JOURNAL OF BACTERIOLOGY, Sept. 1987, p. 4242–4248
0021-9193/87/094242-07$02.00/0

Vol. 169, No. 9

Cytokinin Production by *Agrobacterium* and *Pseudomonas* spp.

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Received 8 December 1986/Accepted 9 June 1987

The production of cytokinins by plant-associated bacteria was examined by radioimmunoassay. Strains producing *trans*-zeatin were identified in the genera *Agrobacterium* and *Pseudomonas*. *Agrobacterium tumefaciens* strains containing nopaline tumor-inducing plasmids, *A. tumefaciens* Lippia isolates, and *Agrobacterium rhizogenes* strains produced *trans*-zeatin in culture at 0.5 to 44 μg/liter. *Pseudomonas solanacearum* and *Pseudomonas syringae* pv. *savastanoi* produced *trans*-zeatin at levels of up to 1 mg/liter. In vitro cytokinin biosynthetic activity was measured for representative strains and was found to correlate with *trans*-zeatin production. The genetic locus for *trans*-zeatin secretion (*tzs*) was cloned from four strains: *A. tumefaciens* T37, *A. rhizogenes* A4, *P. solanacearum* K60, and *P. syringae* pv. *savastanoi* 1006. Southern blot analysis showed substantial homology of the *Agrobacterium tzs* genes to each other but not to the two *Pseudomonas* genes.

In several plant-pathogen interactions, phytohormones have been shown to be involved with virulence (10, 14, 20, 23, 27). For example, cytokinin production by pathogenic strains of *Rhodococcus fascians* (basonym, *Corynebacterium fascians*), the causal agent of fasciation or witches broom, is strongly associated with virulence. Production of auxin by *Xanthomonas campestris* pv. *campestris* and *Pseudomonas solanacearum*, causal agents of black rot and bacterial wilt, respectively, has been reported. The disease symptoms of bacterial wilt are characteristic of the effects associated with increased auxin concentration. Auxin production is, however, not sufficient for virulence since avirulent *P. solanacearum* strains also synthesize auxins (24).

Two well-studied phytopathogens, *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi*, incite hyperplasias on susceptible plant hosts (21–23). Auxin production by *P. syringae* pv. *savastanoi*, the causal agent of olive knot disease, is required for pathogenicity; and increased severity of the disease symptoms is correlated with increased auxin synthesis (32, 33). Cytokinin and auxin production by *P. syringae* pv. *savastanoi* have also been reported to be associated with virulence (33).

Infection of susceptible dicotyledonous hosts by *A. tumefaciens* results in formation of crown gall tumors. Virulent strains contain a tumor-inducing (Ti) plasmid, which has the two regions that are required for tumor formation. The T-DNA contains several oncogenes which are transferred and integrated into the host nuclear DNA. Three of these genes encode for cytokinin (*tmr*) or auxin (*tms-1*, *tms-2*) biosynthetic enzymes (20, 21, 23). The virulence (*vir*) region contains genes essential for virulence, but these genes are not transferred to the plant. In nopaline strains, one *vir* locus (*tzs*) is responsible for *trans*-zeatin (tZ) secretion into the culture medium (25). The *tzs* gene shows significant DNA homology to the *tmr* gene (2, 20). The nopaline strain C58 also produces indoleacetic acid in culture. One locus associated with indoleacetic acid production is located in the *vir* region and is required for tumorigenesis (17).

Since cytokinin production has been previously reported in only a limited number of plant-associated bacteria, a survey was undertaken to determine if cytokinin production is a widespread phenomenon or if it is limited to only a few species. The identification of additional phytopathogens which synthesize tZ may be useful for future studies on the involvement of phytohormones in plant-pathogen interactions.

MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains used in this study are listed in Table 1. Bacteria were maintained on the following media: *Escherichia coli*, L agar (19); agrobacteria, AB minimal medium plus biotin (7); xanthomonas, AB minimal or King B medium (16); rhodococci, AB minimal or NBY (8 g of nutrient broth, 2 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.12 g of $MgSO_4$, and 5 g of glucose per liter) medium; and *P. syringae* pathovars, King B medium. For short-term maintenance, *P. solanacearum* strains were maintained on CPG plates, but otherwise they were stored in water (15). *E. coli* strains carrying recombinant plasmids were maintained on medium containing 200 μg of carbenicillin per ml.

For initial screening of culture medium for tZ content and quantitative analysis of cytokinins in culture medium, bacteria were grown in M9 medium (19) supplemented with 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.01% thiamine, and 2 μg of biotin per liter or in AB minimal medium supplemented with 2 μg of biotin per liter. Bacteria were cultured in silanized test tubes or flasks on a rotary shaker at 25°C, except for *E. coli*, which was grown at 37°C.

tZ secretion assay. Culture media were screened for the presence of tZ by solution radioimmunoassay as described previously (2), with the exception that nonfat milk (final concentration, 0.1% [wt/vol]) (13) was substituted for ovalbumin and gelatin.

Extraction and analysis of cytokinins from culture medium. Wild-type bacteria were grown to the mid- to late-logarithmic (A_{600}, 0.5 to 1.5) phase in defined medium. Recovery of cytokinins was quantitated by the addition of [^{3}H]iso-

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TABLE 1. Survey of bacterial strains for tZ production and DNA hybridization to *tzs* and *tmr* probes

Strain	Characterization	Source	tZ[a]	DNA hybridization to[b]:				
				T37	A4	K60	1006	A6tmr
Agrobacterium tumefaciens								
A136	C58 chromosomal background	E. W. Nester	−	−	−	−	−	−
A348	A136(pTiA6); octopine	E. W. Nester	−	−	++	−	−	+++
ACH5	Wild type; octopine	E. W. Nester	−	−	++	−	−	+++
IIBV7	Wild type; octopine	E. W. Nester	−	−	++	−	ND	+++
AB2/73	Wild type; Lippia; biotype II	E. W. Nester	+	+	+	−	−	−
AB11/73	Wild type; Lippia; biotypeII	E. W. Nester	+	+	ND	−	ND	ND
S7/73	Wild type; Lippia; biotype II	E. W. Nester	+	+	+	−	−	−
A892	A136(pTiAB2/73d)	E. W. Nester	+	ND	ND	ND	ND	ND
C58	Wild type; nopaline	E. W. Nester	+	+++	+++	−	+	+++
T37	Wild type; nopaline	E. W. Nester	+	+++	+++	−	+	+++
A208	A136(pTiT37); nopaline	E. W. Nester	+	+++	ND	−	ND	ND
A281	A136(pTiBo542)	E. W. Nester	−	−	+++	−	ND	+++
Agrobacterium rhizogenes								
A4	Agropine	E. W. Nester	−	+++	+++	−	+	−
K47	Agropine	E. W. Nester	−	+++	+++	ND	+	−
R1000	Agropine	E. W. Nester	−	+++	ND	ND	ND	ND
15834	Agropine	E. W. Nester	−	+++	+++	−	+	−
TR105	Agropine	E. W. Nester	−	+++	+++	ND	+	−
8196	Mannopine	E. W. Nester	+	+++	+++[c]	−	+	−
TR7	Mannopine	E. W. Nester	+	+++	+++[c]	−	+	−
Pseudomonas syringae								
Y30	pv. *phaseolicola*	D. Mills	−	−	−	−	−	−
PA45	pv. *angulata*	M. Obukowicz	−	−	−	−	−	−
11528	pv. *tabaci*	M. A. Schell	−	−	−	−	−	−
2009	pv. *savastanoi*; oleander isolate	T. Kosuge	−	−	−	−	−	−
205	pv. *savastanoi*; oleander isolate	T. Kosuge	+	ND	ND	ND	ND	ND
213-3	pv. *savastanoi*; oleander isolate	T. Kosuge	+	ND	ND	ND	ND	ND
2015	pv. *savastanoi*; oleander isolate	T. Kosuge	−	ND	ND	ND	ND	ND
231	pv. *savastanoi*; olive isolate	T. Kosuge	+	ND	ND	ND	ND	ND
1006	pv. *savastanoi*; olive isolate	T. Kosuge	+	−	−	−	+++	−
TK1050	pv. *savastanoi*; olive isolate	T. Kosuge	+	−	−	−	+++	−
218	pv. *savastanoi*; privet isolate	T. Kosuge	−	ND	ND	ND	ND	ND
Pseudomonas solanacearum								
25(K60)	Race 1; tomato	L. Sequeira	+	−	−	+++	−	−
8	Race 1; *Eupatorium* sp.; Costa Rica	L. Sequeira	+	−	−	+++	−	−
82	Race 1; potato; Colombia	L. Sequeira	−	−	ND	++	ND	ND
248	Race 1; tobacco; Colombia	L. Sequeira	+	−	−	+++	−	−
258	Race 1; potato; Costa Rica	L. Sequeira	+	−	−	+++	−	−
263	Race 1; potato; Peru	L. Sequeira	−	−	−	+++	−	−
273	Race 1; potato; Costa Rica	L. Sequeira	−	−	ND	+++	ND	ND
301	Race 1; potato; Sri Lanka	L. Sequeira	+	−	−	+++	−	−
386	Race 1	L. Sequeira	−	−	−	−	−	−
106-2	Race 1	M. A. Schell	+	−	−	+++	−	−
41	Race 2; banana; Venezuela	M. A. Schell	−	−	−	+++	−	−
388	Race 2; banana; Grenada	M. A. Schell	−	−	−	−	−	−
GH2	Race 3	M. A. Schell	−	−	−	+++	−	−
19	Race 3; potato; Colombia	L. Sequeira	−	−	ND	+++	ND	ND
229	Race 3; potato; Brazil	L. Sequeira	±	−	−	+++	−	−
260	Race 3; potato; Peru	L. Sequeira	±	−	−	+++	−	−
360	Unknown race; mulberry	L. Sequeira	+	−	−	+++	−	−

Continued

pentenyladenosine dialcohol to cultures immediately before centrifugation. Cytokinins were extracted from the culture medium as described previously (25). Briefly, cell-free culture supernatant (25 to 50 ml) was passed through octadecylsilica (0.5 to 1 g; 40 to 60 μm; Sepralyte C_{18}; Analytichem, Harborview, Calif.), and cytokinins were eluted with methanol and subsequently fractionated by high-performance liquid chromatography. By using a liquid chromatograph (model 334; Beckman Instruments, Inc., Palo Alto, Calif.) fitted with a cartridge column (3 by 3 cm; Pecosphere C_{18}; Perkin-Elmer, Norwalk, Conn.), extracts were chromatographed as described previously (2). Cytokinin-containing fractions were collected and dried in vacuo for analysis by radioimmunoassay. The [^{3}H]isopentenyladenosine dialcohol fraction was collected and counted to determine cytokinin recovery.

In vitro cytokinin biosynthesis—dimethylallyl PP_i:5′-AMP dimethylallyl transferase assay. Bacterial cytokinin biosynthetic activity was measured by using modifications of previously published methods (1, 11). Wild-type bacteria

TABLE 1—*Continued*

Strain	Characterization	Source	tZ[a]	DNA hybridization to[b]:				
				T37	A4	K60	1006	A6tmr
Xanthomonas campestris pv. *campestris*								
PHW 120-6		P. H Williams	−	−	−	−	−	−
PHW 47-6		P. H Williams	−	−	−	−	−	−
PHW 117-10		P. H Williams	−	−	−	−	−	−
PHW 116-6		P. H Williams	−	−	−	−	−	−
Rhodococcus fascians[d]								
MW11		E. W. Nester	±	−	−	−	−	−
CF19	Origin; gall formation	A. K. Vidaver	−	−	−	−	−	−
CF107	Origin; gall formation	A. K. Vidaver	−	−	ND	−	ND	ND

[a] Assay of cell-free culture medium for tZ immunoreactive compounds. Symbols: +, tZ production, >4 ng/500 μl; −, undetectable, <0.4 ng/500 μl; and ±, weak to intermediate reaction, 0.8 to 1 ng/500 μl.

[b] Hybridization of total DNA preparations to *tzs* probes described in the text. Symbols: + + +, strong hybridization under high-stringency wash condition; + +, weak hybridization under high-stringency wash conditions; +, hybridization under low-stringency wash conditions only; −, no detectable hybridization; ND, not determined.

[c] Under low-stringency wash conditions, A4 *tzs* probe also hybridized to a 2.2-kb fragment in mannopine strains 8196 and TR7.

[d] Basonym, *Corynebacterium fascians*.

were grown in rich medium to the mid-log phase, harvested by centrifugation, and washed in Tris A buffer, which consisted of 50 mM Tris hydrochloride (pH 7.4), 10 mM $MgCl_2$, 20 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride. Washed cells were suspended at 1 g (wet weight)/5 ml of Tris A buffer, sonicated six times for 30 s each time, centrifuged to remove cell debris, and dialyzed against two 4-liter changes of Tris A buffer. A fraction (200 μl) of the dialyzed protein was removed for protein determination (18), and the remaining protein was assayed for dimethylallyl PP_i:5′-AMP dimethylallyl transferase activity in a reaction volume of 10 ml of Tris A buffer containing [^{3}H]AMP (0.5 μCi, 20 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), dimethylallyl PP_i (1 mg), and 10 mM potassium fluoride. Reactions were gently mixed at room temperature for 90 min and terminated by the addition of EDTA to a final concentration of 50 mM, and [^{3}H]isopentenyladenosine dialcohol was added as a recovery standard. Cytokinins were extracted by adsorption onto C_{18}-silica columns, eluted with methanol, and dried in vacuo. Samples were dissolved in 50 mM Tris hydrochloride (pH 9.1)–15 mM $MgCl_2$, treated with alkaline phosphatase (1 U; Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min, extracted by adsorption onto C_{18}-silica, eluted with methanol, and dried in vacuo. Samples were dissolved in methanol and chromatographed, cytokinin-containing fractions were collected, and radioactivity was determined by scintillation counting.

DNA isolation. Total DNA was isolated from *Agrobacterium* and *Pseudomonas* spp. by the alkaline lysis procedure described by Currier and Nester (8). Total *Rhodococcus* DNA was isolated by the method described by Schiller et al. (29). For hybridization probes, plasmid DNA was isolated by the method described by Birnboim and Doly (6).

Hybridizations. Total bacterial DNA was digested with *Eco*RI, electrophoretically separated, and transferred to nylon membranes (Zeta-Probe; Bio-Rad Laboratories, Richmond, Calif.) as described by Southern (30). Restriction fragments containing the different *tzs* gene probes were gel purified (5) and radioactively labeled by nick translation (26).

Hybridization conditions were slightly modified from those described by Amasino (3). Hybridizations were done in a low-stringency solution containing 0.5 M phosphate (pH 7.2), 7% sodium dodecyl sulfate, 0.25 M NaCl, 1 mM EDTA, 35% formamide, and 10 μg of denatured salmon sperm DNA per ml at 42°C for 14 to 18 h. Nylon membranes were washed twice at 65°C for 50 min in a low-stringency wash solution containing 0.25 M phosphate (pH 7.2), 2.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1.2% sodium dodecyl sulfate, and 1 mM EDTA. Following autoradiography, blots were washed twice in a high-stringency wash solution containing 50 mM phosphate (ph 7.2), 1.2% sodium dodecyl sulfate, and 1 mM EDTA and again autoradiographed.

Hybridization probes. For the *A. tumefaciens* T37 *tzs* probe, an 830-base-pair *Eco*RI fragment from an M13 subclone, M13-8.1 (2), was used. This fragment contained 112 base pairs 5′ to the translational start codon and the entire *tzs* gene minus the last 8 base pairs.

The *Agrobacterium rhizogenes* A4 *tzs* gene was subcloned as a 2.1-kilobase (kb) *Pst*I fragment on the basis of homology to the T37 *tzs* gene. The A4 *tzs* gene is located in the *Hin*dIII 19 fragment, which maps in the *vir* region of the hairy-root-inducing (Ri) plasmid (12). *E. coli* isolates containing this *Pst*I fragment cloned into pUC19 synthesized tZ.

The *tzs* gene from *P. solanacearum* K60 was cloned from a cosmid library of genomic DNA. A 4.5-kb *Pst*I fragment was found to contain a functional *tzs* gene, and a 1.2-kb *Sst*I-*Bam*HI subfragment was subsequently used as probe.

The *tzs* gene from *P. syringae* pv. *savastanoi* 1006 was cloned from a *Sau*3A partial cosmid library of plasmid DNA. *E. coli* containing cosmids were screened by radioimmunoassay for tZ production. Clones containing a functional *tzs* gene were further subcloned as a 3.2-kb *Eco*RI-*Sal*I fragment. A 2-kb *Sst*I-*Bam*HI subfragment was used as a *tzs* hybridization probe.

The *Kpn*I fragment (1.1 kb) from pRA120 (1) was used as the *A. tumefaciens* A6 *tmr* probe. This fragment contained the entire *tmr* coding region and 400 base pairs of the noncoding 3′ region.

RESULTS

Screening of culture media for tZ activity. To determine how widely the ability to synthesize tZ is distributed in

TABLE 2. Cytokinin quantitation and DMA transferase activity

Strain	Amt of the following cytokinin[a]:			DMA transferase activity[b]
	tZ	tZR	iP/iPA	
A. tumefaciens				
A136	0	0	0.1	0.5
A348	0	0	0.7	1.0
C58	2	0	0.4	18
T37	44	2	2	51
AB2/73	6	1	0.3	166
S7/73	3	0.4	0.3	47
A. rhizogenes				
A4	0.05	0.03	0.6	2
15834	0.5	0	0.5	11
TR7	0.8	0.7	0	48
8196	3	0.6	0.1	132
P. syringae pv. *savastanoi*				
2009	0	0	0.09	0
1006	1,050	104	0.9	2,170
TK1050	1,140	170	0.6	ND[c]
P. solanacearum				
8	6	0.3	0.3	ND
19	ND	ND	ND	13
41	ND	ND	ND	12
K60	ND	ND	ND	295
229	0.8	0	0.2	78
248	109	18	2	1,750
258	36	9	0.3	ND
260	2	0.4	0.5	17
263	ND	ND	ND	29
301	26	2	0.7	ND
360	13	0.9	0.5	232
386	ND	ND	ND	0.8
388	ND	ND	ND	0

[a] Nanograms of cytokinin per milliliter per A_{600} unit. Abbreviations: tZ, *trans*-zeatin; tZR, tZ riboside; iP, isopentyladenine; iPA, isopentyladenosine.
[b] Femtomoles of cytokinin per milligram of protein per 90 min. DMA, Dimethylallyl PP_i:5'-AMP dimethylallyl.
[c] ND, Not determined.

nature, culture media from plant pathogens were assayed for tZ content (Table 1). The survey assayed cell-free, unfractionated culture media for tZ-immunoreactive material. Under the conditions used, the assay had a detection limit of 400 pg/500 μl of culture medium. Of the strains tested, 21 were positive (>4 ng/500 μl), 3 were intermediate (0.8 to 1 ng/500 μl), and the remainder were negative (<400 ng/500 μl).

Consistent with results of a previous report (25), our data confirmed that *A. tumefaciens* strains containing nopaline Ti plasmids produce tZ, while octopine strains do not. The present survey was more extensive, however, and revealed tZ production by other *Agrobacterium* strains. Several limited-host-range strains, AB2/73, S7/73 and AB11/73, that were originally isolated from galls on *Lippia canescens* (4) also produced tZ. *A. rhizogenes*, the causal agent of hairy root tumors, has two opine utilization phenotypes, agropine and mannopine. Mannopine strains produced detectable levels of tZ, while agropine strains did not under our screening assay conditions. Analysis of larger quantities of culture medium, however, showed that the agropine strains A4 and 15834 synthesized very small amounts of tZ.

A number of phytopathogenic *Pseudomonas* strains were also screened. Only strains within the species *P. syringae* pv. *savastanoi* and *P. solanacearum* secreted tZ. *P. syringae* pv. *savastanoi* has a host range that is limited to olive, oleander, privet, and ash; but cytokinin production did not correlate with host range or any known metabolic marker. Of the *P. solanacearum* strains examined, nine synthesized tZ. Of these nine, six were classified as race 1, two were classified as race 3, and one was uncharacterized.

None of the limited number of *Rhodococcus* or *Xanthomonas* strains examined produced detectable amounts of tZ under the culture and assay conditions used. In addition, strains from several subspecies of *Rhizobium* and *Erwinia* were also tested, but they did not produce tZ (data not shown). These negative results do not rule out, however, production under different culture conditions or production induced by host plant metabolites.

Further identification and quantitation of cytokinins was performed on selected strains. The predominant cytokinin that was found was tZ (Table 2). tZ riboside typically occurred at only 10 to 20% of the tZ level. In all strains except *A. rhizogenes* TR7, isopentyladenine and its riboside were found at low but detectable levels (0.09 to 2.2 ng/ml per A_{600} unit) during log-phase growth. In those cases in which it was determined, the free base isopentyladenine predominated over its riboside. The levels of tZ found in culture media varied greatly from 0.05 ng/ml per A_{600} unit by agrobacteria to 1,140 ng/ml per A_{600} unit by *P. syringae* pv. *savastanoi*.

Cytokinin biosynthesis in vitro. All strains that tested positive for tZ production also had dimethylallyl PP_i:5'-AMP dimethylallyl transferase activity in vitro (Table 2). Although dimethylallyl PP_i:5'-AMP dimethylallyl transferase levels varied greatly, the two assays showed good correlation with each other. Strains that produced low levels of cytokinins had low levels of enzyme activity, and similarly, strains that produced high levels of cytokinins had high levels of enzyme activity.

DNA homology of *tzs* genes among tZ-producing bacteria. To determine if the *tzs* genes from *Agrobacterium* and *Pseudomonas* strains were conserved, probes for each of the four cloned *tzs* genes were hybridized to *Eco*RI digests of total DNA from a representative sampling of tZ-positive and -negative strains. Under low-stringency hybridization and wash conditions, none of the probes hybridized to total DNA from *Xanthomonas* or *Rhodococcus* strains (data not shown). Under high-stringency wash conditions, the strain T37 *tzs* probe hybridized to a single *Eco*RI fragment in nopaline strains C58, T37, and A208 (6.8 kb) and to *A. rhizogenes* agropine (3.5 kb) and mannopine (2.2 kb) strains (Fig. 1A). Under low-stringency wash conditions, the same probe hybridized to a 5.9-kb fragment in *A. tumefaciens* Lippia strains AB2/73, AB11/73, and S7/73. Even under low-stringency wash conditions, no hybridization to the T37 *tzs* probe was seen in octopine strains.

A. rhizogenes agropine strains showed strong homology to the T37 *tzs* probe. Based on this homology, the *tzs* gene from strain A4 was subcloned as a 2.1-kb *Pst*I fragment which was used as the A4 *tzs* probe (Fig. 1B). Under high-stringency wash conditions, homology was observed to *A. tumefaciens* nopaline strains C58 and T37 (6.8 kb); octopine strains ACH5 and A348 (9.5 kb), A281 (8.1 kb), and IIBV7 (8.1 kb); and *A. rhizogenes* agropine (3.5 kb) and mannopine (3.3 kb) strains. In *A. tumefaciens* nopaline and *A. rhizogenes* agropine strains, the same size *Eco*RI fragment hybridized to the A4 and T37 *tzs* probes. DNA sequence analysis of the A4 *tzs* gene contained on the 2.1-kb *Pst*I fragment confirmed greater than 80% homology to the T37 *tzs* gene (unpublished

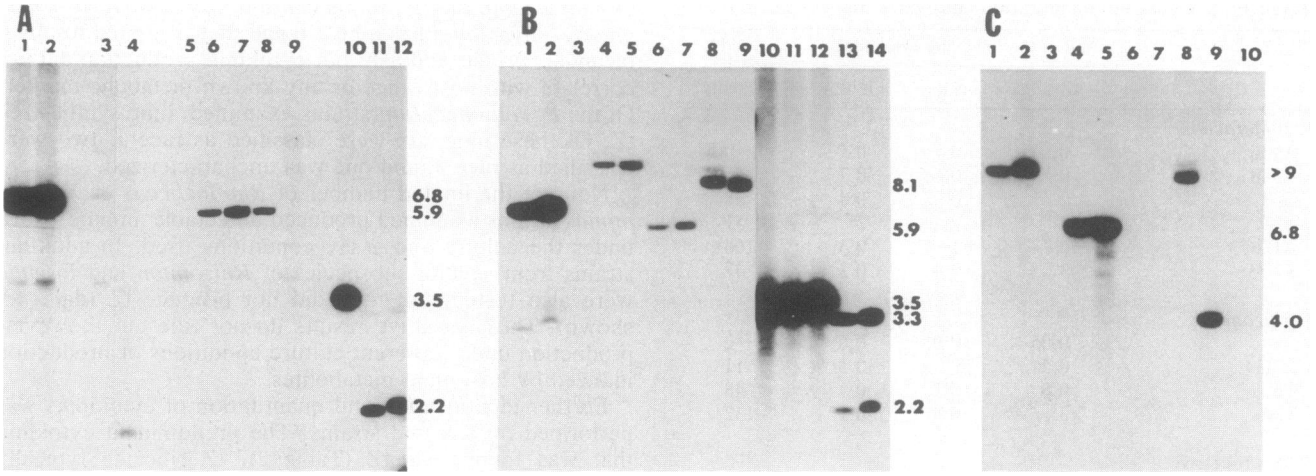

FIG. 1. Hybridization of *tzs* and *tmr* genes to bacterial DNA preparations under low-stringency hybridization and wash conditions. Nick-translated probes were hybridized to total *Agrobacterium* DNA preparations digested with *Eco*RI. (A) *A. tumefaciens* T37 *tzs* probed against T37 (lane 1), C58 (lane 2), A348 (lane 3), IIBV7 (lane 4), A136 (lane 5), AB2/73 (lane 6), AB11/73 (lane 7), S7/73 (lane 8), A281 (lane 9), A4 (lane 10), TR7 (lane 11), 8196 (lane 12). (B) *A. rhizogenes* A4 *tzs* probed against T37 (lane 1), C58 (lane 2), A136 (lane 3), A348 (lane 4), ACH5 (lane 5), AB2/73 (lane 6), S7/73 (lane 7), A281 (lane 8), IIBV7 (lane 9), A4 (lane 10), TR105 (lane 11), K47 (lane 12), TR7 (lane 13), 8196 (lane 14). (C) *A. tumefaciens* A6 *tmr* probed against T37 (lane 1), C58 (lane 2), A136 (lane 3), A348 (lane 4), ACH5 (lane 5), AB2/73 (lane 6), S7/73 (lane 7), A281 (lane 8), IIBV7 (lane 9), A4, (lane 10). Numbers to the right of the gels are in kilobases.

data). Under low-stringency wash conditions, hybridization of the A4 *tzs* probe to a 2.2-kb fragment in mannopine strains and to a 5.9-kb fragment in the Lippia strains AB2/73 and S7/73 was also observed.

Using the *A. tumefaciens* A6 *tmr* probe under high-stringency wash conditions, homology to *A. tumefaciens* IIBV7 (4 kb) and A281 (>9 kb) and also to the octopine (6.8 kb) and nopaline strains (>9 kb) was observed (Fig. 1C). The homology to nopaline strains was not to the *Eco*RI fragment containing the *tzs* gene but rather to the fragment containing the nopaline *tmr* gene, which is highly homologous to the octopine *tmr* gene (9). No homology was seen to either the Lippia strains or to the *A. rhizogenes* strains under low-stringency wash conditions.

The *tzs* probe derived from *P. solanacearum* K60 showed no homology to *Agrobacterium* strains or to *P. syringae* pathovars. DNA sequence analysis of the K60 *tzs* gene contained on the 4.5-kb *Pst*I fragment showed less than 50% homology to the T37 *tzs* gene (unpublished data). Under high-stringency conditions, however, many of the *P. solanacearum* strains hybridized, although not always to *Eco*RI fragments of the same size. Under high-stringency wash conditions, very weak hybridization of the *tzs* gene derived from *P. syringae* pv. *savastanoi* 1006 to *A. tumefaciens* C58 and T37 (6.8 kb) and *A. rhizogenes* A4 (3.5 kb) and 8196 (3.3 kb) was observed. No homology was seen, however, to other *P. syringae* pathovars. Also, strong hybridization was seen to another olive isolate strain, TK1050 (>10 kb), but not to strain 2009, an oleander strain which did not produce tZ.

DISCUSSION

In this study we have confirmed and extended results of previous reports of tZ production by *Agrobacterium* spp. and *P. syringae* pv. *savastanoi* (10, 14, 20). tZ production was found among *Agrobacterium* strains other than those carrying nopaline Ti plasmids. This is the first report of tZ production by *A. rhizogenes* and for the limited host range *A. tumefaciens* Lippia strains. The *tzs* genes from *Agrobacterium* spp. were all plasmidborne. The genes from *A. tumefaciens* T37 and *A. rhizogenes* A4 were located in the *vir* regions of the Ti and Ri plasmids, respectively. The *tzs* gene from *A. tumefaciens* Lippia strain AB2/73 is probably located on the megaplasmid pTiAB2/73d, since transconjugates containing this plasmid also produced tZ. In addition, the *tzs* gene from *P. syringae* pv. *savastanoi* 1006 is located on the larger (90 kb) of the two plasmids (20). The location and linkage of the *tzs* gene in *P. solanacearum* has not yet been determined.

Interestingly, *A. rhizogenes* and *A. tumefaciens* Lippia strains show homology to *tzs* but not to *tmr* (12, 34; this study). Whether this indicates that bacterial tZ production is necessary for virulence will require further study. In fact, cytokinins may only play a secondary role in virulence in crown gall tumorigenesis since octopine strains lack homology to the *tzs* gene and yet are virulent on a wide variety of host plants. Additional evidence suggesting that tZ secretion is not absolutely required for virulence comes from mutagenesis of the strain T37 *tzs* gene by transposon insertional inactivation. Marker exchange of the mutagenized *tzs* gene into the nopaline plasmid pTiC58 did not result in avirulence on *Kalanchoe diagremontiana* leaves and stems or on *Nicotiana tabacum* var. *xanthi* (D. E. Akiyoshi, unpublished data). Therefore, the presence of *tzs* on Ri and nopaline Ti plasmids may not directly affect virulence; but rather, it may have some undetermined effect on host range, efficiency of tumor initiation, or susceptibility of plant tissue to infection. Recently, it has been reported (28) that the *tzs* gene, like many of the other *vir* genes, is inducible by acetosyringone, a compound produced by wounded plant cells (31).

Agrobacterium spp. and *P. syringae* pv. *savastanoi* are similar in that both pathogens incite hyperplasias at wound sites on susceptible host plants (21, 22). In other respects, however, the diseases are different, which may be indicative of differences in their mechanism of infection. Crown gall and hairy root tumors incited by *Agrobacterium* infection

are transformed by the transfer of bacterial T-DNA to the plant cell. In contrast, knots on olive or oleander formed by *P. syringae* pv. *savastanoi* infection are untransformed (22). Both pathogens produce auxin and cytokinin in culture, although at greatly different levels (14, 20, 23, 33; this study). The much lower levels of phytohormones produced by *Agrobacterium* spp. may reflect the hormone auxotrophy of crown gall tissue, which would not require high levels of hormone production by the bacteria for tumor maintenance. In contrast, production of higher levels of phytohormones by *P. syringae* pv. *savastanoi* may be necessary for the maintainenance of rapid gall growth since the plant cells do not produce phytohormones at a level necessary for gall growth.

The occurrence of tZ in culture medium of *P. solanacearum* was unexpected since the disease symptoms of bacterial wilt are indicative of altered auxin levels. Auxin production has been studied previously in *P. solanacearum* (24) and has been shown not to be directly involved in virulence. Thus, the role of cytokinin and auxin production in the pathogenicity of *P. solanacearum* strains is unclear and will require further study.

Although several strains were found to produce tZ in this study, activity was not detected in some bacteria, including *Rhizobium* and *Rhodococcus* spp., in which cytokinin production has been reported previously (10, 14, 20). This may be accounted for in part by the use in this study of different strains, different growth and harvest conditions, and an immunoassay specific for tZ derivatives only.

With the availability of genetic and analytical tools including rapid high-performance liquid chromatography and radioimmunoassay; the role of cytokinins in pathogenesis at the molecular level can now be studied.

ACKNOWLEDGMENTS

This study was supported by grant NP-336B from the American Cancer Society, grant PCM 8413335 from the National Science Foundation, and grant 85-FSTY-9-0141 from the U.S. Department of Agriculture.

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