Medium-dependent Alteration of Lysine Transfer Ribonucleic Acid in Sporulating *Bacillus subtilis* Cells

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The presence of the altered lysine transfer ribonucleic acid (tRNA) in *Bacillus subtilis* spores is strongly dependent on the medium on which the cells were sporulated. Cells sporulated on synthetic media or dilute complex media contain little or none of the new component, whereas those sporulated on concentrated complex media accumulate the altered tRNA. The accumulation begins during the fifth or sixth stage of sporulation, the formation of the tunic, and the appearance of refractility, respectively. Mutants blocked early in sporulation differ in their ability to accumulate the altered tRNA when cultured on the same complex medium. Of the four mutants examined, one failed to accumulate any of the RNA, whereas a second contained the full complement characteristic of spores. The third and fourth mutant contained small amounts of the material. It is tentatively concluded that the accumulation of the altered lysine tRNA is not obligate to sporulation but is an epiphenomenon of the process.

In a previous publication (4), data were presented which showed that the transfer ribonucleic acid (tRNA) obtained from *Bacillus subtilis* spores contained a species of lysine tRNA not found in appreciable amounts in vegetative cells. Recently, alterations of two other tRNA species from *B. subtilis* have been reported. Kaneko and Doi (3) have shown that the proportion of the two valyl-tRNA species discernible in methylated albumin-coated kieselguhr (MAK) elution profiles is altered in the early stages of sporulation but returns again to that characteristic of vegetative cells in late sporulation. A second type of alteration—one dependent on the culture medium—recently was described by the same authors (2). The relative amounts of the three chromatographically separable serine tRNA species of *B. subtilis* vegetative RNA vary depending on the medium used to culture the vegetative cells. Thus, it appears that two types of tRNA modifications exist in *B. subtilis*: one is demonstrable in sporulating cells, and is presumably related to the sporulation process; the other is demonstrable in vegetative cells, and is related to culture conditions.

In the present report, evidence is presented indicating that the modification of lysine tRNA shares properties with both of the above types of

modifications. It is medium-dependent, but manifests itself only in spores or in cells in the last stages of sporulation.

MATERIALS AND METHODS

Organism. B. Subtilis 60015 (*met⁻, ind⁻*) is a derivative of the transformable *B. subtilis* 168. The asporogenous mutants 60651, 60662, 60710, and 60789 were isolated and generously made available to us by Ernst Freese.

Media and culture conditions. Tryptone, yeast extract (TY) medium contained 10 g of tryptone (Difco), 5 g of yeast extract, 5 g of NaCl, 10^{-3} moles of $CaCl_2$, 10^{-5} moles of $MnCl_2$, and 10^{-6} moles of $FeCl_3$ per liter. The media designated ½ × TY and ¼ × TY contained, respectively, one-half and one-quarter the amounts of tryptone and yeast extract, but the full complement of inorganic salts.

Nutrient sporulation medium (NSM) contained 8 g of Nutrient Broth (Difco), 1 g of KCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 10^{-3} moles of $CaCl_2$, 10^{-5} moles of $MnCl_2$, and 10^{-6} moles of $FeCl_3$ per liter (6); 2 × NSM had the same composition, except that it contained 16 g of Nutrient Broth per liter.

Brain-heart infusion (BHI) medium contained 37 g of Difco Brain Heart Infusion per liter.

Synthetic sporulation medium 10 (SSM 10) contained 5.6 g of K_2HPO_4, 2.4 g of KH_2PO_4, 1 g of sodium citrate, 3.6 g of tris(hydroxymethyl)aminomethane (free base), 4.4 g of glutamic acid, 1.5 g of

glucose, 10^{-3} moles of $CaCl_2$, 10^{-3} moles of $MgSO_4$, 5×10^{-5} moles of $ZnCl_2$, 5×10^{-5} moles of $MnCl_2$, and 10^{-5} moles of $FeCl_3$ per liter (Freese, *personal communication*).

Minimal glucose (MG) medium contained 14 g of K_2HPO_4, 6 g of KH_2PO_4, 1 g of sodium citrate·$2H_2O$, 2 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4·7H_2O$, and 5 g of glucose. The synthetic media, SSM 10 and MG, were supplemented with 25 mg of tryptophan and 25 mg of methionine per liter.

The various solid media used to obtain spores had the same composition as those above, but included 20 g of agar per liter.

Spores were prepared and purified as previously described (4). Vegetative and sporulating cells were grown in 200 to 250 ml of liquid medium in a 2-liter Erlenmeyer flask with rotary or reciprocal shaking at 37 C.

Preparation of tRNA. Extracts of spores or sporulating cells were prepared by sonic treatment (4). For large preparations of spore extracts, mechanical disruption with a Vibrogen cell mill was employed. Vegetative extracts were prepared with a French pressure cell. All extracts were clarified by centrifugation at 15,000 rev/min for 10 min. Transfer RNA was prepared from the extracts as before (4), except that the extracts were treated twice with water-saturated phenol and twice with chloroform-isoamyl alcohol (24:1) prior to chromatography on columns of Sephadex G-100. Where noted in the legends or text, RNA preparations not fractionated with Sephadex G-100 were employed.

Preparation of ^{14}C- and ^{3}H-labeled lysyl RNA. The conditions for esterification and purification of lysyl-tRNA were the same as previously described (4), except that the reaction mixture contained 80 μmoles of imidazole·HCl buffer (pH 8.0), 20 μmoles of $MgCl_2$, 4 μmoles of adenosine triphosphate (ATP), 4 μmoles of mercaptoethanol, 0.02 μmoles of ^{14}C-lysine (specific activity, 200 to 250 μc/μmole), 0.025 μmoles each of the 19 other amino acids in nonradioactive form, ca. 0.2 mg of tRNA, and enzyme.

MAK column chromatography. Chromatographic separation of the tRNA species was carried out as before (4). Samples (1 ml) of the fractions were either counted directly with 0.5 ml of 1.5 M NH_4OH and 10 ml of Bray's liquid scintillator (1) or the RNA was precipitated from the samples with trichloroacetic acid counted in the NH_4OH-Bray's scintillator solution.

RESULTS

As noted earlier (4), the MAK elution profiles of lysyl-tRNA from vegetative cells and spores cultured on TY medium are markedly different (Fig. 1). A similar comparison of lysyl-tRNA patterns from vegetative cells and spores obtained on another complex medium, NSM, did not reveal any significant difference (Fig. 2). (The elution positions of the sporal and vegetative lysine tRNA patterns shown in Fig. 2 differ from that of the vegetative pattern shown in Fig. 1. Although frequently less extreme than that
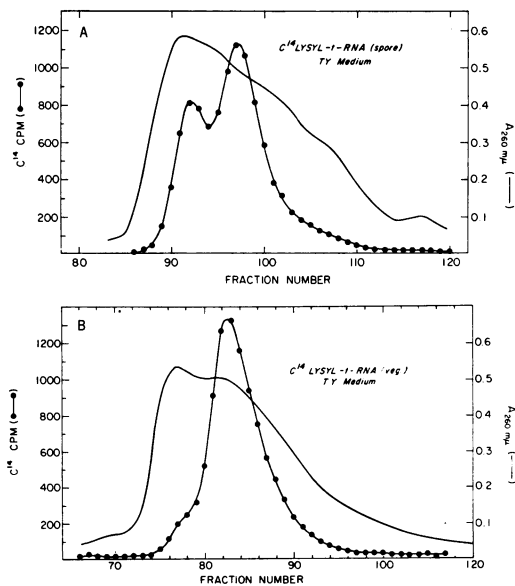

FIG. 1. *Elution profile of ^{14}C-lysyl-tRNA from vegetative cells and spores cultured on TY medium. (A) 3.0 A_{260} of ^{14}C-lysyl spore RNA (25,000 counts/min) and 17 A_{260} of carrier tRNA were applied to MAK column. (B) 2.3 A_{260} of ^{14}C-lysyl vegetative tRNA (21,886 counts/min) and 24 A_{260} of carrier tRNA were applied to MAK columns. The RNA was eluted and counted as described in Materials and Methods.*

shown in Fig. 2, the displacement to the right is often observed with lysine tRNA from NSM-cultured cells. However, the similarity of the vegetative lysine tRNA from the two sources has been confirmed by cochromatography of ^{3}H-lysine tRNA from TY-grown cells and ^{14}C-lysine tRNA from NSM-grown cells.) The failure of lysyl-tRNA from NSM cultured spores to exhibit the new component which is prominently exhibited in the elution profile of tRNA from TY-grown spores can not be easily correlated with differences in the growth rate on these media. In liquid media, the doubling time of *B. subtilis* is 25 to 30 min on both TY and NSM media. However, the levels of terminal growth attainable on the two media differ substantially, and reflect the different concentration of complex nutrients in these media. The possibility that the concentration of nutrients and not the qualitative differences in the media influences the accumulation of the altered lysine RNA during sporulation was examined. *B. subtilis* cultured in TY medium in which the concentration of tryptone and yeast extract were proportionally reduced attained the same growth rate, but entered the sporulation phase at a lower cell density than when cultured in full strength TY (Table 1).
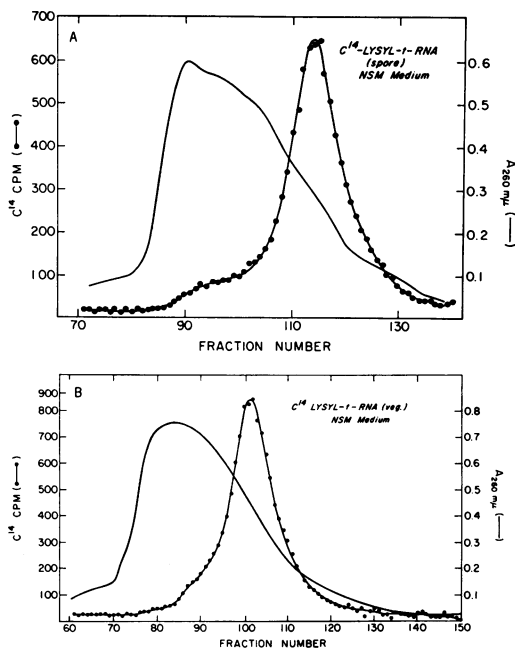
FIG. 2. *Elution profile of ¹⁴C-lysyl-tRNA from vegetative cells and spores cultured on NSM medium. (A) 0.84 A₂₆₀ of ¹⁴C-lysyl spore tRNA (15,330 counts/ min) and 30 A₂₆₀ of carrier RNA applied to MAK column. (B) 0.9 A₂₆₀ of ¹⁴C-lysyl vegetative RNA (20,870 counts/min) and 30 A₂₆₀ of carrier RNA applied to MAK column. RNA was eluted and counted as described in Materials and Methods.*

TABLE 1. *Medium dependence of first lysine t-RNA component*

Medium	Doubling time (min)	Terminal density (cells/ml)	Sporu-lation	Presence of first component
TY........	25–30	12–15 × 10⁸	+	+[a]
½ × TY...	25–30	8–10 × 10⁸	+	+[a]
¼ × TY...	25–30	6 × 10⁸	+	Trace[b]
2 × NSM..	25–30	13 × 10⁸	+	+[a]
NSM......	25–30	5 × 10⁸	+	Trace[b]
SSM10.....	70	11 × 10⁸	+	−[b]
BHI.......	18–20	11 × 10⁸	−	−[b]

[a] tRNA fractionated on Sephadex G-100 employed in profile analysis.
[b] Unfractionated RNA employed in profile analysis.

Similarly, increasing the concentration of nutrient broth in NSM media did not alter the growth rate but only increased the level of terminal growth. Thus, without qualitatively changing the growth media, *B. subtilis* can be sporulated in cultures at different cell densities. RNA was

obtained from spores cultured on diluted TY medium and enriched NSM medium, and the elution patterns of the lysine tRNA were scored for the presence of the first lysine tRNA component. The results shown in Table 1 and Fig. 2 and 3 indicate that the concentration of nutrients does affect the accumulation of this component. *B. subtilis* sporulated in the most dilute media of either type contains only traces of the altered species. Conversely, sporulation in 2 × NSM, ½ × TY, and TY, media which allow a terminal growth of 8 × 10⁸ to 15 × 10⁸ cells/ml, is accompanied by the accumulation of the new lysine tRNA component. However, high terminal growth level alone is not a sufficient condition for the accumulation of the new component. The RNA obtained from spores cultured on a synthetic medium allowing high terminal growth level, SSM 10, does not contain this component (Fig. 4).

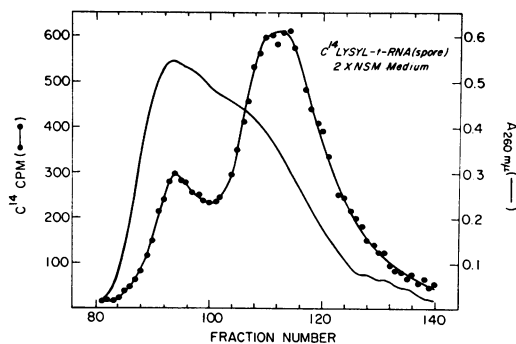The observation that the altered lysine tRNA is accumulated late in sporulation (4 and see

FIG. 3. *Elution profile of ¹⁴C-lysyl tRNA obtained from Bacillus subtilis sporulated on 2 × NSM medium: 2.8 A₂₆₀ of ¹⁴C lysyl tRNA (17,140 counts/min) and 16 A₂₆₀ of carrier applied to MAK column and eluted by the standard method.*
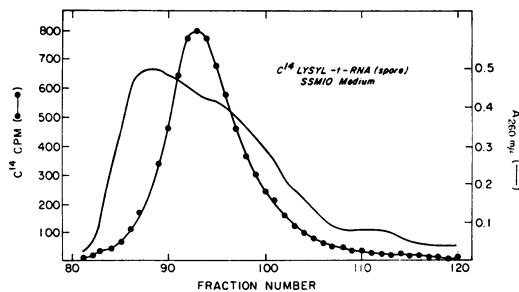

FIG. 4. *Elution profile of ¹⁴C lysyl tRNA obtained from Bacillus subtilis sporulated on SSM10 medium: 6.7 A₂₆₀ of ¹⁴C lysine tRNA (15,640 counts/min) and 15 A₂₆₀ of carrier tRNA chromatographed on MAK column. Unfractionated SSM10 spore RNA was employed.*

below) when the medium has been substantially exhausted raises the question of whether the first component of lysine tRNA is accumulated in response to the depletion of the rich medium and the attendant physiological changes. The results of the stepdown culture experiments indicate that the events leading to the accumulation of the new lysine tRNA component cannot be duplicated by reculturing, even though the transition from rich to poor medium is more severe than in normal terminal growth. Mid-log phase cells grown on TY medium were collected by centrifugation at 15 C and were immediately resuspended in three types of prewarmed synthetic media: minimal medium lacking glucose, in which cell growth does not take place; minimal medium plus glucose, in which growth of resuspended cells commences after a 2.5-hr lag; and SSM 10 medium, in which growth resumes immediately at the slower rate characteristic of this medium. Samples of cells were cultured in minimal glucose medium for 0, 1, 2, and 3 hr before harvesting. Cells suspended in minimal medium lacking glucose and in SSM 10 medium were cultured for 120 and 75 min, respectively,

before harvesting. RNA was prepared from the cells, charged with ^{14}C-lysine, and chromatographed on MAK columns. In none of the elution profiles so obtained were there detectable amounts of the new lysine tRNA component.

The time course of development of the new lysine tRNA in liquid TY medium is depicted in Fig. 5. The spore content of the culture was estimated by determining the number of heat-resistant colony-forming units. Samples of the culture were diluted 100-fold with water and heated for 10 min at 80 C in capped tubes. Dilutions of the heat-treated material were spread on solid complex medium. After incubating for 16 hr at 37 C, the plates were scored for total number of colonies. Portions of the sporulating culture were withdrawn at the times indicated by the arrows, and RNA was prepared from the cells. The results of the chromatographic analyses of the RNA samples obtained from these cultures (Fig. 5B and C) indicate that the accumulation of the first component of lysine tRNA commences at about 90 min before refractility is observed and heat-resistant spores appear in the medium. RNA obtained from cells harvested
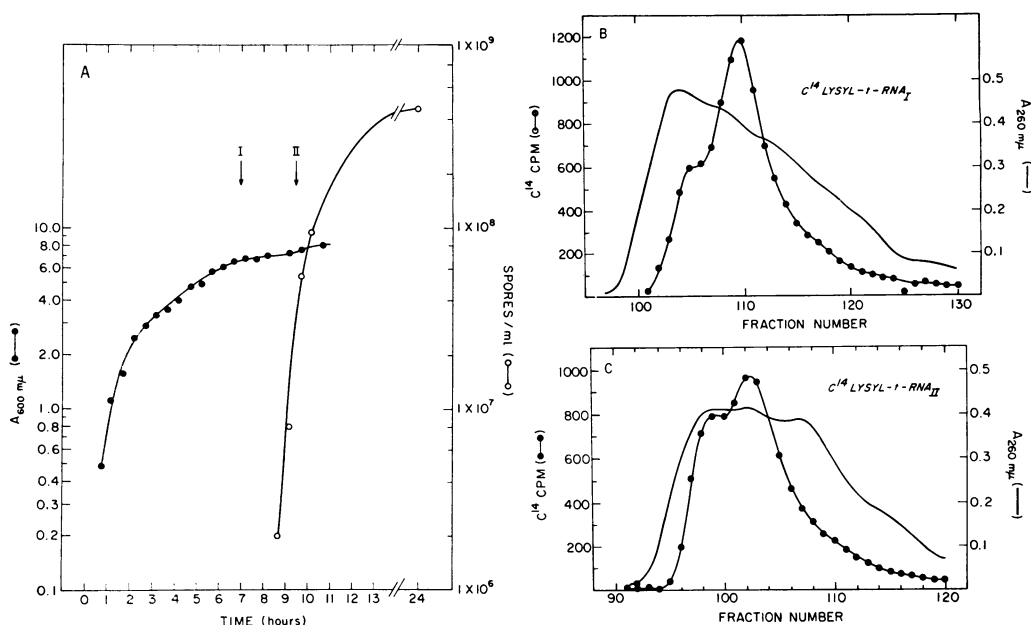

FIG. 5. Time course of sporulation on TY medium and the accumulation of the first lysine tRNA component. (A) Bacillus subtilis cells were cultured on TY medium at 37 C with reciprocal shaking. Vegetative growth was followed turbidimetrically at 600 mμ, and the development of heat-resistant spores was estimated as described in the text. (B) RNA obtained from cells harvested at the time indicated by arrow I was maximally charged with ^{14}C-lysine and chromatographed on an MAK column; 7.5 A_{260} of ^{14}C lysyl tRNA$_I$ (13,620 counts/min) and 13 A_{260} of carrier applied to column. (C) RNA obtained from cells harvested at the time indicated by arrow II was charged with ^{14}C lysine and chromatographed on MAK column; 7.5 A_{260} of ^{14}C lysyl-tRNA$_I$ (25,370 counts/min) and 13.7 A_{260} of carrier tRNA applied to column. RNA samples I and II were not fractionated with Sephadex G-100 prior to esterification with ^{14}C lysine.

after sample II exhibit only a slight enrichment of the first component above that observed in sample II. Some variability in the time of appearance of the new component has been noted. In seemingly identical experiments, the alteration of the lysine tRNA profile was first observed at the time refractility appeared. However, notwithstanding this variability, the new component is accumulated in a semisynchronous manner.

Comparison of the above results with the sequence of morphological stages described for sporulation (6) would place the alteration at stage 5 or 6, the formation of the tunic and the appearance of refractility, respectively. If the first component of lysine tRNA is related to sporulation, then sporulation mutants (*sp⁻*) blocked at stage 4 or earlier would not be expected to accumulate this component, even after prolonged culturing in the stationary phase. Four sporulation mutants which are presumably blocked at an early stage since they do not elaborate antibiotic materials (5) were cultured

FIG. 6. *Elution profiles of lysyl-tRNA obtained from asporogenous mutants. (A) 4.7 A_{260} of ^{14}C-lysyl-tRNA (15,100 counts/min) from mutant 60651 and 17.5 A_{260} of carrier RNA were chromatographed. (B) 7.7 A_{260} of ^{14}C-lysyl-tRNA (16,740 counts/min) from mutant 60710 and 17.5 A_{260} of carrier were chromatographed. The RNA preparations were not fractionated with Sephadex G-100 prior to esterification.*

on TY medium. The cells were harvested at a time corresponding to 10 hr on the growth curve shown in Fig. 5. Culturing the mutants beyond this point was hampered by cell lysis. RNA was prepared from the cells and esterified with ^{14}C-lysine. The results of MAK chromatography revealed that three of the mutants had little or none of the first component. Their elution profiles were either indistinguishable from the vegetative profile (mutant 60662) or were characterized by a slight bulging on the leading edge of the main peak (mutants 60651 and 60789; Fig. 6a). The fourth mutant (60710) exhibited a full complement of the new component (Fig. 6b).

DISCUSSION

The present series of experiments demonstrate that the presence of the new lysine tRNA species in spores is not a general characteristic of spore RNA, but depends on the medium on which sporulation took place. No clear correlation is discernible between the two physiological parameters, growth rate and terminal growth level, and the accumulation of the new tRNA species. The five complex media used in these experiments support the growth of *B. subtilis* at approximately the same rate. However, spores obtained on two of them, NSM and ¼ × TY contained only trace amounts of the new component, whereas spores obtained on the other three media contained substantial amounts. The possibility that in NSM and ¼ × TY media the alteration of the lysine tRNA elution profile is transient [as with the change in valine tRNA during sporulation (3)], thereby escaping detection, is remote. In time-course experiments similar to that shown in Fig. 5, but employing NSM medium, no alteration of the vegetative lysine tRNA pattern was observed.

The fact that *B. subtilis* sporulated on 2 × NSM or TY medium contained the new component, whereas cells sporulated on dilutions of these media contained only traces, indicates that the concentration of complex nutrients influences this accumulation. This concentration dependence is difficult to understand, since the accumulation of the new lysine tRNA commences very late in sporulation, when the medium is substantially exhausted. Trivial explanations for this dependence based upon environmental differences attributable to high cell density (e.g., reduced oxygen tension, waste product accumulation, etc.) seem unlikely. Neither spores cultured on SSM 10 nor late stationary-phase cells from BHI medium contain the altered tRNA, although both media allow a high terminal cell density.
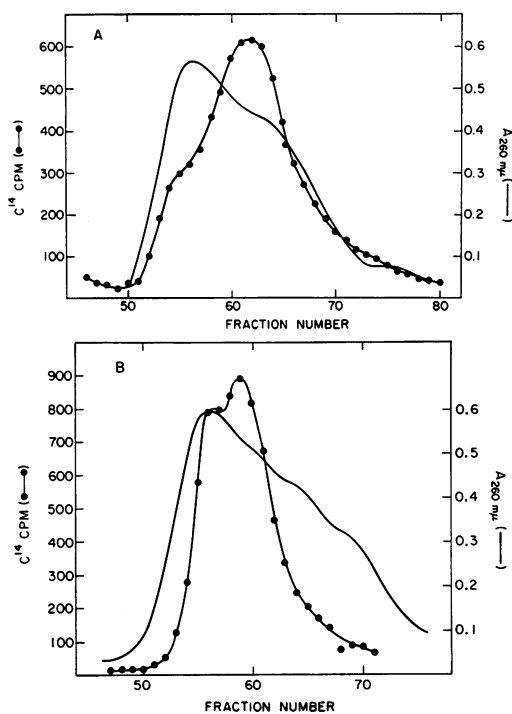
The biological function of the first component

is obscure at present. The observations that this material is localized in spores, accumulates in a semisynchronous manner during sporulation, and is not accumulated by cells cultured on a medium which does not support sporulation suggest that the new material is related to sporulation. However, this relationship is, at best, not obligate, since *B. subtilis* can sporulate on appropriate media in the absence of detectable amounts of this material. Furthermore, the diverse results obtained with the four sporulation mutants, all of which are blocked in the initial stages, indicate that the factors regulating the accumulation of the lysine tRNA may operate independently of the later stages of sporulation. In view of this variable association with sporulation, it is tentatively concluded that the new lysine tRNA is not obligate to sporulation and, like pigmentation, is an epiphenomenon of the process.

ACKNOWLEDGMENTS

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