fivefold ratio (in $A_{260}$ units) of 30S/50S subunits was necessary to obtain the maximum activity for both the vegetative and sporulation ribosomal subunits. The 30S subunits of the latter used in this experiment seem to have a slightly higher activity.

Table 1 shows the phenylalanine-synthesizing activity by homologous and heterologous complementary ribosomal subunits. The 30S sporulation subunits are almost twice as efficient as vegetative 30S subunits, and this is in

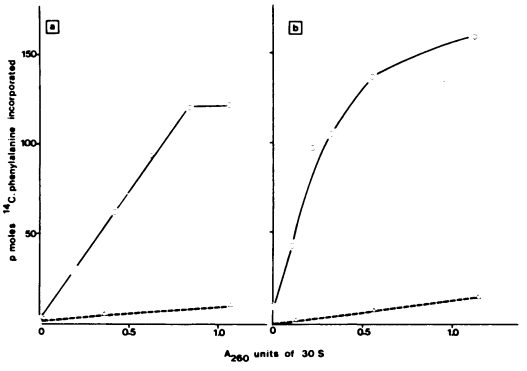


FIG. 1. *Amounts of 30S subunits required to saturate 50S for polyphenylalanine synthesis. (a) Vegetative 30S and 50S subunits; (b) sporulating 30S and 50S subunits. Symbols:* (○) *Incorporation of [$^{14}$C]phenylalanine in the presence of fixed amounts of 50S subunits (0.21 $A_{260}$ units) with increasing amounts of homologous 30S;* (△) *activity in the absence of 50S subunits. Conditions of incubation are described in the footnote to Table 1.*

TABLE 1. *Poly(U)-dependent polyphenylalanine synthesis by homologous and heterologous ribosomal subunits of vegetative and sporulating cells of B. subtilis*[a]

| 30S ribosomal subunit | [14C]phenylalanine incorporated with 50S ribosomal subunits (cpm) | |
|---|---|---|
| | Vegetative | Sporulation |
| Vegetative | 27,650 | 22,880 |
| Sporulation | 51,693 | 41,000 |

[a] The incubation mixture, in a volume of 125 μl, contained: Tris-hydrochloride, pH 7.8, 52 mM; $NH_4Cl$, 60 mM; magnesium acetate, 11 mM; dithiothreitol, 4 mM; adenosine 5'-triphosphate, 1 mM; guanosine 5'-triphosphate, 0.04 mM; phosphoenolpyruvate, 7.2 mM; pyruvate kinase, 2 μg; spermidine, 0.5 mM; tRNA of *B. subtilis*, 50 μg; 19 amino acids except phenylalanine, 0.1 mM; [14C]phenylalanine, 4.8 μmol (1 pmol/330 cpm); poly(U), 100 μg; 150,000 × *g* supernatant, 190 μg of protein. Assays contained, for vegetative and sporulation 30S, 0.71 and 0.74 $A_{260}$ units, respectively, and, for vegetative and sporulation 50S, 0.21 and 0.20 $A_{260}$ units, respectively.

the presence of either vegetative or sporulation 50S particles. Vegetative 50S subunits constantly had a slightly higher activity (about 15 to 25%) than the corresponding sporulation subunits, whatever the source of the 30S.

When the abilities of the same subunits to utilize natural phage φe mRNA were tested (Table 2), the 30S subunits from sporulating cells had a reduced protein-synthesizing activity as compared with their vegetative counterparts. This difference was highly reproducible and was not influenced by whether vegetative or sporulation initiation factors were used.

To show directly that in the presence of phage φe mRNA the source of the initiation factors is without functional significance in the protein-synthesizing system of *B. subtilis*, we assayed the formyl-methionyl-tRNA binding activity by 70S ribosomes and initiation factors isolated from exponential and $t_4$ sporulating cells. Both types of initiation factors were found to have the same activity in the presence of poly(AUG) or phage φe mRNA (Table 3). In this reaction it can also be seen that the ribosomes from sporulating cells have, in the presence of natural mRNA, a reduced formyl-methionyl-tRNA binding capacity as compared with the vegetative ribosomes.

To determine whether the alteration observed in the 30S ribosomal subunits isolated from cells at $t_3$ to $t_4$ is a sporulation-specific event or a general characteristic of stationary-phase ribosomes, the activities of 30S ribosomal subunits from stationary-phase cells of a Spo mutant (*B. subtilis* 12A) were compared with sporulation ribosomes. The 30S stationary ribosomal subunits from this mutant had the same activity in the presence of vegetative or $t_3$ 50S subunits isolated from the wild type and slightly stimulated the activity in the presence of the homologous 50S subunits (Table 4). The

TABLE 2. *Phage φe mRNA-dependent protein synthesis by vegetative or sporulation ribosomal subunits of B. subtilis*[a]

| 30S subunit | Initiation factor | [14C]leucine incorporated with 50S subunits (cpm) | |
|---|---|---|---|
| | | Vegetative | Sporulation |
| Vegetative | Vegetative | 1,754 | 1,642 |
| Sporulation | Vegetative | 1,192 | 1,123 |
| Vegetative | Sporulation | 2,319 | 1,812 |
| Sporulation | Sporulation | 1,465 | 1,028 |

[a] Incubation was carried out for 10 min at 37°C, and the incubation mixture contained, in a total volume of 125 μl: Tris (pH 7.8), 52 mM; $NH_4Cl$, 60 mM; magnesium acetate, 11 mM; adenosine 5'-triphosphate, 1 mM; guanosine 5'-triphosphate, 0.04 mM; phosphoenolpyruvate, 7.2 mM; pyruvate kinase, 2 μg; tRNA (*B. subtilis*), 50 μg; tetrahydrofolic acid, 0.2 mM; 19 amino acids except leucine, 10 nmol of each; [14C]leucine (specific activity, 236 mCi/mmol, 1 pmol/470 cpm), 6.8 μmol (0.2 μCi); 150,000 × *g* supernatant, 385 μg of protein. Ribosomal subunits contained: vegetative 30S, 1.42 $A_{260}$ units, and sporulation 30S, 1.58 $A_{260}$ units; vegetative 50S, 0.42 $A_{260}$ units, and sporulation 50S, 0.51 $A_{260}$ units. Each assay contained 1.43 $A_{260}$ units of phage φe mRNA (15 min). Initiation factors: 25 or 29.6 μg of protein from vegetative or sporulating cells, respectively. Control values in the absence of mRNA were subtracted from each assay. The proportions of 30S and 50S were determined from saturation curves obtained with homologous subunits.

TABLE 3. *Formyl-methionyl-tRNA binding activity by ribosomes and initiation factors from B. subtilis*[a]

| Source of: | | Formyl-[3H]methionyl-tRNA bound (cpm) | |
|---|---|---|---|
| Ribosomes | Initiation factors | AUG dependent | mRNA dependent |
| Vegetative | Vegetative | 2,275 | 2,180 |
| Vegetative | Sporulation | 2,139 | 2,132 |
| Sporulation | Vegetative | 2,560 | 1,433 |
| Sporulation | Sporulation | 2,430 | 1,539 |

[a] Each assay contained 2.0 $A_{260}$ units of high-salt-washed ribosomes and 5 μg of crude initiation factors from either vegetative or sporulating cells, about 9,000 cpm of formyl-[3H]methionyl-tRNA, and 1.0 $A_{260}$ unit of phage φe mRNA (4 min) or 0.5 unit of poly(AUG). Control values in the absence of mRNA were subtracted from each assay.

TABLE 4. *Interaction of ribosomal subunits from the wild type and Spo mutant (12A) in phage φe mRNA-dependent protein synthesis*[a]

| Source of 30S subunits | [14C]leucine incorporated with 50S ribosomal subunits (cpm) | | |
|---|---|---|---|
| | 168M, vegetative | 168M, sporulation | 12A[b] |
| 168M, vegetative | 2,837 | 2,362 | 3,151 |
| 168M, sporulation ($t_3$) | 895 | 759 | 830 |
| Spo12A, $t_3$ | 3,525 | 3,302 | 4,742 |

[a] Incubation was carried out as described in Table 2, with the following exceptions: S150, 600 μg of protein, and phage φe mRNA, 2.2 $A_{260}$ units; vegetative initiation factors, 30 μg of protein; and the following amounts of ribosomal subunits: 30S vegetative, sporulation, and 12A—2.1, 2.1, and 2.2 $A_{260}$ units, respectively; 50S vegetative, sporulation, and 12A—0.41, 0.48, and 0.42 $A_{260}$ units, respectively.

stationary-phase 30S subunits from the 12A mutant behaved in a manner similar to that of the vegetative cells (Table 5).

It has to be emphasized that the variations in the specific activity of the ribosomal subunits observed in the experiments described in Tables 2 and 4 are due to the fact that we are dealing with two different preparations, although they are derived from the same stages of growth and sporulation. Such variations in activity are a frequent phenomenon of ribosomal preparations.

Although the method we used to purify ribosomes prevents contamination by proteolytic enzymes (7), we nevertheless examined the possibility of whether such an enzyme is present in the 30S subunits and could attack the phage φe mRNA-coded [14C]leucine protein without acting on the [14C]polyphenylalanine. This enzyme, if it exists, could be absent in the 12A, protease-negative mutant. To carry out this experiment, we incubated the sporulation 30S subunits in the presence of [35S]methionine-labeled protein isolated from *B. subtilis* cells after infection by phage φe under the conditions described in Materials and Methods. The results (Table 6) clearly show that no detectable proteolytic activity was present in the 30S sporulation subunits. Similarly, we examined these subunits for ribonuclease activity under the conditions described in Materials and Methods. Here, too, the results were negative (Table 6).

In another control experiment, we examined the effect of sporulation 30S ribosomes on vegetative ones in a "mixing" experiment shown in Table 7. The results obtained are not very meaningful, since the mixing of sporulation and vegetative 30S subunits produces the same effect as mixing two equivalents of vegetative subunits (Table 7). In both cases a 14 to 19% decrease in the [14C]leucine incorporation was observed, as could be expected from the cumulative values.

## DISCUSSION

The isolation from vegetative and sporulating cells of ribosomal 30S and 50S subunits

TABLE 5. *Phage φe mRNA-dependent protein synthesis by ribosomal subunits derived from the 12A Spo mutant of B. subtilis*[a]

| 30S ribosomal subunit | [14C]leucine incorporated with 50S ribosomal subunits (cpm) | |
|---|---|---|
| | Vegetative | $t_3$ |
| Vegetative | 5,083 | 7,122 |
| $t_3$ | 6,790 | 7,634 |

[a] Conditions were similar to those described for Table 2, except that the incubation mixture contained 3 mM adenosine 5'-triphosphate and 0.12 mM guanosine 5'-triphosphate; S150, 600 μg of protein; vegetative and stationary 30S ribosomal subunits, 1.86 and 2.1 $A_{260}$ units, respectively; 50S, 0.42 and 0.43 $A_{260}$ units, respectively. The phage φe mRNA (2.16 $A_{260}$ units/assay) was obtained from a culture infected with phage φe (multiplicity of infection, ≈20) and harvested 15 min after infection.

TABLE 6. *Lack of degradative activity in the vegetative and sporulation 30S ribosomal subunits*[a]

| Activity | 30S ribosomal subunits | | |
|---|---|---|---|
| | None | Vegetative | Sporulation |
| Protease | 13,340[b] | 12,920 | 13,980 |
| Nuclease | 5,626 | 5,574 | 5,571 |

[a] Protease and nuclease activities were assayed as indicated in Materials and Methods. Under these conditions the addition of 5 μg of proteinase K (Merck) degraded trichloroacetic acid-precipitable material by 65%, and the addition of 20 μg of ribonuclease (Worthington) degraded [3H]RNA by 50%.

[b] Total counts per minute.

TABLE 7. *Effect of mixing vegetative and sporulation 30S ribosomal subunits from strain 168 cells*

| 30S | [14C]leucine incorporated (cpm) |
|---|---|
| Vegetative | 3,350 |
| Sporulation | 1,170 |
| Vegetative + sporulation | 4,100 |
| Vegetative + vegetative | 5,760 |

[a] Each assay contained, in a total volume of 150 μl: 600 μg of protein of S150; 2.1 $A_{260}$ units of phage φe (15-min) RNA; 0.84 $A_{260}$ unit of 50S subunits; and 1.7 $A_{260}$ units of 30S subunits, from vegetative and/or sporulating cells. The other ingredients were as described in Table 2. Controls in the absence of mRNA were subtracted.

active in protein synthesis in vitro (9) has allowed us to examine the relative role of each one of these subunits in a possible translational control of the sporulation process.

It was first shown here that the 30S subunits from sporulating cells ($t_3$-cells), even though more active than their vegetative counterparts in poly(U)-dependent polyphenylalanine synthesis (Table 1), are less efficient in protein synthesis when natural mRNA is used. The 50S subunits from vegetative or sporulation ribosomes appear to be interchangeable and function with similar efficiency whether the source of 30S subunits is vegetative or $t_3$.

The importance of the 30S ribosomal particle in selection of mRNA has been observed by several authors. By hybridization of ribosomal subunits from *Bacillus stearothermophilus* with those from *Escherichia coli*, Lodish (12) found that the 30S subunit is responsible for the selection of natural mRNA. Martin and Iandolo (13) have hybridized subunits from rapidly growing cells of *Staphylococcus aureus* with those of slowly growing cells from the same organism and have shown that the 30S subunits determine the efficiency of the poly(U)-directed polyphenylalanine synthesis. Legault-Demare and Chambliss (10) and Stallcup et al. (24) have shown that the in vitro *B. subtilis* protein-synthesizing system is restricted in its ability to translate *E. coli* phage mRNA (T4), whereas the *E. coli* system translates with equal efficiency various mRNA's regardless of their source. Moreover, this restriction was not due to the initiation factors, since Legault-Demare and Chambliss (10) have shown that at limiting concentration the initiation factors from *E. coli* and *B. subtilis* are functionally very similar. However, by complementation of *B. subtilis* 70S ribosomes with *E. coli* 30S and 50S ribosomal subunits, these authors have concluded that the 30S subunit of *B. subtilis* ribosomes is most likely responsible for the restriction in template specificity.

The results reported here provide direct evidence for the implication of the *B. subtilis* 30S subunit in the reduction of protein-synthesizing activity by sporulation ribosomes in the presence of phage $\phi$e mRNA. The 50S subunits and the initiation factors are not responsible for this partial "turn-off" of the translational apparatus.

Moreover, we also show here that only the 30S subunits isolated from sporulation ribosomes are affecting the protein-synthesizing capacity of the system, since the homologous subunits isolated from stationary-phase ribosomes of the zero-stage 12A asporogenic mutant of *B. subtilis* show, rather, a stimulation of protein synthesis.

It can therefore be concluded from these experiments that the alteration of the 30S ribosomal subunits of the $t_3$-cells seems to be specific to sporulating cells and not simply to stationary-phase cells in the absence of sporulation.

The method of washing the cells prior to breakage, which removes extracellular protease activity, and the protection afforded by inhibitors during the isolation procedure reduce the activity, if any, of the intracellular proteases to an insignificant level. In addition, $Ca^{2+}$, which is required for the intracellular protease activity in *B. subtilis* (19), is not present in any of the media used here.

Apparently only a small fraction of the 30S subunit population retained their protein-synthesizing activity, since about six times more mole-equivalents of 30S subunits were required to saturate the 40S subunits. This fragility, already observed by others (20, 26, 27), is a general feature of the 30S subunits from *B. subtilis* and is independent of the growth phase of the cells from which the ribosomes were isolated (vegetative or stationary) and also independent of the bacteria's ability to sporulate.

An alteration in the ribosomal proteins responsible for the reduced translational capacity of sporulation 30S subunits cannot be excluded, although these proteins apparently do not differ from those isolated from vegetative ribosomes, as shown by two-dimensional gel electrophoresis (7). The possibility that one of the proteins from the 30S sporulation subunit may have some nuclease activity towards natural mRNA and such activity might be absent in the subunits of the asporogenic mutant was excluded by testing ribonuclease activity on the 30S subunits in the presence of $^3$H-labeled phage $\phi$e mRNA as substrate (see Table 6). We have also discarded the possibility that the 30S sporulation subunits contain a proteolytic enzyme that could degrade the phage $\phi$e mRNA-coded protein without acting on the polyphenylalanine. Such a contaminating enzyme would be absent in ribosomes from the 12A protease-negative mutant. Also, a nicking of the 16S rRNA by a colicin-like enzyme (1), which might alter the 30S subunits, can be excluded since such an alteration leads to a loss of the polyphenylalanine-synthesizing activity, which is not the case here.

Another alternative is that the change encountered resides in the 3'-terminal sequence of the 16S ribosomal RNA, which, according to Shine and Dalgarno (21), may be involved in

the recognition of the ribosomal binding site on an mRNA. This hypothesis, though controversial (1, 15), appears nevertheless attractive in the present case.

It has to be emphasized that the importance of the 30S ribosomal subunit in a possible control of sporulation at the translational level provided by this work is based on experiments using mRNA isolated from *B. subtilis* cells infected by phage $\phi$e. We do not know yet whether the discrimination by the 30S subunits concerns specifically the phage $\phi$e mRNA or is also valid for sporulation-specific mRNA. Until now no such mRNA has been isolated; therefore, the control of sporulation at the level of the 30S ribosomal subunits or at the level of initiation factors (3) remains to be proven.

## ACKNOWLEDGMENTS

This work was supported by grants from the Centre National de la Recherche Scientifique (A.T.P. "Différenciation cellulaire," no. 1854), the Commissariat à l'Energie Atomique, France, and the Fondation pour la Recherche Médicale Française.

We are grateful to S. Sedgwick for helpful reading of the manuscript.

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