TRANS-ZEATIN IN CULTURE FILTRATES OF *AGROBACTERIUM TUMEFACIENS*

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SUMMARY:

Cytokinin-active bases and nucleosides have been isolated from the culture filtrates of *Agrobacterium tumefaciens* by trace enrichment onto octadecyl-silica and have been identified by GLC-MS of their permethyl and trimethylsilyl derivatives. Besides the expected 6-(3-methylbut-2-enylamino) purine, the filtrate contained zeatin (85% *trans*, 15% *cis*), 2-methylthio-ribosylzeatin and smaller quantities of ribosylzeatin and other cytokinin-active nucleosides.

INTRODUCTION:

The production of cytokinins by plant pathogenic or symbiotic bacteria is now well documented. *Rhizobium* strains are known to produce a zeatin-like substance (1) and *Corynebacterium fascians* produces two cytokinins identified as i^6Ade and *cis*-zeatin(*c*-io^6Ade) (2,3). Two reports have appeared (4,5) suggesting that the plant pathogen *Agrobacterium tumefaciens*, the causative agent of crown-gall disease, produces i^6Ade. In both cases, identification was based upon chromatographic data and the presence of biological activity. More recently, however (6), there has been a suggestion that the cytokinin produced by *A. tumefaciens* resembles *trans*-ribosylzeatin (*t*-io^6Ado) rather than i^6Ade in its effects upon a specific moss bioassay.

ABBREVIATIONS:

HPLC, high performance liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; f^6Ade, 6-furfurylamino purine; i^6Ade, 6-(3-methylbut-2-enylamino) purine; *c*-io^6Ade, *t*-io^6Ade, *cis*- and *trans*-6-(4-hydroxy-3-methylbut-2-enylamino) purine (*cis*- and *trans*-zeatin); ms^2i^6Ade, 6-(3-methylbut-2-enylamino)-2-methylthio-purine; ms^2io^6Ade, 6-(4-hydroxy-3-methylbut-2-enylamino-2-methylthio purine; i^6Ado and ms^2io^6Ado are the corresponding 9-β-ribofuranosides of the above compounds.

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We have demonstrated recently (7) that the tRNA of *A. tumefaciens*, contains *trans*-ribosylzeatin, and we now report that *trans*-zeatin (io^6Ade), i^6Ade, ms^2io^6Ado, and a variety of other cytokinins may be isolated from culture filtrates of this organism.

MATERIALS AND METHODS:

Growth and harvest of cultures. *Agrobacterium tumefaciens* (Strain C58, virulent, plasmid-positive) was grown to early stationary phase (24 hours) in a defined medium modified from that of Valera and Alexander (8) by the use of sucrose (15 g l^{-1}) as carbon source, by the addition of biotin (1 mg l^{-1}) and by increasing the $(NH_4)_2SO_4$ to 2 g l^{-1}. The cells were harvested to give a cell-free culture filtrate and a cell pellet from which tRNA was prepared.

Isolation of cytokinins. Cytokinins in the culture filtrate (48.5 l) were extracted by trace-enrichment on C_{18}/Porasil B, fractionated on μBondapak/C_{18} (Waters Assoc., Waltham, Mass.) and bioassayed as described previously (9). The material in fractions containing biological activity was permethylated or trimethylsilylated (10,11) and examined by GLC-MS.

In order to distinguish between *cis* and *trans*-zeatin, the permethylated isomers were separated by capillary GLC and identified in the mass spectrometer.

Transfer RNA was prepared from the cell pellet and hydrolyzed in the usual way (7). Cytokinins in the hydrolysate were identified by the above techniques.

RESULTS:

Trace enrichment, HPLC upon microparticulate octadecyl-substituted silica and subsequent bioassay revealed that both the culture filtrate and tRNA of *A. tumefaciens* contained cytokinins (Fig. 1). Three peaks of cytokinin activity were present in the chromatogram from the culture filtrate (Fig. 1A). They corresponded in mobility (see Fig. 1C) to authentic io^6Ade and io^6Ado (Fraction A1), i^6Ade, and i^6Ado (Fraction A2) and ms^2i^6Ado (Fraction A3). The chromatogram of the tRNA hydrolysate (Fig. 1B) contained one prominent peak of activity (Fraction B2) which did not correspond in mobility to any of the standards. Other minor peaks of activity were present and corresponded in mobility to ribosyl zeatin (io^6Ado, B1), i^6Ado (B3), and ms^2i^6Ado (B4).

Permethylation followed by GLC-MS showed that fraction A1 contained

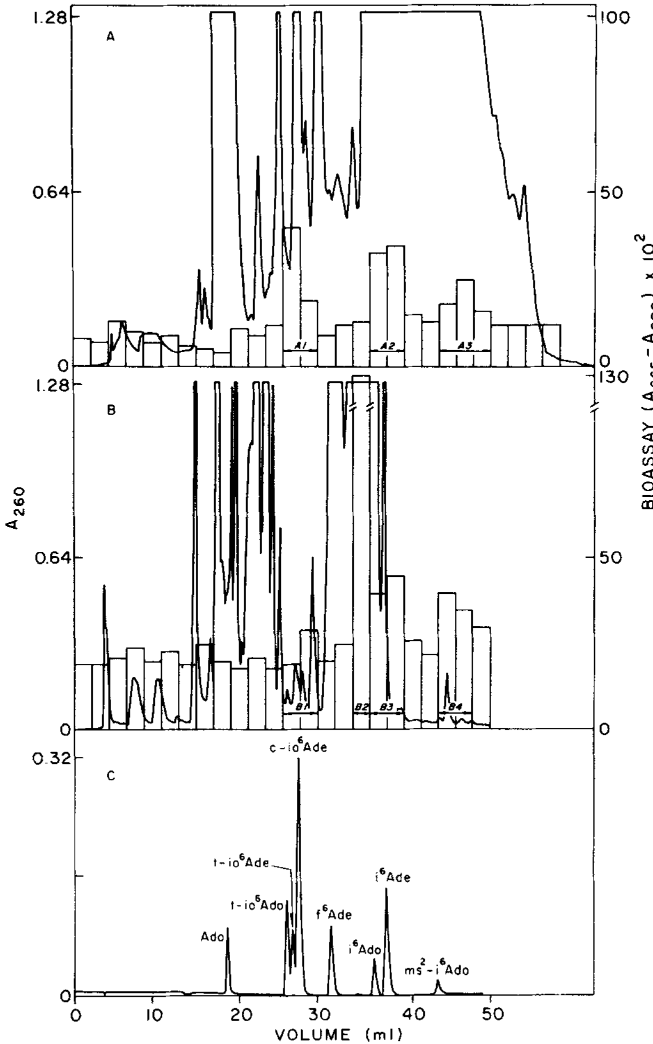

Fig. 1. High performance liquid chromatography of cytokinins from culture filtrate and tRNA hydrolysate on μBondapak/C_{18}.

A. Cytokinins from culture filtrate.
B. Cytokinins from tRNA hydrolysate.
C. Standard cytokinin mixture.

two known cytokinins: io^{6}Ade (whose mass spectrum is shown in Fig. 2A) and io^{6}Ado. In order to determine which geometric isomer of io^{6}Ade was present, an aliquot of A1 was permethylated and subjected to GLC-MS on a Dexsil 300 support-coated open glass capillary. The base peak of the

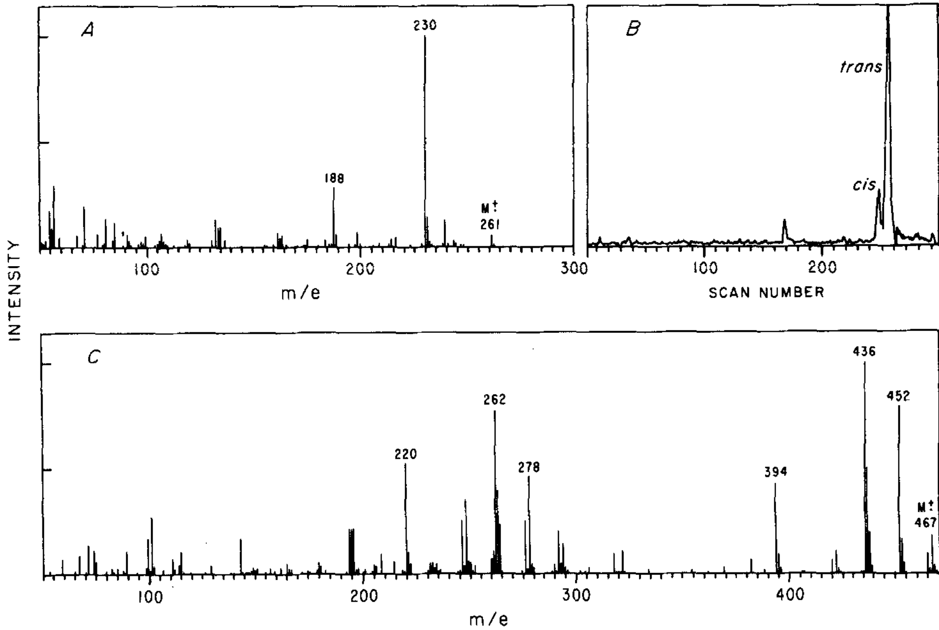

Fig. 2. A. Mass spectrum of permethylated zeatin (io^{6}Ade) from culture filtrate, fraction A1.
B. Reconstructed mass chromatogram of permethylated zeatin showing *cis*- and *trans*-isomers. m/e = 230.
C. Mass spectrum of permethylated ms^{2}io^{6}Ado, Fraction B2.

permethyl zeatin mass spectrum (m/e = 230) was monitored as a function of time giving the reconstructed mass chromatogram shown in Fig. 2B. Two peaks were present having retention times and mass spectra identical to authentic *cis*- and *trans*-zeatin, respectively. Integration of the peak areas showed that the *trans*-isomer predominated (85%).

Fraction A2 contained two components with GLC retention times and mass spectra (not shown) identical to those of authentic i^{6}Ade and i^{6}Ado. In addition, a third component was present which was also present in fraction B2 of the tRNA hydrolysate. Its mass spectrum is shown in Fig. 2C. It had a molecular ion at m/e = 467 and major fragment ions at m/e = 452 (-$CH_3^{\bullet}$), 436 (-$OCH_3^{\bullet}$), 394 (side chain cyclization), 378 (-$CH_3^{\bullet}$ from Base H^+) and 262 (-$OCH_3^{\bullet}$ from BH^+). The fragmentation pattern was almost

TABLE 1. Summary of the occurrence of cytokinins in tRNA and culture filtrates of *A. tumefaciens*

Cytokinin	Source: tRNA[1] mole %	Culture Filtrate[2] μg/g cells
i^6Ade	--	0.28
i^6Ado	0.11	0.09
c-io^6Ade	--	0.036
t-io^6Ade	--	0.204
t-io^6Ado	0.011	0.08
ms^2i^6Ado	0.015	0.02
ms^2io^6Ado	0.048	0.21

[1]Yield of tRNA from 45.87 g cells = 80.3 mg.
[2]One liter of medium gave 1.01 g of cells.

identical to that of io^6Ado except that major fragment ions were shifted upward by 46 mass units, the mass of a methylthio group. The same phenomenon is seen when the spectra of ms^2i^6Ado is compared to that of i^6Ado. The fragmentation pattern together with the expected facile loss of $CH_3^{\bullet}$ from $M^{\ddagger}$ to give m/e = 452 indicated the presence of ms^2io^6Ado both in the culture filtrate and tRNA.

The remaining fractions contained the following cytokinins, identified on the basis of gas chromatographic retention times and mass spectra. A3, ms^2i^6Ado; B1, *t*-io^6Ado; B3, i^6Ado; B4, ms^2i^6Ado. The level of each cytokinin was estimated by integration of peak areas with results presented in Table 1. It will be noted that the predominant cytokinins of the culture filtrate were i^6Ade, *t*-io^6Ade, and ms^2io^6Ado which, to-

gether, made up 75% of the total present. Not all cytokinins were represented, however, since neither ms^2i^6Ade was found nor was ms^2io^6Ade present.

DISCUSSION:

The presence of *trans*-zeatin in the culture filtrate of *A. tumefaciens* confirms that this prokaryote, like *C. fascians* (3) can, hydroxylate the methylbutenyl side chain to produce zeatin. However, the two organisms differ in that *A. tumefaciens* produces primarily the *trans* isomer of zeatin whereas *C. fascians* produces primarily the *cis* isomer (3). Presumably, the reason lies in the specificity of the mixed function oxidases thought to catalyze side chain hydroxylation (12).

Besides *trans*-zeatin, *A. tumefaciens* strain C58 secretes significant amounts of i^6Ade, previously identified in strain B6 by chromatography and bioassay (4,5), and ms^2io^6Ado the free base of which (ms^2io^6Ade) has been found to be produced by *C. fascians* (13). The total free cytokinin concentration measured here is about 1 μg l^{-1}, comparable to the value reported for *C. fascians* (3).

The presence of ribosides in the free cytokinin population suggests that the ultimate source of these compounds is the tRNA. Free cytokinins might be produced by tRNA degradation following cell lysis either during isolation or growth, or they might be produced by normal catabolism of tRNA during log or stationary phase. The first possibility is not considered likely since stringent precautions were taken to avoid cell lysis during harvest. Furthermore, no significant decrease in viable cell count was observed during progression of late log phase cultures through stationary phase to harvest. With respect to normal tRNA catabolism, Hahn et al. (5) reported significant tRNA turnover in *A. tumefaciens* strain B6 and considered that it was sufficient to account for the i^6Ade produced. In strain C58, however, we have not observed any significant

tRNA turnover during stationary phase (unpublished experiments). Experiments designed to test for the presence of a tRNA-independent pathway as postulated by Burrows (12) are currently underway.

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