

DIFFERENCES IN LYSINE-SRNA FROM SPORE AND VEGETATIVE CELLS OF *BACILLUS SUBTILIS*

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The changes in the syntheses of macromolecules that accompany sporulation of *Bacillus subtilis* have been the subject of several recent reports. In brief, the available data show that as a rapidly growing culture of *B. subtilis* departs from strict logarithmic growth, the net synthesis of RNA is abruptly arrested although the protein and DNA content approximately double after that time.¹ *De novo* RNA synthesis, however, measured by the incorporation of labeled precursors, continues throughout the stationary and sporulation phase but is just offset by a degradation of cellular RNA. The RNA synthesized during this time appears not only in the messenger fraction but also in soluble and ribosomal fractions.¹⁻³ Despite this active turnover of soluble and ribosomal RNA during sporulation, the RNA composition of *B. subtilis* spores appears to be only slightly different from that of vegetative cells. The ratio of soluble to ribosomal RNA increases during stationary phase, but the base compositions of these species are not significantly different from their counterparts in vegetative cells.^{4, 5} However, Doi⁶ has observed an asymmetry between the elution profiles from methylated albumin kieselguhr columns of soluble RNA from vegetative cells and spores, indicating an alteration of this fraction during sporulation.

In the present communication, the abilities of sRNA from spores and vegetative cells of *B. subtilis* to accept several amino acids are compared. Of the six amino acids tested, all but lysine were incorporated into spore sRNA to a lesser extent than into vegetative sRNA. The incorporation of lysine into spore sRNA was more than twice that observed when vegetative sRNA was employed as an acceptor. Comparison of the elution profiles of spore and vegetative lysyl-sRNA shows that spore sRNA contains a component not found in appreciable amounts in vegetative sRNA.

Materials and Methods.—*Organisms and culture conditions:* All organisms employed were derivatives of the transformable *Bacillus subtilis*, Marburg strain. *B. subtilis* 60015 (Met⁻, Ind⁻) and *B. subtilis* 60005 (prototroph) were originally obtained from Dr. E. Nester. *B. subtilis* 60021 (Ind⁻) and 60253 (constitutive for tryptophan synthetase) were obtained from Drs. Spizizen and Anagnostopoulos, respectively.

The growth medium employed, TY, contained 10 gm Bacto-Tryptone, 5 gm yeast extract, 5 gm sodium chloride, 1×10^{-3} moles CaCl₂, and 1×10^{-6} moles MnCl₂ per liter. The solid medium had the same composition as TY but included 20 gm agar per liter.

Vegetative cells were grown on liquid TY with forced aeration at 37° and harvested before the turbidity reached 1.0 absorbance units at 600 mμ. Under these growth conditions the average doubling time is 30 min, and cultures attain a final (stationary phase) turbidity of 5.0–7.0 absorbance units at 600 mμ. Spores were prepared by seeding TY agar plates (22 × 34 cm) containing 400 ml of medium with vegetative cells. After 48–72 hr incubation at 37°, the felts of spores and vegetative debris were scraped from the agar, suspended in cold distilled water, and washed twice by centrifugation and decantation. The washed spores and debris were suspended in 0.01 M Tris, pH 7.0, containing 0.15 mg/ml lysozyme and incubated for 30 min at 37°. DNase was then added to a final concentration of 10 μg/ml and the suspension incubated for an additional 30 min.

To ensure lysis of vegetative cells and maximum solubilization of vegetative debris, sodium dodecyl sulfate was added to a final concentration of 1% and incubation continued for an additional 30 min. Spores were separated from the suspension by centrifugation at $7000 \times g$ for 10 min. The sedimented spores were then washed by centrifugation eight times with distilled water. After each centrifugation the pellet was rinsed with distilled water to remove vegetative debris which formed a fluffy layer on top of the densely packed spores.

Preparation of amino acid activating enzymes: A crude extract of vegetative cells prepared by lysozyme treatment was adjusted to a protein concentration of 25–30 mg/ml by dilution with 0.02 *M* Tris, pH 7.5. A warm (40°C) protamine sulfate solution (10 mg/ml) was added slowly to the rapidly stirred crude extract until 5 mg of protamine had been added for every 100 mg of bacterial protein. The precipitate was allowed to coagulate for 15 min at ice bath temperature and then was removed by centrifugation. An aliquot of the slightly turbid supernatant fluid containing 0.4 gm of protein was applied directly to a 1.8×20 -cm DEAE-cellulose column previously equilibrated with 0.01 *M* potassium phosphate, pH 7.5, containing 0.003 *M* mercaptoethanol. The column was washed with 50–100 ml of the starting buffer to remove unabsorbed protein and then developed with a linear gradient formed between 200 ml of 0.01 *M* potassium phosphate, pH 7.5, containing 0.003 *M* mercaptoethanol and 200 ml of a solution containing 0.01 *M* potassium phosphate, pH 7.5, 0.5 *M* NaCl, and 0.003 *M* mercaptoethanol. Fractions containing maximum activity for each of the synthetases employed were either frozen directly or concentrated by pervaporation and dialyzed. The positions in the elution profile corresponding to maximal activity were: phenylalanine, 240 ml effluent vol; leucine, 245 ml; arginine, 255 ml; glutamate, 264 ml; lysine and valine, 285 ml.

Preparation of soluble RNA: Soluble RNA was prepared from frozen vegetative cells by the method of Fleissner and Borek⁷ with the following modifications. The buffer used to extract the alumina-ground cells contained 0.01 *M* MgCl₂ and 0.05 *M* KCl. The aqueous phase from the phenol treatment was further deproteinized with chloroform/isoamylalcohol (24/1). The resulting RNA solution was adjusted to 1 *M* NaCl and applied to a Sephadex G-100 column (2.8×80 cm) equilibrated with 1 *M* NaCl. Fractions containing the soluble RNA were combined and mixed with 2.5 vol of ethanol. The resulting precipitate was collected by centrifugation, dissolved in a minimal volume of 0.01 *M* Tris, pH 7.5, and dialyzed against the same buffer.

Extracts of spores were prepared by sonication of a slurry containing 1 part spores and 4 parts glass beads with a Branson probe sonifier. After sonication, the mixture was diluted with 0.05 *M* Tris, pH 7.0, and the extract separated from the bulk of the glass beads by decantation. The beads were washed twice with a minimal volume of buffer. The combined supernatant fluid and washes were centrifuged at $25,000 \times g$ for 20 min. The clarified supernatant fluid was deproteinized first with phenol and then with chloroform/isoamylalcohol. The soluble RNA in the resulting solution was separated from ribosomal debris by passage through a Sephadex G-100 column equilibrated with 1 *M* NaCl as described for the vegetative soluble RNA.

Estimation of amino acid acceptance of soluble RNA: The conditions employed for amino acid incorporation into sRNA were the same as those previously reported⁸ except that the reaction mixtures contained 0.05–0.1 mg sRNA and the C¹⁴-amino acids had specific activities of 220–333 $\mu\text{C}/\mu\text{mole}$. The enzyme fractions used were those fractions from a DEAE-cellulose column which had maximal synthetase activity for the particular amino acid. Estimations of maximal amino acid acceptance by preparations of sRNA were made from time courses in which the incorporation into TCA-precipitable material of labeled amino acids was followed for 1 hr. Generally, 90% of maximal incorporation was observed within 15 min.

Preparation of C¹⁴-lysyl-sRNA: The reaction conditions for the preparation of lysyl-sRNA were the same as those described above, except that the reactions were carried out on a 5–10 times larger scale. The reaction was terminated by the addition of $1/10$ vol of 0.5 *M* potassium succinate, pH 5.0, and 1 vol of phenol. The suspension was shaken, cooled in an ice bath, and centrifuged at $12,000 \times g$ for 10 min. The upper phase containing the lysyl-sRNA was freed of phenol and unesterified lysine by passage through a Sephadex G-25 column (2.2×50 cm) equilibrated with 0.005 *M* potassium succinate, pH 5.0.

Chromatography on methylated albumin-kieselguhr (MAK) columns: MAK chromatographic analysis was carried out essentially as previously reported,⁹ except that a slower flow rate (1.5 ml/min) was employed.

TABLE 1
AMINO ACID INCORPORATION INTO sRNA FROM *B. subtilis*

Amino acid	Spore sRNA		Vegetative sRNA	
	Incorporation ($\mu\text{moles}/A_{260} \text{ m}\mu$)	Ratio to leucine	Incorporation ($\mu\text{moles}/A_{260} \text{ m}\mu$)	Ratio to leucine
Leucine	50.7	1	88.5	1
Lysine	59	1.2	26.0	0.29
Phenylalanine	24.6	0.49	27.5	0.31
Arginine	31.1	0.61	40.3	0.47
Valine	41.5	0.80	52.6	0.60
Glutamic acid	62.2	1.2	80.5	0.91

Results and Discussion.—The maximal incorporations of several amino acids into sRNA from spores and vegetative cells of *B. subtilis* 60015 are shown in Table 1. The levels of incorporation for leucine, phenylalanine, arginine, valine, and glutamic acid obtained with sporal sRNA are significantly less than those obtained with vegetative sRNA. In contrast, the incorporation of lysine into sporal sRNA is more than twice that observed into vegetative sRNA. This specific increase in lysine acceptance is seen more clearly when the data are presented as the ratios of incorporation for each amino acid to that of a standard amino acid, leucine. Expressed in this way, the results are not complicated by factors, such as the presence of inactive RNA, which affect the values for each amino acid proportionally.

The ratios of incorporations for the amino acids are remarkably constant between different vegetative sRNA preparations from the same *B. subtilis* strain and between at least three different strains of *B. subtilis*. The ratios obtained with spore sRNA varied somewhat between different preparations, even though aliquots of the same spore paste were used as the starting material. This variation is traceable to a variation in the level of leucine incorporation. The range of values for the lysine to leucine ratios obtained with RNA preparations from spores of three different strains of *B. subtilis* was 0.9–1.2.

The possibility that the apparent increase in lysine incorporation into spore sRNA is due to the presence of acceptor molecules other than sRNA or the incorporation of labeled compounds other than lysine seems unlikely. The inclusion of the other 19 amino acids in nonradioactive form in the incorporation assay does not diminish the incorporation of C^{14} -lysine. Furthermore, chromatography of an alkaline hydrolysate of the product on a Stein and Moore amino acid analyzer shows that the labeled material could not be resolved from lysine by this technique. The results of the two experiments shown in Table 2 indicate that the product of lysine

TABLE 2
SENSITIVITY OF SPORE LYSYL-sRNA

	Expt. No.	
	I (cpm)	II (cpm)
C^{14} -Lysyl-sRNA	702	1030
+ RNase	39*	30†
+ 1 M NH_2OH ‡	29	42
+ 0.1 M Glycine, pH 10.3 ‡	65	59

C^{14} -lysine-charged purified sRNA ($0.07 A_{260}$) and unfractionated RNA ($0.19 A_{260}$) were employed in experiments I and II, respectively. The RNA and the indicated reagents in a total volume of 0.2 ml (expt. I) or 1.0 ml (expt. II) were incubated for 30 min at 37° . The reaction was terminated with TCA. The precipitated RNA was collected on Millipore filters and the radioactivity measured in a liquid scintillation counter.

* 10 $\mu\text{g}/\text{ml}$ RNase.

† 50 $\mu\text{g}/\text{ml}$ RNase.

‡ Final concentrations indicated.

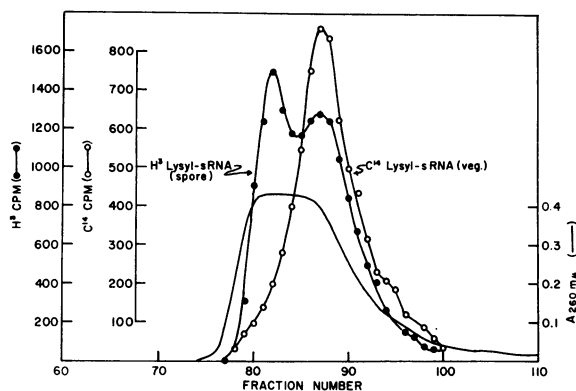


FIG. 1.—MAK elution profile of H^3 -lysyl-sRNA from spore and C^{14} -lysyl-sRNA from vegetative cells. The MAK column was charged with 1.3 A_{260} of H^3 -lysyl-sRNA (20,100 cpm) from spores, 3.2 A_{260} C^{14} -lysyl-sRNA (9,700 cpm) from vegetative cells and 11.0 A_{260} of carrier vegetative sRNA. Two-ml fractions were collected, mixed with 10 A_{260} of carrier RNA, and precipitated by adding 5 ml ethanol. Precipitated RNA was recovered by centrifugation and dissolved in 0.5 ml 1.5 M NH_4OH and 10 ml of Bray's¹⁸ liquid scintillation fluid and entire sample transferred to a counting vial. Recovery of radioactivity from column: H^3 , 83%; C^{14} , 73%.

incorporation exhibits the same sensitivity to ribonuclease, alkaline pH, and hydroxylamine that would be expected of lysyl-sRNA. The sensitivity to alkaline pH and hydroxylamine also provides evidence that the attachment of lysine is not through the N^6 position of adenine residues as in the class of sRNA derivatives recently described by Hall.^{10, 11} Thus, the higher incorporation of lysine into spore sRNA preparations reflects an increase in content of lysine-sRNA rather than the presence of non-RNA acceptor molecules or an increase in the modes of attachment of the amino acid to the acceptor RNA.

The MAK elution profiles of lysyl-sRNA from spore and vegetative cells are compared in Figure 1. Soluble RNA from spores esterified with H^3 -lysine was mixed with vegetative sRNA containing C^{14} -lysine, applied to a methylated albumin-kieselguhr column, and eluted with a linear salt gradient. The eluted fractions were monitored for C^{14} and H^3 radioactivity and UV-absorbing material. The resultant elution profiles (Fig. 1) clearly show that the spore sRNA contains a species of lysine-sRNA not found in appreciable amounts in the vegetative preparation. The presence of this species also has been observed in sRNA prepared from spores of two other strains of *B. subtilis*, 60005 and 60021.

The presence of the new species of lysine-sRNA in spore preparations raises the question of whether the new form is an artifact arising from differences in the enzymic complement in crude extracts of vegetative cells and spores. Nishimura and Nomura^{12, 13} have shown that there are at least two ribonucleases elaborated by stationary phase and sporulating cultures of *Bacillus subtilis* that are not found during logarithmic growth. Furthermore, Nishimura and Novelli¹⁴ reported that certain species of *E. coli* sRNA maintain their competence to accept amino acids after digestion with *B. subtilis* RNase, but exhibit grossly altered MAK elution profiles after treatment with the enzyme. Were the "native" spore sRNA identical to vegetative, but enzymically altered in the course of the purification of

sRNA, it should be possible to mimic these events by treating purified vegetative sRNA with a spore extract. Such treatment would be expected to have a twofold effect; change the ratio of lysine to leucine incorporation to that characteristic of spore sRNA and alter the MAK elution profile. Incubation of vegetative sRNA with a crude extract of spores leads to a decrease in the acceptance of leucine. However, lysine acceptance decreases by an equivalent amount, and the ratio of lysine to leucine acceptance is not significantly altered. The MAK elution profiles of two samples in which lysine acceptance had decreased 27 per cent and 65 per cent are compared to an untreated control in Figure 2. Although equivalent amounts of vegetative RNA were applied to each column, the elution profiles can be compared for qualitative differences only since the recovery of radioactive material from the column varied and the samples contained different amounts of spore sRNA derived from the extract. Nonetheless, it can be seen that treatment of vegetative RNA with spore enzymes does not lead to the formation of the new component of lysine-sRNA.

The magnitude of the enrichment of lysine-sRNA and the appearance of the new species in spores invites speculation concerning the biochemical function of this additional sRNA. Since this material is found only in spores or sporulating cells, it seems reasonable to assume, at this juncture, that it is related to a spore-specific process. The results of preliminary experiments indicate that the alteration of lysine-sRNA commences late in sporulation. Comparison of these results with the temporal sequence of morphological¹⁵ and biochemical¹⁶ events accompanying sporulation would place the alteration at stage 5 or 6, the formation of spore wall and the appearance of refractility, respectively. Thus, it seems likely that the new lysine-sRNA participates in one or more of the biochemical events occurring during these phases. Speculation beyond this point is severely hampered by our ignorance of the events, other than the morphological ones, occurring at these stages.

While this manuscript was in preparation, Kaneko and Doi¹⁷ reported a specific alteration of valine-sRNA occurring during the sporulation of a nontransformable strain of *B. subtilis*, W23. Although MAK elution profiles of lysyl-sRNA from sporulating and vegetative cells were compared by them, no significant difference was observed. The failure to observe the alteration in lysyl-sRNA can be attributed to the fact that it occurs very late in sporulation. The sRNA preparations em-

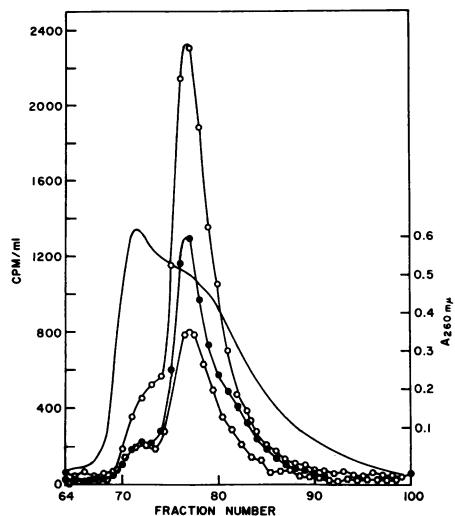


FIG. 2.—Effect of treatment with spore extract on the elution profiles of lysyl-sRNA from vegetative cells. *Open circles*: elution profile of 2.45 A_{260} untreated vegetative sRNA. *Closed circles* and *open hexagons*: elution profiles of 2.45 A_{260} after treatment with spore extract sufficient to lower lysine acceptance 27% and 65%, respectively. Recoveries of radioactivity from columns: *upper curve*, 75%; *middle curve*, 59%; *lower curve*, 49%.

ployed by Kaneko and Doi were obtained from cells at stage 4 and earlier.

Summary.—A comparison of the ability of sporal and vegetative sRNA of *B. subtilis* to accept several amino acids reveals a marked enrichment of lysine-specific sRNA in sporal preparations. Chromatography on methylated albumin-kieselguhr of spore and vegetative lysyl-sRNA showed that the enrichment of lysine-sRNA in the spore sRNA was, at least in part, accounted for by the presence of a species of lysyl-sRNA not found in appreciable amounts in vegetative cells.

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Abbreviations used: RNA, ribonucleic acid; sRNA, soluble ribonucleic acid; MAK, methylated albumin-coated kieselguhr; RNase, ribonuclease.

¹ Spotts, C. R., and J. Szulmajster, *Biochim. Biophys. Acta*, **61**, 635 (1962).

² Balassa, G., *Biochim. Biophys. Acta*, **76**, 410 (1963).

³ Balassa, G., in *Mécanismes de Régulation des Activités Cellulaires chez les Microorganismes*, Colloques Internationaux du Centre National de la Recherche Scientifique, Paris, no. 124 (1965), p. 565.

⁴ Doi, R. H., and R. T. Igarashi, *Nature*, **203**, 1092 (1964).

⁵ Doi, R. H., and R. T. Igarashi, *J. Bacteriol.*, **87**, 323 (1964).

⁶ Doi, R. H., in *Spores III*, ed. L. L. Campbell and H. O. Halvorson (Ann Arbor: American Society for Microbiology, 1965), p. 111.

⁷ Fleissner, E., and E. Borek, these PROCEEDINGS, **48**, 1199 (1962).

⁸ Lazzarini, R. A., and A. H. Mehler, *Biochemistry*, **3**, 1445 (1964).

⁹ Lazzarini, R. A., and A. Peterkofsky, these PROCEEDINGS, **53**, 549 (1965).

¹⁰ Hall, R. H., *Biochemistry*, **3**, 769 (1964).

¹¹ Hall, R. H., and G. B. Chheda, *J. Biol. Chem.*, **240**, PC 2754 (1965).

¹² Nishimura, S., and M. Nomura, *Biochim. Biophys. Acta*, **30**, 430 (1958).

¹³ Nishimura, S., and M. Nomura, *J. Biochem.*, **46**, 161 (1959).

¹⁴ Nishimura, S., and G. D. Novelli, *Biochim. Biophys. Acta*, **80**, 574 (1964).

¹⁵ Schaeffer, P., H. Ionesco, A. Ryter, and G. Balassa, in *Mécanismes de Régulation des Activités Cellulaires chez les Microorganismes*, Colloques Internationaux du Centre National de la Recherche Scientifique, Paris, no. 124 (1965), p. 553.

¹⁶ Halvorson, H. O., in *Function and Structure in Microorganisms*, 15th Symposium of the Society for General Microbiology, London (1965), p. 343.

¹⁷ Kaneko, I., and R. H. Doi, these PROCEEDINGS, **55**, 565 (1966).

¹⁸ Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).