CULTURE CONDITION DEPENDENT CHANGES OF 4-THIOURIDINE IN THE TRANSFER RNA OF *AZOTOBACTER VINELANDII*

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ABSTRACT

4-Thiouridine, a thionucleoside present in the transfer RNA of the free living, nitrogen-fixing bacterium *Azotobacter vinelandii* shows a culture condition dependent change. When the bacterium is grown in the absence of any fixed nitrogen the tRNA contains 4-thiouridine to the extent of 45% of the total sulphur incorporated. This gets reduced to 5% when the bacterium is grown in the presence of excess of ammonium salt. Instead, a new thionucleoside which appears to be a derivative of 4-thiouridine is found in the tRNA to the extent of 28% of the total sulphur incorporated.

INTRODUCTION

THE metabolism of some of the modified nucleosides in the tRNA of microorganisms has been found to be sensitive to a variety of culture conditions in that their relative proportions vary with the conditions. The culture conditions that have been found to influence the level of modification include degree of aeration, temperature of growth, different phases of growth and availability of nutrients[1-11]. Thionucleosides which have been reported to undergo culture condition dependent changes include 2-methylthioisopentenyladenosine (ms^2i^6A),[1,2,4,9,10] 2-methylthioribosylzeatin (ms^2io^6A),[3,6] 5-methyl-2-thiouridine (m^5s^2U)[7], and 5-methyl aminomethyl-2-thiouridine (mnm^5s^2U)[8]. 4-Thiouridine (s^4U), widely distributed in the tRNA of bacteria has not so far been reported to change in its relative proportions dependent on culture conditions. Besides, no other derivative of 4-thiouridine has so far been shown to be present in tRNA although several derivatives of 2-thiouridine are known. This paper gives the first report of the change in the relative proportion of 4-thiouridine in the tRNA of the free-living, nitrogen fixing bacterium *Azotobacter vinelandii* dependent upon culture conditions.

MATERIALS AND METHODS

Carrier-free $H_2{}^{35}SO_4$ was obtained from Bhabha Atomic Research Centre, Bombay, India. Thin layer microcrystalline cellulose plates were from Macherey-Nagel, West Germany. 4-Thiouridine-5′-phosphate was from Sigma Chemical Company, St. Louis, U.S.A.

A. vinelandii (OP) Wisconsin strain was grown in Burk's modified nitrogen-free medium[12]. For growth in the presence of ammonium salt, 2.8 mg N/ml equivalent of ammonium chloride was added to the medium. The concentration of ammonium salt used in the present studies has been shown to be sufficient to completely repress the nitrogenase genes[13]. Radioactive medium contained all the normal ingredients with only one-tenth of the normal amount of sulphate, in addition to 5mCi of $H_2{}^{35}SO_4$ per 50 ml medium. Cells were grown as shake culture at 30°C to late log phase and harvested. ^{35}S-Labelled total tRNA was isolated, purified, deacylated and converted to nucleotides as described earlier[11].

High voltage paper electrophoresis of the ^{35}S-nucleotides was carried out on Whatman 3 MM paper in acetic acid-pyridine-EDTA buffer, pH 3.5 (5% acetic acid containing 5 mM disodium EDTA adjusted to pH 3.5 with pyridine), at 60 V/cm of the paper using xylene cyanol and acid fuchsin as dye markers[14]. The nucleotides were eluted out of the paper using water according to the method of Heppel[15]. The nucleotides were converted into nucleosides using bacterial alkaline phosphatase. The radioactive nucleotides were detected by autoradiography and their relative proportions were determined by cutting and counting. R_f values of the thionucleosides were determined on Whatman 3 MM paper in different solvent systems. Spectra of tRNA samples were taken in the range 300–360 nm in 10 mM tris-HCl buffer, pH 7.0, using Shimadzu UV-180 recording spectrophotometer.

RESULTS

High voltage paper electrophoresis of ^{35}S-labelled nucleotides from the tRNA of *A. vinelandii* cells grown

in the absence of ammonium salt revealed four prominent spots (figure 1A). The relative proportion of the spots 1, 2, 3 and 4 amounted to 24.7%, 7.8%, 13.5% and 45.1% respectively (table 1). The electrophoretic pattern of the thionucleotides from the tRNA of the

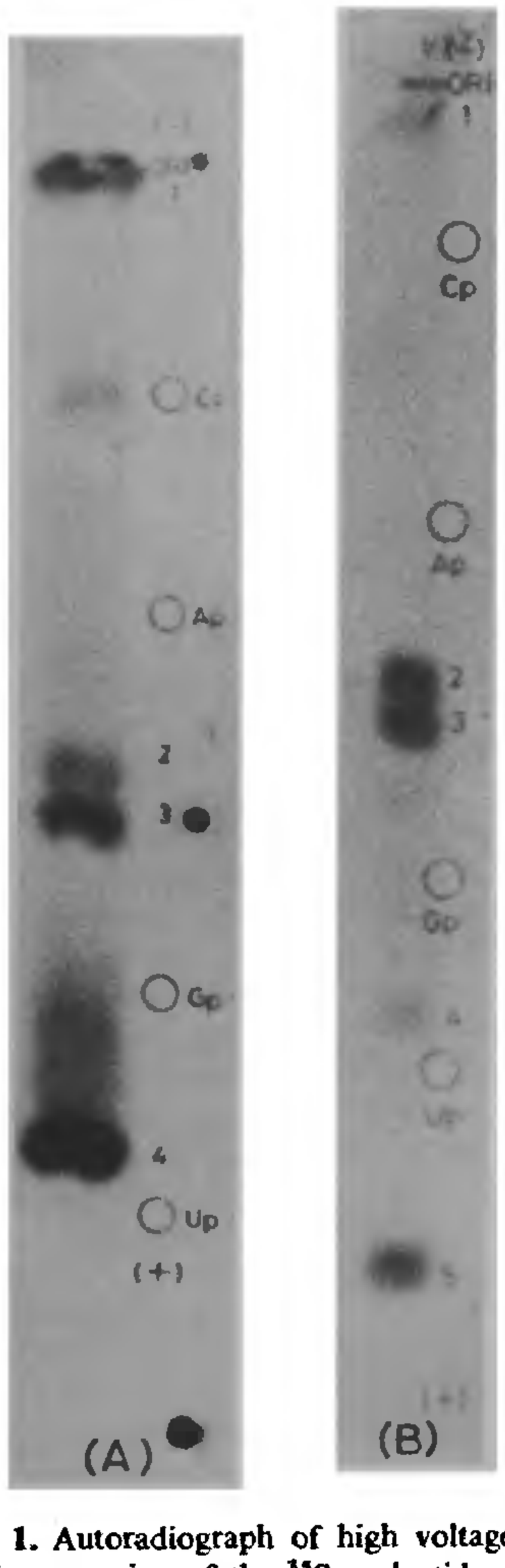

Figure 1. Autoradiograph of high voltage electrophoretic separation of the ^{35}S-nucleotides from the tRNA of *A. vinelandii* cells grown in the absence of ammonium salt (A) as well as presence of excess of ammonium salt (B). ^{35}S-thionucleotides from the two samples of tRNA were subjected to electrophoresis at pH 3.5, Whatman 3 MM paper at 60 volts per cm.

Table 1 *Relative proportions of the thionucleotides in the tRNA of A. vinelandii cells grown in the absence as well as the presence of ammonium salt*

Thionucleotide	Percent of total sulphur incorporated: in the absence of ammonium salt	Percent of total sulphur incorporated: in the presence of ammonium salt
5-Methylamino-methyl-2-thio-uridine-3′-phosphate (mnm^5s^2Up)	24.7	4.3
2-Methylthioiso-pentenyl-adenosine-3′-phosphate (ms^2i^6Ap)	7.8	29.0
2-Methylthiozeatin ri-botide (ms^2io^6Ap)	13.5	29.0
4-Thiouridine-3′-phosphate (s^4Up)	45.1	5.1
Xp (unidentified)	—	28.4

^{35}S-Labelled tRNA samples prepared from cells grown under the two conditions were digested with RNase T_2 and subjected to high voltage paper electrophoresis. The radioactive spots were cut and counted. Several experiments were carried out. Essentially the same results were obtained in all cases. The results of a representative experiment are presented.

bacteria grown in the presence of excess of ammonium salt was entirely different. The most striking difference was the drastic decrease in the proportion of the 4th spot and the appearance of a new thionucleotide having mobility higher than that of Up (figure 1B). The relative proportions of the other thionucleotides were also different. They amounted to 4.3%, 29%, 29%, 5.1% and 28% respectively for spots 1 to 5 (table 1). The specific activities of the sulphur incorporated under the two conditions were almost equal, 56000 CPM per A_{260} unit of tRNA. The decrease in the relative proportion of the 4th spot and the appearance of the fifth spot suggested a possible relationship between them.

Comparison of the two electrophoretic patterns of the thionucleotides of the tRNA with a standard pattern[8] indicated spots 1 to 4 to be 5-methylaminomethyl-2-thiourdine-3′-phosphate ($mnm^5 s^2U_p$), 2-methylthioisopentenyl adenosine-3′-phosphate (ms^2i^6Ap), 2-methylthiozeatinribotide (ms^2io^6Ap), and 4-thiouridine-3′-phosphate (s^4Up) respectively. The fifth spot represented an unknown thionucleotide. A change in s^4Up, dependent upon culture conditions, was quite unexpected. Hence a detailed study of the 4th and the 5th spots was carried out. Data on further characterisation of the spots 1 to 3

and their culture condition dependent variations are being published elsewhere. Spots No. 4 and 5 (figure 1) were eluted out of the paper and converted to nucleosides using bacterial alkaline phosphatase. The nucleoside corresponding to spot No. 4 was subjected to paper chromatography along with an authentic sample of 4-thiouridine prepared by the dephosphorylation of s^4Up. Radioactivity in the spot was found to comigrate with the authentic sample of 4-thiouridine in all the three solvent systems tried (table 2). This established the fourth spot to be s^4Up. The nucleoside corresponding to spot No. 5 gave R_f values of 0.49 in the solvent system C (table 2) and 0.26 in another solvent system, isobutyric acid: 0.5 M ammonium hydroxide, 5:3 (v/v) which showed a close correspondence with the R_f values reported for uridine-5-oxyacetic acid, 0.43 and 0.32 respectively in the above two solvent systems[16]. This indicated the possibility that the spot No. 5 might be a derivative of uridine-5-oxyacetic acid. However further characterisation is required to come to a definite conclusion.

Transfer RNA containing s^4U show a characteristic absorption at 338 nm[17]. No other nucleotide has been reported to have absorption in this region. The spectrum of a sample of tRNA (50 A_{260} units per ml) prepared from cells grown in the nitrogen-free medium taken in the 300–360 nm range showed the characteristic absorption at 338 nm as expected. It was quite surprising that the tRNA prepared from cells grown in the presence of ammonium salt also gave the characteristic absorption at 338 nm (figure 2). The intensity of absorption at 338 nm of this sample was at least five to seven times greater than that expected from the proportion of 5% of s^4U in the sample (table 3). From the absorbance data the proportion of s^4U in the tRNA from cells grown in the presence of ammonium salt was about 80% of that present in the tRNA from cells grown in the absence of ammonium salt. On the other hand, from the incorporation experiments the proportion of s^4U in the former was only about 5% (table 1). Since the specific activities of the sulphur incorporated in the two cases were almost the same the counts in each spot could very well be compared. These results suggested the presence of a 4-thiouridine derivative other than 4-thiouridine itself. The unknown 5th spot (figure 1B) may be this derivative. As

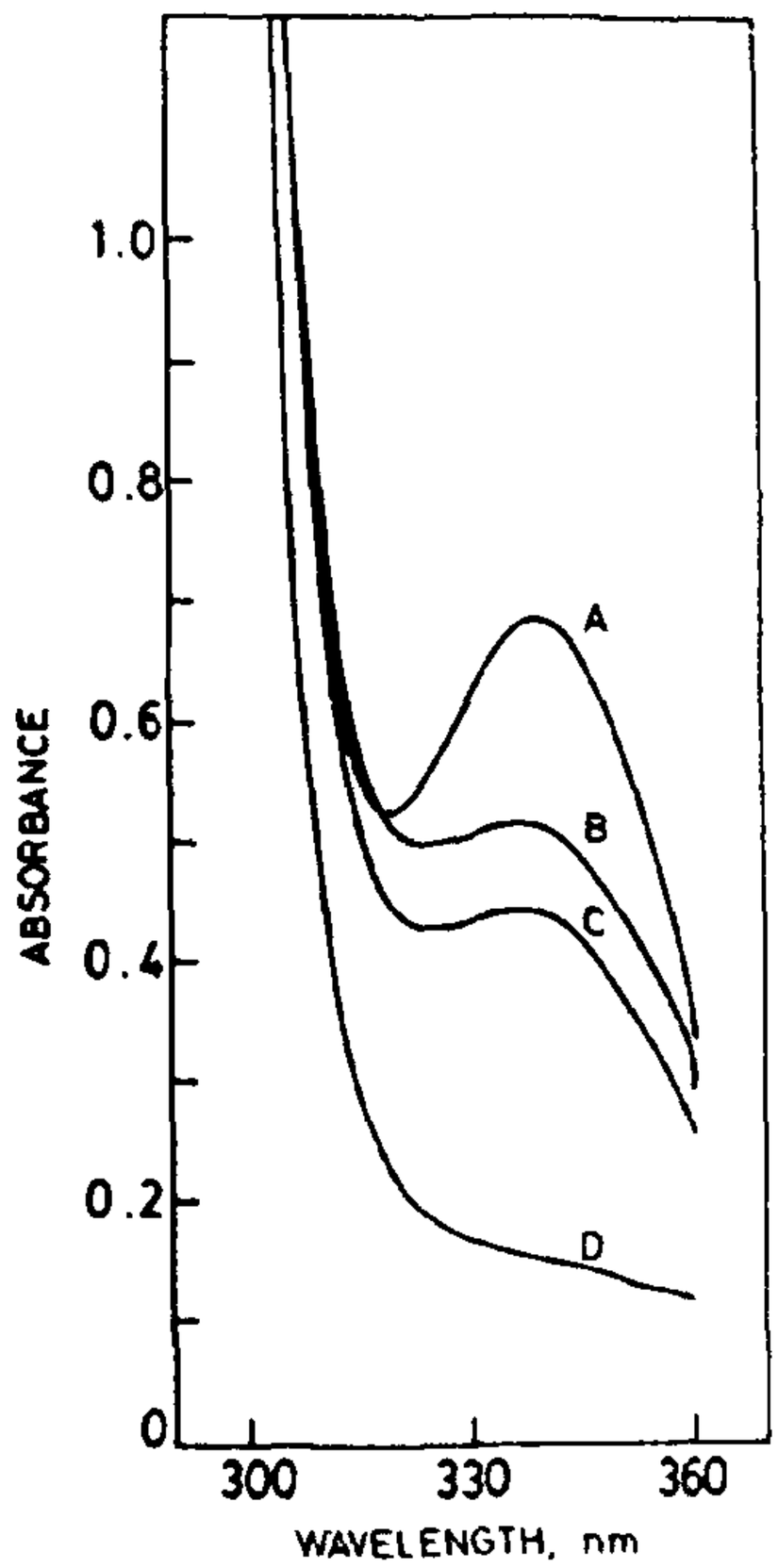

Figure 2. Absorption spectra of the total tRNA of *A. vinelandii* cells grown in the absence as well as presence of ammonium salt. Spectra of total tRNA from *E. coli* and chick embryo were taken for comparison.

Fifty A_{260} units of the four tRNA samples in 0.01 M Tris-HCl buffer, pH 7.4, were used for the spectral measurement in the range 300 to 360 nm. **A.** *E. coli* tRNA. **B.** *A. vinelandii* tRNA from cells grown in the absence of ammonium salt. **C.** *A. vinelandii* tRNA from cells grown in the presence of ammonium salt. **D.** Chick embryo tRNA.

Table 2 *R_f values of the nucleoside corresponding to spot No. 4 (figure 1A) in three solvent systems*

Nucleoside	R_f value (Solvent systems) A	B	C
Thionucleoside corresponding to spot No. 4 of figure 1A	0.58	0.55	0.68
Authentic sample of 4-thiouridine	0.60	0.53	0.71

Solvent systems: A—Isopropanol: Conc. NH_4OH: water, 7:1:2 (v/v/v); B—*n*-butanol: water, 86:14 (v/v/); C—isopropanol: 1% ammonium sulphate, 2:1 (v/v)

the fifth spot was present in very small quantities and in the absence of an authentic sample its further characterization could not be done.

When *A. vinelandii* cells labelled with radioactive sulphate in the presence of excess of ammonium salt was transferred to the nitrogen-free medium and grown for five generations, the radioactivity in the fifth spot was reduced from 28% to 4%, while the proportion of s^4Up spot was increased from 4% to 32%. On the other hand, in the reverse experiment in which the cells were first labelled in the absence of ammonium salt in the medium and then grown for five generations in the medium containing ammonium salt, no change in the relative proportion of s^4Up and the fifth spot was noticed. These observations further suggested a relationship between s^4Up and the fifth thionucleotide spot.

DISCUSSION

The experimental results presented here show that the thionucleotide pattern in the tRNA of *A. vinelandii* depends on the culture conditions. The thionucleotide which shows the maximum variation in this case is 4-thiouridine which has not so far been shown to undergo drastic changes in its relative proportion in an organism. The variation noted in the present studies is nearly ten-fold, 5% of ^{35}S-sulphur incorporated when the bacterium is grown in the presence of ammonium salt and 45% when it is grown in the absence of ammonium salt (table 1). Incorporation of sulphur per A_{260} unit of tRNA has been found to be almost the same (approximately 56000 CPM under the two experimental conditions used).

4-thiouridine is the only thionucleotide which has been shown to have absorption at 338 nm. The present studies suggest that *A. vinelandii* tRNA isolated from cells grown in the presence of ammonium salt contains a new thionucleotide which has absorption at 338 nm. As absorption at 338 nm is characteristic of 4-thiouridine, we speculate that the unknown thionucleotide is a 4-thiouridine derivative which has a higher electrophoretic mobility at pH 3.5 as compared to 4-thiouridine itself. It may be related to uridine-5-oxyacetic acid. Further work is required to identify the unknown thionucleoside.

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