Zeatin Ribonucleosides in the Transfer Ribonucleic Acid of 
*Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, 
*Corynebacterium fascians*, and *Erwinia amylovora*

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Until recently, the presence in transfer ribonucleic acid (tRNA) of the hydroxylated cytokinin ribosylzeatin [N^6-(4-hydroxy-3-methylbut-2-enyl)adenosine] was thought to be unique to higher plants. This extension of work from several laboratories indicates the presence of 2-methylthioribosylzeatin in the tRNA of the plant-associated bacteria *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, and *Corynebacterium fascians*, but not in that of *Erwinia amylovora*. This cytokinin has the cis configuration, as is normally found in the tRNA’s of plants. The tRNA thionucleotide patterns in these bacteria are different from those of *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium*, which contain the unhydroxylated analogs of ribosylzeatin or 2-methylthioribosylzeatin.

The N^6-substituted adenosines, named cytokinins, are found in the transfer ribonucleic acid (tRNA) of all classes of cells. The usual substitution is N^6-isopentenyl-adenosine (i^6A), found in yeast, animals, some plants, and most bacteria (5). In some bacteria, however, it is often further modified to 2-methylthio-i^6A (ms^2i^6A) (3). In higher plants, the isopentenyl side chain is hydroxylated, yielding N^6-(4-hydroxy-3-methylbut-2-enyl)adenosine (io^6A), also called ribosylzeatin (2, 6, 16, 18).

The cytokinins have a special function aside from their role in facilitating codon-anticodon interactions in tRNA. When administered exogenously to plants in various test systems, they stimulate growth, promote cell division, and stimulate germination and budding (5). The ability to hydroxylate the isopentenyl side chain of i^6A to form io^6A in the tRNA was long thought to be restricted to higher plants (2, 6, 16, 18). Recently, however, hydroxylated cytokinins have been reported in the tRNA of plant-associated bacteria. The presence of 2-methylthio-io^6A (ms^2io^6A) has been detected in *Pseudomonas aeruginosa* tRNA (17), and trans-io^6A has been detected in *Agrobacterium tumefaciens* tRNA (4). The latter is a plant pathogen, as are some species of *Pseudomonas*, although not *P. aeruginosa*. In the present study, we extend these observations to the tRNA of a few other plant-associated bacteria. We investigated the general thionucleoside pattern and the occurrence of ms^2io^6A in the tRNA of *Rhizobium leguminosarum*, *A. tumefaciens*, *Corynebacterium fascians*, and *Erwinia amylovora*. All of these species were found to contain the hydroxylated cytokinin ms^2io^6A except *E. amylovora*, which is also—curiously enough—the only one in the list that does not stimulate plant growth.

MATERIALS AND METHODS

The strains *E. amylovora* ATCC 19382, *R. leguminosarum* ATCC 10314, *A. tumefaciens* ATCC 15955, and *C. fascians* ATCC 12974 were from the American Type Culture Collection, Rockville, Md. *Escherichia coli* K-12 was obtained from Alan Peterkofsky. A sample of cis-ms^2io^6A was a gift from Nelson J. Leonard.

Preparation of 35S-labeled tRNA. The bacteria were grown in synthetic medium in which most of the sulfate was replaced with chloride so that the sulfate content was 2 to 4 mg/liter. Five to 10 mCi of carrier-free [35S]H$_2$SO$_4$ per liter was added after autoclaving. *E. amylovora*, *A. tumefaciens*, and *C. fascians* were grown in the medium used by Klambt et al. (7) for *C. fascians*, except that, in the case of *E. amylovora*, 50 mg of nicotinic acid per liter was also added (15). Growth was good in all instances. The growth medium for *R. leguminosarum* was that of Phillips and Torrey (11), with the modifications mentioned above. Culture flasks were shaken in the dark at room temperature (23 to 26°C), and the cells were harvested after 20 to 30 h. *E. coli* was grown at 37°C in minimal medium with a low sulfate concentration.

tRNA was isolated by extraction of the cells with

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phenol, followed by chromatography on diethylaminoethyl-cellulose (1). The sample eluted from diethylaminoethyl-cellulose was deacylated with trishydroxymethyl)aminomethane buffer (pH 8.8) for 30 min and filtered on a Sephadex G-100 column. Fractions containing tRNA were pooled and concentrated on small diethylaminoethyl-cellulose columns. Incorporation of $^{35}$S into these bacterial tRNA's was of the order of 200,000 to 400,000 cpm/mg, approximately $\frac{1}{10}$ that of E. coli tRNA grown under the same conditions. Carrier E. coli tRNA was added whenever necessary.

As a control for the inadvertent carry-over of the nucleoside $[^{35}$S]$ms^{2}io^{6}A$ from the culture medium into the final chromatography, labeled C. fascians tRNA, purified in the usual way, was hydrolyzed with alkali and neutralized, and the nucleotide digest was examined by paper electrophoresis. Less than 5% of the radioactivity migrated outside the nucleotide region (details not included).

Chromatography. The purified, deacylated radioactive tRNA was converted to nucleosides by alkaline hydrolysis and dephosphorylation, and the digest was chromatographed on a fresh phosphocellulose column (0.9 by 85 cm) as described earlier (13). In the chromatography pattern, the first radioactive peak would include any unconverted nucleotides, 2'-O-methyl dinucleoside monophosphates, if present, and free sulfate that might have escaped the purification procedure (see above). Thin-layer chromatography was performed on glass plates coated with cellulose or silica gel (Analtech Inc., Newark, Del.).

RESULTS

The chromatographic pattern of the nucleoside digest prepared from $^{35}$S-labeled tRNA of E. coli K-12 is used as a reference. The elution pattern from a phosphocellulose column shows that the four major ribonucleosides were well separated (Fig. 1a) and also indicates six peaks of radioactivity (Fig. 1b). The components of the first (see above) and last peaks are not known; the others were earlier identified as (in order): 4-thiouridine (s$^{4}$U); ms$^{2}$i$^{6}$A; 2-thiocytidine (s$^{2}$C); and 5-methylaminomethyl-2-thiouridine (s$^{2}$U*) (13). The elution position of ms-ribosylzeatin is shown by the arrow.

The thionucleoside pattern of E. amylovora (Fig. 1c) was somewhat similar to that of E. coli, except for a minor peak eluting after the s$^{4}$U peak. There is no radioactive peak in the position of ms$^{2}$io$^{6}$A, and the Rf of the unknown material eluting just after s$^{4}$U was different from that of ms$^{2}$io$^{6}$A on thin-layer chromatography in four systems.

In the tRNA's of R. leguminosarum, A. tumefaciens, and C. fascians (Fig. 2), ms$^{2}$io$^{6}$A was present as one of the major thionucleoside species. R. japonicum gave similar results (data not included). The radioactive material eluting ahead of guanosine (see arrow) moved with an authentic sample of cis-ms$^{2}$io$^{6}$A upon thin-layer chromatography in four solvent systems (Table 1). Since solvent system D separates the geometrical isomers of ribosylzeatins (12), the identical Rf values of the radioactivity and known ms-ribosyl-cis-zeatin suggest the presence of the cis isomer in all three bacterial tRNA's.

Comparison of the phosphocellulose chromatographic patterns indicated the presence of s$^{4}$U, ms$^{2}$i$^{6}$A, s$^{2}$C, and s$^{2}$U* in E. amylovora, R. leguminosarum, and A. tumefaciens, although the relative proportions of the various thionucleosides differed (Fig. 1 and 2). C. fascians tRNA lacked all the thionucleosides present in E. coli except s$^{4}$U (Fig. 2d), the major thionucleoside in E. coli, Bacillus subtilis, and Salmonella typhimurium (8, 14). This pattern was obtained in at least six experiments. The pat-
tern for *R. leguminosarum* showed the presence of not only the five known thionucleosides, but possibly as many as five additional minor components.

**DISCUSSION**

It is clear from the present studies that not all plant-pathogenic bacteria contain ms\(^{3}i\)A in their tRNA. It is interesting that this constituent is present in the tRNA of all three bacteria which promote growth in plants: *R. leguminosarum* is well known for its symbiotic action on plants, *A. tumefaciens* is well known for induction of plant tumors, and *C. fascians* is well known for producing abnormal growth in plants. *E. amylovora*, on the other hand, lacks ms\(^{3}i\)A in its tRNA and also retards plant growth through fire blight disease. Obviously, the series must be extended before this correlation can be validly made.

Evidence obtained in the present studies shows that the cis isomer of ms\(^{3}i\)A is present in the tRNA of plant-associated bacteria. The cis isomer of i\(^{3}\)A is normally found in plant tRNA, although the presence of both isomers has been reported (18). The studies of Chapman et al. (4) show that the nonthiolated ribosylzeatin in *A. tumefaciens* tRNA is of the trans configuration. At present it is not clear whether both isomers of thiolated and nonthiolated ribosylzeatins are present in the tRNA from plant-associated bacteria.

**ACKNOWLEDGMENTS**

We are grateful to N. J. Leonard for a sample of synthetic cis-ms\(^{3}i\)A.

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**LITERATURE CITED**


**TABLE 1.** R\(_{t}\) values of ms-ribosylzeatin peak

<table>
<thead>
<tr>
<th>Organism</th>
<th>R(_{t}) value</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
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<tbody>
<tr>
<td><em>R. leguminosarum</em></td>
<td>0.84</td>
<td>0.91</td>
<td>0.87</td>
<td>0.28</td>
<td></td>
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<tr>
<td><em>A. tumefaciens</em></td>
<td>0.85</td>
<td>0.92</td>
<td>0.88</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td><em>C. fascians</em></td>
<td>0.83</td>
<td>0.90</td>
<td>0.90</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

* The fractions under the radioactivity peak marked by arrow (Fig. 2) were pooled, evaporated to dryness under vacuum, and dissolved in a small volume of water. Portions were subjected to thin-layer chromatography along with the authentic sample. The matrix at the ultraviolet spot was scraped off and counted in a liquid scintillation counter. Solvent systems used with cellulose were: (A) isopropanol–concentrated NH\(_{4}\)OH–water (7:1:2, vol/vol/vol); (B) ethanol–1.0 M NH\(_{4}\) acetate, pH 7.5 (7:3, vol/vol); (C) n-butanol–water (86:14, vol/vol). The solvent system used with silica gel was (D) chloroform–methanol (9:1, vol/vol).

[FIG. 2. Chromatography of \(^{35}S\)-labeled nucleosides from tRNA of different bacteria. Chromatography was performed as described in the legend to Fig. 1, except that less radioactivity was used with *C. fascians* tRNA. (a) Absorbance at 260 nm (A\(_{260}\)); (b) radioactivity for *R. leguminosarum*; (c) radioactivity for *A. tumefaciens*; (d) radioactivity for *C. fascians*.]


