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Two *Agrobacterium tumefaciens* genes for cytokinin biosynthesis: Ti plasmid-coded isopentenyltransferases adapted for function in prokaryotic or eukaryotic cells

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Summary. *Tzs* and *ipt* are two Ti plasmid genes coding for proteins with isopentenyltransferase (IPT) activity in vitro. We cloned both genes for protein expression in *Escherichia coli* and in *Agrobacterium tumefaciens*, and we investigated differences between the two genes by analysing the properties of the proteins in vitro and in vivo. In vitro, extracts with *tzs* or *ipt*-coded proteins had high IPT activity, and the enzymes were identical in most properties. The most important difference was detected in vivo: the *tzs*-encoded protein was very active in cytokinin production, while the *ipt* protein required overexpression in order to obtain measurable activity in bacteria. In both cases, *trans*-zeatin was the major product of the gene activity. Formation of this cytokinin requires a hydroxylase function in addition to the IPT reaction. No such activity could be ascribed to *tzs* or *ipt*-encoded proteins in vitro or in vivo, but cytokinin hydroxylase activity was detected in cells and extracts of *E. coli*, regardless of the presence or absence of the cytokinin genes. Based on these results it is proposed that both genes code for a single enzyme activity (isopentenyltransferase), that the genes and proteins are adapted for function either in bacteria (*tzs*) or in transformed plant cells (*ipt*), and that in both prokaryotic and eukaryotic cells hydroxylation to *trans*-zeatin is a function contributed by host enzymes.

Key words: *Agrobacterium* – Ti plasmids – Cytokinin biosynthesis – Isopentenyltransferase – *trans*-zeatin

Introduction

Cytokinins are considered as important and essential growth regulators in plants. They are also produced and secreted by many microorganisms living in contact with plants (Morris 1986). Among the bacteria, *Agrobacterium tumefaciens* has been studied in most detail. It appears that Ti plasmids in these bacteria contain at least two genes responsible for cytokinin biosynthesis:

A. A gene in octopine and nopaline type plasmids, which is located in the T region, is transferred to plant cells during tumour induction, and results in cytokinin autonomy of the transformed cells. The protein product has been identified as isopentenyltransferase (IPT) which synthesizes iPMP from 5′-AMP and DMAPP (reaction A in Fig. 1), (Akiyoshi et al. 1984; Barry et al. 1984; Buchmann et al. 1985), and accordingly the gene has been named *ipt*.

B. A gene in nopaline type plasmids (e.g. T37 and C58) which is responsible for *trans*-Z secretion in *A. tumefaciens*, and is accordingly named *tzs*. At the time of discovery it was concluded from this phenotype that it might encode a *trans*-Z permease, a *cis*/*trans*-isomerase, or a cytokinin hydroxylase (reaction B in Fig. 1), (Regier and Morris 1982). Later analysis has shown that this locus is very close to the *vir* region and that it probably contains a single gene with a single protein product. This gene is functional in *Escherichia coli* (phenotype: *trans*-Z secretion). Both gene and protein share a high degree of homology with *ipt*, and, most importantly, the *tzs*-encoded protein also has IPT activity (Akiyoshi et al. 1985; Barry et al. 1985; Beaty et al. 1986).

The case of *tzs* raises an interesting question. The phenotype predicts that the protein either synthesizes *trans*-Z (by isomerization of *cis*-Z, or by hydroxylation of iP, iPA, or iPMP), or that it transports *trans*-Z into the medium (as a permease type enzyme). In vitro, however, the protein acts as IPT, just like the enzyme encoded in *ipt*. What then is the difference between *tzs* and *ipt*? Does *tzs* code for a bifunctional protein, while *ipt* does not? If so, it would seem most likely that the second function is a cytokinin hydroxylase which produces *trans*-Z from an iP-type cytokinin synthesized in the IPT reaction. This follows from the observation that the *tzs* phenotype is expressed in *E. coli*, an organism that has not previously been expected to encode a cytokinin hydroxylase activity (Buck et al. 1982; Janzer et al. 1982), although this would be necessary if the second function were a *cis*/*trans*-Z-isomerase or a zeatin permease.

We investigated the functions encoded in *tzs* and *ipt* in two ways: (i) in vitro, by comparison of the properties of the IPTs expressed in *E. coli*, and by searching for cytoki-

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Abbreviations: DMAPP, dimethylallylpyrophosphate; iP, isopentenyladenine; iPA, isopentenyladenosine; iPMP, isopentenyladenosine 5′-monophosphate; IPT, isopentenyltransferase; *trans*-Z, *trans*-zeatin

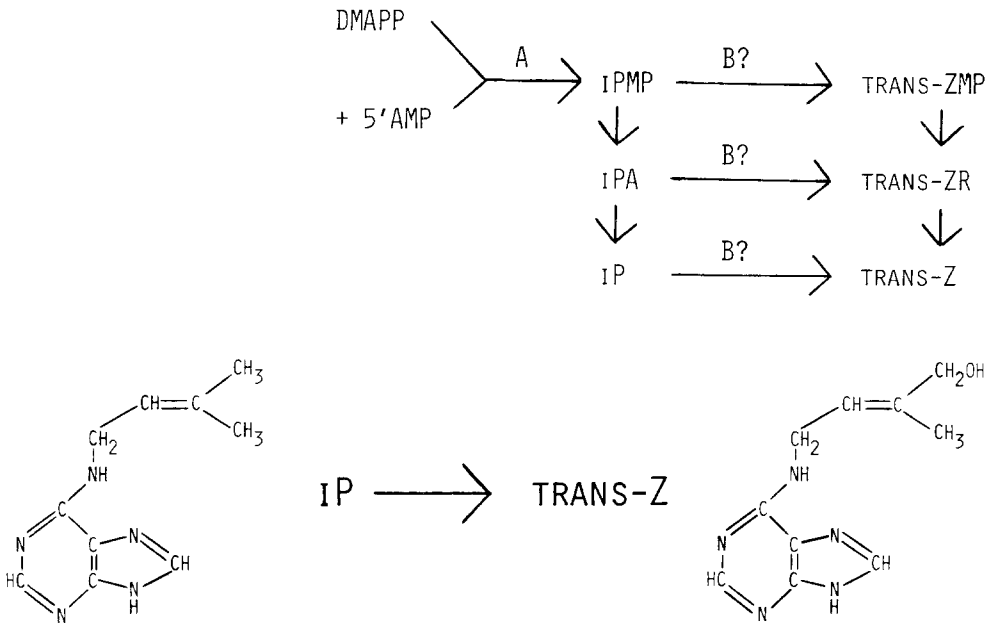

Fig. 1. Biosynthesis of cytokinins. A: Isopentenyltransferase (IPT); B: cytokinin hydroxylase; the *question marks* indicate that the precise substrates are not known. Abbreviations (according to Morris 1986): DMAPP: dimethylallylpyrophosphate, (Δ^2-isopentenylpyrophosphate); iPMP: N^6-(Δ^2-isopentenyl)-5'-AMP; iPA: N^6-(Δ^2-isopentenyl)-adenosine; iP: N^6-(Δ^2-isopentenyl)-adenine; ZMP: zeatin riboside 5'-monophosphate; ZR: zeatin riboside; Z: zeatin

nin hydroxylase activity; and (ii) in vivo, by analysis of *tzs* and *ipt*-induced cytokinin formation in *E. coli* and in *A. tumefaciens*. The results suggest that the proteins expressed from both genes have a single function, namely IPT activity, and that hydroxylation to *trans*-Z is a step performed by endogenous host-cell enzymes.

Materials and methods

Cloning and expression of genes. Table 1 summarizes the plasmids and strains, and it provides the references for those from other sources. Cloning procedures and miniscale plasmid analyses were performed as described (Maniatis et al. 1982), and enzymes were used as recommended by the manufacturers (Boehringer Mannheim; Bethesda Research Laboratories; New England Biolabs). Protocols for the minicell system and analysis of the proteins labelled with ^{35}S-methionine (37 TBq/mmol, Amersham Buchler) by sodium dodecylsulfate polyacrylamide gel electrophoresis have been published (Schröder et al. 1983). Oligonucleotides (SO1, SO2, Fig. 2) were synthesized by the method of Markham et al. (1980) and were phosphorylated prior to use with T4 polynucleotide kinase. DNA sequencing was performed on DNA fragments subcloned into phage M13 by the method of Sanger et al. (1977) with kits supplied by Amersham Buchler.

Cell-free enzyme extracts and assay for IPT activity. Pellets from 9 ml cultures (A_{660}, 1.0–1.2) were suspended in 0.625 ml buffer A (8 mM Tris-HCl, pH 8.0, 1.3 mM EDTA, 10 mM 2-mercaptoethanol, 27 mM KCl, 2.7 mM $MgCl_2$). After addition of lysozyme to 1 mg/ml and incubation for 10 min on ice, extracts were dialyzed for 4×0.5 h against 250 ml buffer B (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10% glycerol). They were then adjusted to pH 7.0 by adding 0.25 M HEPES-KOH to a final concentration of 50 mM, and assays were performed immediately. Protein concentrations were 2.5–2.8 mg/ml.

Incubations contained 10 µl enzyme extract, 10 µl [2-^{3}H] 5'-AMP (480 GBq/mmol, Amersham Buchler; 6×10^4 cpm, final concentration 0.2 µM), 10 µl 0.8 mM DMAPP, and 10 µl buffer B or other additions (as described in the text

Table 1. List of plasmids and bacterial strains

Plasmid	Relevant property	Reference
pGS207.2	*ipt* from Ach5 in pINIIA	Buchmann et al. (1985)
pGS208.2	*ipt* from C58 in pACYC184	Buchmann et al. (1985)
pDTi401.1	*ipt* from Ach5, overproducer	This report
pT159.7	*ipt* from Ach5 in pSUP104	This report
pT160.9	*ipt* from C58 in pSUP104	This report
pGV0348	*tzs* in large clone of C58 *Vir* region	Depicker et al. (1980)
pVIR18.3	*tzs* in subclone of pGV0348	This report
pVIR35b.9	*tzs* in subclone of pVIR18.3	This report
pVIR36.2	*tzs* in subclone of pVIR35b.9	This report
pVIR158.2	*tzs* from pVIR35b.9, in pSUP104	This report
pGS214.2	No cytokinin gene (control)[a]	Unpublished
pDTi503.2	No cytokinin gene (control)[a]	Unpublished
pGV2260	Derivative of Ach5 (T region deleted)	Deblaere et al. (1985)
pGV3850	Derivative of C58 (deletion in T region)	Zambryski et al. (1983)
Escherichia coli DS410	Minicell producer	Schröder et al. (1983)
APF1, APF2	Plasmid-free *Agrobacterium* strains	Hynes et al. (1985)

[a] Contain Ach5 T region DNA other than *ipt*

or legends to the figures). Standard assays contained 8 mM $MgCl_2$; they were performed at 30° C for 10 min and were stopped by heating for 5 min at 65° C which completely inactivates IPT.

Quantitative evaluation of IPT activity. The quantification was based on measuring the product of the IPT reaction as iP. Reactions were stopped by heating for 5 min at 65° C; then 4.4 Units of alkaline phosphatase (Boehringer Mannheim) and 20 µl of *E. coli* control extracts (free of IPT) were added, and the incubations were continued for 1 h at 37° C. In this time, the iPMP synthesized in the initial reaction was quantitatively converted to iP by removal of the phosphate group (phosphatase) and of the ribose (bacterial enzymes). Macromolecules were removed by isopropanol precipitation (Maniatis et al. 1982), and iP in the supernatant fluid was extracted by partitioning into ethylacetate. After concentration in vacuo to 50 µl, 3.5 µg each of unlabelled iP, adenine, and adenosine were added, and the samples were analyzed by TLC (silica plates with fluorescence indicator, Merck; solvent: chloroform: glacial acetic acid, 2:3, v/v). Under these conditions iP ran at R_f, 0.52, well separated from adenine (R_f, 0.30) and other non-identified radioactive reaction products (R_f, 0.1–0.25). The iP spot was identified under UV light, scraped off the plates, and the iP eluted with methanol was counted in 5 ml scintillation cocktail containing Triton X-100. The following comments should be noted. Recovery of radioactive iP added before starting the enzyme incubation was 90%–95% with this analysis procedure. No radioactive substance at the position of iP was detected with control extracts from *E. coli* cells free of cloned cytokinin genes. The procedure was developed for extracts containing high IPT activity (e.g. pDTi401.1 or pVIR35b.9). Simultaneous determination of *trans*-Z (R_f, 0.33) was not possible since it migrates close to adenine in the TLC system. This was not, however, a significant limitation, since further conversion of iP-type cytokinins to *trans*-Z occurs at negligible rates under standard assay conditions (see the results in Table 2A).

Cytokinin hydroxylation in vitro. Extracts were prepared by lysis of cells in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 5 mM 2-mercaptoethanol. They were dialyzed for 4×0.5 h against 20 mM Tris-HCl, pH 7.5, with 5 mM 2-mercaptoethanol, and used immediately. Incubations contained 90 µl extract and 1.1×10^5 cpm [8-^{14}C] iP (0.4 GBq/mmol; a gift from Dr. R. Horgan). Samples were incubated for 30 or 60 min at 30° C and then analysed as described for IPT. In this case, however, a different solvent (chloroform:methanol, 8:2, v/v) was used which gives a better resolution of IP (R_f, 0.54) and *trans*-Z (R_f, 0.28). Experiments analysed with immunoassays were performed with 10 µM unlabelled iP or iPA. Coupled IPT/cytokinin hydroxylase reactions were performed with extracts prepared according to the protocol for IPT (details in the text or in the legend to Table 2).

Cytokinin production in vivo by E. coli *and* A. tumefaciens. Precultures were grown overnight in medium containing 10 g Bactotryptone and 5 g NaCl (both per 1,000 ml); they were then diluted 1:100 into fresh medium and harvested after growth for 5.5 h (*E. coli*) or other times (*Agrobacterium*, see Table 4). Cytokinins in the supernatants or the cells were measured by immunoassay. When iP and/or iPA were tested as precursors, they were added at a final concentration of 60 µM at the start of the main culture.

Table 2. Coupled isopentenyltransferase/cytokinin hydroxylase reactions in *Escherichia coli* extracts. Assays contained 15 µM 5′-AMP, 0.2 mM DMAPP, 8 mM $MgCl_2$, and 0.35 ml extract in a final volume of 0.55 ml. Duplicate incubations were performed at 30° C. The results are presented as pmoles/assay, as determined by immunoasay

Specific assay conditions	Plasmid	Cytokinin gene	Cytokinins detected	
			iP	*trans*-Z
A.				
Closed tubes	pVIR35b.9	*tzs* from C58	657	13.0
no shaking	pDTi401.1	*ipt* from Ach5	1795	19.1
(standard con-	pINIIA	–	10	<0.1[a]
ditions)	Buffer	–	<0.1[a]	<0.1[a]
B.				
Open tubes	pVIR35b.9	*tzs* from C58	7.1	97.1
vigorous shaking	pDTi401.1	*ipt* from Ach5	19.6	107.3
(200 rpm)	pINIIA	–	7.5	32.7
	Buffer	–	<0.1[a]	<0.1[a]

[a] Detection limit

Cytokinin enzyme immunoassays. The quantification of *trans*-Z by enzyme immunoassay was performed with monoclonal antibody J3-1-B3 (Eberle et al. 1986). iP was analyzed with a similar enzyme immunoassay, based on monoclonal antibody J40-4-C4 (Eberle et al. in preparation). Briefly, this IgG_1 antibody exhibits high affinity (K_a, 8×10^8 M^{-1} for iPA), sensitivity (measuring range of ELISA, 0.1–5 pmol), and selectivity (cross reactions: 53% for iP; 0.7% for dihydrozeatin-riboside; 0.01% for *cis*-Z).

Identification of trans-Z *by HPLC. Escherichia coli* cells containing pVIR35b.9 were grown overnight to stationary phase; the cells were pelleted by centrifugation, and cytokinins in the medium were analysed as described (Botz 1985). Briefly, *trans*-Z was purified through adsorption to silica gel C18, chromatography on polyvinylpyrrolidone, and TLC. Separation of *cis*-Z and *trans*-Z was achieved by HPLC (RP-18, column 4×250 mm; mobile phase: 15% methanol and 5% ethylacetate in 25 mM triethanolamine, pH 8.0). The standards were *cis*-Z and *trans*-Z (mixed isomers, Sigma Biochemicals).

Broad host range vector and mobilization to Agrobacterium. Plasmid pSUP104, its mobilization from *E. coli* to agrobacteria, and the plasmid-free *Agrobacterium* strains APF1 and APF2 have been described (Simon et al. 1983, 1986; Hynes et al. 1985).

Results

Cloning and expression of ipt *and* tzs *genes*

In order to obtain an improved expression level of the *ipt* gene product, the procedure outlined in Fig. 2 was adopted

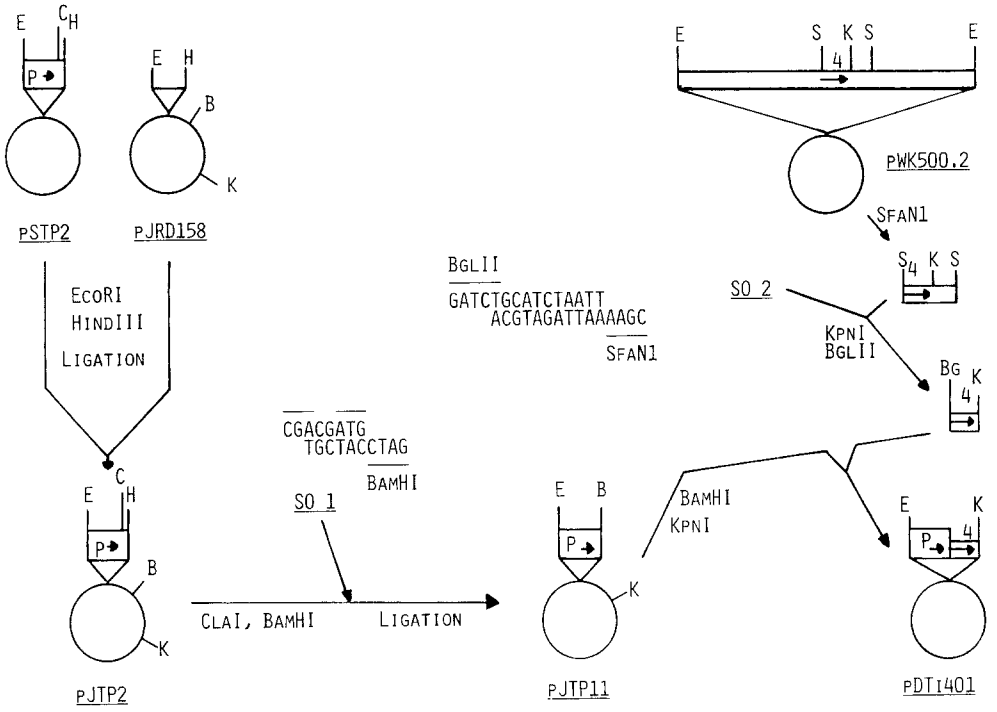

Fig. 2. Procedure used to generate high expression of *ipt* in *E. coli*; see the text for description. Abbreviations: SO1 and SO2, synthetic linkers; E, *Eco*RI; H, *Hin*dIII; S, *Sfa*N1; K, *Kpn*I; C, *Cla*I; B, *Bam*HI; Bg, *Bgl*II; P, synthetic *trp* promoter; 4, coding region of *ipt*

to place the gene from pTiAch5 under the control of a synthetic *E. coli trp* promoter (Windass et al. 1982). Firstly, the *trp* promoter was sub-cloned as an *Eco*RI/*Hin*dIII fragment into the "restriction site bank vector" pJRD158 (Davison et al. 1984) to produce pJTP2, a new *trp* promoter vector. This plasmid was further modified by insertion of a linker fragment (SO1), comprised of two synthetic oligonucleotides, between the *Cla*I and *Bam*HI sites. In this way, plasmid pJTP11 was generated, carrying a translation initiation codon (ATG) followed immediately by a unique *Bam*HI site 26 bp from the *trp* promoter transcription start site.

The *ipt* gene from pTiAch5 was inserted into pJTP11 as follows: a *Sfa*N1 fragment containing the majority of the gene (Gielen et al. 1984) was isolated from plasmid pWK500.2 (Schröder et al. 1981) and ligated to a second synthetic linker fragment (SO2). This linker replaces the codons deleted from the 5′ end of the *ipt* gene by *Sfa*N1 and provides a convenient *Bgl*II cloning site. After ligation, *Bgl*II and *Kpn*I digestion was used to release an *ipt* coding fragment which was cloned between the *Bam*HI and *Kpn*I sites of pJTP11. The key structural features of the resulting plasmid, pDTi401.1, were verified by DNA sequencing. Plasmid pDTi401.1 contains the complete *ipt* gene coding sequence fused to an *E. coli trp* promoter by an efficient translation initiation sequence.

Minicells containing pDTi401.1 express high levels of a 27000 M_r protein, consistent with that expected for the *ipt* protein (Fig. 3, lane E). Immunoprecipitation with antiserum specific for a decapeptide derived from the DNA sequence of the *ipt* gene (Buchmann et al. 1985) confirmed the identity of this product (Fig. 3, lane F). Whole cell extracts from *E. coli* containing this plasmid clearly display the *ipt* gene product in Coomassie brilliant blue stained polyacrylamide gels (not shown). Analysis of such gels by scanning densitometry indicates the level of the *ipt* product to be between 2% and 3% of the total cell protein.

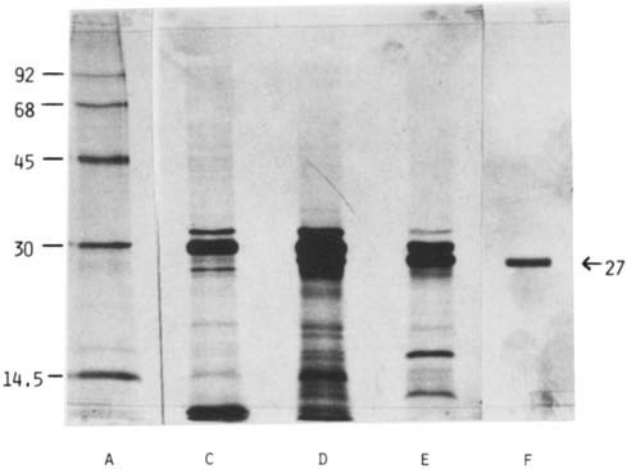

Fig. 3. Proteins expressed from cytokinin genes. Polypeptides were labelled in *Escherichia coli* minicells and analysed by sodium dodecylsulfate bpolyacrylamide gel electrophoresis. Lanes: A, marker proteins (sizes in $M_r \times 10^{-3}$ at the left-hand side); C, vector control (pINIIA); D, pVIR35b.9 (*tzs*); E, pDTi401.1 (*ipt* from pTiAch5); F, immunoprecipitate from E with the decapeptide antiserum against the *ipt* protein (Buchmann et al. 1985). 27: size of the *ipt*-encoded protein ($M_r \times 10^{-3}$)

The *tzs* gene from nopaline plasmid pTiC58 was identified by its capacity to induce secretion of *trans*-Z in *E. coli*, and this phenotype was used as the selection in subcloning of the gene. The results are summarized in Fig. 4; they essentially confirm previous experiments on the location of the gene (Akiyoshi et al. 1985; Barry et al. 1985; Beaty et al. 1986). The plasmid most active in *trans*-Z formation (pVIR35b.9, see Table 3) synthesized in minicells a protein of M_r 27000 (Fig. 3, lane D). The polypeptide is not visible in Coomassie brilliant blue stained polyacrylamide gels, in-

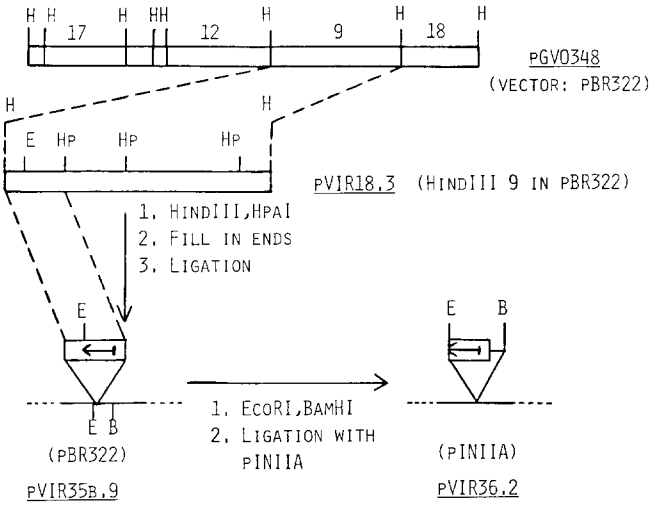

Fig. 4. Subcloning of the *tzs* gene of pTiC58. Abbreviations: H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; Hp, *Hpa*I. Lower part: the lines with arrow within the restriction fragments indicate the position of the *tzs* coding region

Table 3. Cytokinin formation in vivo in *Escherichia coli*. The bacteria were grown for 5 h at 37° C, and cytokinins were determined in the supernatant after removal of the bacteria by centrifugation. Data are given in pmoles/ml, as measured by immunoassay

Plasmid	Cytokinin gene	iP	*trans*-Z
pGV0348	*tzs* from C58	12.7	1.9
pVIR18.3	*tzs* from C58	22.8	35.7
pVIR35b.9	*tzs* from C58	33.2	78.0
pVIR36.2	*tzs* from C58	23.5	16.8
pDTi401.1	*ipt* from Ach5	13.1	1.6
pGS207.2	*ipt* from Ach5	11.1	<0.1[a]
pGS208.2	*ipt* from C58	11.9	<0.1[a]
pINIIA	–	10.4	<0.1[a]
pGS214.2	–	11.9	<0.1[a]
pDTi503.2	–	10.5	<0.1[a]

[a] Detection limit

dicating that it is expressed at a much lower level than the *ipt* protein from pDTi401.1.

Properties of ipt *and* tzs*-encoded IPTs in vitro*

Extracts from cells containing either pDTi401.1 or pVIR35b.9 show similar IPT activity when compared on the basis of total extract protein employed in the assays (Fig. 5), although expression into protein is much higher from *ipt* than from *tzs*. This could be explained by large differences in activity per enzyme molecule, but other possibilities are not excluded (see the Discussion). To compare the enzyme properties in more detail, we used a newly developed quantitative assay which is suitable for simultaneous evaluation of large numbers of samples (see the Materials and methods). Control incubations with extracts from *E. coli* free of cloned cytokinin genes were routinely included; they did not show significant enzyme activity under any of the conditions tested. IPT activities encoded by both *ipt* and *tzs* were completely dependent on DMAPP. The rate of iP formation was linear with time up to about 15 min (Fig. 5A), but the protein dependence was non-linear (Fig. 5B). A possible reason is the presence of phosphatases which inactivate the phosphorylated substrates DMAPP and 5′-AMP in competing reactions. Some attempts to purify IPT failed for two reasons: (i) the activity was very unstable, and (ii) much of the protein appeared in the membrane pellet after centrifugation of extracts; similar results have been reported for many foreign proteins expressed in *E. coli* (reviewed by Marston 1986). In order to measure all of the enzyme activity as directly as possible, we therefore continued with crude, dialyzed extracts. Since pDTi401.1 and pVIR35b.9 are expressed in the same *E. coli* strain (DS410), competing reactions should be equal in both extracts, and the effects were minimized by employing low protein concentrations (15 µg/assay) and short incubation times (10 min). The results can be summarized as follows:

(a) *$MgCl_2$*. No distinct optimum was detected between 4 and 30 mM. Omission led to complete loss of activity, but about 50% of maximum activity was obtained with 0.5 mM $MgCl_2$. No significant differences between the two enzymes were observed.

(b) *pH*. Both enzymes showed a broad optimum between pH 6.0 and 8.5.

(c) *Temperature* (Fig. 5C). The enzyme from *ipt* revealed a fairly sharp optimum at 32° C, while the *tzs* protein showed a broader optimum between 30° and 37° C. This difference was observed in three independent experiments.

(d) *K_m for 5′-AMP* (Fig. 5D). Experiments were performed at 30° C and with saturating concentrations of DMAPP (0.2 mM). Both enzymes showed an apparent K_m of 5 µM, with variations between 3 and 7 µM in repeated experiments.

(e) *K_m for DMAPP*. The concentration of radioactive 5′-AMP was held at 1 µM for technical reasons, since addition of unlabelled 5′-AMP (to reach saturating substrate concentrations) reduced the incorporation of radioactivity into the product so much that quantification was no longer possible. The apparent K_m for both enzymes was about 15 µM, and no significant differences were observed. The absolute values should be considered with caution, since the DMAPP was not 100% pure (D. Akiyoshi, personal communication), and since a non-saturating concentration of 5′-AMP was used.

(f) *Effects of divalent cations and sodium phosphate*. Assays were performed with 0.4 mM $MgCl_2$, and the salts were added just before starting the incubations. $FeSO_4$ (Fe^{2+}) or $FeCl_3$ (Fe^{3+}), both at 0.2 mM, or 0.5 mM $ZnSO_4$ (Zn^{2+}) were without effect and did not restore full activity; higher concentrations of $ZnSO_4$ were inhibitory. $CaCl_2$ (Ca^{2+}), (2–10 mM) and 2 mM $MnCl_2$ (Mn^{2+}) restored full IPT activity, and higher concentrations of $MnCl_2$ even led to an additional stimulation. Sodium phosphate (in the presence of 8 mM $MgCl_2$) in concentrations up to 20 mM did not inhibit the enzyme. The two enzymes showed no obvious differences.

(g) *Alternative substrates*. Radioactive adenosine was not a substrate for either enzyme. Unlabelled adenosine in concentrations up to 0.1 mM had no significant effect on the reaction with the standard substrate, 5′-AMP. Neither ADP or ATP could be tested as substrate or as inhibitor, since partial conversion to the standard substrate 5′-AMP could not be excluded.

Taken together, these results indicate that the IPT activities encoded in *ipt* and *tzs* have very similar properties in vitro. The only significant differences were in the appar-

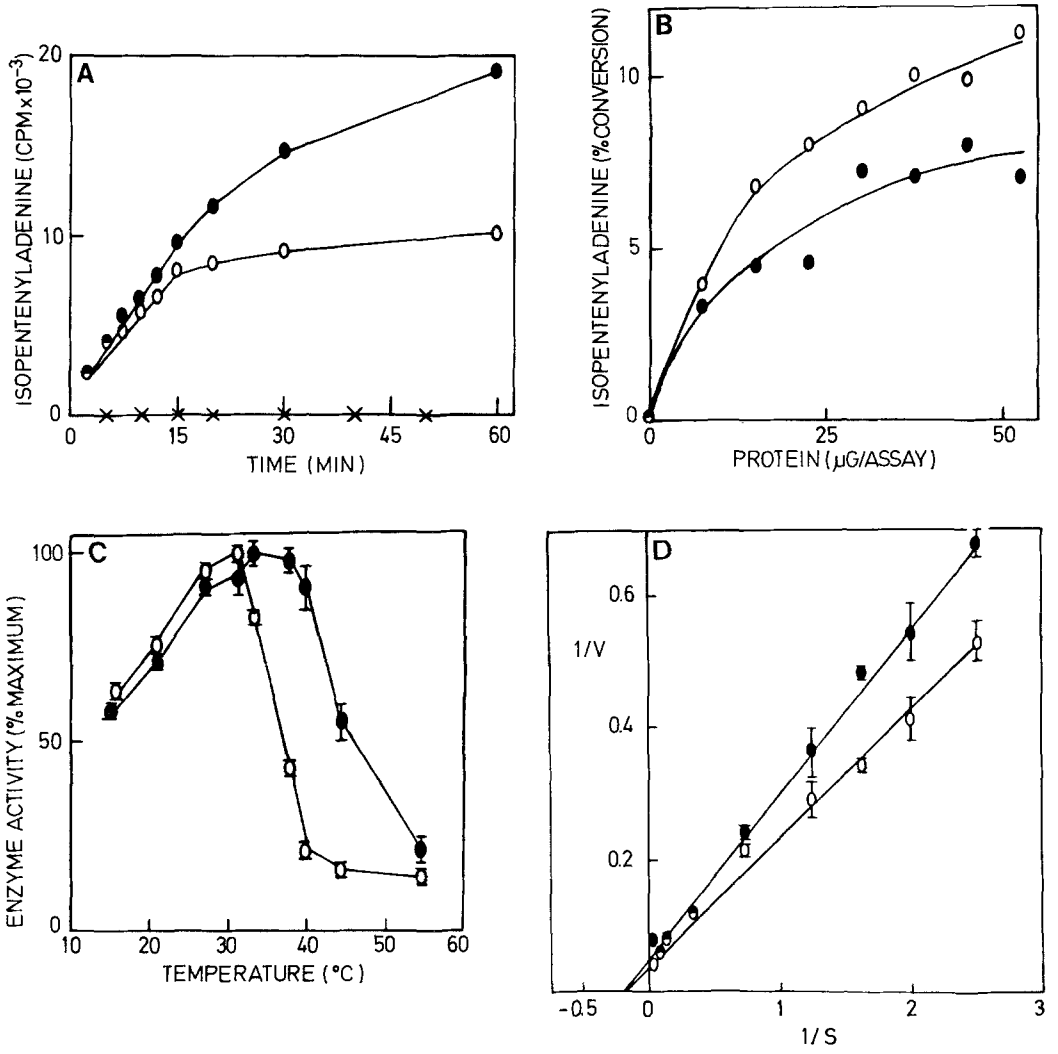

Fig. 5. In vitro properties of the IPT activities encoded in *tzs* (•, pVIR35b.9) and *ipt* (o, pDTi401.1). **A.** Time course of the reaction. Incubations were performed under standard assay conditions, with 10 µl (pDTi401.1) or 20 µl (pVIR35b.9) extract. x, 20 µl extract from control cells (pINIIA) without cloned cytokinin genes. **B.** Dependence on protein concentration. Assays were incubated for 10 min under standard conditions. **C.** Temperature dependence. Assays contained 15 µg protein and were incubated for 10 min. *Vertical bars* indicate the variation in duplicate assays. **D.** K_m for 5′-AMP (Lineweaver-Burk plot). Assays were incubated for 10 min at 30° C in the presence of 15 µg protein

ent activity per enzyme molecule and in the shape of the temperature optimum curve.

Cytokinin hydroxylase activity in E. coli *extracts*

Since the phenotype of *tzs* is formation of *trans*-Z, either the protein itself or an *E. coli* function is capable of hydroxylating iP-type cytokinins to *trans*-Z. We attempted in a large number of experiments to demonstrate such activity in vitro, either with ^{14}C-labelled iP or with unlabelled iP or iPA, and *trans*-Z was measured after TLC or by immunassay. The results showed that all extracts contained traces of *trans*-Z, but it was not possible to demonstrate hydroxylase activity in vitro.

The direct product of the IPT reaction is iPMP (Fig. 1), and this may be the true substrate. Since iPMP was not available, we used *ipt* and *tzs* to synthesize unlabelled iPMP directly in the incubation, and cytokinins were then determined by immunoassay. Table 2A shows that extracts with *ipt* and *tzs* contained not only iP, but also small amounts of *trans*-Z, while controls contained very little iP (endogenous to *E. coli*, see in vivo data) and no detectable *trans*-Z. Since O_2 might have been limiting, the experiments were repeated with vigorous shaking. Table 2B shows that the amount of total cytokinins was now substantially reduced (indicating that IPT was inhibited either by shaking or by O_2), but *trans*-Z was the predominant cytokinin with *tzs* or *ipt*. However, high levels of *trans*-Z were also detected with the extract from cells which contained no cloned cytokinin gene and no IPT activity. The simplest explanation for these data is that extracts from normal *E. coli* cells possess enzymes capable of cytokinin hydroxylation, and that the high concentration of *trans*-Z results from increased tRNA degradation followed by hydroxylation of iP-type nucleotides. The higher yields with pVIR35b.9 and pDTi401.1 most likely reflect the fact that *tzs* and *ipt*-coded IPT increased the substrate concentration for the *E. coli* hydroxylase. Taken together, the results do not allow a conclusion that a hydroxylase function is encoded in the Ti plasmid genes.

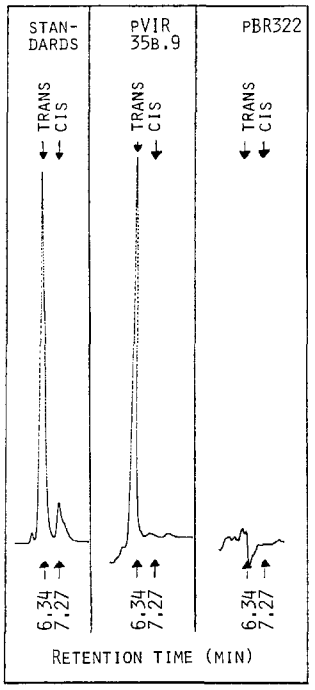

Fig. 6. HPLC identification of *trans*-Zeatin isolated from supernatants of *Escherichia coli* cells containing the *tzs* gene in pVIR35b.9

Cytokinin production in vivo in E. coli

Since the in vitro experiments did not reveal any major differences, we investigated cytokinin formation in vivo, and the significant results are summarized in Table 3. Most of the plasmids with *tzs* led to increased secretion of iP, and all showed significant production of *trans*-Z, when compared with the controls. The precise reason for the quantitative differences between the plasmids is not clear, but they correlate with the subcloning of the gene on smaller fragments. In most of the experiments, cytokinins were measured by immunoassays, but in the case of pVIR35b.9, which produced very high levels of *trans*-Z, this cytokinin was also purified and identified by HPLC (Fig. 6). These results confirm and extend previous findings (Akiyoshi et al. 1985; Barry et al. 1985; Beaty et al. 1986).

More importantly, Table 3 also shows that *ipt* genes from pTiAch5 (in pGS207.2) or pTiC58 (in pGS208.2) do not lead to increased secretion of IP or *trans*-Z, although the proteins are expressed and functional in vitro (Buchmann et al. 1985). The exception was the protein overproducer pDTi401.1, which shows a small but significant increase of *trans*-Z; however, even this was not always seen in other, independent experiments. Since the in vitro assays had shown that a temperature of 37° C might not be optimal for the enzyme from *ipt* (Fig. 5C), comparable experiments were performed at 30° and 25° C but did not give significantly different results. We measured concentrations inside the cells, but there was no indication that cytokinins were synthesized but not secreted. We also analysed hydroxylase activity in vivo in feeding experiments with iP and iPA. The results showed that both cytokinins were converted with low efficiency into *trans*-Z, but the presence of the cytokinin genes made no significant difference.

Several conclusions emerge from these studies: (i) all *E. coli* cells secrete measurable amounts of iP into the medium; (ii) the proteins encoded in *tzs* and *ipt* are different: *tzs* is highly active in vivo, while *ipt* shows very little, if any, activity; (iii) the little activity of *ipt* results in *trans*-Z production, just like with *tzs*; (iv) *E. coli* can hydroxylate iP-type cytokinins to *trans*-Z.

Cytokinin formation in vivo in Agrobacterium

Since the plasmids used so far are based on pBR322 or its derivatives, it was necessary to reclone the genes into a broad host vector which could be mobilized into *Agrobacterium*. We used pSUP104 (Simon et al. 1983), and the *ipt* genes from pTiAch5 (in pGS207.2) and pTiC58 (in pGS208.2) were cloned as *Eco*RI fragments into the *Eco*RI site of the chloramphenicol acetyltransferase gene (CAT) in such orientation that they are transcribed from the CAT promoter. The *tzs* gene was inserted into the same site by the following procedure. It was excised from pVIR35b.9 with *Bam*HI and *Cla*I; the fragment was end-filled and then ligated into *Eco*RI-digested, end-filled pSUP104. Constructions were in *E. coli*; they were confirmed by restriction analysis both before and after mobilization.

We first investigated whether resident Ti plasmids influence cytokinin production by *tzs* on a separate plasmid. We used pGV2260 and pGV3850 since both carry large deletions in the T region, including *ipt* (see Table 1), and this avoids confusion with possible endogenous *ipt* gene activity. *Agrobacterium* containing only pGV2260 secretes

Table 4. Cytokinin formation in vivo in *Agrobacterium*. Cells were grown at 28° C, and cytokinins were determined by immunoassay in the supernatants after removal of the bacteria by centrifugation. Results are presented in pmoles/ml

Introduced plasmid (cytokinin gene)	Strain	Resident Ti plasmid	iP			*trans*-Z		
			13 h	17 h	22 h	13 h	17 h	22 h
–	C58C1	pGV2260	8.3	6.3	8.0	<0.1[a]	<0.1[a]	<0.1[a]
pVIR158.2 (*tzs* C58)	C58C1	pGV2260	17.5	18.7	17.5	8.5	4.7	2.5
–	C58C1	pGV3850	13.2	13.8	17.2	<0.1[a]	<0.1[a]	2.9
pVIR158.2 (*tzs* C58)	C58C1	pGV3850	17.4	5.7	5.8	4.7	4.6	4.6
–	APF1	–	6.2	7.4	5.9	<0.1[a]	<0.1[a]	<0.1[a]
–	APF2	–	7.5	5.8	5.0	<0.1[a]	<0.1[a]	<0.1[a]
pVIR158.2 (*tzs* C58)	APF2	–	4.3	4.6	5.9	5.0	8.5	<0.1[a]
pT159.7 (*ipt* Ach5)	APF2	–	5.5	4.6	5.3	<0.1[a]	<0.1[a]	<0.1[a]
pT160.9 (*ipt* C58)	APF2	–	5.2	14.6	18.0	<0.1[a]	<0.1[a]	2.9

[a] Detection limit

iP but not *trans*-Z (Table 4). Introduction of *tzs* leads to increased amounts of iP and to *trans*-Z, indicating that the gene is functional. Plasmid pGV3850 (which carries the *tzs* gene from pTiC58) leads to *trans*-Z secretion late in the growth cycle. This is consistent with previous results that *trans*-Z production is highest in the stationary phase (Weiler and Spanier 1981). Introduction of the additional *tzs* gene results in *trans*-Z secretion throughout the cell cycle. These results establish that re-introduced *tzs* is functional in *Agrobacterium*, regardless of the presence of other plasmids; they also show that pGV2260, an octopine plasmid derivative, carries no functions interfering with the activity of *tzs*.

When *Agrobacterium* strains free of detectable plasmids became available (Hynes et al. 1985), we also introduced the *ipt* genes. Plasmid-free APF1 or APF2 secreted no *trans*-Z but small amounts of iP into the medium. Introduction of *tzs* leads to *trans*-Z secretion in the early growth phase without significant change in iP concentrations (Table 4). The *ipt* gene from pTiAch5 appears to be inactive, but the gene from pTiC58 induces increased concentrations of iP, and *trans*-Z was detected in the stationary phase of growth. The results indicate that at least the *ipt* protein from nopaline plasmid pTiC58 is functional in *Agrobacterium*, and that this activity not only produces iP but also *trans*-Z. By definition this is the phenotype of a *tzs* gene.

Discussion

The experiments described here demonstrate a major difference between the two cytokinin genes expressed in *E. coli*: the *tzs* protein is highly active in vivo, while the *ipt* protein has little if any activity. However, in the single case where *ipt* activity is detectable, it also leads to *trans*-Z, not only iP (Table 3, pDTi401.1). Similar results were obtained when the genes were re-introduced into *Agrobacterium*. The *ipt* gene from pTiAch5 appears to be inactive, but the corresponding gene from pTiC58 leads to increased iP concentrations and, most importantly, to the appearance of *trans*-Z, if only in the late stage of growth (Table 4). Thus, if *ipt* or *tzs* proteins are functional at all in vivo, both produce the *tzs* phenotype.

The proteins encoded by *ipt* and *tzs* possess IPT activity in vitro. It is commonly assumed that formation of *trans*-Z requires a second enzyme activity (reaction B in Fig. 1) acting on iP-type cytokinins. If the *ipt* and *tzs* proteins active in vivo both lead to a *tzs* phenotype, the mechanism of hydroxylation becomes an interesting question. The answer could be provided by at least two different mechanisms: either the proteins are bifunctional, or hydroxylation is performed by a second protein (a host enzyme or an additional polypeptide product of the *ipt* and *tzs* genes). Although other possibilities are not rigorously excluded, our results clearly favour hydroxylation of iP-type cytokinins by host enzymes. Such activities were present in cells and extracts of *E. coli* that were free of cloned cytokinin genes. Neither presence nor expression of *tzs* or *ipt*-coded proteins increased the rate of conversion of iP, iPA, or iPMP to zeatin-type cytokinins, and it was not possible to ascribe an additional hydroxylase activity to either one of the enzymes. A final answer could be given with highly purified proteins, but our attempts at purification were thwarted by the instability and insolubility of the enzymes.

The properties of *ipt* and *tzs*-encoded IPTs are very similar in vitro, and it is not clear whether the two observed differences are sufficient to explain the drastic discrepancies in vivo: (i) The apparent differences in activity per enzyme molecule could reflect true properties of the proteins. However, in this case one would also expect significant differences in the kinetic analysis (e.g. K_m), but this was not observed. It seems, therefore, equally likely that overexpression of the *ipt* protein led to enzymatically inactive aggregates, and that only a fraction of the enzyme was available in active form. Similar findings have been reported for many foreign proteins expressed in *E. coli* (Marston 1986). It would be tempting to speculate that differential solubility might be responsible for the differences between *ipt* and *tzs* proteins in vivo. Although this is not excluded, comparisons of hydropathy profiles do not reveal large differences that would support such a hypothesis. (ii) The difference in the temperature optimum curve (Fig. 5C) appears equally unlikely as explanation, since growing the cells at 30° C instead of at 37° C does not increase cytokinin production by *ipt* proteins in vivo. The molecular basis for the differences observed in vivo presumably must lie in the amino acid sequences, and in fact *tzs* and *ipt* proteins do show such differences. However, *ipt* proteins from different Ti plasmids also reveal a significant number of amino acid exchanges (reviewed in Morris 1986), and the two *tzs* proteins whose genes have been sequenced are not identical either (Akiyoshi et al. 1985; Beaty et al. 1986). In this context, it is also important that a recently described cytokinin gene from *Pseudomonas savastanoi* possesses typical *tzs* properties (*ptz*, Powell and Morris 1986). It confers the *tzs* phenotype to *E. coli*, and in vitro the enzyme has IPT activity. However, the protein sequence reveals only 48% perfect matches with *tzs*, and this figure increases to only 61% with allowance for presumably conservative exchanges (Powell and Morris 1986). The functional significance of specific amino acid exchanges is not understood, and therefore it is difficult to pinpoint the difference between *ipt* and *tzs* that is responsible for the altered enzyme activity in vivo. With respect to the *ipt* genes it may be important that they were originally defined by their function in transformed plant cells (cytokinin autonomy). It seems a plausible hypothesis that the *ipt* enzymes are adapted to function in eukaryotic cells rather than in bacteria. Plant cells are known to possess active cytokinin hydroxylases, and *trans*-Z and its further derivatives are the predominant cytokinins produced by crown gall cells (reviewed by Morris 1986). Thus, *ipt*-coded IPT activity in plant cells probably leads to a *tzs* phenotype in the same way as *tzs*-coded IPT in bacteria. It would be interesting to see whether a *tzs* gene provided with eukaryotic expression signals produces a protein with IPT activity in vivo in plant cells.

Our results suggest that cytokinin hydroxylation is contributed by host enzymes, but the necessity for this step is not clear. *E. coli* does contain iP, but not *trans*-Z, as part of certain tRNAs (Buck et al. 1982; Janzer et al. 1982), and low amounts of iP-type cytokinins, presumably the result of tRNA turnover, are secreted into the medium. In many bacteria, tRNA-bound cytokinins participate in regulatory mechanisms (Buck and Ames 1984; Starzyk 1984), but free cytokinins are not known to exert similar effects. It is possible, however, that drastic increases of free iP, caused by *tzs* or *ipt*-coded IPT activity, may interfere with the functions of bound cytokinins. Hydroxylation of iP and secretion of *trans*-Z may represent a mechanism to remove excess free cytokinins from the cells. In this context it is

of interest that the highly active *tzs* gene from *Pseudomonas savastanoi* could not be stably maintained on multicopy plasmids in *E. coli*, presumably because of excess cytokinin production (Powell and Morris 1986), and this indicates that very high cytokinin concentrations may be detrimental to bacteria.

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