ISOACCEPTING PHENYLALANINE tRNAs FROM *BACILLUS SUBTILIS* AS A FUNCTION OF GROWTH CONDITIONS
Differences in the content of modified nucleosides

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1. Introduction

The formation of additional tRNA isoacceptors has been observed in *Escherichia coli* growing under conditions of unbalanced growth [1–5]. In *Bacillus subtilis* important changes in the elution profiles for a variety of isoaccepting tRNAs were noted when tRNA from vegetative cells, from stationary phase and from spores were compared [6–11]. Previous papers described that in the presence of pactamycin submethylated tRNA accumulates [12–14]. A distinct change in the ratio of two phenylalanine specific tRNAs was found in *B. subtilis* treated with pactamycin [12,14]. A relative increase in the amount of the early eluting $tRNA^{Phe}$ species of *B. subtilis* was observed also when parameters of the growth conditions were changed [15].

The formation of additional species of tRNA-isoacceptors under certain growth conditions can be the result of an additional post-transcriptional modification of the normal tRNA species or the undermodification of the normal tRNA. In a recent work one of us demonstrated that the two species of $tRNA^{Tyr}$ in *B. subtilis* W168 differ in the degree of modification of one hypermodified adenosine [16]. An alternative hypothesis can be advanced, that the presence of additional tRNA could be derived by transcription of 'silent' tRNA genes, which are normally not expressed.

These studies were undertaken to define the differences between the two phenylalanine tRNA species from *B. subtilis*. We have isolated both tRNAs and as a first step, analyzed their nucleoside composition with particular respect to the content of modified nucleosides. Our results show that the phenylalanine tRNAs in *B. subtilis* differ in two posttranscriptional modifications, the lack of 2′-*O*-methylated guanosine and the lack of the thiomethyl group of ms^2i^6A. The composition of the other minor nucleosides and of the four main nucleosides seems to be unaltered.

2. Materials and methods

Chemicals were obtained from the following sources: Cellulose coated aluminium foil for the thin-layer chromatography and all solvents used, Merck AG., Darmstadt. [^{14}C]Phenylalanine (532 Ci/mol),

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Radiochemical Centre, Amersham. The materials used for column chromatographies were from sources as already described [17].

B. subtilis W23 was grown either in glucose salt medium [18] or in enriched medium, pH 7.5 [19].

Nucleic acids were extracted from the cells by phenol–SDS buffer [19]. tRNA was prepared as described [17]. The most abundant species $tRNA_{II}^{Phe}$, was purified by three column chromatographic steps on (1) BD-cellulose pH 7.5 (2) RPC-5 column, pH 7.5 (3) RPC-5 column pH 4.5. All procedures have already been described in detail [17].

The additional species, called $tRNA_{I}^{Phe}$, was purified from bulk tRNA extracted from vigorously aerated cultures of *B. subtilis* grown in minimal medium. The first BD-cellulose column chromatography, at pH 7.5, was used for total separation of $tRNA_{I}^{Phe}$ and $tRNA_{II}^{Phe}$. The $tRNA_{I}^{Phe}$ was eluted within the salt gradient and further purified by chromatography on a Sepharose 4B column. Elution was carried out with a decreasing gradient of ammonium-sulfate as described [20]. Collected fractions containing $tRNA_{I}^{Phe}$ were finally aminoacylated with [^{14}C]phenylalanine (48 Ci/mol) and chromatographed on a BD-cellulose column, at pH 4.5. All tRNA species except charged phenylalanyl-$tRNA_{I}^{Phe}$ were eluted with 1.5 M sodium chloride containing buffer, whereas Phe-$tRNA_{II}^{Phe}$ was eluted in an ethanol gradient (0–30%).

The phenylalanine acceptances of purified $tRNA_{I}^{Phe}$ and $tRNA_{II}^{Phe}$ were 1520 and 1600 pmol/A_{260} unit respectively.

For the determination of their nucleoside composition both phenylalanine specific tRNAs have been analyzed according to Rogg et al. [21]. Approximately 6–8 A_{260} units of each tRNA were used for the analysis. The nucleosides on the thin-layer chromatograms were detected at 254 nm. For the measurement of dihydrouridine the area, where dihydrouridine is normally placed, was scraped from the thin-layer chromatographic plate and the time-dependent loss of absorption at 230 nm in 0.1 M KOH served as index for the concentration of dihydrouridine [22]. All other spots visible at 254 nm were scraped from the thin-layer chromatographic plate and the nucleosides eluted in 0.5 ml water. In order to determine the relative amounts, spectra in acidic and alkaline solutions were drawn.

3. Results

The difference of chromatographic behaviour of the two species of phenylalanine tRNAs from *B. subtilis* can be shown by RPC-5 column chromatographies. Figure 1 shows the elution profiles of $tRNA_{I}^{Phe}$ and $tRNA_{II}^{Phe}$ after chromatography of bulk tRNA extracted from *B. subtilis* grown in normal aerated maximal medium respectively in vigorously aerated glucose minimal medium. In cells grown in maximal medium at any aeration or in normal aerated minimal medium (not shown) $tRNA_{II}^{Phe}$ exists, whereas the additional $tRNA_{I}^{Phe}$ only appears in cells grown in vigorously aerated minimal medium. The appearance of $tRNA_{I}^{Phe}$ seems to be a consequence of the differences in aeration but not a consequence of differences in the media. A new species arises also in cells treated with pactamycin [12,14]. This species seems to be identical with $tRNA_{I}^{Phe}$ as can be seen from co-chromatography of tRNA isolated from inhibitor treated cells and tRNA from cells grown in minimal medium under optimal aeration (results not shown). Both phenylalanine isoacceptors are eluted from the column as sharp symmetrical peaks, indicating that the tRNAs consist of homogenous material without any aggregates.

For the analysis of $tRNA_{I}^{Phe}$ and $tRNA_{II}^{Phe}$ we have isolated both species by chromatography starting with 40 000 A_{260} units of bulk tRNA prepared as described under Material and methods. The purification procedure described above allows total separation of both phenylalanyl-tRNAs without any vice versa contamination.

As can be seen from the nucleoside analysis of the enzymatic hydrolysate (see fig.2) $tRNA_{II}^{Phe}$ contains beside the main bases eight modified nucleosides: one ribothymidine, one 7-methylguanosine, one ms^2i^6A, one ribose methylated guanosine, two dihydrouridine and two pseudouridines. This result confirms the analysis of nucleotides of $tRNA_{II}^{Phe}$, which we have published previously [16]. As indicated in figs.2 and 3, $tRNA_{I}^{Phe}$ and $tRNA_{II}^{Phe}$ differ in two nucleosides. The thin-layer chromatogram of the digest of $tRNA_{I}^{Phe}$ shows the lack of O'-methylated guanosine and instead of the ms^2i^6A spot the presence of a slightly different moving spot near the solvent front in the first dimension. This very non polar nucleoside corresponds to 6-(Δ^2-isopentenyl) adenosine as can

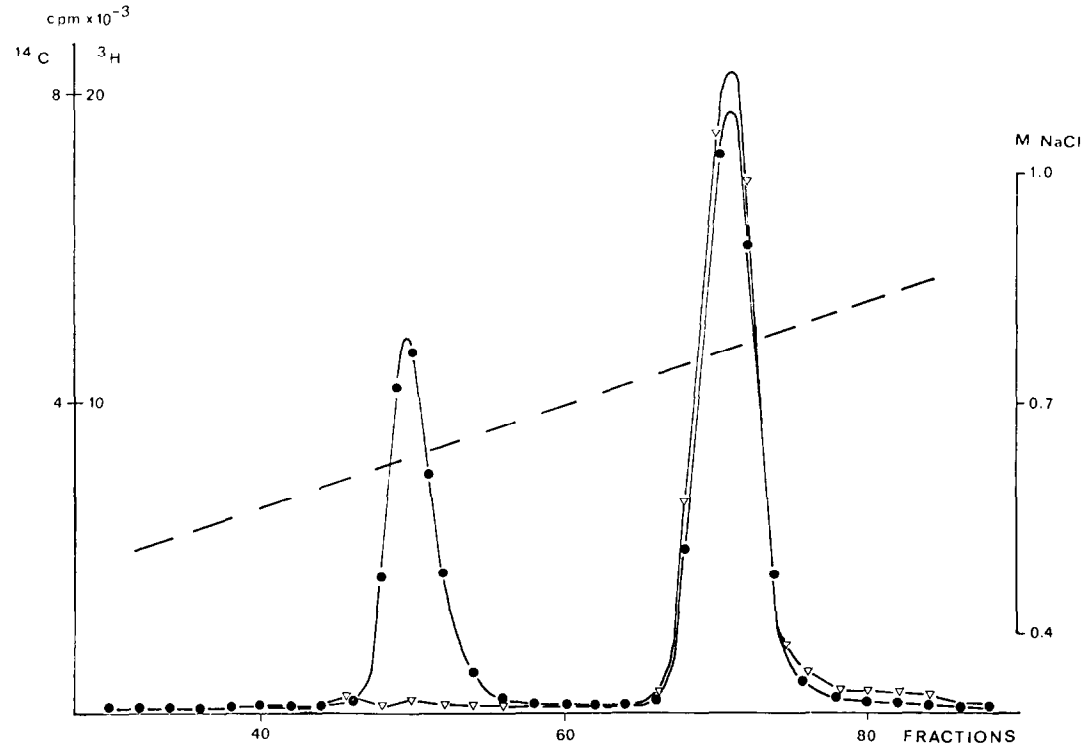

Fig.1. Co-chromatography of *B. subtilis* tRNA on a RPC-5 column under conditions as described [13]. Filled circles, [^{14}C]phenylalanyl-tRNA^{Phe} from cells grown in glucose salt medium under vigorous aeration. Open triangles, [^{3}H]phenylalanyl-tRNA^{Phe} from cells grown in maximal broth medium under normal aeration. Dashed line, sodium chloride gradient. Aminoacylation of the applied tRNA was carried out in 0.5 ml reaction mixture containing 100 mM Hepes, pH 8.0, 20 mM magnesium acetate, 20 mM potassium chloride, 1 mM ATP, 20 μM [^{14}C]phenylalanine (520 Ci/mol) or [^{3}H]phenylalanine (2 Ci/mmol) 3–5 A_{260} units tRNA and sufficient amounts of S100 enzyme extracts. Incubation was for 30 min at 37°C.

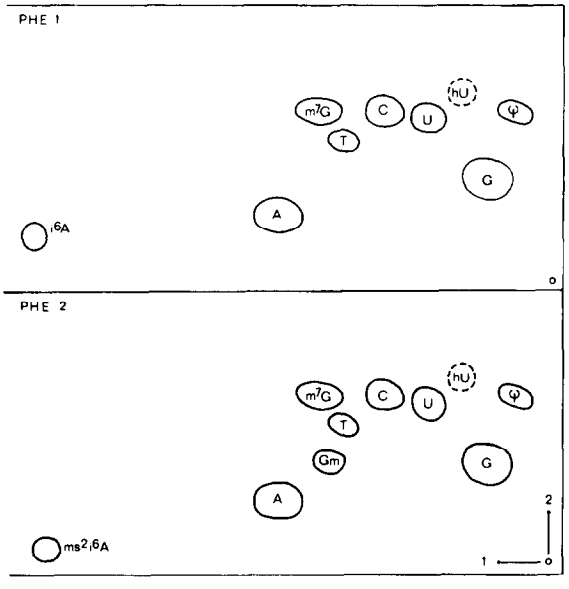

Fig.2. Chromatographies on cellulose thin-layer plates of the nucleosides from tRNA$_{I}^{Phe}$ and tRNA$_{II}^{Phe}$ of *B. subtilis*. Digestion and chromatographic conditions are described under Materials and methods. First dimension, right to left. Second dimension, bottom to top. All spots except ribose 2′-*O*-methylated guanosine and ms^{2}i^{6}A or i^{6}A were found in equivalent amounts in both tRNA digests. The phenylalanine specific tRNAs contain 22–24 guanosines, 12–14 adenosines, about 20 cytidines, about 12 uridines, 1 ribothymidine, 2 pseudouridines, 1 m^7-methylguanosine, 2 dihydrouridines. The tRNA$_{I}^{Phe}$ contains in addition 1 isopentenyladenosine, tRNA$_{II}^{Phe}$ contains ms^{2}i^{6}A instead of i^{6}A and in addition 1 ribose 2′-*O*-methylated guanosine.

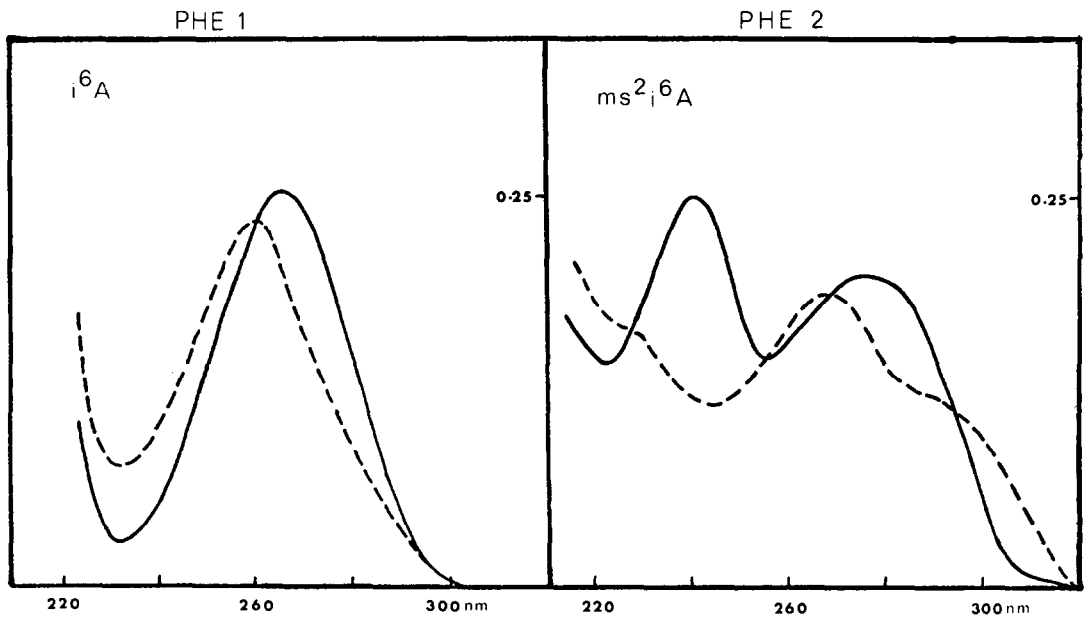

Fig.3. Ultraviolet absorption spectra of i^6A and ms^2i^6A isolated from $tRNA_I^{Phe}$ and $tRNA_{II}^{Phe}$ respectively. Full lines pH 13, dotted lines, pH 1.0.

be seen from the ultraviolet spectra, at pH 1 and pH 13, determined after elution from the thin layer chromatographic plate (fig.3). The numbers of the other nucleoside components seem to be equivalent in both phenylalanine tRNAs (results are given in the legend to fig.2).

4. Discussion

The conditions under which $tRNA_I^{Phe}$ accumulates in *B. subtilis* have been studied in detail and will be published elsewhere.

The results presented here indicate that $tRNA_I^{Phe}$ differs from $tRNA_{II}^{Phe}$ in the extent of post-transcriptional modifications. Whereas the normally abundant species $tRNA_{II}^{Phe}$ contains eight modified nucleosides, $tRNA_I^{Phe}$ is lacking the methylation of a ribose moiety of guanosine and the thiomethyl group of ms^2i^6A. As we can conclude from our analysis the composition of the other nucleosides appears to be not different in both phenylalanine tRNAs. The $tRNA_I^{Phe}$ can therefore be considered as an under-modified intermediate in the biosynthesis of $tRNA_{II}^{Phe}$. Whether both tRNA species are really the product of the same gene has to be proven by direct sequence analysis which is underway.

Our data agree well with the results described by Singhal and Vold, who have found varying amounts of methyl groups and an unaltered net composition of total tRNA of *B. subtilis* at different growth phases [23]. The difference between the two tyrosine specific tRNAs was restricted exclusively to the modification of i^6A to ms^2i^6A [16]. For the two phenylalanine isoacceptors we have found an additional lack of modification concerning a ribose methylation of guanosine. Both nucleosides which have not become modified are located in the anticodon loop of $tRNA_I^{Phe}$. This is concluded from the oligonucleotide A–C–U–Gm–A–A–ms^2i^6A–A–Ψ–C–C–G obtained from a RNAase T_1 digest of $tRNA_{II}^{Phe}$, which contains the anticodon sequence for phenylalanine, Gm–A–A (preliminary results of sequence studies).

The absence of both modifications increases strongly the polarity of $tRNA_I^{Phe}$ in comparison to $tRNA_{II}^{Phe}$ as can be seen from the early elution position on BD-cellulose [17] or RPC-5 columns (fig.1). Whether this altered property of $tRNA^{Phe}$ has any influence on its function in protein synthesis has yet not been examined.

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