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Study of Tyrosine Transfer Ribonucleic Acid Modification in Relation to Sporulation in *Bacillus subtilis*

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A reversal in the relative amounts of the two major species of tyrosine transfer ribonucleic acid ($tRNA^{Tyr}$) (I and II) has been previously observed by others during the development of *Bacillus subtilis*. These species have been purified by benzoylated diethylaminoethyl-cellulose chromatography and were shown to differ by the modification of an adenosine residue (species I contains i^6A and species II ms^2i^6A). As suggested by competitive hybridization assays, they might possess the same nucleotide sequence. A $tRNA^{Tyr}$ species lacking isopentenyl and methylthio moieties was not detected. The structural difference between species I and II was shown to be important for ribosome binding but not for charging. The extent of alteration during growth was studied in parallel with physiological events. Like sporulation, $tRNA^{Tyr}$ change is iron dependent. Moreover, when sporulation is prevented by an excess of glucose, the $tRNA^{Tyr}$ change is delayed as is the synthesis of enzymatic systems required for the onset of sporulation. $tRNA^{Tyr}$ change also demands unceasing protein synthesis.

The variability in transfer ribonucleic acid (tRNA) populations under various physiological conditions has led many to postulate that tRNA may play a regulating role in cell differentiation (30). Alteration of tRNA patterns during the development of *Bacillus subtilis* has received particular attention since sporogenesis may be considered as a relatively simple model of cellular differentiation. By analyzing chromatographic elution profiles of aminoacyl-tRNA's from exponentially growing cells and spores, Vold (32) found changes in 10 isoaccepting groups. For three groups (tyrosyl-, leucyl-, and tryptophanyl-tRNA's), a transition in the relative amounts of isoaccepting species was shown to occur at the onset of stationary phase (33) and might, therefore, be related to the commitment to sporulation.

B. subtilis tyrosine tRNA ($tRNA^{Tyr}$) can be separated by chromatography into two major species, I and II, and some minor species (17, 32). The modification of *B. subtilis* $tRNA^{Tyr}$, first reported by Arceneaux and Sueoka (1) for cells grown in a complex medium, consists of a reversal in the relative amounts of the two major species, $tRNA^{Tyr}$ I being predominant in vegetative cells and $tRNA^{Tyr}$ II in stationary-phase cells and spores. McMillian and Arceneaux (17) extended this study by comparing $tRNA^{Tyr}$ patterns of a wild-type strain grown in various media and of asporogenous mutants. According to this analysis, the extent of $tRNA^{Tyr}$ modification does not seem to be directly correlated with the ability of cells to sporulate.

However, chromatographic studies cannot differentiate between the possible causes for the variations in isoacceptor levels and, therefore, do not allow any conclusions as to their biological significance. To compare the structure and properties of the two major species of *B. subtilis* $tRNA^{Tyr}$, we have purified them, as described here. As a first step, nucleoside composition was determined. From a previous study (12), $tRNA^{Tyr}$ I and $tRNA^{Tyr}$ II were found to differ in the extent of modification of an adenosine, species I having the partially modified 6-(Δ^2-isopentenyl) adenosine (i^6A) and species II having the fully modified 2-methylthio-6-(Δ^2-isopentenyl) adenosine (ms^2i^6A). Therefore, the modification of $tRNA^{Tyr}$ during the development of *B. subtilis* cells might correspond only to the addition or to the release of a methylthio group from a unique $tRNA^{Tyr}$ species.

This study continues our observations in an attempt to answer the following questions: are $tRNA^{Tyr}$ I and II transcribed on the same deoxyribonucleic acid (DNA) site? Does the structural difference between species I and II lead to differences in their biological properties? Does $tRNA^{Tyr}$ modification require protein synthesis and could it be related to sporulation-linked events?

(This work was taken in part from a thesis to be submitted by B.M. to the Faculty of Sciences, University of Paris XI, Orsay, France.)

MATERIALS AND METHODS

Strains. *B. subtilis* W168, *B. subtilis* W23 D obtained from R. H. Doi, and W23 S obtained from P. Schaeffer were stored as spore suspensions.

Growth conditions. Cells were cultivated at 37 C with vigorous agitation on different media: nutrient broth medium (28); Penassay broth (Difco); SCM medium (11) (synthetic medium supplemented with Casamino Acids [Difco]); MS medium (29) (synthetic medium with 0.2% NH_4Cl as a nitrogen source and 0.5% malate as a carbon source).

MSS medium (8), synthetic sporulation medium supplemented with or without iron, contains (in grams per liter): sodium citrate, 2.0; sodium acetate, 2.0; glutamic acid, 2.0; NH_4Cl, 0.5; glycerol, 0.5. Iron was extracted from a 5×-concentrated stock solution with chloroform-8-hydroxyquinoline by the method of Waring and Werkman (35). Before use, $MnCl_2$ and $(NO_3)_2Ca$, filter sterilized, were added from stock solutions to give final concentrations of 10 μM and 0.5 mM, respectively. This medium was supplemented with or without 1 ml of 1 mM SO_4Fe per liter, filter sterilized.

Experimental cultures were inoculated from growing precultures in the same medium, themselves already having been inoculated with cells from overnight cultures at 30 C in agar-nutrient broth medium.

Sporulation frequency determination. The sporulation frequency represents the ratio of viable cells at t2 to spores number at t24 (to designate the end of exponential growth and t_1, t_2, etc., the time in hours after the exponential growth had stopped). The amount of viable cells was determined after plating on nutrient agar medium. A correction factor was introduced to take into account the proportion of chains of two or more bacteria determined in a given preparation after septa staining. Spore counts were carried out by plating on nutrient agar medium; before plating, samples were incubated for 10 min at 37 C in the presence of lysozyme (100 μg/ml) and then heated at 80 C for 10 min in sealed ampoules.

Preparation of bulk tRNA and aminoacyl-tRNA ligase. Bulk tRNA was prepared according to the previously described method (6).

Crude aminoacyl-tRNA ligase was prepared according to Yamane and Sueoka (37) from cells (strain W168) harvested at the end of the exponential growth phase on nutrient broth medium.

Aminoacylation was carried out for 30 min at 37 C. The reaction mixture contained, per milliliter: tris(hydroxymethyl)aminomethane - hydrochloride (pH 7.2), 100 μmol; $MgCl_2$, 15 μmol; adenosine 5'-triphosphate, 5 μmol; radioactive amino acid, 0.05 μmol; 0.05 μmol of each of the other unlabeled amino acids; crude aminoacyl-tRNA ligase corresponding to 180 μg of protein; 30 to 50 absorbancy units at 260 nm (A_{260}) of tRNA. After 30 min of incubation, potassium acetate (pH 5) was added to a final concentration of 2%, and aminoacyl-tRNA's were isolated by phenol extraction, precipitated by ethanol, and dissolved in 1 mM potassium acetate (pH 5). The extent of aminoacylation was determined by the acid-precipitable radioactivity.

In large-scale aminoacylation reactions, only unlabeled amino acid was used; however, a parallel microreaction was carried out with radioactive amino acid at the same concentration to provide a tracer for the aminoacyl-tRNA during subsequent steps.

Chromatography of tRNA. Methylated albumin Kieselguhr (MAK) columns were prepared with 15 g of Hyflo Supercel and 6 ml of 1% methylated serum albumin. Chromatography was carried out at room temperature. tRNA was eluted with a linear gradient of 170 ml each of 0.3 and 0.7 M NaCl in 0.05 M phosphate buffer, pH 6.5.

The RPC5 system, as described by Pearson et al. (23), was used. Columns (50 by 1 cm) were equilibrated with a solution containing 10 mM potassium acetate, pH 4.5, 0.4 M NaCl, 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol. tRNA was eluted with a 400-ml linear salt gradient (0.4 to 1.0 M NaCl) containing the other constituents of the equilibration solution.

Chromatography on benzoylated diethylaminoethyl (BD)-cellulose (column 40 by 2.5 cm) was performed as described by Maxwell et al. (20). Selected fractions were concentrated by passage through a diethylaminoethyl-cellulose column as described by Nishimura et al. (22) or, in the case of small volumes, put into a dialysis bag and concentrated against solid polyethylene glycol.

tRNA-DNA hybridization. DNA was extracted from *B. subtilis* W168 harvested in late exponential phase by the method of Marmur (19). Native DNA preparations were denatured by alkali according to the conditions described by Pigott and Midgley (24).

Hybridization was carried out for 3 h at 33 C in liquid medium: 50% formamide, 2× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate), pH 5.3. DNA was added to a concentration of 75 μg/ml. Duplicates, but without DNA, were prepared to substract the background counts for each point. After hybridization, the incubation mixture was diluted 100 times with 6× SSC and filtered on nitrocellulose membranes previously washed with 6× SSC. It was necessary to lower the formamide concentration to retain DNA on the filters. After filtration, the filters were washed with 50 ml of 6× SSC solution and then incubated for 30 min at room temperature in 3× SSC containing ribonuclease T1 (2.5 μg/ml), washed again wtih 3× SSC, and dried. Finally, the radioactivity retained on the filters was determined.

Permanganate treatment of tRNA. Permanganate treatment was carried out according to the method of Kline et al. (14).

Binding of tyrosyl-tRNA to ribosomes. Ribosomal preparations and binding experiments were performed as described by Roy and Söll (26). The incubation mixture (0.05 ml) contained 0.1 M tris(hydroxymethyl)aminomethane acetate, pH 7.2, 0.05 M KCl, 0.02 M magnesium acetate, 2.5 A_{260}

units of ribosomes, 15 nmol of polyuridylate, -adenylate, -cytidylate [poly(U, A, C)] (in base residues), and about 10 pmol of tyrosyl-tRNA. Incubation was carried out at 25 C for 20 min.

Materials. Radioactive L-amino acids were purchased from CEA, Saclay, France. Specific activities (curies/millimole) were as follows: [^{3}H]tyrosine, 40; [^{14}C]tyrosine, 0.18. Plaskon impregnated with Adogen 464, used for the RPC5 system, and random copolymer poly(U, A, C) were purchased from Miles Laboratories.

RESULTS

Chromatographic profiles of tyrosyl-tRNA from exponential- and stationary-phase cells. The reversal in the two major species of tRNA^{Tyr} during the growth of *B. subtilis* was first described by Arceneaux and Sueoka (1) by comparing, on a MAK column, tyrosyl-tRNA from exponential- and stationary-phase cells grown on rich medium (Penassay). By the use of an RPC5 column, McMillian and Arceneaux (17) later confirmed this result.

Figure 1 shows that the same modification occurred when cells were cultivated in nutrient broth medium. The ratio of tyrosyl-tRNA I/tyrosyl-tRNA II, which was about 3.3 in exponential phase, shifted to 0.3 at t2. As mentioned by Vold (32), the peak corresponding to species II presents an important shoulder, designated as peak III, when chromatography was carried out on an RPC5 column (Fig. 1A). The same profiles were obtained by charging tRNA after chromatography.

Chromatography on the MAK column (Fig. 1B) yielded a poor resolution and a recovery of tyrosyl-tRNA of only 20 to 30%. However, the relative amount of each species was identical to that on an RPC5 column.

Moreover, when chromatographed on a MAK column, some preparations of tRNA from cells grown in nutrient broth medium showed an additional small peak designated as peak F, which was eluted ahead of tyrosyl-tRNA I, in the front part of the optical density profile. This peak was of variable size, but it did not account for more than 10 to 15% of the total tyrosyl-tRNA eluted. Form F is not growth phase dependent, since it was observed in both exponential- and stationary-phase preparations. It was also detected by aminoacylation with radioactive tyrosine after chromatography and therefore corresponds to an active form.

Purification of tRNA^{Tyr} I and tRNA^{Tyr} II. *B. subtilis* tRNA^{Tyr} I and II were purified using modifications of the method described by Maxwell et al. (20) for yeast tryptophan and tyrosine tRNA. This method is based on the fact that these uncharged tRNA's may be eluted from BD-cellulose columns by salt solution, whereas the elution of tRNA's charged with their corresponding aromatic amino acids required the addition of ethanol to the salt solution. The purification steps for *B. subtilis* tRNA^{Tyr} species are given in Table 1.

tRNA^{Tyr} I. *B. subtilis* tRNA^{Tyr} I was sufficiently purified for our further studies in two simple chromatographic steps. tRNA extracted from exponential-phase cells was first chromatographed on BD-cellulose at room temperature. Fractions rich in tRNA^{Tyr}, which were eluted between 0.65 and 0.9 M NaCl, were selected. tRNA from these fractions was then charged with [^{14}C]tyrosine and rechromatographed on BD-cellulose at 4 C (Fig. 2). The main part of the optical density was eluted with a salt gradient of NaCl from 0.4 to 1.2 M, followed by a solution of 1.2 M NaCl. Tyrosyl-tRNA was then

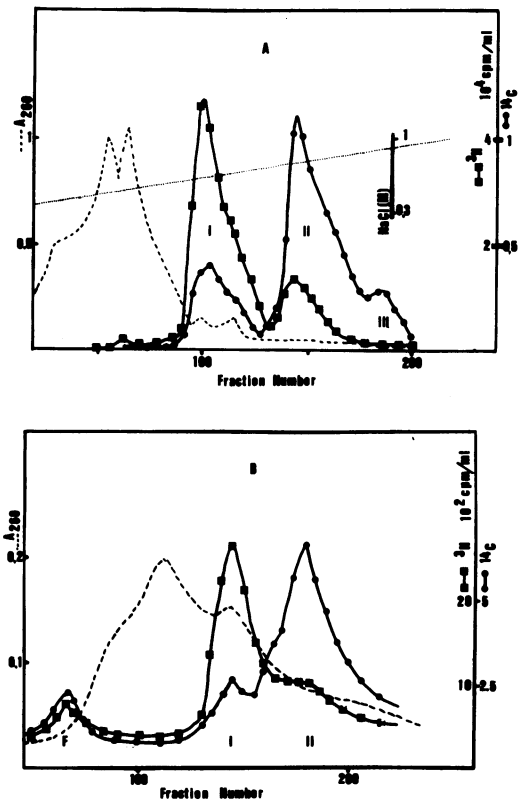

FIG. 1. *Cochromatography of B. subtilis tRNA from vegetative and early stationary-phase cells (strain W168) grown in nutrient broth. Symbols: (■) [^{3}H]tyrosyl-tRNA from vegetative cells; (●) [^{14}C]tyrosyl-tRNA from stationary cells. (A) RPC5 chromatography of 20 A_{260} units of exponential-phase tRNA and 57 A_{260} units of stationary-phase tRNA; (B) MAK chromatography of 6 A_{260} units of exponential-phase tRNA and 16 A_{260} units of stationary-phase tRNA. Volume of fractions, 1.5 ml.*

TABLE 1. *Purification of tRNA^{Tyr} I and tRNA^{Tyr} II*

Stage of purification	A_{260} units		Tyrosine acceptor activity		Amino acid[a] acceptance activity/A_{260} unit (pmol of amino acid)			
	Total	% Initial	Total (10^3 pmol of Tyr)	% Initial	Tyr	Phe	Leu	Ser
tRNA^{Tyr} I								
Starting material	16,000	100	890	100	55	60		
1st BD cellulose	5,170	32.3	665	74.7	128	70		
I_A	182	1.1	293	32.9	1,610	20	50	20
2nd BD cellulose								
I_B	114	0.7	190	21.3	1,670	100	10	0
tRNA^{Tyr} II								
Starting material	17,000	100	680	100	40	60		
1st BD cellulose	3,500	20.5	450	66.2	128	235		
2nd BD cellulose	150	0.9	150	22.0	1,000	250		
3rd BD cellulose								
Fraction a (final purified tRNA II)	38	0.2	69	10.2	1,828	10	0	0
Fraction b	60	0.3	62	9.1	1,030	450		
RPC 5 (chromatography of fraction b)								
tRNA^{Tyr} II	20	0.1	30	4.4	1,500	105		

[a] Cysteine and tryptophan acceptance activities, also tested, were not detected in purified preparations.

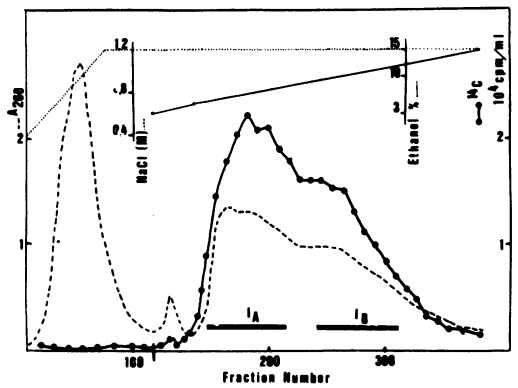

FIG. 2. *Purification of tRNA^{Tyr} I, second BD-cellulose column. tRNA from selected fractions eluted from the first BD-cellulose column was aminoacylated with [^{14}C]tyrosine and unlabeled tyrosine and rechromatographed on BD-cellulose at 4 C. Steps of elution: salt gradient from 0.5 to 1.2 M NaCl (total volume, 1 liter); solution of 1.2 M NaCl (0.5 liter); ethanol gradient from 0 to 15% in 1.2 M NaCl (total volume, 1 liter).*

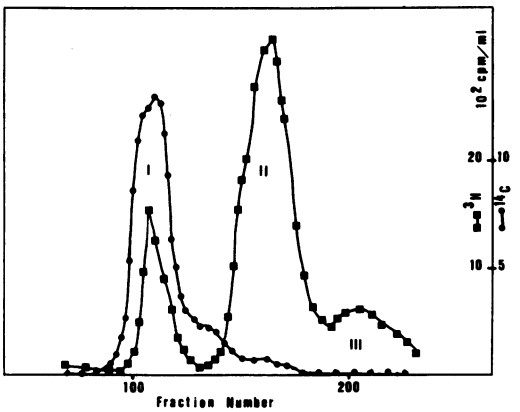

FIG. 3. *Cochromatography on an RPC5 column of purified tRNA^{Tyr} IA and IB and bulk tRNA. Samples of tRNA^{Tyr} IA and tRNA^{Tyr} IB in equal amounts were aminoacylated with [^{14}C]tyrosine (●). Bulk stationary-phase tRNA was aminoacylated with [^{3}H]tyrosine (■).*

eluted with a gradient of ethanol, 0 to 15% in 1.2 M NaCl. It showed a biphasic profile and was therefore separated into two fractions designated I_A and I_B. When cochromatographed on an RPC5 column with crude tRNA charged with [^{3}H]tyrosine, fractions I_A and I_B were eluted together in a single peak superimposed with [^{3}H]tyrosyl-tRNA^{Tyr} I and showed no contamination with tRNA^{Tyr} II (Fig. 3). The tRNA^{Tyr} I preparations showed about 40 times the acceptor activity per A_{260} unit for tyrosine than did untreated tRNA. They were tested for contamination by tRNA reading codons beginning with U, which tend to bind tightly with BD-cellulose (34). The acceptor activities for contaminant tRNA's, expressed as a percentage of that for tyrosine, were, respectively, 5.6 and 6.6 for tRNA^{Tyr} I_A and I_B. Purification was

achieved with a good yield, since about 54% of the initial tyrosine acceptor activity was recovered by adding the activity of I_A and I_B.

tRNA^{Tyr} II. The purification of *B. subtilis* tRNA^{Tyr} II was made more complicated by the fact that it has nearly the same affinity for BD-cellulose as tRNA^{Phe}. Moreover, the chromatographic behavior of these tRNA's varied during the course of purification.

The first two steps of purification were carried out as for tRNA^{Tyr} I. When tRNA extracted from early stationary-phase cells was chromatographed on BD-cellulose, tRNA^{Tyr} was recovered with tRNA^{Phe} in fractions eluted with salt solution between 0.85 and 1 M NaCl. Chromatography at 4 C of tRNA from these fractions, aminoacylated with radioactive tyrosine, allowed the separation of tyrosyl-tRNA II from the contaminant tyrosyl-tRNA I (Fig. 4), but tRNA^{Phe} was also recovered in the ethanol fraction. On a third chromatographic run, tRNA^{Tyr} II, although stripped, remained bound to BD-cellulose at a salt concentration of 1.2 M NaCl. Its elution required the addition of ethanol. The peak of acceptor activity was divided into two parts (Fig. 5). The first part showed a high tyrosine acceptor activity and was nearly free of tRNA^{Phe} and other tRNA's having a high affinity for BD-cellulose (34). This fraction was used for the analysis of the hydrolysis products. The aminoacylation of tRNA from the second part with phenylalanine before an additional chromatography on BD-cellulose did not improve in a significant fashion the separation of tRNA^{Tyr}.

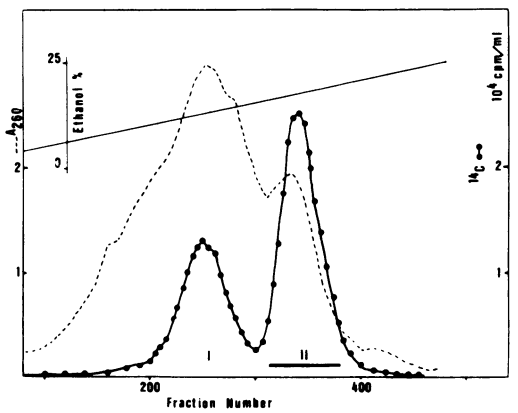

FIG. 4. *Purification of tRNA^{Tyr} II. tRNA from selected fractions eluted from the first BD-cellulose column was aminoacylated with [^{14}C]tyrosine and unlabeled tyrosine and rechromatographed on BD-cellulose at 4 C. Steps of elution: salt gradient from 0.4 to 1.2 M NaCl (total volume, 1 liter); solution of 1.2 M NaCl (1.5 liters); ethanol gradient from 0 to 25% in 1.2 M NaCl (total volume, 1 liter).*

However, further purification of tRNA^{Tyr} from this fraction charged with radioactive tyrosine was obtained with a poor yield by chromatography on the RPC5 column (Fig. 6); as in the case of nonpurified tRNA, the radioactive profile showed an additional peak.

Structural studies on tRNA^{Tyr} species. Purified tRNA^{Tyr} I (A and B) and tRNA^{Tyr} II species were previously subjected to complete nucleoside analysis (12). They showed identical composition, with the exception that species I (A and B) contained i^6A in place of ms^2i^6A in spe-

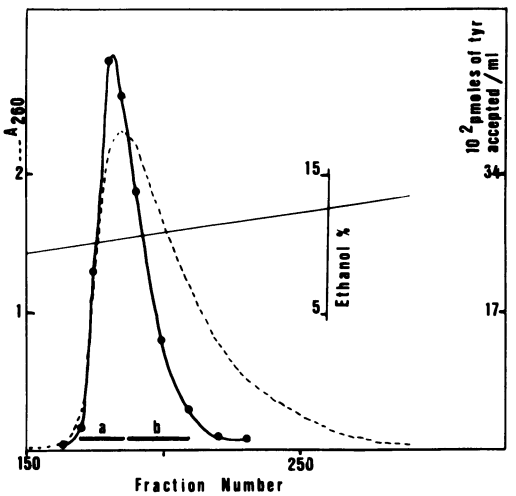

FIG. 5. *Purification of tRNA^{Tyr} II. tRNA^{Tyr} II eluted from the second BD-cellulose column (Fig. 4) was stripped and rechromatographed on BD-cellulose at room temperature. Steps of elution: salt gradient form 0.6 to 1.2 M NaCl (total volume, 1 liter); solution of 1.2 M NaCl (0.5 liter); ethanol gradient from 0 to 15% in 1.2 M NaCl (total volume, 1 liter). Fraction a corresponds to final purified tRNA^{Tyr} II.*

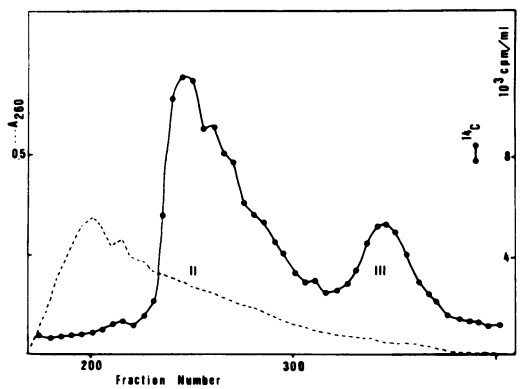

FIG. 6. *Chromatography on an RPC5 column of partially purified tRNA^{Tyr} II, eluted from the third BD-cellulose column. tRNA designated as fraction b (in Fig. 5) was aminoacylated with [^{14}C]tyrosine.*

cies II. As with other modified compounds, both species contain the base Q, one ribothymine, one methyladenosine, two pseudouridine, one s^4U, and probably one dihydrouridine. Peak III tRNA^{Tyr} could not be purified sufficiently to be analyzed.

(i) Filtration on Sephadex G100. Loehr and Keller (16) have observed that a part of tRNA^{Ala} can exist as a dimer separable from the monomer by chromatography on BD-cellulose and reverse-phase columns and by filtration on Sephadex G100. This does not seem to be the case for tRNA^{Tyr} I_B and tRNA^{Tyr} III: when filtered on Sephadex G100 with 0.1 A_{260} unit of [^{3}H]tyrosyl-tRNA^{Tyr} I_A as a marker, tRNA^{Tyr} I_B or tRNA^{Tyr} III (5 A_{260} units) gives one peak of optical density superimposable with the radioactive peak (not shown here). Nevertheless, it is possible that dimers or aggregates formed during chromatography then might be easily disrupted like the unstable, concentration-dependent aggregates characterized by Millar and Mackenzie (21).

(ii) Permanganate treatment of tRNA^{Tyr} species. A chromatographic study of permanganate-treated tRNA^{Tyr} species of *B. subtilis* was performed in an attempt to determine whether the peak F detected by chromatography on MAK could coreespond to an unmodified species.

A mild permanganate treatment was shown by Kline et al. (14) to remove the Δ2-iospentenyl group of tRNA, leaving an adenosine residue; it therefore might cause a shift in chromatographic position. The hypothesis proposed by Juarez et al. (10) of a precursor relationship between different species of tRNA^{Phe}, which were presumed to differ in the degree of modification of an adenosine, was in part supported by the interpretation of the chromatographic behavior of permanganate-treated tRNA.

Purified tRNA^{Tyr} I_A and II were aminoacylated with [^{3}H]tyrosine after permangante treatment. This treatment reduced the tyrosine acceptor activity 25% for tRNA^{Tyr} I and 40% for tRNA^{Tyr} II.

When cochromatographed on a MAK column with bulk tRNA charged with [^{14}C]tyrosine, treated species I was not eluted with form F but was eluted only two to three fractions sooner than nontreated species I (Fig. 7). The nature of form F, therefore, was not elucidated by this experiment.

Since the nature of the modification of species II was known, it was of interest to examine the chromatographic behavior of this species after permanganate treatment. To improve the resolution, the chromatographic study of treated and nontreated tRNA^{Tyr} species was performed with the RPC5 system (Fig. 8). Treated tRNA^{Tyr} II is mostly eluted from the RPC5 column like treated tRNA^{Tyr} I, but the peak is more heterogenous and possesses a shoulder located after that of the untreated species I. This chromatographic behavior suggests that the permanganate oxidation not only removes the isopentenyl chain, but also modifies or removes the methylthio group. The heterogeneity of the peaks corresponding to treated species could depend upon incomplete release or modification of the side chains or on side reactions.

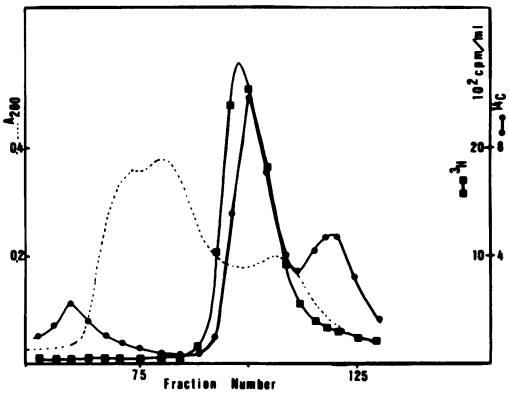

FIG. 7. *Cochromatography on MAK column of purified MnO_4K-treated tRNA^{Tyr} I and bulk tRNA. MnO_4K-treated tRNA^{Tyr} IA was aminoacylated with [^{3}H]tyrosine (■). Bulk tRNA from late exponential-phase cells grown on nutrient broth was aminoacylated with [^{14}C]tyrosine (●).*

The shift in the position of species II after treatment does not depend on the action of an excess of bisulfite, since we observed that the chromatographic behavior of the tRNA was not modified by a short treatment with bisulfite (0.01%) before the aminoacylation.

The fact that ms^2i^6A (species II) and i^6A (species I) were eluted at nearly the same position when treated with permanganate must, therefore, be taken into account to interpret the chromatographic changes caused by this treatment.

(iii) Hybridization competition experiments. The results of the total hydrolysis of tRNA^{Tyr} species suggest that they differ only in the extent of a modified adenosine residue. If they are the product of the same gene, they might hybridize with *B. subtilis* DNA in a competitive fashion.

We first determined the concentration of each species necessary to achieve the saturation of the DNA site. Increasing amounts of [^{3}H]tyrosyl-tRNA I (A and B) or II were incubated with 75 μg of DNA under annealing con-

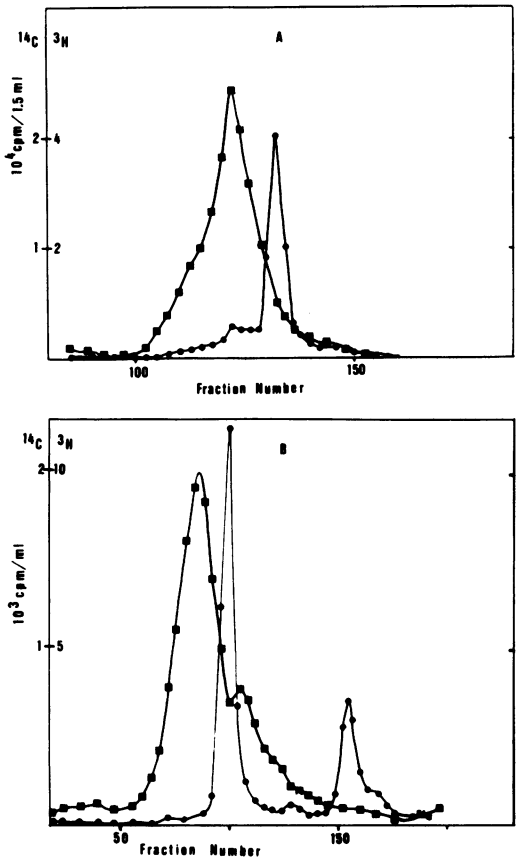

FIG. 8. *Effect of MnO_4K treatment on RPC5 chromatographic profiles of purified $tRNA^{Tyr}$ species. (A) Cochromatography of [^{14}C]tyrosyl-$tRNA^{Tyr}$ I_A (●) and MnO_4K-treated $tRNA^{Tyr}$ I_A aminoacylated with [^{3}H]tyrosine (■). (B) Cochromatography of bulk tRNA from exponential-phase cells aminoacylated with [^{14}C]tyrosine (●) and purified MnO_4K-treated $tRNA^{Tyr}$ II aminoacylated with [^{3}H]tyrosine (■).*

ditions. In the presence of about 30 pmol of tyrosyl-$tRNA^{Tyr}$, the same plateau value (about 0.02 pmol of tyrosyl-tRNA hybridized) was obtained for the two forms (Fig. 9A). The low deacylation (about 2%) that occurs during the annealing period was neglected.

Competition between the $tRNA^{Tyr}$'s for *B. subtilis* DNA is shown in Fig. 9B. DNA was mixed with [^{3}H]tyrosyl-$tRNA^{Tyr}$ II in a ratio sufficient to give the saturation level, and uncharged $tRNA^{Tyr}$ I (A and B) was added in varying amounts as the competitor. The decrease in radioactive counts in the hybridized material shows the inhibition of the hybridization of $tRNA^{Tyr}$ II by uncharged $tRNA^{Tyr}$I.

One additional test of competition was performed according to the method of Chuang et al. (2) by adding saturating levels of charged $tRNA^{Tyr}$ I (A and B) and II separately or simultaneously to the hybridization reaction mixture. In both cases, nearly the same hybridization value was obtained, showing that competition occurs between species I and species II (Table 2).

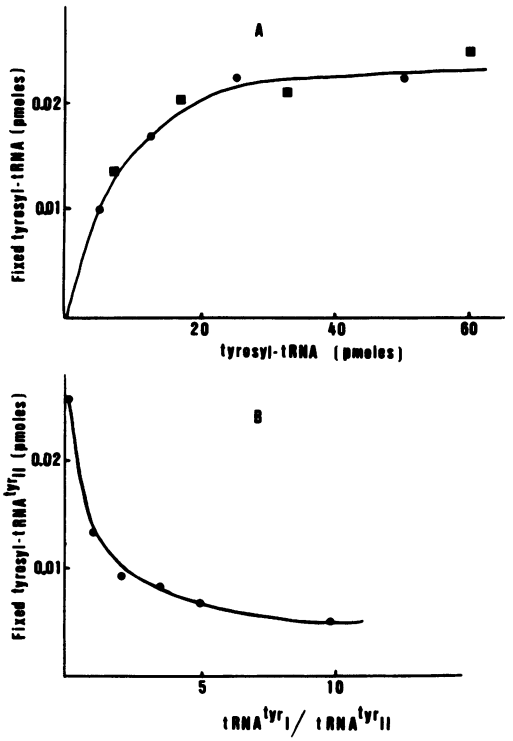

FIG. 9. *Hybridization $tRNA^{Tyr}$-DNA. (A) Hybridization of tyrosyl tRNA I (A and B) (●) and II (■) with B. subtilis DNA as a function of the tyrosyl-tRNA concentration. (B) Competitive hybridization assay between [^{14}C]tyrosyl-tRNA II and $tRNA^{Tyr}$ I (A and B) with B. subtilis DNA.*

TABLE 2. *Competitive hybridization assay between tyrosyl-tRNA species*[a]

$tRNA^{Tyr}$ species	$tRNA^{Tyr}$ input (pmol)	$tRNA^{Tyr}$ fixed on filter (10^{-2} pmol)
I	30	2.3
	60	2.4
II	33	2.0
	66	2.7
I	30	
+		2.4
II	33	
I	60	
+		2.8
II	66	

[a] Purified $tRNA^{Tyr}$ I (A and B) and II were aminoacylated with [^{3}H]tyrosine and hybridized with 75 μg of *B. subtilis* DNA.

These results suggest that tRNA^{Tyr}'s I_A, I_B, and II are transcribed from the same site and, therefore, might possess the same nucleotide sequence.

Functional comparisons of tyrosine tRNA species. Gefter and Russell (5) identified in ϕ80 dsu^+_{III}-infected *Escherichia coli* three forms of tRNA^{Tyr} that have the same nucleotide sequence but differ in the extent of modification of the Ap residue adjacent to the 3' end of the anticodon. Form I has an unmodified A, form II has an isopentenyl group, and form III contains, in addition, a 2-thiomethyl group. These forms were shown to differ in their abilities to support in vitro protein synthesis and to bind to ribosomes: the more tRNA^{Tyr} that is modified, the more efficiency is observed. However, the three forms do not differ with respect to the rate at which they can be charged with tyrosine.

Since the differences between the two tRNA^{Tyr} species of *B. subtilis* also involve an adenosine residue modification, we compared their binding abilities in the presence of poly(U, A, C). Results given in Table 3 show that tRNA^{Tyr} II is more than two times as efficient as tRNA^{Tyr} I (A and B) for binding to ribosomes extracted from either exponentially growing cells of *B. subtilis* or early stationary-phase cells of *E. coli*. No significant binding was observed with early stationary-phase ribosomes of *B. subtilis* prepared in parallel with exponential-phase ribosomes. This last result possibly could be due to an alteration of the native structure of stationary-phase ribosomes or to some partial degradation by proteases synthetized by sporulating cells.

To determine whether tRNA^{Tyr} species of *B. subtilis* differ in their rate of charging, the following experiment was performed. Bulk tRNA extracted from late exponential-phase cells, and therefore possessing the two tRNA^{Tyr} species in significant amounts, was charged, on the one hand, with an excess of [^{14}C]tyrosine and, on the other, with [^{3}H]tyrosine in limiting concentrations, so that the aminoacylation was 20-fold lower with [^{3}H]tyrosine than with [^{14}C]tyrosine. When cochromatographed on an RPC5 column (not shown), the two preparations showed the same elution profile, which indicates the same level of charge for the two species. From this result we can conclude, in agreement with Arceneaux and Sueoka (1), that the two species do not differ in their kinetics of aminoacylation.

It thus appears that the structural feature that differentiates the two tRNA^{Tyr}'s of *B. subtilis* is important for ribosomes binding but not for charging, as was found for the suppressor tyrosine tRNA's species of *E. coli* (5).

TABLE 3. *Binding of [^{14}C]tyrosyl-tRNA (species I_A, I_B, and II) to ribosomes in the presence of poly(U,A,C)*[a]

Source of ribosomes	tRNA^{Tyr} species	tRNA^{Tyr} bound (counts/min)		% Input tRNA^{Tyr} bound specifically
		Minus template	Plus template	
E. coli	I_A	360	756	14.0
	I_B	340	725	17.3
	II	315	1,010	35.0
B. subtilis (exponential phase)	I_A	366	811	15.8
	I_B	295	740	15.5
	II	355	1,160	40.2
B. subtilis (stationary phase)	I_A	54	75	0.7
	I_B	70	82	0.5
	II	100	199	4.9

[a] The input radioactivities for I_A, I_B, and II were 2,820, 2,220, and 2,000 counts/min, respectively ([^{14}C]tyrosine, 285 counts/min per pmol).

Patterns of tyrosyl-tRNA from cells grown in different media. (i) Growth rate. It has been reported that the composition of the growth medium affects the tyrosyl-tRNA pattern of *B. subtilis* (17). We thus determined the ratio of species I and II in tRNA extracted from cells cultivated in media, allowing different growth rates and different sporulation frequencies.

No variation in the relative amount of each species was found as a function of the growth rate. Table 4 shows nearly identical tyrosyl-tRNA patterns in exponential-phase cells grown at a rate varying between 0.7 and 2.1 generations/h.

(ii) Effect of iron on tyrosyl-tRNA modification and sporulation. Table 4 reveals that the modification of tRNA^{Tyr} occurs at nearly the same extent during growth in the different media tested, whatever the sporulation frequency of the culture, except in the case of iron-limited medium. The alteration of tRNA^{Tyr} does not occur in cells grown in MSS medium that was not supplemented with iron after treatment with 8-hydroxyquinoline (Fig. 10). The presence of iron is also important for sporulation: in iron-supplemented medium, the frequency of sporulation is 500 times higher than in iron-free medium. As reported by Rosenberg and Gefter (25), the methylthiolation of the adenosine located at the 3' end of the anticodon of several *E. coli* tRNA species is also dependent on the presence of iron in the growth medium.

(iii) Tyrosyl-tRNA patterns from cells grown in SCM medium. The results from Arceneaux and co-workers (1, 17) do not agree with

TABLE 4. *Ratios of tyrosyl-tRNA's (I/II) in tRNA from the strain W168 grown in various media*[a]

Medium	Exponential growth rate (doublings/h)	Viable cells/ml at t2	Spores/ml at t24	$tRNA^{Tyr}$ I/$tRNA^{Tyr}$ II	
				Exponential phase	Stationary phase
Nutrient broth	2.0	4.3×10^8	3.2 ± 10^8	3.32	0.27 (t2)
Penassay	2.1	13.0×10^8	3.1×10^8	4.0	0.26 (t2)
SCM	1.7	9.0×10^8	9.5×10^7	2.72	0.18 (t1)
MS-malate	0.7	2.1×10^8	3.0×10^6	3.75	0.77 (t2)
MSS (iron supplemented	0.8	4.5×10^8	3.6×10^8	2.51	0.25 (t2)
MSS (iron free)	0.7	2.5×10^8	5.0×10^5	6.32	9.31 (t2)

[a] Ratios were calculated from the area under each peak eluted from an RPC5 column. The shoulder designated as peak III was included in the measurement of tyrosyl-tRNA II.

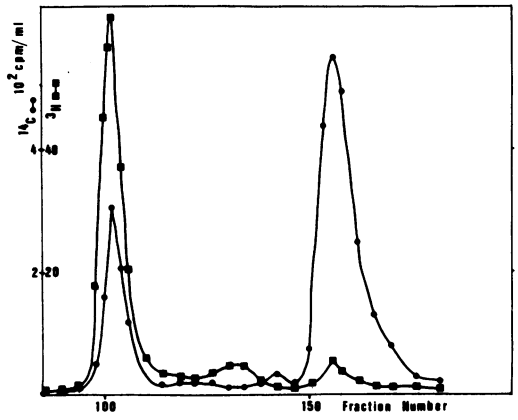

FIG. 10. *RPC5 chromatography of tyrosyl-tRNA extracted from cells in iron-free and iron-supplemented growth medium. Bacteria were harvested 2 h after the end of exponential growth phase. Symbols: (■) [^{3}H]tyrosyl-tRNA from cells grown in iron-free medium; (●) [^{14}C]tyrosyl-tRNA from cells grown in iron-supplemented medium.*

ours in the case of tyrosyl-tRNA of *B. subtilis* grown in SCM medium. We found that tyrosyl-tRNA from strains W168 and W23 D undergoes the same dramatic change during growth in SCM medium as first described by Arceneaux and Sueoka (1) in the case of tyrosyl-tRNA from cells grown in Penassay medium. According to their chromatographic study on a MAK column, they found that exponential-phase tRNA isolated from strain W168 grown in SCM medium contained tyrosyl-tRNA I as the major species and stationary-phase tRNA (from cells harvested 5 h after the culture reached the maximal turbidity) contained approximately equal amounts of species I and II. However, in a further paper, McMillian and Arceneaux (17) reported that species I remains predominant in stationary-phase cells (strain W168) grown in SCM medium: the amount of tyrosyl-tRNA II relative to tyrosyl-tRNA I only shifted from 0.09 in exponential phase to 0.33 in stationary phase. This last study was performed by RPC5 chromatography. We were unable to define the cause for the discrepancy between these results. In the different studies, tRNA was extracted from cells harvested at about the same stage during exponential or stationary phase. The possibilities of differential removal of tyrosine or incomplete charging are unlikely. The aminoacyl ester bond of the two species has the same stability at pH 6.5, since the profile of tyrosyl-tRNA was not modified by the deacylation of 70 to 80% that occurred during MAK chromatography, as shown above, nor does it seem that the two species differ by their aminoacylation rate. One possible explanation is that the extent of $tRNA^{Tyr}$ alteration depends on the concentration of a component that could vary from one batch of SCM medium to another. This component might be iron. As suggested by Wettstein and Stent (36), the concentration of metal ions such as Fe^{2+}, which tend to form insoluble hydroxides, might change upon aging. According to these authors, the same new $tRNA^{Phe}$ species was observed in tRNA from *E. coli* grown in aged medium and that grown in iron-limited medium. As shown above, the alteration of $tRNA^{Tyr}$ of *B. subtilis* is also iron dependent.

(iv) Glucose effect. The addition of an excess of glucose in nutrient medium was shown to inhibit the commitment to sporulation (4, 31). Growth of strains W168 and W23 D in nutrient broth supplemented with 3% glucose resulted in greater than 99.9% inhibition of spore formation. However, the sporulation of strain W23 S was stimulated by the addition of glucose (7). The sporulation frequency of W23 in the presence and absence of glucose was, respectively, 0.9 and 0.4 (Table 5). Strain W23 S is not defective for the transport of glucose since the uptake of glucose was the same, up to t1, as in strain W23 D; thereafter, it declined in strain W23 D and increased further in strain W23 S,

although the cell division stopped. It was therefore of interest to analyze tRNA^{Tyr} patterns in both strains, in parallel with biochemical changes related to the commitment to sporulation.

Significant changes in the synthesis of enzymatic systems, such as reduced nicotinamide adenine dinucleotide oxidase and tricarboxylic acid cycle enzymes, occur during sporulation of *B. subtilis*, and mutants devoid of components of these systems are asporogenic or oligosporogenic (3, 31). As in sporulation, several of the enzymes that have been observed to increase after the end of exponential phase are subject to catabolic repression. When the complex sporulation medium is supplemented with an excess of glucose, cells sporulate at a very low frequency and the increase of enzymatic activities is delayed until the time when catabolic repression is released by some nutrient limitation.

We measured reduced nicotinamide adenine dinucleotide oxidase and isocitric dehydrogenase activities during exponential and early stationary growth phases of strains W23 D and W23 S grown in nutrient broth supplemented with or without 3% glucose.

Our data (Table 5) concerning strain W23 D were in agreement with those already reported. However, in strain W23 S, enzymatic synthesis is not affected by the presence of glucose; the increase occurs at the same time as in its absence. Strain W23 S thus possess a mechanism, which remains to be elucidated, responsible for the suppression of the glucose effect.

We observed that the modification of tRNA^{Tyr} occurred in parallel with variations in the levels of tested enzymes. tRNA^{Tyr} I was still the major species at stage t1 in tRNA from strain W23 D grown in the presence of glucose, whereas the extent of modification was not changed in strain W23 S by the addition of glucose (Table 5). However, tRNA^{Tyr} patterns were the same in both strains at t4. Therefore, there are correlations between the expression of enzymes required for the onset of sporulation and tRNA^{Tyr} modification.

tRNA^{Tyr} pattern of chloramphenicol-treated cells. tRNA extracted from a culture in late exponential phase, incubated for 2 h in the presence of chloramphenicol at 100 μg/ml, showed the same tyrosyl-tRNA profile as tRNA extracted before chloramphenicol treatment (Fig. 11). Chloramphenicol thus completely blocked the transformation of tRNA^{Tyr}, although it was already initiated. This result indicates that the formation of tRNA^{Tyr} II requires continuous protein synthesis.

We did not observe an accumulation of tRNA in chloramphenicol-treated *B. subtilis* cells, in contrast with that reported for *E. coli*. It was shown that tRNA synthesis continues in *E. coli*

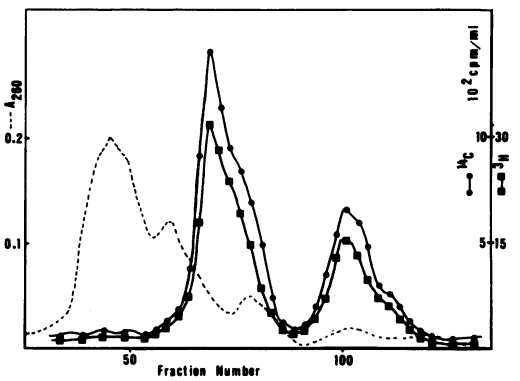

FIG. 11. *RPC5 chromatography of tyrosyl-tRNA extracted from cells treated with chloramphenicol or untreated. A late exponential-phase culture was divided in half; tRNA extracted from the cells, harvested immediately, was aminoacylated with [^{14}C]tyrosine (●). tRNA extracted from the cells treated with chloramphenicol for 2 h before harvesting was aminoacylated with [^{3}H]tyrosine (■).*

TABLE 5. *Some properties of strains W23 D and W23 S as a function of growth in nutrient broth medium supplemented with 3% glucose or not supplemented*[a]

Strain	Glucose in medium	S/V	NADH oxidase			Isocitric dehydrogenase			tRNA^{Tyr} I/tRNA^{Tyr} II		
			Exponential	t1	t4	Exponential	t1	t4	Exponential	t1	t4
W23 D	0	8×10^{-1}	0.07	0.15	0.30	0.36	1.20	0.74	3.3	0.4	ϵ
	+	4×10^{-5}	0.08	0.09	0.20	0.30	0.37	1.00	3.5	1.5	0.2
W23S	0	4×10^{-1}	0.06	0.15	0.23	0.21	0.95	1.10	3.5	0.5	ϵ
	+	9×10^{-1}	0.10	0.25	0.31	0.25	0.80	0.64	3.5	0.6	ϵ

[a] Enzyme activities were determined by standard methods in extract prepared according to the procedure described by Szulmajster (31). Specific activities are expressed as change in optical density at 240 nm/mg of protein per min. Ratios of tyrosyl-tRNA's were determined as described in the footnote to Table 4. S/V represents the ratio of viable cells per milliliter (V) at t2 to spore number per milliliter (S) at t24. NADH, Nicotinamide adenine dinucleotide, reduced form.

cells during prolonged incubation in chloramphenicol (15); according to the chromatographic profiles, several new $tRNA^{Phe}$ species produced were presumed to differ from normal species by lack of modifications such as the methylthio group, isopentenyl group, or both (10). We have observed that tRNA extracted from an exponential culture of *E. coli* (strain 1014), incubated under the same conditions as the culture of *B. subtilis*, was 3.5 times more abundant than tRNA extracted from an untreated culture. The cessation of tRNA synthesis in *B. subtilis* treated with chloramphenicol is in keeping with the absence of modification of the tyrosyl-tRNA pattern: i.e., an increase of species I or the appearance of unmodified species.

DISCUSSION

According to our results, the accumulation of one $tRNA^{Tyr}$ species, accompanied by a corresponding decrease in the amount of the other, depends on a post-transcriptional modification. The analysis of the hydrolysis products of purified $tRNA^{Tyr}$ species I and II previously showed that they differ from each other in the degree of modification of an adenosine residue, species I having i^6A and species II having ms^2i^6A. Since the purified $tRNA^{Tyr}$ I and II hybridize with *B. subtilis* DNA in a competitive fashion, they might have the same nucleotide sequence. This aspect will be fully elucidated from primary sequence studies currently in progress.

Some additional minor peaks were observed in elution profiles of tyrosyl-tRNA. Peak III $tRNA^{Tyr}$ might correspond to aggregates; however, this could not be demonstrated. The significance of the small peak of variable size eluted ahead of the optical density profile on the MAK column also remains obscure. It does not seem to correspond to an unmodified form lacking isopentenyl or methylthio moieties, since it was not eluted at the position corresponding to species I treated with MnO_4K, a process that has been shown to remove the isopentenyl side chain and, according to our results, also removes or modifies the methylthio group. Our inability to detect unmodified $tRNA^{Tyr}$ species suggests that the isopentenylation occurred as soon as $tRNA^{Tyr}$ was synthetized. According to Schaefer et al. (27), the *E. coli* $tRNA^{Tyr}$ precursor already contains i^6A in low molar yields.

The modified adenosine residue of $tRNA^{Tyr}$ species is most likely adjacent to the anticodon, as is found in all cases where the sequence is known. Undermodification of this residue in *E. coli* $tRNA^{Tyr}$ was shown to impair the codon recognition properties (5). In the same way, we found that the reading capacity of *B. subtilis* $tRNA^{Tyr}$ I was two to three times less efficient than that of species II, as measured by the ribosome binding assay. The modification of $tRNA^{Tyr}$ that occurs when cells enter the sporulation period could therefore play a role in regulating translation during this stage.

Accumulation of tRNA species containing i^6A in place of ms^2i^6A was demonstrated in *E. coli* cells grown in a low-iron medium (25) and through methionine deprivation (9). According to chromatographic studies, the same phenomenon occurs during the cessation of protein synthesis through chloramphenicol treatment and in altered growth conditions such as limited aeration (36), phosphate limitation, and low growth rate (18). However, in the case of *B. subtilis*, i^6A was found in tRNA from well-aerated cultures during the period of the most active protein synthesis, whereas ms^2i^6A appears during the slowing down of growth. The presence of ms^2i^6A in tRNA at this stage thus constitutes an intriguing problem.

Studies performed with asporogenous strains and with cells cultivated under various conditions were designed in an attempt to determine whether $tRNA^{Tyr}$ modification could be related to sporogenesis. Chromatographic patterns of $tRNA^{Tyr}$ from asporogenous strains were analyzed by Vold (33) and by McMillian and Arceneaux (17). Several strains showed the same $tRNA^{Tyr}$ change as in the parent strains. However, a set of asporogenous mutants was found to be defective to variable degrees for $tRNA^{Tyr}$ modification (17). In exponential phase, these mutants showed nearly the same pattern as that observed with the wild strain; however, in stationary phase the amount of $tRNA^{Tyr}$ II relative to $tRNA^{Tyr}$ I was 11.5 in the wild strain, whereas it varied between 3.79 and 0.49 in mutant cells.

Under the growth conditions described here, the $tRNA^{Tyr}$ modification can occur even when the sporulation frequency is very low. However, in iron-limited medium, the only situation in which we did not observe the modification, massive sporulation does not occur. We have also observed that full tRNA modification is delayed when the commitment to sporulation is prevented by an excess of glucose. This is also the case for the increase of synthesis of enzymatic systems, such as reduced nicotinamide adenine dinucleotide oxidase and isocitrate dehydrogenase, required for sporulation. The fact that both phenomena seem to be regulated concomitantly does not necessarily mean that they are related to one another. Nevertheless, these observations, along with the occurrence of asporogenous strains defective for $tRNA^{Tyr}$ change,

make plausible the hypothesis that the methylthiolation of tRNA^{Tyr} could be included in events required for spore formation.

The modification of tRNA^{Tyr} at the end of the exponential growth phase also requires protein synthesis, since the addition of chloramphenicol to the medium blocked the change completely. As showed by Kersten and Kersten (13), the appearance of the stationary tRNA^{Tyr} pattern is also prevented by pactamycin, an inhibitor of protein synthesis. This synthesis could involve the thiomethylation enzyme or some protein regulating the pattern of methylating enzyme. It is therefore of interest to determine the level of methylthiolation enzyme as a function of growth; this investigation is currently in progress.

ms^{2}i^{6}A detected in tRNA^{Tyr} II corresponds very likely to the unidentified methylated nucleotide found by Kersten and Kersten (13) to be characteristic for *B. subtilis* stationary-phase tRNA. According to these authors, this nucleotide constitutes 10% of all methylated tRNA nucleotides. However, ms^{2}i^{6}A of tRNA^{Tyr} could not account for such an amount. It is therefore possible that the same modification occurs in other tRNA's, since Vold (32) has reported, in stationary-phase tRNA of *B. subtilis*, the increase or appearance of tryptophanyl- and leucyl-tRNA species liable to possess ms^{2}i^{6}A.

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