JOURNAL OF BACTERIOLOGY, June 1970, p. 711-715
Vol. 102, No. 3


# Comparison of Lysyl-Transfer Ribonucleic Acid Species from Vegetative Cells and Spores of *Bacillus subtilis* by Methylated Albumin-Kieselguhr and Reversed-Phase Chromatography

BARBARA S. VOLD

*Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

Received for publication 23 March 1970

Lysyl-transfer ribonucleic acid (tRNA) species from a spore-forming strain of *Bacillus subtilis* (168 *trp2*$^-$) and an early blocked asporogenous mutant (*spoA* 12) were compared on reversed-phase and methylated albumin-kieselguhr columns. Lysyl-tRNA species from spores and the asporogenous mutant in stationary phase both exhibited altered chromatographic profiles compared to that of log-phase cells. The major peak in spore lysyl-tRNA species eluted later than that characteristic of vegetative cells, whereas the major peak of the lysyl-tRNA species from the asporogenous mutant in stationary phase eluted earlier. Although the early eluting lysyl-tRNA species was observable on methylated albumin columns, the late eluting peak was not detectable by that column technique. By using a shallower gradient on an RPC-2 column, the resolution of all lysyl-tRNA species increased. Several subspecies were revealed. The chromatographic comparisons clearly show that both the spore-forming strain and the asporogenous mutant undergo relative increases in different lysyl-tRNA species when grown to late stationary phase. No new species seem to be involved but rather altered amounts of minor species existing in log-phase cells. The experiments also demonstrate the usefulness of reversed-phase columns for such comparisons.

Alterations in the chromatographic profile of transfer ribonucleic acid (tRNA) species which are correlated with the sporulation process in *Bacillus subtilis* have been reported for lysyl-tRNA species (4) and valyl-tRNA species (3). Both studies employed methylated albumin-kieselguhr (MAK) columns for fractionating the aminoacylated tRNA species. Now that columns with higher resolving power and a wider variety of well-defined *B. subtilis* sporulation mutants are available, we are attempting to re-investigate the problem of tRNA changes that accompany sporulation.

In this paper, we report one alteration in lysyl-tRNA species which was found in spores and one found in an asporogenous mutant. The alterations were characterized by their elution position on reversed-phase columns (RPC-2) relative to lysyl-tRNA species from log-phase cells of a tryptophan-requiring mutant of parent strain 168. We then compare their chromatographic elution patterns on MAK. Running the RPC-2 with a very shallow gradient covering only the lysyl-tRNA region subfractionated the region into a number of reproducible peaks. Clearly, the relative amounts of lysyl-tRNA species changed in both the spore-forming strain and early blocked asporogenous mutant when log-phase cells were grown to late stationary phase. Both alterations in lysyl-tRNA species appear to be different from the one which was originally reported by Lazzarini (4). The possible biological significance of these alterations is discussed.

## MATERIALS AND METHODS

**Growth of bacteria.** *B. subtilis* strains were grown at 37 C with shaking in 1 liter of tryptone-yeast extract medium (4) with 40 μg of L-tryptophan per ml. All bacterial strains were obtained from the laboratory of John Spizizen. Spores were isolated from liquid cultures which had been grown for 24 hr. Cultures were inoculated either from a loop of asporogenous bacteria or a sample of heat-shocked spores.

**Extraction of tRNA species from vegetative cells.** Harvested cells were washed once with cold 10 mM $MgCl_2$ and 10 mM tris(hydroxymethyl)aminomethane (Tris), *p*H 7.5, and suspended in the same buffer plus 500 $\mu$g of lysozyme and 20 $\mu$g of electrophoretically purified deoxyribonuclease per ml. After a 30-min incubation at 37 C with gentle shaking, one-tenth volume of 4 M NaCl, one-tenth volume of 10% sodium lauryl sulfate, and an equal volume of water-saturated phenol were added, and the incubation was continued for another 30 min. The phases were separated by centrifugation; the aqueous phase was saved, and the phenol phase was washed once with water. The combined aqueous phases were extracted once with an equal volume of $CHCl_3$:isoamylalcohol (24:1, v/v) and precipitated with 3 volumes of cold 95% ethanol. The precipitate was collected by centrifugation and fractionated on a diethylaminoethyl (DEAE)-cellulose column. The RNA from the pooled 0.7 M NaCl eluate was recovered by ethanol precipitation, dissolved in 10 mM $MgCl_2$, 2 mM $Na_2S_2O_3$, and 10 mM Tris (*p*H 7.5), and stored frozen.

**Extraction of tRNA species from spores.** Spores were purified from vegetative cells and debris by the washing procedure of Lazzarini (4) followed by sedimentation through 60% renographin (RENOgrafin-76; E. R. Squibb & Sons, New York, N.Y.). Transfer RNA species were extracted from these purified spores by the following procedure which was modified from a method originally developed for deoxyribonucleic acid (DNA) extraction by Audrey Evans in the Laboratory of John Spizizen (Audrey Evans, Ph.D. thesis, University of California at San Diego, 1970).

Purified spores (38 g, wet weight) were suspended in 100 ml of 8 M urea and 10% 2-mercaptoethanol (*p*H 3.0) and allowed to stand at room temperature for 1 hr. The spores were then centrifuged at 6,000 $\times$ *g* for 10 min in a refrigerated Sorvall centrifuge and washed five times with 10 mM NaCl and 50 mM sodium citrate, *p*H 7.0 (SSC). After the final wash, the spores were suspended in 100 ml of SSC and incubated with shaking at 37 C with 1 mg of Pronase B per ml for 30 min. The suspension was made 1% in sodium lauryl sulfate and incubated another 30 min. The spores were then washed by centrifugation three times with SSC and once with 10 mM Tris and 10 mM $MgCl_2$, *p*H 7.5 (TM buffer). The washed pellet was suspended in 50 ml of TM buffer and 500 $\mu$g of lysozyme per ml and incubated with shaking at 37 C for 1.5 hr, after which the suspension was centrifuged as above and the supernatant fluid was withdrawn and saved. The pellet was resuspended in 50 ml of TM buffer plus 2 mg of lysozyme per ml and 5 $\mu$g of electrophoretically purified deoxyribonuclease per ml. After incubation with shaking at 37 C for 30 min, one-tenth volume of 4 M NaCl, one-tenth volume of 10% sodium lauryl sulfate, and an equal volume of water-saturated phenol were added, and the incubation was continued another 30 min. The phases were separated by centrifugation; the aqueous phase was saved and the phenol phase was washed once with water. The combined aqueous phases were extracted once with an equal volume of $CHCl_3$:isoamylalcohol (24:1, v/v) and precipitated with 3 volumes of cold 95% ethanol. The supernatant saved from the first lysozyme incubation was extracted once with phenol and once with $CHCl_3$:isoamylalcohol, precipitated with ethanol, and combined with the ethanol precipitate from the second lysozyme incubation.

The precipitate was recovered by centrifugation and dissolved in 1 M NaCl, 10 mM Tris, 1 mM ethylenedinitrilotetraacetic acid (EDTA) (*p*H 7.5), and 1% methanol. This solution was applied to a Sephadex G-100 column (2.5 by 92 cm) which was equilibrated and eluted with the same buffer. Eluant fractions from the 4*S* region were combined and precipitated with ethanol. The resulting precipitate was collected by centrifugation, dissolved in 10 mM $MgCl_2$, 2 mM $Na_2S_2O_3$, and 10 mM Tris (*p*H 7.5), and stored frozen. The yield was 80 $A_{260}$ units. The 240/260 absorbance ratio was 0.650 and the 280/260 ratio was 0.494.

**Aminoacylation.** The following 1-ml reaction was used: 50 to 600 $\mu$g of tRNA, 5 mM adenosine triphosphate, 0.1 mM cytosine triphosphate, 10 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol, 50 mM sodium cacodylate buffer (*p*H 7.4), 10 nmoles each of 15 unlabeled L-amino acids without lysine, 200 $\mu$g of protein (crude aminoacyl-RNA synthetase fraction from DEAE-cellulose), and either 1 nmole of $^{14}$C-L-lysine (312 mc/mmole; BioResearch Inc., Orangeburg, N.Y.) or 0.15 nmole of $^{3}$H-L-lysine (16 c/mmole, Schwarz) plus 1 nmole of unlabeled L-lysine. The reaction was incubated at 37 C for 20 min. The reaction mixtures were then combined and extracted once with water-saturated phenol and once with $CHCl_3$:isoamylalcohol (24:1, v/v), and the aqueous phase was run over DEAE cellulose. The tRNA fraction from DEAE was then adjusted to the appropriate NaCl and buffer concentrations for chromatography.

**Chromatography on reversed-phase columns.** The RPC 2 was prepared by the method of Weiss and Kelmers (9). All columns (RPC-2) were run at 24 C in jacketed glass columns at a flow rate of 1 ml/min. Elution solutions were made in buffer X which was composed of 10 mM $MgCl_2$, 1 mM EDTA, 2 mM 2-mercaptoethanol, 10 mM sodium acetate (*p*H 4.5), and 0.1 ml/liter of isoamylacetate. Fractions (10 ml) were collected. After the absorbance had been measured, the fractions were precipitated by the addition of one drop of 10 mg of bovine serum albumin per ml and 1 ml of 50% trichloroacetic acid. After about 15 min at 4 C, the precipitated samples were collected on glass fiber filters (Whatman GF/C), washed with 95% ethanol, and dried under an infrared lamp. The dried filters were placed in a liquid scintillator composed of 4 g of 2,5-diphenyloxazole and 0.1 g of dimethyl-1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene, and the radioactivity was measured in a scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). Each column was regenerated by washing with at least one column volume of 1.2 M NaCl in buffer X and then reequilibrated with starting buffer. Average recovery of absorbance at 260 nm was 90% and of radioactivity was 70%. Under these conditions, 20 to 30% of the lysyl-tRNA aminoacyl bonds hydrolyzed during the time required for a column run (15 to 30 hr).

Two variations of the basic chromatographic procedure were used. (i) Low resolution for scanning the entire absorption profile at 260 nm. The tRNA was loaded with 5 mg of carrier tRNA onto a column (1 by 240 cm) equilibrated with 0.40 M NaCl in buffer X. After the column was washed with 0.40 M NaCl in buffer X, the tRNA species were eluted in a linear gradient formed from 1 liter of 0.40 M NaCl in buffer X and 1 liter of 0.60 M NaCl in buffer X. (ii) High resolution for preferentially resolving the lysyl-tRNA range. The tRNA was loaded without carrier onto a column (0.9 by 65 cm) equilibrated with 0.34 M NaCl in buffer X. The tRNA species were eluted, without washing, with a linear gradient formed from 500 ml of 0.34 M NaCl in buffer X and 500 ml of either 0.39 or 0.45 M NaCl in buffer X.

**Chromatography on MAK columns.** An MAK column was prepared by the method of Mandell and Hershey (7), except that Celite 545 was used in place of kieselguhr and the buffers were adjusted to *p*H 6.0. The latter modifications were used by Lazzarini and Peterkofsky (5) and were adapted by us so that our results with lysyl-tRNA species on MAK could be compared to those of Lazzarini and his colleagues.

The column was equilibrated at room temperature with 0.40 M NaCl and 0.05 M sodium phosphate (*p*H 6.0). The tRNA species were loaded and washed with the same buffered solution and then eluted in a linear gradient formed from 500 ml each of 0.40 and 500 ml of 0.80 M NaCl in 0.05 M sodium phosphate (*p*H 6.0). The flow rate was 1 ml/min, and 5-ml fractions were collected and processed as described above. The column was regenerated by washing with at least one column volume of 1.2 M NaCl in 0.05 M sodium phosphate (*p*H 6.0) and then reequilibrated with 0.40 M NaCl in phosphate buffer. The average recovery of absorbance at 260 nm was 90% and of radioactivity was 45%. The loss of radioactivity was due to hydrolysis of the aminoacyl bond.

## RESULTS

**Lysyl-tRNA species from log-phase cells and spores of 168 trp2⁻.** The results of chromatographic comparison of lysyl-tRNA species from log-phase cells and spores of *B. subtilis* 168 *trp2*⁻ are shown in Fig. 1. The upper section of the figure shows the pattern obtained on the reversed-phase column, RPC-2. The major species of lysyl-tRNA from spores elutes later than the major species from log-phase cells. The same profiles were seen when the isotopic labels were reversed or when Chromosorb G was used as a supporting matrix instead of Chromosorb W. Only alternate fractions of the elution pattern shown in the upper section of Fig. 1 were precipitated for the measurement of radioactivity. The remaining fractions which contained lysyl-tRNA were combined, concentrated by use of a small DEAE-cellulose column, and chromatographed on an MAK column after the addition of 600 μg of a tRNA carrier. These results are presented in the lower section of Fig. 1.

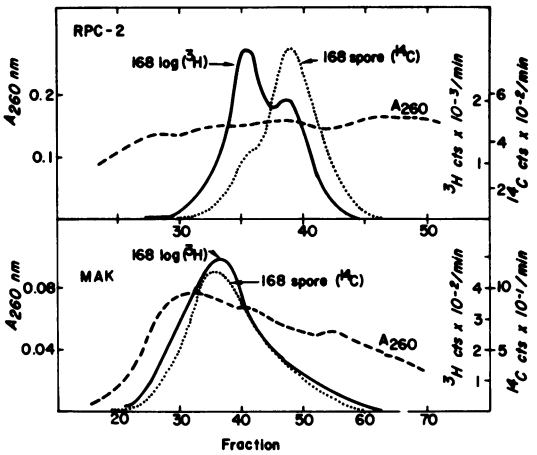


FIG. 1. *Co-chromatography of lysyl-tRNA species from log-phase cells and spores of B. subtilis 168 trp2⁻ on RPC-2 (1 by 240 cm) and MAK. Procedural details are given in Materials and Methods.*

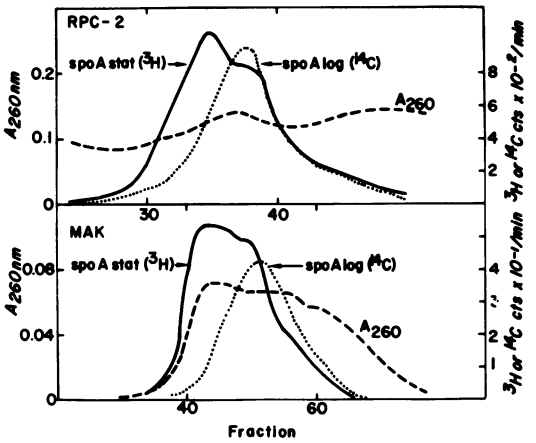


FIG. 2. *Co-chromatography of lysyl-tRNA species from log-phase and stationary-phase cells of B. subtilis spoA 12 on RPC-2 (1 by 240 cm) and MAK.*

The two lysyl-tRNA peaks which were resolved by the RPC-2 column were not detected as separate peaks on the MAK column even though the identical lysyl-tRNA species were chromatographed.

**Lysyl-tRNA species from log-phase cells and the late stationary-phase cells of spoA 12.** Having established a difference in the RPC-2 elution profiles from log-phase cells and spores of 168 *trp2*⁻, we investigated the patterns from an asporogenous mutant. We chose *B. subtilis spo*A 12 because it is blocked very early in the sporulation cycle by a single mutational event. It does not form extracellular protease or antibiotic, and it is

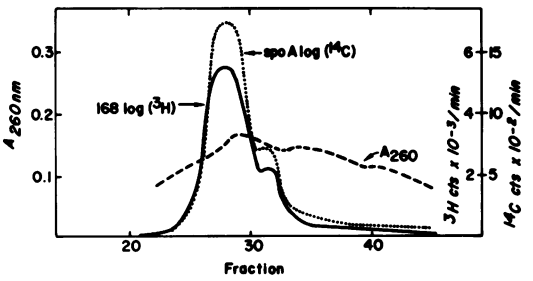


FIG. 3. *Co-chromatography of lysyl-tRNA species from log-phase cells of B. subtilis 168 trp2⁻ and spoA 12 on RPC-2 (1 by 240 cm).*

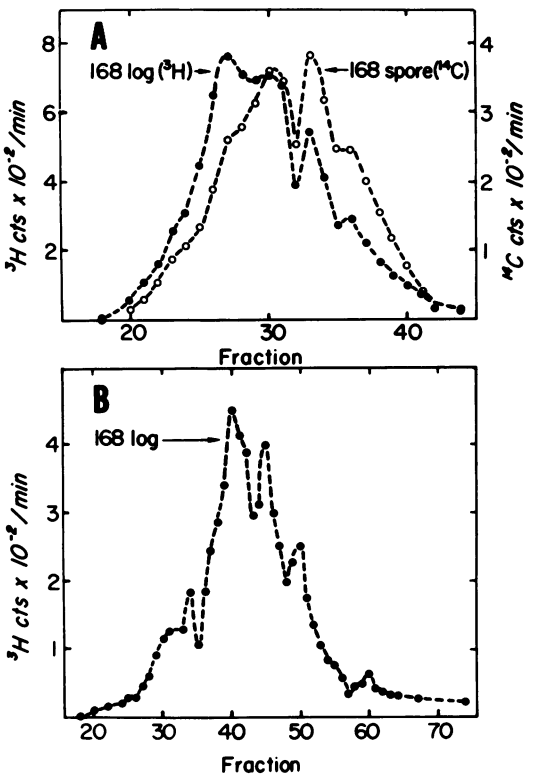


FIG. 4. *Co-chromatography of lysyl-tRNA species. A, Log-phase cells and spores of B. subtilis 168 trp2⁻ on a RPC-2 column (0.9 by 65 cm) eluted with 0.34 and 0.45 M NaCl. B, Log-phase cells of 168 trp2⁻ on the same column as in part A, eluted with 0.34 and 0.39 M NaCl.*

sensitive to $\phi$15. Mutants of this type are described by Hoch and Spizizen (2).

Although the mutational block in *spo*A 12 is a very early one and the cells cannot go through a normal sporulation cycle, they can continue to grow vegetatively for several hours after exponential growth under the conditions used in our experiments. The cells were harvested after 24 hr.

The results of a chromatographic comparison of the lysyl-tRNA species from log-phase and late stationary-phase cells of *spo*A 12 is shown in Fig. 2. The upper section of Fig. 2 shows the chromatography on RPC-2. As described in the previous section, alternate fractions from the lysyl-tRNA region of the RPC-2 were combined, concentrated, and chromatographed on MAK. The results from the latter column are shown in the lower section of Fig. 2. From both columns, the major lysyl-tRNA species of stationary-phase cells eluted earlier than that from log-phase cells.

**Lysyl-tRNA species from log-phase cells of 168 trp2⁻ and spoA 12.** Since we had log-phase cells from two different organisms in Fig. 1 and 2, we ran a control experiment, co-chromatographing lysyl-tRNA species from log-phase cells of 168 *trp2*⁻ and *spo*A 12 (Fig. 3). The profiles are identical, establishing the validity of comparing the peak positions of the two organisms relative to log-phase cells from either organism, as one would expect.

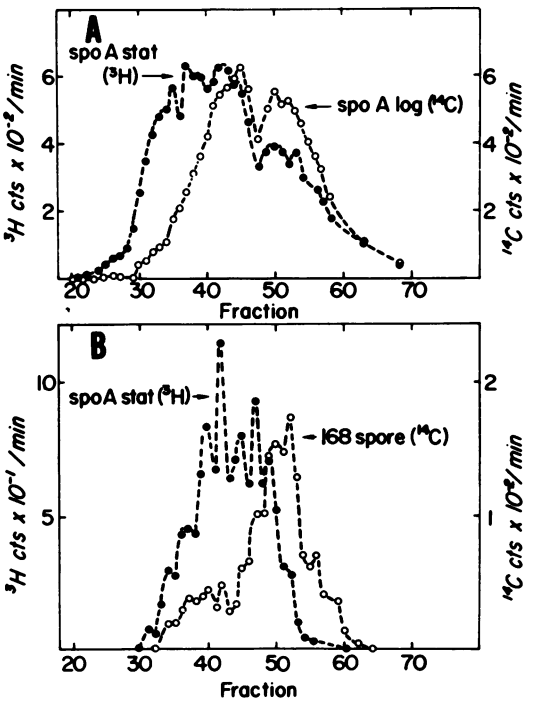


FIG. 5. *Co-chromatography of lysyl-tRNA species on RPC-2 (0.9 by 65 cm) eluted with 0.34 and 0.39 M NaCl. A, Log-phase cells and stationary-phase cells of B. subtilis spoA 12. B, Stationary-phase cells of spoA 12 and spores of 168 trp2⁻.*

**Chromatography at high resolution on RPC-2.** To separate the peaks more clearly, we employed a more shallow gradient for RPC-2 chromatography to give a higher resolution covering only the lysyl-tRNA area. Figure 4A shows the resolution of lysyl-tRNA species from log-phase cells and spores of 168 *trp2*$^-$ by using a 1-liter linear gradient formed from 0.34 and 0.45 M NaCl each in buffer X. Obviously, the resolution is improved over that shown in Fig. 1. An even shallower gradient with a 1 liter linear gradient formed from 0.34 and 0.39 M NaCl each in buffer X is shown in Fig. 4B. Since no other major peaks are resolved, we thought this resolution was sufficient and the column could be loaded, eluted with the gradient, washed with 1.2 M NaCl in buffer X, and reequilibrated with starting buffer in less than 24 hr. Figure 5A shows the resolution of lysyl-tRNA species from late stationary-phase cells of *spo*A and log-phase cells at high resolution. Figure 5B shows the resolution of lysyl-tRNA species from late stationary-phase cells of *spo*A 12 and spores of 168 *trp2*$^-$. The late stationary-phase *spo*A 12 material was resolved into several peaks.

## DISCUSSION

Clearly, both sporulating *B. subtilis* 168 *trp2*$^-$ and the spore-defective mutant *spo*A 12 show altered chromatographic profiles relative to log-phase cells when the cells are allowed to sporulate or to grow to a very late stationary phase, respectively. None of these alterations seems to be the same as that reported by Lazzarini (4) and Lazzarini and Santangelo (6), which they concluded was not obligate to sporulation. As we have shown, they would not have detected our late eluting species from RPC-2 by the MAK technique. Our early eluting species did chromatograph on MAK in the same elution position relative to log-phase species as theirs and thus looked similar; however, we have never found this as a major species in spores. We have only found these early eluting species as major components in early blocked mutants grown to late stationary phase.

Although more experiments are needed to prove the biological significance of the alterations we have established in this report, we suggest that two processes are involved. The late eluting species increased only in spores and may be related to the process of sporulation. The early eluting species increased only in the early blocked asporogenous mutant grown to late stationary phase and may be related to the process of aging. For example, a shift to an earlier elution position is a characteristic of at least some undermodified tRNA species (1, 8), and these early eluting species may represent a variety of undermodified lysyl-tRNA species which were produced in old asporogenous cells because they lacked the appropriate biochemical functions. We are in the process of testing those hypotheses.

The high resolution data from RPC-2 show that no new lysyl-tRNA species are involved but rather increase in different lysyl-tRNA species relative to those which predominate in log-phase cells. These results demonstrate the changing relative amounts of isoaccepting tRNA species which can occur when the bacterial cell is exposed to different metabolic challenges.

### ACKNOWLEDGMENTS

I thank G. David Novelli for his helpful suggestions concerning the use of reversed-phase columns, John Spizizen for the *B. subtilis* strains, and Gail M. Clinton for excellent technical assistance.

This investigation was supported by Public Health Service fellowship 5-F2-GM-23,736-02 from the National Institute of General Medical Sciences and by grant HD 02807 from the National Institute of Child Health and Human Development.

## ADDENDUM IN PROOF

Subsequent to the preparation of this manuscript, we have found that the spore lysyl-tRNA species will elute in an early position relative to log-phase species if the RPC-2 column is run in the presence of urea. This observation raises the possibility that the major lysyl-tRNA species from spores and the asporogenous mutant are the same. The late-eluting position of the spore material without urea may be due to an artifact of the extraction procedure which is corrected by urea. It is also possible that the co-chromatography with urea is fortuitous.

### LITERATURE CITED

1. Gefter, M. L., and R. L. Russel. 1969. Role of modifications in tyrosine transfer RNA: a modified base affecting ribosome binding. J. Mol. Biol. 39:145–157.
2. Hoch, J., and J. Spizizen. 1969. Genetic control of some early events in sporulation of *Bacillus subtilis* 168, p. 112–120. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores IV. American Society for Microbiology, Ann Arbor, Michigan.
3. Kaneko, I., and R. H. Doi. 1966. Alterations of valyl-sRNA during sporulation of *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. 55:546–571.
4. Lazzarini, R. A. 1966. Differences in lysine-sRNA from spore and vegetative cells of *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. 56:185–190.
5. Lazzarini, R. A., and A. Peterkofsky. 1965. The characterization of a new species of leucyl-sRNA formed during methionine deprivation of *Escherichia coli* with relaxed control. Proc. Nat. Acad. Sci. U.S.A. 53:549–556.
6. Lazzarini, R. A., and E. Santangelo. 1967. Medium-dependent alterations of lysine-transfer ribonucleic acid in sporulation *Bacillus subtilis* cells. J. Bacteriol. 94:125–130.
7. Mandell, J. D., and A. D. Hershey. 1960. A fractionating column for analysis of nucleic acids. Anal. Biochem. 1:66–77.
8. Shugart, L., G. D. Novelli, and M. P. Stulberg. 1968. Isolation and properties of undermethylated phenylalanine transfer ribonucleic acids from a relaxed mutant of *Escherichia coli*. Biochim. Biophys. Acta 157:83–90.
9. Weiss, J. F., and A. D. Kelmers. 1967. A new chromatographic system for increased resolution of transfer ribonucleic acids. Biochemistry 6:2507–2513.