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Secretion of Zeatin, Ribosylzeatin, and Ribosyl-1″-Methylzeatin by *Pseudomonas savastanoi*[1]

PLASMID-CODED CYTOKININ BIOSYNTHESIS

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ELIZABETH M. S. MACDONALD, GARY K. POWELL, DEAN A. REGIER[2],
N. LOUISE GLASS, FRANCISO ROBERTO, TSUNE KOSUGE, AND ROY O. MORRIS*
Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331 (E.M.S.MacD., G.K.P., D.A.R., R.O.M.); *and Department of Plant Pathology, University of California, Davis, California 95616* (N.L.G., F.R, T.K.)

ABSTRACT

Cytokinin production by strains of the phytopathogenic bacterium *Pseudomonas syringae* pv *savastanoi* was measured by immunoaffinity chromatography of the culture medium on immobilized anti-cytokinin antibodies, followed by high performance liquid chromatography, radioimmunoassay and mass spectrometry. *P. savastanoi* strain PB213-2 secretes zeatin (80 nanograms per milliliter) and ribosylzeatin (80 nanograms per milliliter). Even higher levels of zeatin (400 nanograms per milliliter) are produced by the olive-specific strain EW1006, which also produces 180 nanograms per milliliter of the recently identified cytokinin, ribosyl-l″-methylzeatin. The amounts secreted were approximately 1000 times greater than those secreted by *Agrobacterium tumefaciens* (DA Regier, RO Morris 1982 Biochem Biophys Res Commun 104: 1560–1566). Examination of cytokinin production by plasmid deletion mutants of PB213-2 and EW1006 indicated that cytokinin biosynthesis was specified, at least in part, by plasmid-borne genes. A fragment of the 105 kilobase pair plasmid from EW1006 was cloned into *Escherichia coli* where its expression resulted in dimethylallyl transferase activity and the secretion of zeatin.

Gall-forming phytopathogenic bacteria have long been known to produce plant hormones (11, 28) and there has been speculation, supported by recent evidence, that pathogenesis may be hormone-dependent. It is now known that the bacterial pathogens *Pseudomonas syringae* pv *savastanoi* and *Agrobacterium tumefaciens* contain genes which specify the synthesis of IAA. *P. savastanoi*, the causative organism of olive and oleander knot, contains two plasmid-born genes which specify IAA biosynthesis (6, 7, 17); the first, *iaa*M, encodes the enzyme tryptophan monooxygenase (12) while the second, *iaa*H, encodes an indoleacetamide hydrolase. Both genes are required for effective pathogenesis (31). *A. tumefaciens*, the causative agent of crown gall disease, also contains plasmid-born genes (*tms*1 and *tms*2) which specify IAA biosynthesis and which are expressed in the plant (16). They have substantial homology with *iaa*M and *iaa*H (34) and are required for tumorigenesis (10, 25).

Cytokinins are also produced by these organisms. *P. savastanoi* secretes a number of cytokinins including the novel methylated derivative, 6-(4-hydroxy-1,3-dimethylbut-2-*trans*-enylamine-9-β-D-ribofuranosyl)purine (ribosyl-l″-methylzeatin) (32, 33). Although some strains of *A. tumefaciens* secrete zeatin (14), the situation is more complicated than in *P. savastanoi*. All *A. tumefaciens* strains examined thus far contain *ipt*, a locus within the T-DNA which specifies the cytokinin biosynthetic enzyme, dimethylallylpyrophosphate:5′AMP transferase (DMA transferase) (3, 5, 18, 24), but not all strains secrete zeatin. *Ipt* is integrated into the host plant genome during tumorigenesis and is partly responsible for tumor morphology (10, 25) and cytokinin content (1). The gene responsible for bacterial zeatin secretion, *tzs*, is carried only on nopaline Ti plasmids (2, 4, 14, 27). It also specifies a DMA transferase (2) but its role in tumorigenesis has not yet been defined.

We show here that two strains of *P. savastanoi* produce zeatin at very high levels and that one strain also produces high levels of ribosyl-l″-methylzeatin. Genes specifying cytokinin production were found to be carried on *P. savastanoi* plasmids and have been cloned and expressed in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial Strains and Growth. *Pseudomonas savastanoi* strains PB213-2, PB1050, and EW1006 have been described previously (7). *Pseudomonas syringae* pv *myricae* isolates (7118 and 7120) were obtained from the Plant Diseases Division Culture Collection, New Zealand Department of Scientific and Industrial Research. Both sets were grown in King-B medium (15) or Miller's Minimal A medium (22) containing 0.2% (w/v) glucose and 0.05% (w/v) casamino acids. Since strains readily lost plasmids by segregation, stocks were maintained in the presence of 50% (v/v) glycerol at −70°C. For cytokinin analysis, cultures were grown at 25°C in silanized glassware and harvested at mid-log phase (A_{600} = 0.6). Prior to analysis, the number and size of the plasmids was determined as described below.

Plasmid Isolation. Plasmid DNA was isolated by modification of the high temperature alkaline lysis procedure of Kado and Liu (13). Cultures were grown overnight in King-B broth, aliquots (0.75 ml) were harvested by centrifugation at 15,000*g* for 5 min, and the cells were resuspended in 0.1 ml of lysis buffer

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[2] Present address: Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195.

(50 mM Tris, 3% SDS (v/v); pH 12.6) at 60°C for 45 min. NaCl (5 M) was added to a final concentration of 0.5 M and the solution was shaken with an equal volume of phenol:chloroform (1:1, v/v). The two phases were separated by centrifugation and the aqueous phase was analyzed directly by gel electrophoresis.

Radioimmunoassay. Direct RIA[3] of culture filtrates was carried out as described previously (19). Cells were grown overnight to late log phase and the supernatant solutions were harvested by centrifugation at 15,000*g* for 5 min. Radioimmunoassay was performed directly on aliquots (0.35 ml) of supernatant solution using monoclonal anti-tZR antibody and [^{3}H]zeatin trialcohol (5 nCi; 2.7 Ci/mmol). Estimation of the amount of total zeatin-like material present was made by comparison with standards assayed under identical conditions.

Immunoaffinity, HPLC, RIA, and MS of Cytokinins. Aliquots (0.5, 1, or 100 ml) of midlog phase culture filtrates were passed sequentially through connected columns of DEAE cellulose (DE-52, 2 or 20 ml) and an immunoaffinity matrix (20) comprised of a mixture of 1.5 ml of anti-tZR antibody-cellulose and 1 ml of anti-iPA antibody-cellulose equilibrated with 40 mM ammonium acetate buffer (pH 6.5) (Buffer A, 2 volumes). The capacity of such columns was approximately 113 ng of ribosylzeatin and 36 ng of *iso*-pentenyladenosine. Samples were applied in a total volume of 3 ml (diluted with buffer A); the columns were washed with buffer A (10 volumes); and the DE-52 column was then discarded. The immunoaffinity column was washed with buffer A (5 volumes), buffer A containing 0.5 M NaCl and 2% v/v DMSO (2.5 volumes), buffer A (5 volumes), and the cytokinins were eluted with 3 ml of methanol. Eluates were evaporated to dryness *in vacuo* and dissolved in a methanol:triethylamine acetate buffer (pH 3.35) (1:1, v/v) for HPLC.

HPLC and RIA was performed as described previously (20). Recoveries were measured in replicate samples which contained [^{3}H]ribosylzeatin or [^{3}H]*iso*-pentenyladenosine (5 Ci/mmol). Cytokinin levels expressed in the text have been corrected for recovery (usually 67–71%). Individual HPLC fractions were evaporated to dryness *in vacuo* and permethylated for MS as described previously (23). Spectra were acquired on a Finnegan 402 quadrupole mass spectrometer at an electron energy of 35 eV.

Cloning of Cytokinin Biosynthetic Genes. *P. savastanoi* plasmid DNA was isolated from 1 g of pelleted cells. Cells were lysed in 100 ml of lysis buffer and extractions were performed as described above. Plasmids were purified by CsCl density gradient centrifugation (9). After partial digestion with restriction endonuclease *Sau*3A, fragments were ligated to the *Bgl*II-digested, alkaline phosphatase-treated, wide host range vector pRK290 (8) and used to transform *E. coli* HB101. Transformants were selected on LB plates containing tetracycline (25 mg/L) and recombinant plasmid DNA was isolated by standard procedures (21).

DMA Transferase Extraction and Assays. Cell pellets from 250 ml of stationary phase cultures were resuspended in 7.5 ml of ice-cold extraction buffer (50 mM Tris-Cl [pH 7.0], 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 100 mM KCl, and 15% glycerol [v/v]) and sonicated for 2 min at 0°C. Cell debris was removed by centrifugation at 30,000*g* for 30 min. The extracts were dialyzed against two changes of extraction buffer and assayed immediately.

Assay mixtures contained (in 1.2 ml) 50 mM Tris-Cl (pH 7.0), 500 mM KCl, 10 mM $CaCl_2$, 10 mM 2-mercaptoethanol, 5 μM unlabeled 5′AMP, 10 μCi [^{3}H]5′AMP (New England Nuclear, 15 Ci/mmol), 1.9 mM dimethylallylpyrophosphate, and 0.1 ml of dialyzed enzyme extract. After incubating at 25°C for 2 h, the pH was lowered to 5.0, 1 mg of wheat germ acid phosphatase was added, and the mixture was incubated 1 h at 37°C. The pH was then raised to 6.5 and cytokinins present in the assay mixture were purified by DE-52 and immunoaffinity chromatography as described above.

RESULTS

Cytokinin Production by *P. savastanoi*. Preliminary RIA of unfractionated culture filtrates from a number of virulent *P. savastanoi* (PB1050, EW1006, and PB213) isolates indicated that they contained approximately 1000 times more zeatin-like cytokinins than filtrates from *A. tumefaciens* strain C58 (27). Two strains were selected for further study: EW1006 (isolated from olive knot) and PB213-2, a deletion mutant of PB213 (originally isolated from oleander knot) that has lost part of the pIAA1 plasmid and which no longer secretes IAA (7). To identify the cytokinins and to estimate the levels present, aliquots of culture filtrates from both strains were applied to immunoaffinity columns containing immobilized mixtures of monoclonal antibodies raised against ribosyl *trans*-zeatin and *iso*-pentenyladenosine (20). Following elution, the purified cytokinin mixtures were analyzed by HPLC and RIA.

The results of analysis of PB213-2 are shown in Figure 1, A and B. Two major UV-absorbing peaks, eluting at 15.2 min and 19.8 min, cross-reacted with anti-tZR antibody. Retention times of these peaks were identical to those of *trans*-zeatin and ribosyl *trans*-zeatin. A much smaller peak at 10.2 min also cross-reacted with anti-tZR antibody. Its retention time was identical to that of zeatin-9-glucoside but it was not characterized further. A fourth peak at 33.5 min cross-reacted with anti-iPA antibody.

Cytokinins were also present in culture filtrates of EW1006 (Fig. 1, C and D). The A_{254} trace displayed few peaks (attesting to the efficacy of the immunoaffinity purification), of which four cross-reacted with anti-tZR antibody and one with anti-iPA antibody. Those cross-reacting with anti-tZR antibody had retention times corresponding to zeatin (14.5 min), dihydrozeatin (16.5 min), ribosylzeatin (18.8 min), and ribosyldihydrozeatin (20.6 min). Small but significant amounts of *iso*-pentenyladenosine were detected at 33.3 min.

In addition to known cytokinins, EW1006 filtrates contained a substantial amount of a UV-absorbing substance (peak I, Fig. 1C) at 25.9 min which cross-reacted weakly with anti-tZR antibody. Its retention time on HPLC was indicative of a polarity intermediate between that of ribosylzeatin and *iso*-pentenyladenosine which would be consistent with the properties of the recently identified novel cytokinin ribosyl-l″-methylzeatin (33).

Confirmation of Cytokinin Identity by Mass Spectrometry. To confirm the presence of zeatin and ribosylzeatin, and determine the identity of peak I, appropriate HPLC fractions were permethylated and examined by MS. Permethylation of the zeatin fractions from PB213-2 or EW1006 gave derivatives whose mass spectral properties were identical to those of authentic permethylzeatin (23). All major ions were present at the expected intensities including the molecular ion at m/z = 261 and fragment ions at m/z = 230, 216, 199, 174, 162, 164, and 134. Retention times of zeatin on HPLC and of permethylzeatin on GLC (data not shown), were identical to those of standards.

Permethylation of the ribosylzeatin fraction from PB213-2 gave a product whose mass spectrum (Fig. 2A) was identical to that of authentic permethylribosylzeatin. The molecular ion and prominent fragment ions were present at m/z = 421, 390, 348, 246, 230, 216, and 174. HPLC and GLC retention times were identical to those of *trans*-ribosylzeatin or its permethyl derivative.

Permethylation and perdeuteromethylation of peak I from

[3] Abbreviations: RIA, radioimmunoassay; anti-tZR antibody, antibody raised against ribosyl *trans*-zeatin-bovine serum albumin conjugate; anti-iPA antibody, antibody raised against *iso*-pentenyladenosine-bovine serum albumin conjugate; kb, kilobase pair(s); amu, atomic mass unit; DMA, dimethylallyl.

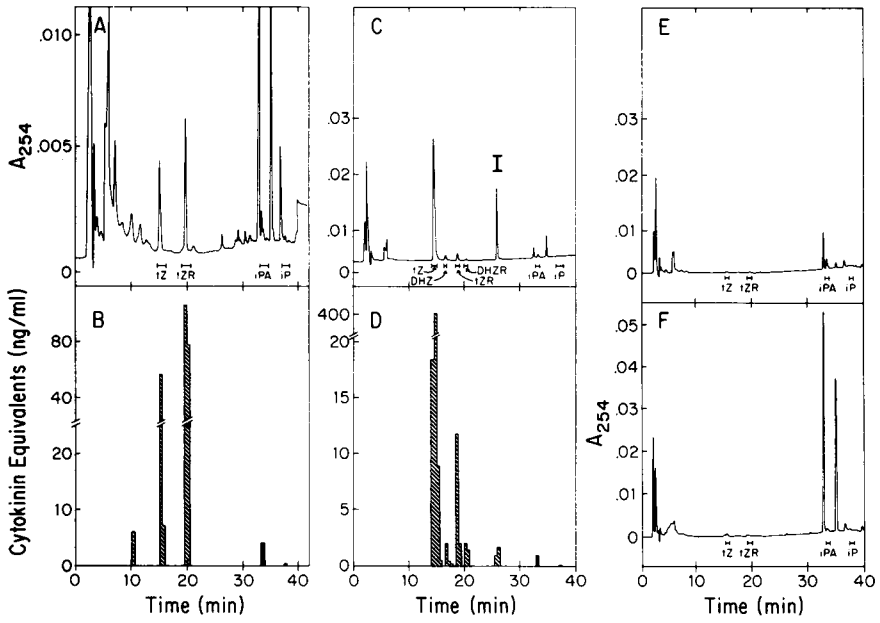

FIG. 1. Cytokinin secretion by *P. savastanoi* strains: analysis by HPLC and RIA. *A* at 254 nm: A, PB213-2 (0.5 ml culture filtrate); C, EW1006 (1 ml); E, PB213-2-4 (100 ml); F, EW1006-2 (100 ml). RIAs: B, PB213-2; D, EW1006. Cytokinins present in the growth media from midlog phase cultures were purified by immunoaffinity chromatography on mixed anticytokinin monoclonal antibodies and fractionated by HPLC. Individual fractions were assayed by RIA using anti-tZR antibody (hatched bars) or anti-iPA antibody (solid bars). Retention times of cytokinin standards differed slighty between runs due to variations in chromatographic conditions and are indicated at the bottom of A, C, and E. tZ, *trans*-zeatin; DHZ, dihydrozeatin; tZR, ribosyl *trans*-zeatin; DHZR, ribosyldihydrozeatin; iPA, *iso*-pentenyladenosine; iP, *iso*-pentenyladenine. The retention time of peak I did not correspond to that of any available cytokinin standard.

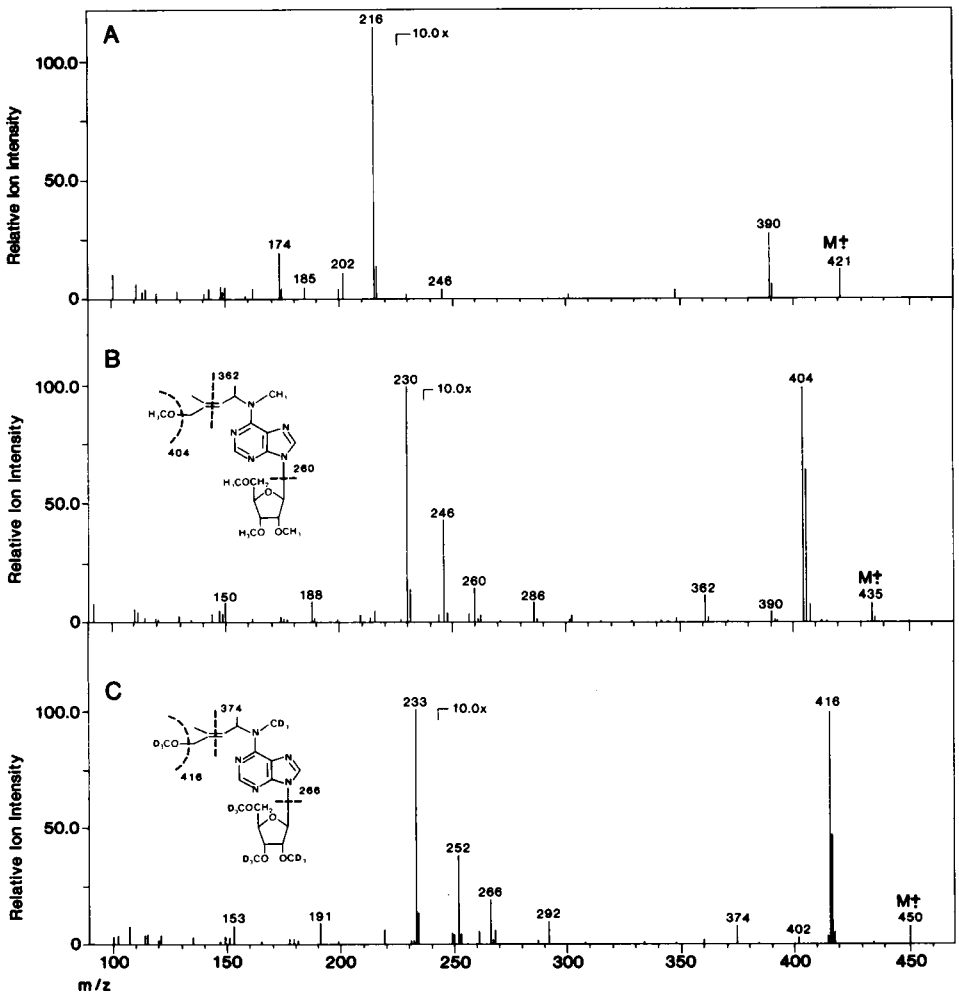

FIG. 2. Mass spectra of permethylated and perdeuteromethylated cytokinins from *P. savastanoi* strain EW1006. A, Permethylribosylzeatin; B, permethylated peak I (see Fig. 1); C, perdeuteromethylated peak I.

EW1006 gave the spectra shown in Figure 2, B and C. There was an obvious molecular ion at $m/z = 435$ and fragment ions at $m/z = 404$, 390, 362, 286, 260, 246, 230, and 188. The spectrum was almost identical to that of permethylribosylzeatin (Fig. 2A) except that clearly homologous fragment ions in Figure 2A were increased in mass by 14 amu over those in Figure 2B, indicating the presence of an extra methyl group. The position of this extra methyl group could be determined by examination of the fragmentation pattern. The peak I fragment ion at m/z 188 (Fig. 2B) corresponds to the ribosylzeatin fragment ion at $m/z = 174$ (Fig. 1A) which is a tricyclic structure (29) resulting from side-chain cleavage (with loss of side chain carbons 3, 4, and 5) and loss of

ribose. The mass increase of 14 amu indicated that the extra methyl group must be present either on the ring or on the 1″ or 2″ positions of the side chain. The mass spectrum of underivatized peak I (data not shown) contained fragment ions at m/z = 148 and 119 which have been assigned to 6-methyleneiminopurinyl and purinyl cations, respectively (30). Analogous ring-methylated ions were not present indicating that the methyl group must be attached to the side chain. Peak I appears, therefore, to be ribosyl-l″-methylzeatin as proposed by Surico *et al.* (33). The spectrum of the perdeuteromethyl derivative confirmed this structural assignment. The molecular and fragment ion masses increased by the expected amounts in all cases.

Correlation between Cytokinin Secretion and the Presence of Specific Plasmids. The genes for IAA biosynthesis in *P. savastanoi* are carried on large plasmids in PB213 and PB2009 (6, 7). To determine whether cytokinin biosynthesis was also plasmid-encoded, spontaneous plasmid deletion mutants of PB213-2 and EW1006 were isolated and assayed for their ability to produce cytokinins. As illustrated in Figure 3, PB213-2 contains 5 plasmids: 38, 42, 48, 56, and 64 kb and secretes high levels of zeatins into the culture medium. Three spontaneous deletion mutants (PB213-2-1, PB213-2-2, and PB213-2-3) had lost the 56 kb plasmid, but still secreted zeatins. However, loss of the 56 kb and 42 kb plasmids (PB 213-2-4, 213-2-5) abolished zeatin production.

A similar situation prevailed in EW1006. Wild type EW1006 secreted very high levels of zeatins, as did a deletion mutant (EW1006-1) from which the smaller of the two plasmids (81 kb) had been lost (Fig. 3). However, EW1006-2, from which 40 kb of the 105 kb plasmid had been deleted, no longer secreted zeatin.

Immunoaffinity chromatography, HPLC, and RIA of PB213-2, EW1006, and deletion mutants confirmed the results of the solution RIAs (Fig. 1, E and F). Production of zeatin and ribosylzeatin was dependent on the presence of the 42 kb plasmid of PB213-2 and the 105 kb plasmid of EW1006. Strain PB213-2 secreted approximately 80 ng/ml each of zeatin and ribosyl-

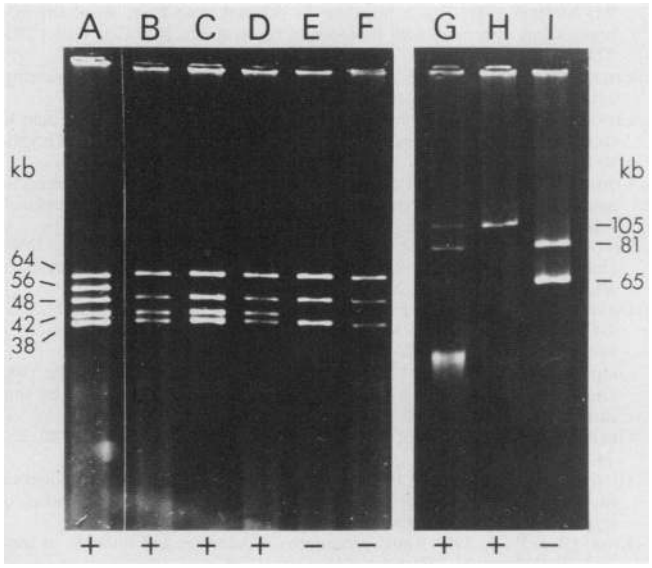

FIG. 3. Cytokinin production and plasmid status. Agarose gel electrophoresis (0.7% agarose; Tris-borate buffer, 0.089 M [pH 8.0]) of plasmids from *P. savastanoi* strains. A, PB213-2 and G, EW1006, and their respective deletion mutants; B, 213-2-1; C, 213-2-2; D, 213-2-3; E, 213-2-4; F, 213-2-5; H, 1006-1; I, 1006-2. On the left and right are marked the estimated sizes (kb) of the plasmids (7). Zeatin production was estimated in unfractionated culture supernatant solutions: +, >150 ng/ml; −, less than 500 pg/ml; detection limit, 300 pg/ml.

zeatin, whereas its deletion mutant PB213-2-4 secreted less than 300 pg/ml. Concomitantly, the amount of *iso*-pentenyladenosine decreased from 8 ng/ml down to 0.4 ng/ml. Deletion of 40 kb of the 105 kb plasmid of EW1006 similarly reduced zeatin levels (from 400 ng/ml down to 12 ng/ml), ribosylzeatin levels (20 ng/ml to undetectable), and ribosyl-l″-methylzeatin levels (180 ng/ml to undetectable), but did not change the amount of *iso*-pentenyladenosine secreted (approximately 5 ng/ml).

Cloning and Expression of the EW1006 Cytokinin Biosynthetic Genes. To isolate and characterize the cytokinin biosynthetic gene(s), a *Sau*3A partial digest of EW1006 plasmid DNA was prepared and the resulting fragments were ligated to the wide host range vector pRK290. Transformation of *E. coli* HB101 with this DNA resulted in one clone, HB101(pPS001), which secreted cytokinins during growth in liquid culture. The recombinant plasmid pPS001 contained a 14.5 kb fragment derived from the large plasmid of EW1006. The *Pseudomonas transzeatin* secretion locus (*ptz*), was further localized by digestion of pPS001 with *Eco*RI, followed by religation. This gave rise to a cytokinin-secreting clone HB101(pPS002) which had lost a 3.7 kb *Eco*Ri fragment from pPS001. Further subcloning of the *ptz* locus was accomplished by excision of a *Bgl*II-*Bam*HI fragment, yielding pPS003 (26). pPS003 contained 5.2 kb of EW1006 DNA and clones bearing this plasmid secreted cytokinins.

To characterize the nature of the cytokinins produced by the EW1006 plasmid fragment, the culture filtrate of HB101(pPS001) was fractionated by HPLC (Fig. 4). A peak at 13.6 min corresponded in retention time to zeatin. Permethylation of this fraction gave a derivative whose mass spectrum was identical to that of authentic permethylzeatin (data not shown). In addition to zeatin, two peaks, with retention times of 33.4 and 35.5 min (corresponding to authentic iPA and iP), and cross-reacting with anti-iPA antibody, were also observed. No evidence was found for synthesis of ribosyl-l″-methylzeatin. Since, in addition to its role in *trans*-zeatin secretion, the *tzs* gene product from *A. tumefaciens* has been found to specify DMA transferase activity, it seemed likely that the cytokinin biosynthetic locus from *P. savastanoi* might exhibit this characteristic as well. This prediction was confirmed by DMA transferase measurements on extracts from HB101(pPS003). Over 7.7 pmol of iPA was formed/hr·mg protein. No detectable DMA transferase activity was found in *E. coli* HB101(pRK290), a strain lacking the *ptz* locus.

DISCUSSION

In summary, *P. savastanoi* strains secreted extraordinarily high levels of zeatin and other cytokinins. Culture filtrates of EW1006 contained 400 ng/ml of zeatin (about 2 μM), while those of PB213-2 contained 80 ng/ml each of zeatin and ribosylzeatin. In addition, EW1006 produced dihydrozeatin, ribosyldihydrozeatin, and high levels of the newly identified cytokinin, ribosyl-l″-methylzeatin. These experiments demonstrate the utility of immunoaffinity chromatography in the isolation and purification of cytokinins. Correspondence between cytokinin levels measured by integration of the A_{254} absorbance trace and by RIA was excellent for zeatin and ribosylzeatin, but demonstrably poor for ribosyl-l″-methylzeatin as would be expected from the presence of an extra methyl group on the isoprenoid side chain. In fact, we have found that *P. savastanoi* secretes a number of novel methylated zeatin derivatives whose binding affinities for anti-tZR antibody are too low to allow them to be isolated by immunoaffinity chromatography (EMS MacDonald, RO Morris, unpublished data). Immunoaffinity chromatography with existing antibodies is therefore valuable for isolation of many, but not all, cytokinins. Accurate quantitation of methylated cytokinin levels must await the production of further antibodies. Nevertheless, the finding that *P. savastanoi* produces high levels of

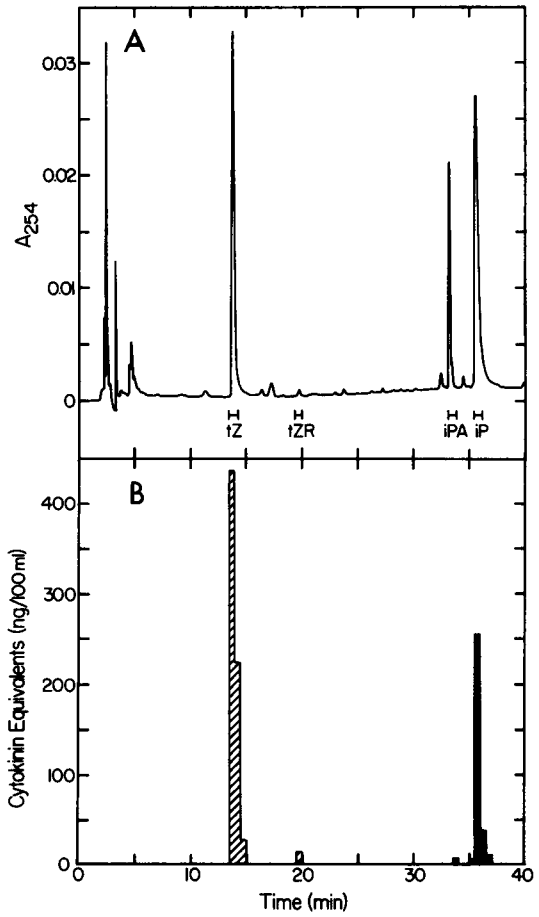

FIG. 4. Production of cytokinins by *E. coli* HB101 (pPS001). Cytokinins present in 400 ml of media from stationary phase cultures of HB101(pPS001) were isolated by passage over columns of DEAE-cellulose (15 ml), octadecyl silica (2 ml) and subsequently over a mixture of anti-tZR (1 ml) and anti-iPA (1 ml) antibodies immobilized on cellulose. Cytokinins were eluted from the immunoaffinity matrix with methanol and fractionated by HPLC as described in the text. Both A_{254} (A) and radioimmunoassay (B) data are shown. Abbreviations and RIA conditions are identical to those in Figure 1.

zeatin and other cytokinins remains valid.

Cytokinin production is specified by plasmid-born genes. Following the precedent established (7), we propose that the 42 kb plasmid of PB213-2 (which is also present in the wild-type PB213) be named pCK1 and that the 105 kb plasmid of EW1006 be named pCK2.

Although the nature of the cytokinin biosynthetic locus remains to be rigorously determined, it is likely that it encodes DMA transferase because: (a) deletion of pCK1 from PB213-2 causes a 10-fold decrease in secretion of *iso*-pentenyladenosine, (b) cytokinin biosynthesis is induced in *E. coli* by the presence of the 4.5 kb fragment of pCK2, and (c) HB101(pPS003) expresses substantial levels of DMA transferase activity. Although homology between the DMA transferase genes of *A. tumefaciens* (*ipt* or *tzs*) and *P. savastanoi* was not detected by hybridization, nucleotide sequence analysis revealed greater than 50% homology between the two sets of genes (26).

The mechanism of synthesis of the six carbon side chain of ribosyl-l″-methylzeatin is not immediately apparent. This novel cytokinin may arise by direct methylation of the five carbon isoprenoid side chain of known cytokinins or it may be formed by an independent route. The location and nature of the genes and enzymes which specify methylation (if indeed methylation does occur) and the effect of methylation on cytokinin biological activity remain to be determined.

The similarities between the phytohormone biosynthetic mechanisms of *P. savastanoi* and *A. tumefaciens* are considerable. Both organisms have genes that specify IAA and cytokinin biosynthesis and IAA production plays a role in pathogenesis by both (1, 10, 24, 25, 31). There is a demonstrable degree of structural similarity between the IAA biosynthetic genes of *P. savastanoi* and those of *A. tumefaciens* (34). Cytokinin biosynthesis is necessary for tumor formation by *A. tumefaciens* (1, 10) and it seems reasonable that it should also be required for pathogenesis by *P. savastanoi*. In fact, recent experiments (F Roberto, T Kosuge, unpublished data) have shown that deletion mutants lacking a functional *ptz* gene but possessing active *iaa*M and *iaa*H genes, have lost the ability to produce galls on olives. There is, therefore, strong evidence that cytokinin biosynthesis plays a role in the pathogenic process.

Although *A. tumefaciens* phytohormone biosynthetic genes have evolved to function in the eukaryotic environment, those of *P. savastanoi* function in the free-living bacteria. In light of the close structural and functional relationship between the two sets of genes, the question as to their origin and acquisition becomes of great interest.

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