JOURNAL OF BACTERIOLOGY, Apr. 1980, p. 355-358
0021-9193/80/04-0355/04$02.00/0

Vol. 142, No. 1

# Methylation of Ribosomal Proteins in *Bacillus subtilis*

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We measured the methylation of ribosomal proteins from the 30S and 50S subunits of *Bacillus subtilis* after growing the cells in the presence of [1-$^{14}$C]methionine and [*methyl*-$^{3}$H]methionine. Two-dimensional polyacrylamide gel electrophoretic analysis revealed a preferential methylation of the 50S ribosomal proteins. Proteins L11 and L16, and possibly L9, L10, L18, and L20, were methylated. On the other hand, only two possibly methylated proteins were found on the 30S subunit. A comparison of these results with those for *Escherichia coli* suggests a common methylation pattern for the bacterial ribosomal proteins.

Methylation of ribosomal proteins both in *Escherichia coli* and in eucaryotes has been reported (1, 3, 5-7, 9-11, 15, 19, 22, 25, 28, 29). Extensive studies done with *E. coli* (1, 6, 7, 9, 10) have indicated that this post-translational modification occurs predominantly in the proteins of the larger ribosomal subunits (7, 9). The biological significance of this methylation is not clearly understood. However, some of the methylated proteins are known to have important ribosomal functions in *E. coli*. Proteins L7 and L12 are essential for translation in protein biosynthesis (21), and protein L11 seems to be involved in the stringent response (23). Protein L11 and *E. coli* methylated proteins L16 and L18 have all been implicated in the peptidyltransferase center of the ribosome (24). Also, it has been suggested that methylation of ribosomal components might be important for the assembly of the particle (2).

Information on methylated ribosomal proteins from procaryotes other than *E. coli* was unavailable until recently (4). We have analyzed and identified the ribosomal proteins of *Bacillus subtilis* that are methylated. The methylation pattern is remarkably similar to that of *E. coli* (1, 6, 7, 9, 10), and some of the proteins that are known to be homologous are methylated in both species.

*Bacillus subtilis* 168 was grown in 50 ml of Spizizen's medium (27) containing 20 mg of L-tryptophan per liter, with 75 μCi of [1-$^{14}$C]methionine (Amersham Corp., 60 mCi/mmol) and 1 mCi of [*methyl*-$^{3}$H]methionine (Amersham Corp., 14 Ci/mmol) to give a final methionine concentration of 6 μg/ml. The cells were harvested at mid-log phase, and 500 mg of carrier *B. subtilis* cells was added. Cells were then washed with 1 M KCl to reduce proteases (20).

For the preparation of ribosomes the cells were suspended in 2.5 volumes of a buffer containing 50 mM tris-hydrochloride (pH 7.5), 20 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA, 6 mM 2-mercaptoethanol, 3.5 mM phenylmethylsulfonyl fluoride, and 5% glycerol. In addition, 8 mg of Macaloid (National Lead Co., Baroid Division, Houston, Tex.) per g of cells was added. The suspension was then incubated in the presence of 1 mg of lysozyme per ml for 30 min at 37°C, followed by a further incubation in the presence of 10 μg of RNase-free DNase per ml for 10 min at 37°C. Ribosomes were obtained and washed with 0.5 M $NH_4Cl$ as previously described (17). Ribosomal subunits were prepared essentially as described by Guha and Szulmajster (13).

Total ribosomal proteins were extracted from the subunits by acetic acid (14) containing 3 mM phenylmethylsulfonyl fluoride. The acetic acid supernatant containing the ribosomal proteins was then dialyzed for 2 h against successive changes of 50, 25, 12.5, and 2% acetic acid containing 1 mM phenylmethylsulfonyl fluoride. The final protein solution was lyophilized and used for polyacrylamide gel electrophoresis. Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins was carried out as described in reference 17 by the standard method of Kaltschmidt and Wittmann (18). The individual stained protein spots were excised from the gel with a scalpel blade.

For determination of radioactivity, the gel slices were treated with 0.4 ml of 30% $H_2O_2$ for 12 h at 55°C in closed scintillation vials and counted in a toluene-Triton X-100-2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid in a Nuclear-Chicago Mark II scintillation spectrometer. Methylation of the different ribosomal proteins was determined by

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the method of Chang et al. (9). When cells are grown in the presence of [1-$^{14}$C]methionine and [*methyl*-$^{3}$H]methionine, there is an equal probability that both isotopes will be incorporated into proteins. If a protein is methylated, the $^{3}$H/$^{14}$C ratio will be higher than that in a protein that is not methylated (9).

Ribosomal proteins from *B. subtilis* 168 have been previously characterized by two-dimensional polyacrylamide gel electrophoresis (12). We have used the same nomenclature described. Figure 1 shows the *B. subtilis* ribosomal proteins from the 30S and 50S subunits resolved by two-dimensional polyacrylamide gel electrophoresis.

Table 1 shows the $^{3}$H/$^{14}$C ratios obtained for the ribosomal proteins from the 50S subunits. The $^{3}$H/$^{14}$C ratio for the total unfractionated proteins was 4.00:1, and an increase over this ratio for any given protein was taken as a measure of the methylation level (4, 7, 9, 15). Accordingly, proteins L11 and L16 had a high level of methylation, whereas the slightly elevated $^{3}$H/$^{14}$C ratios for proteins L18 and L20 indicated low levels of methylation. Our results were essentially reproducible in four independent experiments, using either separated ribosomal subunits or 70S ribosomes. Protein L11 was considered to be the most heavily methylated of the *B. subtilis* ribosomal proteins, since it had the highest $^{3}$H/$^{14}$C ratio. It is interesting that protein L11 from *E. coli* is also the most heavily methylated protein in the *E. coli* ribosome (1, 9). Protein L11 from both species appears to be involved in the stringent response (23, 26), and both proteins are immunologically related, indicating that they are structurally and functionally homologous (26).

Proteins L9 and L10 were also possibly methylated. Protein L9 from *B. subtilis* is equivalent to L7L12 of *E. coli* (16, 17) and appears to be methylated like the latter (7, 8, 29), since the method used to detect methylation is more sen-

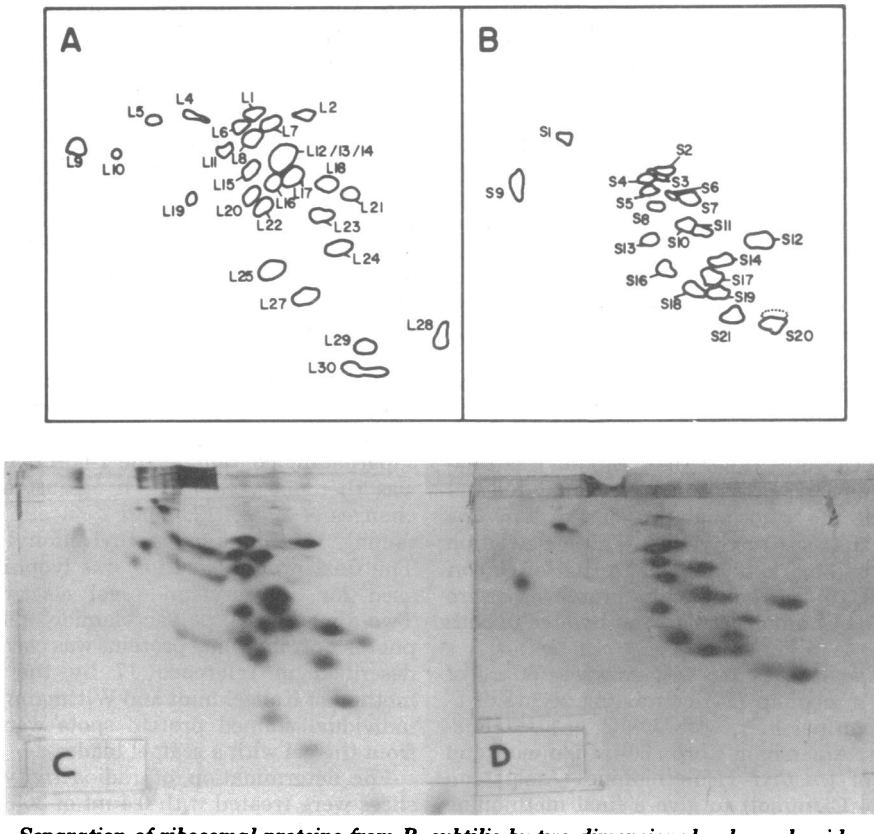


FIG. 1. *Separation of ribosomal proteins from B. subtilis by two-dimensional polyacrylamide gel electrophoresis. Ribosomal proteins from 30S and 50S subunits were separated (18) and were numbered according to Geisser et al. (12). (A) Diagrammatic representation of 50S proteins separated in (C). Proteins L3, L19, and L26, which have been numbered by Geisser et al. (12), were not detected with the standard method used (18). (B) Diagrammatic representation of 30S proteins separated in (D). Protein S15 described by Geisser et al. (12) could not be detected in the stained gel.*

sitive when the protein analyzed has a low methionine content (9). Protein L9 from *B. subtilis* has recently been sequenced (16) and does not contain methionine, indicating that all the incorporation is probably due to methylation from [*methyl*-$^3$H]methionine. Therefore, a disproportionately high $^3$H/$^{14}$C ratio would be expected for this protein. The low incorporation of radioactivity into protein L9 indicates that it might have a very low level of methylation. A similar finding has been reported for protein L7 from *E. coli*, in which the in vivo incorporation of methyl groups is only 1/70 the value recorded for *E. coli* L11 (7). The lack of incorporation of [1-$^{14}$C]methionine into protein L10 suggests that, similar to L9, it may not contain methionine but may be methylated.

Table 2 shows the results obtained for the methylation of the 30S ribosomal proteins from *B. subtilis*. Clearly, the methylation pattern is very different from that of the 50S subunit. Only proteins S8 and S13 show slightly elevated $^3$H/$^{14}$C ratios. This may indicate that the in vivo methylation of ribosomal proteins occurs preferentially, if not exclusively, on the 50S subunit, as is the case for the *E. coli* ribosome (1, 7, 9).

TABLE 1. *Methylation of the ribosomal proteins of the 50S subunit*[a]

| Protein | $^3$H (kdpm) | $^{14}$C (kdpm) | $^3$H/$^{14}$C ratio |
|---|---|---|---|
| L1 | 42.36 | 9.89 | 4.28 |
| L2 | 26.59 | 6.66 | 3.99 |
| L4 | 6.55 | 1.52 | 4.31 |
| L5 | 7.79 | 1.63 | 4.78 |
| L6 | 55.79 | 13.47 | 4.14 |
| L7 | 55.95 | 13.35 | 4.19 |
| L8 | 31.07 | 7.63 | 4.07 |
| L9 | 0.60 | <0.01 | |
| L10 | 1.20 | <0.01 | |
| L11 | 45.18 | 4.24 | 10.66* |
| L12/13/14 | 115.32 | 30.97 | 3.72 |
| L15 | 35.19 | 10.07 | 3.49 |
| L16 | 11.51 | 1.76 | 6.54* |
| L17 | 40.60 | 8.98 | 4.52 |
| L18 | 9.20 | 1.72 | 5.34* |
| L20 | 9.70 | 1.66 | 5.84* |
| L21 | 12.52 | 3.22 | 3.88 |
| L22 | 32.51 | 8.37 | 3.88 |
| L23 | 29.30 | 6.92 | 4.23 |
| L24 | 0.99 | 0.55 | |
| L25 | 13.87 | 4.05 | 3.42 |
| L27 | 6.06 | 2.05 | 2.96 |
| L28 | 2.53 | 0.67 | 3.78 |
| L29 | 8.04 | 2.27 | 3.54 |
| L30 | 8.69 | 2.25 | 3.86 |

[a] *B. subtilis* was grown in the presence of [*methyl*-$^3$H]methionine and [1-$^{14}$C]methionine. Proteins from the 50S subunit were isolated and separated as in Fig. 1. Proteins L12/13/14 were considered as a single spot since they are not resolved in the standard electrophoretic system (12). The gel slices were counted for both $^3$H and $^{14}$C as described in the text. The $^3$H/$^{14}$C ratio for the total unfractionated ribosomal proteins was 4.00:1. Proteins in which methyl groups are present in excess of the methionine present are marked by an asterisk. $^3$H/$^{14}$C ratios were not calculated for those proteins with very low levels of radioactive label incorporation since their estimation is not accurate.

TABLE 2. *Methylation of the ribosomal proteins of the 30S subunit*[a]

| Protein | $^3$H (kdpm) | $^{14}$C (kdpm) | $^3$H/$^{14}$C ratio |
|---|---|---|---|
| S1 | 15.41 | 3.22 | 4.79 |
| S2 + S3 | 16.35 | 5.40 | 3.03 |
| S4 | 15.53 | 3.50 | 4.44 |
| S5 | 25.64 | 6.11 | 4.20 |
| S6 + S7 | 46.61 | 10.82 | 4.31 |
| S8 | 21.48 | 3.72 | 5.77* |
| S9 | 11.69 | 2.61 | 4.48 |
| S10 | 10.69 | 3.14 | 3.40 |
| S11 | 4.50 | 1.31 | 3.44 |
| S12 | 5.67 | 1.62 | 3.50 |
| S13 | 14.78 | 2.87 | 5.15* |
| S14 | 0.95 | 0.39 | |
| S16 | 18.97 | 4.00 | 4.74 |
| S17 | 14.07 | 3.82 | 3.68 |
| S18 | 9.44 | 2.91 | 3.24 |
| S19 | 12.51 | 2.98 | 4.20 |
| S20 | 8.04 | 3.10 | 2.59 |
| S21 | 4.65 | 1.18 | 3.94 |

[a] Methylation was determined as in Table 1. Geisser et al. (12) described two spots for proteins S1, S9, and S20. Since they are not completely separated, they were excised from the gels as one spot. Proteins S2 and S3 and S6 and S7 were also taken together. Proteins in which methyl groups are present in excess of the methionine present are marked by an asterisk.

Comparison of the 50S methylated ribosomal proteins from *E. coli* and *B. subtilis* on two-dimensional polyacrylamide gels indicated that, with few exceptions, their electrophoretic mobilities are remarkably similar. This suggests that a common pattern of protein methylation may exist for the procaryotic ribosome. Our results indicate also that the methylation of the proteins known to be functionally and immunologically homologous (L7L12 from *E. coli*, L9 from *B. subtilis*, and L11 from both species) may be conserved, at least in these two bacteria. Whether the other *B. subtilis* methylated ribosomal proteins are homologous to the *E. coli* methylated proteins with similar electrophoretic properties remains to be established. An analysis of the amino acids that are methylated in *B. subtilis* and other procaryotes will be necessary to confirm our observations. While preparing this report, we learned that protein L11 from *Bacillus megaterium*, which is homologous to *E. coli* L11, is also heavily methylated (4) and

that protein L11 is absent from 70S ribosomes of thiostrepton-resistant mutants of *B. subtilis* (26) and *B. megaterium* (4).

This work was supported by grants from UNDP/UNESCO RLA 75/047 and project no. B169-784 from the University of Chile.

We express our appreciation to N. Brot, S. Ramagopal, and P. Masler for reading the manuscript.

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