THE OCCURRENCE OF RIBOTHYMIDINE, 1-METHYLADENOSINE, METHYLATED GUANOSINES AND THE CORRESPONDING METHYLTRANSFERASES IN *E. COLI* AND *BACILLUS SUBTILIS*

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1. Introduction

The sequence analyses of different species of tRNA's from *E. coli* revealed certain homologies, namely the sequence GT Ψ CG in one loop [1–4]. This sequence was also found to be present in tRNA's from yeast and from other eukaryotic tRNA's so far studied. The common pentanucleotide sequence was suggested to be involved in the mRNA-dependent binding of tRNA's to ribosomes since oligonucleotides, that contain this sequence, inhibit the binding *in vitro* [5–7]. If GT Ψ CG is really a common sequence for most tRNA's species from various bacterial sources the relative frequency of ribothymidine, i.e. m^5U in tRNA populations and the m^5U-transferase activities from various microorganisms are expected to be relatively high and should not vary significantly. The results presented here show that ribothymidine is absent in most species of tRNA's of *B. subtilis*. Uracil residues in *B. subtilis* tRNA's can be methylated by the m^5U transferase from *E. coli* to the same extent occurring in *E. coli* tRNA's. Vice versa adenosine residues in several species of *E. coli* tRNA's can be methylated to m^1A to a relative frequency corresponding to that of *B. subtilis* tRNA's. The results indicate that GT Ψ CG is not a common sequence for all tRNA's and that heterologous methylations do not occur randomly.

2. Materials and methods

2.1. *Chemicals*

The chemicals were from the following sources: [$^{14}CH_3$] S-adenosyl-methionine (specific activity = 53 μCi/umole); [^{14}C] l-methionine (specific activity = 52–56 mCi/mmole); Radiochemical Centre Amersham; β-mercaptoethanol, Sigma Chemical Company; DEAE-cellulose, Whatman; methylated nucleosides, Cyclochemical Corporation, Los Angeles, California, USA (the free bases were obtained by hydrolysis in $HClO_4$, subsequent neutralisation with KOH and separation from $KClO_4$).

2.2. *Bacterial strains*

E. coli MRE 600 was grown in a minimal medium containing per litre: 1 g NaCl; 2.5 g $(HN_4)_2HPO_4$; 1.5 g KH_2PO_4; 3.0 g Na-glutaminate; 0.1 g $MgSO_4 \cdot 7\,H_2O$; 3.0 g glucose. *B. subtilis* W 23 was grown in a medium containing per litre: 6 g KH_2PO_4; 11.5 g $Na_2HPO_4 \cdot H_2O$; 2.0 g $(NH_4)_2SO_4$; 2.5 g Na-glutaminate; 0.33 mg $MnSO_4 \cdot H_2O$; 0.2 g $MgSO_4 \cdot 7\,H_2O$; 4.0 g glucose. Cell cultures of 100 ml were grown in the presence of [^{14}C] methyl-l-methionine (100 μCi = 7 μmoles) up to the early stationary phase ($A_{578\ nm}$ = 1.8; d = 1 cm). At this time 15–20% of the added radioactivity was found in the medium.

2.3. *$^{14}CH_3$-labelled tRNA's*

The cell walls of *B. subtilis* cannot be disrupted by phenol. Therefore about 0.5 g (wet weight) cells of *E. coli* or *B. subtilis* were ground with a 2-fold amount of Alcoa at 0–4°C for 5 min and extracted

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with 2–3 ml buffer: 0.01 M Tris–HCl pH 7.8; 0.01 M Mg acetate, 0.06 M mercaptoethanol, 0.06 M KCl. After centrifugation at 12000 *g* for 15 min the supernatant was extracted with an equal volume of buffer saturated phenol. The phenol extract was purified by DEAE-cellulose chromatography on a column 1 × 2.5 cm. At least 50 ml of 0.01 M Mg acetate, 0.001 M EDTA-buffer pH 4.8 containing 0.25 M NaCl were passed through the column. The tRNA's were eluted quantitatively with 3 ml of the same buffer containing 0.5 M NaCl and subsequently dialysed and precipitated with 2.5 vol of 90% ethanol, containing 2% potassium acetate.

Purity was checked by electrophoresis on polyacrylamide-gel, showing a tRNA preparation free from 16 S and 23 S RNA. However, a minor contamination by fragments of ribosomal RNA's, resulting from their slight degradation, cannot be excluded.

2.4. *tRNA-methylation*

Sub-methylated tRNA's from *E. coli* were obtained from *E. coli* meth$^-$ Rel$^-$ 168/21 according to Fleissner and Borek [8]; sub-methylated tRNA's from *B. subtilis* according to Kersten et al. [9]. 20 A_{260} tRNA purified by DEAE-Sephadex chromatography were incubated in a total volume of 1.7 ml with 0.2 μmoles SAM (53 mCi/mmoles), 25 μmoles Tris–HCl pH 0.8; 50 μmoles triethanolamine; 10 μmoles mercaptoethanol; 5 μmoles $MgCl_2$ and 0.5 ml of crude enzyme (= 8 mg protein of S-100 supernatant of *E. coli* or *B. subtilis*). After 2 hr of incubation at 37°C tRNA's were purified on DEAE-cellulose, eluted and precipitated as described above.

2.5. *Analysis of the modified bases*

The precipitated tRNA's were dissolved in 0.5 ml of 70% $HClO_4$ and hydrolyzed in sealed tubes for 60 min at 100°C. The hydrolysate was purified by passing through columns of charcoal as described by Lakings and Gehrke [10]. The recovery for all individual bases was at least 90%. The extracts were found to be free from ribose, $HClO_4$ and phosphate. Two-dimensional chromatography was carried out at room temperature on cellulose plates from Merck 20 × 20 cm. For each sample two plates were run in the first dimension with solvent A (methanol/HCl/water, 65:17:18) for 3 hr. In the second dimension two different systems were used. Solvent B (*n*-butanol/acetic acid/water 4:1:1) with one plate and solvent C (saturated $(NH_4)_2SO_4$/isopropanol/0.1 M phosphate pH 6.0,79:2:1) for the second plate. System B separates especially m^5C and m^3C and system C separates m^1G and m^7G [11]. The methylated bases were localized by autoradiography on X-Ray film Agfa Osray T 4 after 6–14 days exposure. The localized spots were scraped off, dissolved in 100 μl of 0.01 M HCl and counted in a dioxan scintillation cocktail [12]. The spots were further identified by chromatography with authentic markers in one dimensional chromatography according to Hacker [13] with solvent system D (2-propanol/12 M HCl/water, 68:17.6:14.3).

3. Results and discussion

E. coli and *B. subtilis* tRNA's were labelled with $^{14}CH_3$ groups by growing the cells in the presence of [$^{14}CH_3$]methionine up to the stationary phase. From the analysis of the methylated bases (fig. 1 A and B and table 1) it is evident that tRNA's from *E. coli* and *B. subtilis* have in common m^1G, m^7G, m^2G, m^6A, m^2A, m^5U, (m^3C), (m^5C) and the as yet unidentified base X. (Those methylated bases which occur in trace amounts < 1% cannot be seen on the photograph. The spots were identified on the film and found to be labelled to an extent of 100–200 cpm, which is significant.) The modified nucleosides m_2^6A, m^1A and m^3U were absent in tRNA's from *E. coli* but present in tRNA's of *B. subtilis*, whereas tRNA's of *B. subtilis* did not contain Z (unidentified). The relative frequency of the methylated bases m^1G + m^7G and m^2A were nearly the same in the tRNA's from both strains. Striking variations were found in the abundance of m^5U and m^1A. Ribothymidine occurred only in trace amounts in *B. subtilis* tRNA's whereas it comprises 44% of the total amount of labelled-CH_3 groups incorporated into *E. coli* tRNA. On the other hand m^1A which is not present in *E. coli* tRNA's comprises 23% of the total CH_3-groups incorporated into tRNA's of *B. subtilis*. These results suggest that *B. subtilis* cells do not contain the 5-methyluracil-transferase activity which converts uracil to thymine in a GU Ψ CG sequence. Extracts of *B. subtilis* however exhibit 1-methyl-adenine transferase activity normally not present in *E. coli*

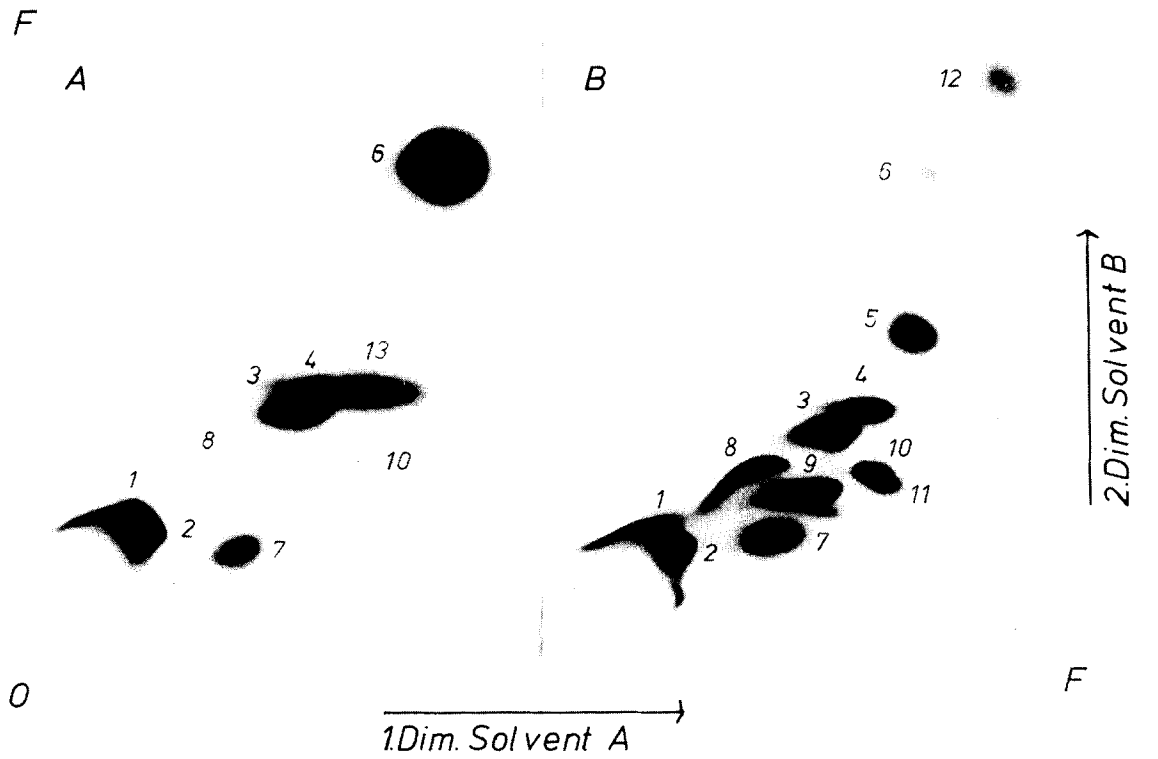

Fig. 1. Autoradiography of the pattern of $^{14}CH_3$-labelled methylated bases of tRNA's isolated from *E. coli* (A) and *B. subtilis* (B). Labelling of the tRNA's, extraction, hydrolysis, conditions for two dimensional chromatography, identification and further details are described under Materials and methods. The identity and relative distribution of the methylated bases are summarized in table 1.

Table 1
Relative amounts of methylated bases in tRNA of *E. coli* and *B. subtilis*.[a]

Code no.	Compounds	Percentage of total radioactivity recovered	
		E. coli	*B. subtilis*
1	1-Methylguanine	4.1	10.1
2	7-Methylguanine	30	32.2
3	2-Methyladenine	13	10.7
4	6-Methyladenine	2.1	5.0
5	6-Dimethyladenine	–	3.0
6	5-Methyluracil	43.5	2.5
7	X unidentified	5.7	3.2
8	2-Methylguanine	< 1.0	5.5
9	1-Methyladenine	–	23
10	5-Methylcytosine	< 1.0	2.0
11	3-Methylcytosine	< 1.0	< 1.0
12	3-Methyluracil	< 1.0	< 1.0
13	Z unidentified	2.2	–

[a] The total radioactivity applied to the TLC was 5×10^4 cpm for *E. coli* and 3×10^4 cpm for *B. subtilis*. The recovery from each plate was 85% at least.

extracts. The enzyme, 1-methyladenine-transferase has been observed in yeast and other eukaryotic cells [14]. In the following experiments submethylated tRNA's from *E. coli* and submethylated tRNA's from *B. subtilis* (obtained according to Kersten et al. [9] were used as substrates for the heterologous methyltransferases. Obviously the patterns of methylated bases of *in vitro* modified *B. subtilis* tRNA agree with the normal methylation patterns of *E. coli* tRNA (figs. 1A and 2B). Vice versa the patterns of methylated bases from *in vitro* modified *E. coli* tRNA with the *B. subtilis* enzymes are similar to the normal methylation patterns of *B. subtilis* tRNA (figs. 2A and 1B). The heterologous enzymes incorporate nearly as many m^1A respectively m^5U bases into *E. coli* tRNA's, respectively into *B. subtilis* tRNA's as are found to be incorporated when the tRNA's were labelled within the cells (table 2). Furthermore the degree of heterolog-

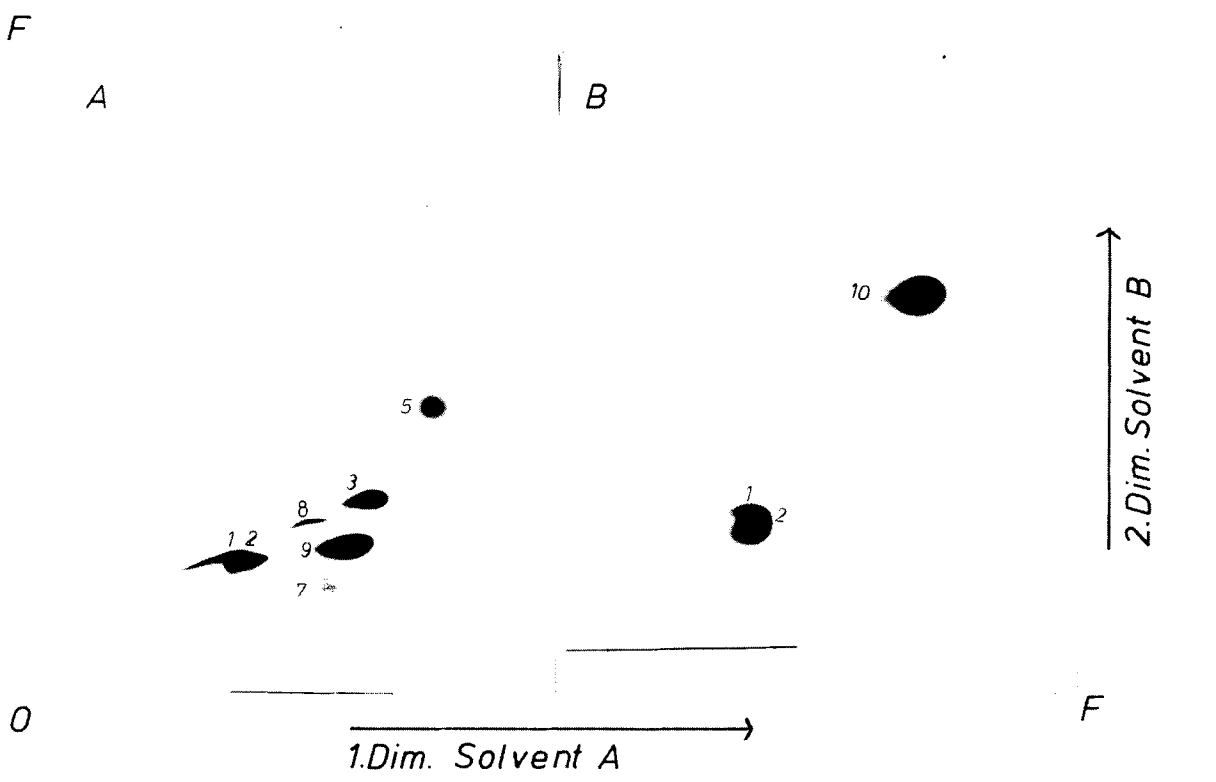

Fig. 2. Autoradiography of the pattern of $^{14}CH_3$-labelled methylated bases of *in vitro* re-methylated tRNA's: A. Sub-methylated tRNA's from *E. coli* meth$^-$, re-methylated with *B. subtilis* methyltransferases, B) sub-methylated tRNA's from *B. subtilis* W 23 re-methylated with *E. coli* methyltransferases. The identity and relative distribution of the methylated bases are summarized in table 2.

Table 2
Relative amounts of methylated bases incorporated in sub-methylated tRNA's of *B. subtilis* and *E. coli* with the heterologous methyltransferases.

Code	Compounds	Percentage of total radioactivity recovered	
		tRNA of *E. coli* Enzyme of *B. subtilis*	tRNA of *B. subtilis* Enzyme of *E. coli*
1	1-Methylguanosine	54.0	45.0
2	7-Methylguanosine		
3	2-Methyladenine	11.0	< 1.0
4	6-Dimethyladenine	4.4	
5	–	–	–
6	–	–	–
7	X unidentified	2.9	< 1.0
8	2-Methylguanine	1.7	< 1.0
9	1-Methyladenine	26.0	< 1.0
10	5-Methyluracil	< 1.0	54.0

ous methylation of G to $m^1G + m^7G$ of the sub-methylated tRNA's from both sources corresponds well with the normal occurrence of these modified bases.

The modified nucleoside m^5U is generally considered to be a universal base in tRNA's except for tRNA's from primitive organisms such as *Mycoplasma*, tRNA's not involved in protein synthesis or initiator $tRNA_f^{met}$ from eukaryotes [14–18]. All tRNA's sequenced up to now contain m^5U at position 23 from the 3'-end [1]. *E. coli* tRNA's contain an average of 2.3 methylated bases per tRNA [19]. Assuming that ribothymidine occurs once in each tRNA species it was calculated that m^5U comprised of 43% of all methylated bases.

A comparison of this calculation with the experimental data described here (44% m^5U in *E. coli* tRNA) shows the absolute accuracy of the analytical method used.

If we accept that methyltransferases are able to recognize certain sequences [20] we must conclude that homologous sequences occur in tRNA's of *B. subtilis* and *E. coli*. Thus the *B. subtilis* sequence in which U is methylated to m^5U might be GU Ψ CG. The modified base m^1A found in *B. subtilis* has been shown to occur in tRNA-Phe of eukaryotes adjacent to CG in the GT Ψ CG loop [14]. Therefore one can speculate that during the evolution of prokaryotic tRNA's a common GU Ψ CGA sequence can either be modified by methylation of adenine to m^1A adjacent to CG or methylation of uracil to m^5U adjacent to Ψ. In favour of this view are the results of Klagsbrun [21] who found m^1A abundant in tRNA's of the Gram-positive bacteria such as *Mycobacterium lysodeicticus* and *B. subtilis* in which ribothymidine is either missing or occurs in trace amounts.

References

[1] Zachau, H.G. (1969) Angew. Chemie 81, 645.
[2] Kerr, S.J. and Borek, E. (1972) Advan. Enzymol. 36, 1.
[3] Zamir, A, Holley, R.W. and Marquisee, M. (1965) J. Biol. Chem. 240, 1267.
[4] Sanger, F., Brownlee, G.G. and Barrell, B.G. (1965) J. Mol. Biol. 13, 373.
[5] Shimizu, N., Hayashi, H. and Miura, K.I. (1970) J. Biochem. 67, 373.
[6] Ofengand, J. and Henes, C. (1969) J. Biol. Chem. 244, 6241.
[7] Henes, C., Krauskopf, M. and Ofengand, J. (1969) Biochemistry 8, 3024.
[8] Fleissner, E. and Borek, E. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1199.
[9] Kersten, H., Chandra, P., Tanck, W., Wiedenhöver, W. and Kersten, W. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 659.
[10] Lakings, D.B. and Gehrke, C.W. (1971) J. Chromatog. 62, 347.
[11] Kahle, P., Hoppe-Seyler, P. and Kröger, H. (1971) Biochim. Biophys. Acta 240, 384.
[12] Bray, G.A. (1960) Anal. Biochem. 1, 279.
[13] Hacker, B. and Mandel, L.R. (1969) Biochim. Biophys. Acta 190, 38.
[14] Keith, G., Picaud, F., Wissenbach, J., Ebel, J.P., Petrissant, G. and Dirkheimer, G. (1973) FEBS Letters 31, 345.
[15] Piper, P.W. and Clark, B.F.C. (1973) FEBS Letters 30, 265.
[16] Simsek, M., Ziegenmeyer, J., Heckmann, J. and Raj-Bhandary, U.L. (1973) Proc. Natl. Acad. Sci. U.S. 7, 1041.
[17] Petrissant, G. (1973) Proc. Natl. Acad. Sci. U.S. 71, 1046.
[18] Johnson, L., Hayashi, H. and Söll, D. (1970) Biochemistry 9, 2823.
[19] Hayashi, Y., Osawa, S. and Miura, K. (1966) Biochim. Biophys. Acta 129, 519.
[20] Baguley, B.C., Wehrly, W. and Staehelin, M. (1970) Biochemistry 9, 1645.
[21] Klagsbrun, M. (1973) J. Biol. Chem. 248, 2612.