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Diversity of Opines and Opine-Catabolizing Bacteria Isolated from Naturally Occurring Crown Gall Tumors

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The diversity of opines from 43 naturally occurring crown gall tumors on several plant species was analyzed for the presence of agropine, chrysopine, iminodiacid, an unidentified leucinopine-like iminodiacid (IDA-B), mannopine, octopine, nopaline, DL- and LL-succinamopine, leucinopine and heliopine. Opine utilization patterns of agrobacteria and fluorescent pseudomonads resident in a tumor were then analyzed and compared for agreement with the opine isolated from that tumor. Nopaline was the most common opine found and was detected in tumors from cherry, blackberry, grape, and plum. Octopine was not found, although octopine-catabolizing bacteria were isolated from several tumors. A new, previously undescribed iminodiacid of the succinamopine-leucinopine type (provisionally designated IDA-B) was isolated from tumors of wild blackberry. Field tumors from apple, blueberry and grape yielded no detectable opines, even though opine-utilizing bacteria were present. Bacterial isolates from plum and cherry showed the best correspondence between the opine in tumors (nopaline) and the presence of bacteria that catabolized that opine. However, several unusual opine catabolic combinations were identified, including isolates that catabolized a variety of opines but were nonpathogenic. More variability was observed among isolates from field tumors on the remaining plant species. We isolated novel mannopine-nopaline type agrobacteria from field tumors of cherry, plum and blackberry that induced tumors containing either mannopine (plus agropine) or nopaline, but not both. Epidemiologically, the galled plants from an area were not of clonal origin (same Ti plasmid), indicating that the field tumors from a small area were incited by more than one type of Ti plasmid.

Plant tumors known as crown gall are incited by pathogenic, soil-inhabiting *Agrobacterium* species. These bacteria carry a large plasmid referred to as a tumor-inducing (Ti) plasmid. Oncogenesis results from attachment of pathogenic agrobacteria to cells in a plant wound followed by transfer of a portion (T-DNA) of the Ti plasmid into the plant nucleus. The T-DNA subsequently integrates into a host chromosome where it is maintained and transcribed (16). Two classes of genes on the T-DNA have been defined: (i) ONC genes responsible for the cancerous growth of the infected plant cells and (ii) a region that encodes synthesis of opines.

Opines are small, novel metabolites whose structures are specifically determined by the inciting *Agrobacterium* strain (44). They occur rarely in nature but are found in crown gall tumors. The Ti plasmid of the inciting bacterium is biochemically distinguishable by the family of opines whose synthesis it encodes.

Chemically, the opines are a diverse group that fall into two structural classes. Agrocinopines are sugar-phosphodiesters. The other opines are secondary amine derivatives, two of which, octopine and nopaline, are synthesized through reductive condensation of arginine with pyruvate and alpha-ketoglutarate, respectively. A group of four other secondary amines are designated as the mannityl opines (27). A variety of opines have been identified, including octopine, lysopine, nopaline, succinamopine, leucinopine, cucumopine, heliopine, chrysopine, mikimopine, mannopine, agropine, and the agrocinopines (15, 22, 27). Further examples of opine diversity are the incompletely characterized vitopine (47), pseudonopaline (22), and opine X (19).

Opine catabolism by the inciting *Agrobacterium* strain is mediated by Ti-plasmid genes not transferred to the plant cell and is typically specific to the opine or opines produced in the induced tumor (40). This nutritional specificity generally means that the inciting strain of *Agrobacterium* catabolizes only those opines produced by the incited tumor. Since opines have been reported to account for as much as 7% of dry weight of a tumor in the absence of catabolizing bacteria (29), it has been proposed that opine production by crown gall tumors provides the inciting *Agrobacterium* strain with a selective growth substrate favoring its propagation (i.e., the "opine concept" [48]). Thus, opines are thought to play a major role in the epidemiology of crown gall and the ecology of *Agrobacterium* spp. (8). Opines not only serve as carbon and/or nitrogen sources for the tumor-inducing bacterium, but a few of the opines also can induce conjugal transfer of the Ti plasmid to neighboring nontumorigenic agrobacteria (25, 30, 31). These features could contribute to the dissemination of the inciting Ti plasmid and favor growth of its bacterial host.

Although the agrobacteria arguably create an exclusive niche for themselves within a very specialized tumor environment, the use of a medium containing opines as the sole carbon/nitrogen source did not favor growth of *Agrobacterium* over other microbes (12, 43). Isolations are hampered by the growth of associated bacteria and fungi, especially pseudomonads, as observed earlier (41). Subsequent reports demonstrated that opine-utilizing non-*Agrobacterium* species were common to soil, rhizosphere, and tumors (2, 9, 23). Other opine-utilizing microorganisms include fluorescent and nonfluorescent pseudomonads, coryneforms, *Arthrobacter*, and several fungal species (1, 3, 50), demonstrating conclusively that opine utilization is not limited to *Agrobacterium.*

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This catabolism of opines by diverse microorganisms raises interesting questions about the role of opines in the ecology of *Agrobacterium*. Does the opine produced by the tumor dictate the population type of *Agrobacterium* and *Pseudomonas* which subsequently colonize that tumor? Do tumors on host plants from the same field or location synthesize identical opines, suggesting that a common T-DNA region is encoded on a Ti plasmid homogenous in the *Agrobacterium* population?

To address these questions, we (i) assayed the kinds of opines present in naturally occurring field tumors from a variety of host plants, (ii) characterized the opine-catabolic patterns of *Agrobacterium* and *Pseudomonas* isolates associated with each tumor, (iii) determined whether there was agreement between opine type(s) synthesized in field tumors and in lab tumors incited by pathogens isolated from the field tumor, and (iv) determined whether pathogens from tumors containing no detectable opines could incite lab tumors that synthesize a known opine.

MATERIALS AND METHODS

Collecting and processing field tumors. Naturally infected plants of apple, blueberry, cherry, grape, plum, and wild blackberry, each bearing a crown gall tumor, were collected from broadly dispersed locations in OR, WA, and MI (Table 1). To compare the homogeneity of opines and resident bacteria in tumors from the same plant species (Table 2), galled specimens were harvested within the same geographical location except for two sets of galled apple trees, each collected from a different site. Distances between galled plants varied, ranging from 0.2 to 45 m. In all cases, soil and debris were washed from the tumor surface under a stream of tap water. Resident bacteria were isolated from a portion of each tumor as described below; the remaining tumor tissue was diced, suspended in 95% ethanol, and stored at −20°C until opine analysis was performed.

Identification of opines in tumors. Samples (ca. 400 mg) of freshly excised tumors were stored in 2 ml of 95% ethanol for up to 1 week. Samples were then removed, air-dried at room temperature, ground to a fine powder with a mortar and pestle, and resuspended in the original ethanol to which 0.5 ml of water was added. After 24 h at room temperature extracts were clarified by filtration, concentrated on a rotary evaporator, and analyzed for the presence of opines by high-voltage paper electrophoresis (HVPE) at pH 1.8 and 4.0 (17–19). At pH 1.8 nopaline exists as a cation, while at pH 4.0 it exists as an anion. Extracts were scored as positive for nopaline if they contained a phenanthrenequinone-stainable metabolite comigrating with nopaline standard at these two pHs. The loading level was generally equivalent to at least 20 mg of tumor tissue (dry weight). Given detection sensitivities of 0.5 μg for octopine and nopaline with phenanthrenequinone, 1 μg for mannopine with silver nitrate, and 3 μg for succinamopine with silver nitrate-mannitol, the lower limit of detection is about 25 ppm (dry weight) for nopaline, 50 ppm for mannopine, and 150 ppm for succinamopine. Nopaline is present in tumors incited by *A. tumefaciens* C58 at about 5 to 10,000 ppm on a dry weight basis. Agropine has been reported in a tumor at 7% dry weight (70,000 ppm) (29).

Two new opines, chrysopine and deoxyfructosyl glutamine (8), are detectable with silver nitrate and comigrate at pH 1.8 with agropine and mannopine, respectively. To detect these newer opines, tumor extracts were analyzed by HVPE at pH 1.8 with detection by triphenyl tetrazolium chloride, which detects only chrysopine and deoxyfructosyl glutamine (22). This procedure confirmed that all isolates previously identified as agropine strains were agropine strains and not chrysopine strains. However, lab tumors induced by all agropine strains which were retested did contain deoxyfructosyl glutamine, an intermediate in the biosynthesis of agropine and mannopine (28).

An enrichment procedure was added for tumors in which opines were not detected initially. The potential opine-containing amino acid fraction was enriched by absorption onto Dowex 50 cation exchange resin, eluted with ammonia, and concentrated on a rotary evaporator before HVPE to increase detection sensitivity. The iminodiacid opine (IDA-B) in blackberry tumors was further purified by preparative electrophoresis at pH 2.8. Purified IDA-B was examined on long electrophoretic runs at pH 1.8 and 2.8 to compare it to the threo- and erythro- isomers of succinamopine and leucinopine.

There were no tests performed for the opines mikinopine (34, 35) and cucumopine (24), which were published after the analyses reported herein.

Isolation of bacteria from tumors. After washing as described above, a 0.1- to 1-g sample (depending on the size of the tumor) was cut from the tumor, diced, and suspended in sterile water for 30 min. The vortexed suspension was then serially diluted before streaking or spreading to duplicate plates of Kerr and Brisbane's selective media for *Agrobacterium* Bv 1 and 2 (10), Roy and Sasser's *Agrobacterium* Bv 3 medium (45), King's Medium B for fluorescent pseudomonads (37) and a battery of defined media containing opines as the sole carbon (C) and nitrogen (N) source, opine + C (glucose), and opine + N [$(NH_4)_2SO_4$] (13). Opines used in these tests included mannopine, octopine, nopaline, and a 1:1 mixture of DL- and LL-isomers of succinamopine (octopine, nopaline, and mannopine were purchased from Sigma Chemical Co., St. Louis, Mo.; the isomers of succinamopine were synthesized in this study [18]). Opine media were solidified with Noble agar (20.0 g liter^{-1}; Difco Laboratories, Detroit, Mich.) or Gelrite (4 g liter^{-1}; Gelrite gellan gum, Kelco Div., Merck & Co., Inc., San Diego, Calif.). Control plates were glucose plus $(NH_4)_2SO_4$. Isolation plates were incubated at 26°C for 1 week. Colonies were selected from each plate and streaked onto King's medium B. Colonies that produced a diffusible fluorescent pigment and colonies with a morphology similar to *Agrobacterium* were transferred to mannitol glutamate medium (36) amended with 1 g of yeast extract liter^{-1} (MGY). Colonies were suspended in sterile distilled water, restreaked to purity on fresh MGY, and then stored at 4°C on potato dextrose agar slants supplemented with 0.05% $CaCO_3$.

Isolates of *Agrobacterium* from field tumors on wild blackberry were difficult to culture because they grew slowly or not at all on all media tested. Improved growth was obtained on MGY modified to include the following ingredients (33) (grams liter^{-1}): mannitol, 10.0; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract, 0.4; L-glutamic acid (monosodium salt), 2.0; K_2HPO_4, 0.65; KH_2PO_4, 1.0; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.01; 1 ml each of micronutrient stocks A and B (Stock A [grams liter^{-1}]: $MnSO_4 \cdot H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02; H_3BO_3, 0.002; $CoCl_2$, 0.002; Na_2MoO_4, 0.02; Stock B: biotin, 0.01; Ca pantothenate, 0.01; thiamine, 0.01.) The medium was adjusted to pH 7.0, 15 g of agar was added, and the medium was autoclaved.

Bacterial patterns of opine catabolism. All bacterial isolates were first screened for putative opine catabolism on a basal salts medium supplemented with 5 mM octopine, nopaline, mannopine, or succinamopine and solidified by addition of (4 g liter^{-1}) Gelrite gellan gum. Suspected opine catabolism by bacteria that grew on the solid medium was confirmed in a liquid medium containing opine as the sole carbon and nitrogen source (12). Opine catabolism was indicated by an increase in cell density as measured in a Spectronic 20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) at 600 nM. A colorimeter reading of 0.20 or greater absorbance was rated positive for growth. Controls consisted of pathogenic *Agrobacterium* sp. strains B49c/83 (mannopine and nopaline utilizer), B6 (mannopine and octopine utilizer), and A518 (succinamopine utilizer).

Disappearance of opines from liquid medium inoculated with bacterial strains also was measured to monitor opine catabolism and, in the case of succinamopine, to determine which isomer was catabolized. Octopine, nopaline, and mannopine were analyzed by HVPE at pH 1.8. Detection reagents and buffers were prepared as previously described (17–19). Phenanthrenequinone was used for detection of octopine and nopaline, and silver nitrate was used for detection of mannopine. Agropinic acid, DL-succinamopine, and LL-succinamopine were analyzed by HVPE at pH 2.8 and detected with silver nitrate-mannitol. Catabolic tests were conducted in Bergersen's medium (7) plus 1 mg of test opine(s) ml^{-1} and 2 mg of ammonium sulfate ml^{-1} to which 0.5 mg of mannitol ml^{-1} was added to prove viability of bacteria in cases where no opine was utilized. Because of the large number of isolates and opines to be tested, several analytically compatible opines were added to the same medium (a "cocktail"). Opine cocktail 1 contained 1 mg of octopine ml^{-1}, DL- and LL-succinamopine, and agropinic acid. Cocktail 2 contained 1 mg of mannopine and nopaline ml^{-1}. In some cases opines were retested for catabolism individually to distinguish those which were noninducing, catabolizable substrates. Control strains of *A. tumefaciens* A208 (nopaline), A277 (octopine), A281 (mannopine, agropinic acid, and LL-succinamopine), and A519 (DL-succinamopine) utilized the appropriate opine within 48 h.

Pathogenicity tests. Each isolate of *Agrobacterium* from field tumors was tested for pathogenicity on three tomato seedlings (*Lycopersicon esculentum* Mill. 'Bonnie Best') (13). Strains that produced no tumors on tomato were inoculated to sunflower seedlings (*Helianthus annus* L. 'Mammoth'). Controls consisted of (i) known pathogenic (C58) and nonpathogenic (K84) strains of *Agrobacterium* inoculated to tomato and sunflower seedlings, (ii) wounded, noninoculated seedlings, and (iii) nonwounded plants. All seedlings were grown and maintained in a greenhouse according to standard methods (12) and observed for the presence of tumors after 4 and 8 weeks. An isolate was scored pathogenic if one or more seedlings developed tumors. Micropropagated blackberry plants were grown in tissue culture and inoculated according to the culture conditions and inoculation methods described by Belanger et al. (4).

Opines from lab tumors. Pathogenic isolates from each of the field tumors were inoculated to greenhouse-grown tomato and sunflower seedlings or *in vitro* plantlets in tissue culture to answer the following questions. (i) Would these isolates incite lab tumors with the same opine as the parent field tumor? (ii) Would opines be synthesized in tumors initiated by pathogens isolated from field tumors which contained no detectable opines? Individual lab tumors from 71 seedlings or *in vitro* plantlets were analyzed for the presence of agropine, mannopine, octopine, nopaline, DL-succinamopine, and LL-leucinopine in the same manner as described for parent field tumors.

TABLE 1. Tumor host plant and collection site

Host	Species	Tumor location	Collection site[a]
Apple	*Malus* × *domestica* Borkh.	Root	Central WA
Apple	*Malus* × *domestica* Borkh.	Aerial	Northwestern OR
Blackberry	Wild blackberry (*Rubus* sp.)	Aerial	Southwestern OR
Blueberry	*Vaccinium corymbosum* Linn.	Root	Western MI
Cherry	*Prunus avium* Linn.	Root	Central WA
Grape	*Vitis vinifera*	Aerial	Central WA
Plum	*Prunus cerasifera* Ehrh.	Root	Northwestern OR

[a] Tumor samples, except those from blackberry, were collected from at least five different commercial nurseries and vineyards. Blackberry tumors were removed from canes of wild plants growing in a noncultivated mountainous region of southwestern Oregon.

RESULTS

Field tumors. (i) Opines present. A total of 43 tumors from six plant species were examined (Table 1). Nopaline was the predominant opine found in extracts from 12 of these field tumors (Table 2). The field collection of blackberry tumors consisted of one nopaline tumor and four other tumors containing a previously undescribed iminodiacid of the succinamopine-leucinopine type. The opine was not present in healthy blackberry tissue. For the purpose of this study, the new iminodiacid opine of blackberry tumors is provisionally designated IDA-B pending structural characterization.

One cherry tumor contained agropine. No octopine was detected in extracts from any of the 43 field tumors, even though octopine-catabolizing strains were isolated from some of these tumors. None of the field tumors from apple and blueberry and only one of seven tumors from grape yielded any known opine (Table 2). To determine whether one of the known opines was present, but at a very low level, subsequent electrophoretograms were analyzed using 20 times the tissue equivalents (400 mg) used for routine analyses, but none of the opines known at the time of analysis was detected. In addition, field tumors were pooled to make an even larger sample that was partially purified by absorption onto cation exchange resin. No opines were detected after this enrichment step either. Two unknown substances were detected in the six grapevine tumors that did not contain any of the opines in our routine analysis. These two substances migrated closely together and responded as silver nitrate-chelating anions on pH 2.8 electrophoresis. Both substances were retained on a cation exchange resin and partially eluted in both the distilled water wash and the ammonia eluate. Weak retention and elution by distilled water are characteristic of iminodiacids (18, 20, 21). These substances had electrophoretic mobility at pH 2.8 (PE 2806) and staining characteristics subsequently reported for vitopine (47). It is unknown whether these substances are actually vitopine or another new iminodiacid opine.

(ii) Pathogenicity and opine catabolism of bacterial isolates. A mixture of pathogenic and nonpathogenic agrobacteria (10^5 to 10^7 CFU/g of tumor tissue) was recovered from each of the field tumors except for those from grapevine (Table 2). Despite the absence of analyzed opines in the apple, blueberry, and most of the grape field tumors, numerous opine-catabolizing agrobacteria and psuedomonads were present. Most of the *Agrobacterium* isolates were nonpathogenic except for two from blueberry and six from apple. A substantial number of the avirulent and all of the virulent *Agrobacterium* strains tested catabolized at least one of the opines used in this study except some blackberry isolates that catabolized a new opine (Table 3).

A common and rather striking finding was the isolation of bacteria from field tumors with the capability of utilizing a different opine than those present in the tumor, or bacterial isolates that catabolized opines when no opines were detected in the tumor (Table 2). For example, octopine-catabolizing strains of both *Agrobacterium* and fluorescent *Pseudomonas*

TABLE 2. Opines in naturally occurring crown gall field tumors and patterns of opine catabolism by *Agrobacterium* and *Pseudomonas* strains isolated from the tumors

Host plant[a]	Field tumors[b]	Opine content[c]	Pathogenic *Agrobacterium*[d]	Opine catabolism[e]	
				Agrobacterium	*Pseudomonas*
Cherry	5	NOP	84/87	77 NOP; 1 OCT; 1 MOP+NOP	11 NOP; 1 NOP+SAP
	1	AGR	NT[f]	NT	NT
Plum	5	NOP	45/74	38 NOP; 9 MOP+NOP; 1 MOP; 1 SAP	9 NOP; 1 MOP+NOP; 1 NOP+SAP
Blackberry	1	NOP	5/26	4 NOP; 1 NOP+OCT	18 NOP; 1 NOP+OCT; 2 NOP+SAP
	4	IDA-B	29/62	1 NOP; 1 NOP+OCT; 3 MOP+NOP; 1 MOP+OCT; 1 MOP; 1 OCT; 1 NOP+SAP	13 NOP; 2 NOP+OCT; 6 NOP+SAP
Blueberry	10	None	3/120	40 NOP; 3 OCT; 1 NOP+NOL+SAP	3 NOP; 1 OCT
Apple	10	None	6/63	2 OCT; 1 NOP; 9 MOP	9 OCT
Grape	1	NOP	0/46	1 OCT	8 OCT; 1 NOP; 7 NOP+OCT
	6	None			

[a] Common name of naturally infected host plant. One tumor from each plant was sampled, extracted and analyzed for opines. The opines identified are presented in column three. Samples of healthy tissue from each plant were analyzed for opine content as controls, but no opines were detected.

[b] The number of tumors (each from a different plant) yielding a particular opine, except for blueberry, apple and most grapevine tumors where no opines were detected.

[c] All tumors and healthy control tissues were analyzed for the presence of agropine (AGR), chrysopine (CHR), iminodiacid (IDA), an unidentified leucinopine-like iminodiacid (IDA-B), mannopine (MOP), octopine (OCT), nopaline (NOP), DL- and LL-succinamopine (SAP), leucinopine and heliopine using HVPE analytical paper electrophoresis. None means no opines were detected. Extracts from small tumor samples were subjected to ion exchange enrichment before being electrophoresed.

[d] Denominator is the number of isolates of *Agrobacterium* spp. isolated from the specified class of field tumors that were evaluated for pathogenicity on tomato or sunflower seedlings. Numerator is the number of these isolates that were pathogenic on one of the two hosts.

[e] *Agrobacterium* and *Pseudomonas* isolates were cultivated in liquid medium containing individual opines as the sole source of carbon and nitrogen to determine opine catabolism patterns. Catabolism of opines was measured either as an increase in optical density (600 nM) coincident with bacterial multiplication or as the disappearance of opines using HVPE. The numeral in front of the opine(s) indicates the number of strains catabolizing that opine(s). The "+" sign between opines indicates that more than one opine was catabolized. Control strains with known opine catabolic patterns were run at the same time and were always in agreement with predicted usage.

[f] NT, not tested.

TABLE 3. Opine content in laboratory tumors incited by pathogenic *Agrobacterium* strains originally isolated from wild blackberry field tumors

Field tumor	Field tumor opines	Pathogenic *Agrobacterium* isolate	Bacterial opine catabolism[a]	Lab tumor[b]	
				Hosts	Opines
B-1	NOP	B204/85	None	1 BB	IDA-B
		B206a/85	MOP+NOP	1 BB	AGR+MOP
B-2	IDA-B	B42a/85	MOP+OCT	1 BB	AGR+MOP[c]
		B209b/85	None	1 BB	IDA-B
		B210/85	NOP+DL-SAP	2 SF	IDA-B
		B212/85	MOP	1 BB	None
B-3	IDA-B	B216a/85	MOP	1 BB	AGR+MOP[c]
B-4	IDA-B	B221/85	None	2 SF	IDA-B
		B222/85	MOP+NOP	1 BB	IDA-B
		B222a/85	MOP+NOP	1 BB	AGR+MOP[c]
		B224/85	None	2 SF	IDA-B
B-5	IDA-B	B227/85	None	1 SF	IDA-B
		B227a/85	None	1 BB	IDA-B
		B230/85	None	1 BB	IDA-B
		A4 (control)[d]	AGR+MOP	2 SF	AGR+MOP
		B6 (control)[d]	OCT	3 T	OCT

[a] *Agrobacterium* strains were tested for the ability to catabolize opines (5 mM) as the sole source of carbon and nitrogen in liquid medium. Opine abbreviations: agropine (AGR), mannopine (MOP), nopaline (NOP); octopine (OCT); an unidentified leucinopine-like iminodiacid (IDA-B).

[b] Opine content in extracts from lab tumors incited by a particular pathogenic strain of *Agrobacterium*, as shown in third column, when inoculated to blackberry plantlets grown in tissue culture (BB), tomato (T) and/or sunflower (SF) seedlings and analyzed electrophoretically for the presence of agropine, mannopine, octopine, nopaline, DL-succinamopine, LL-leucinopine, and the unidentified leucinopine-like iminodiacid in the same manner as described for parent field tumors. The number preceding the host abbreviation is the number of tumors yielding the opine(s). None means no opines were detectable.

[c] These tumors also contained deoxyfructosylglutamine, but not chrysopine.

[d] Control strains were rhizogenic strain A4 and tumorigenic strain B6.

were isolated from many of these tumors, even though octopine was not detected in any field tumor.

In general, the opine-catabolizing patterns for *Pseudomonas* isolates were similar to those of the agrobacteria isolated from the same tumor. The interesting differences were the *Pseudomonas* isolates from cherry, plum, and blackberry tumors that could catabolize DL-succinamopine, an opine not present in the gall and not catabolized by the *Agrobacterium* strains coinhabiting the galls (Table 2). An additional difference was that none of the pseudomonads tested, except one from a plum tumor, could catabolize mannopine. This inability also was noted by Tremblay (50) with the exception of *Pseudomonas putida* NA513, one of six *P. putida* soil isolates that could utilize mannopine and mannopinic acid (43).

There was good agreement between the kind of opine present in cherry and plum tumors (nopaline) and the opine catabolic pattern of pathogenic *Agrobacterium* isolated from these tumors. However, several unusual opine catabolic combinations also were identified (see Tables 2, 3, and 5). Some *Agrobacterium* strains from nopaline-yielding tumors atypically catabolized both nopaline and mannopine. Others catabolized a variety of opines but were nonpathogenic, and some did not catabolize any of the opines or utilized opines that were not detected in the field tumor from which they were isolated.

Agrobacterium isolates from blackberry tumors exhibited seven different patterns of opine catabolism, and *Pseudomonas* isolates had three patterns (Table 2), making these the most diverse group of isolates from field tumors. Seven isolates from four blackberry tumors did not catabolize any of the opines tested (Table 3). Although no opines were detected in any of the 10 field tumors from blueberry (Table 2), one tumor

TABLE 4. Opine content in laboratory tumors incited by *Agrobacterium* strains originally isolated from blueberry field tumors from which no opines could be detected

Field tumor	Pathogenic *Agrobacterium* isolate	Opine catabolism[a]	Lab tumor[b]	
			Hosts	Opines
D-3	D50/85	OCT	1 SF	NOP
			1 SF	None
	D100/85	NOP	2 SF	NOP
			2 SF	None
	A4 (control)[c]	AGR+MOP	2 SF	AGR+MOP
	B6 (control)[c]	OCT	3 T	OCT

[a] *Agrobacterium* strains were tested for ability to catabolize opines (5 mM) as the sole source of carbon and nitogen in liquid medium. Opine abbreviations: agropine (AGR), mannopine (MOP), nopaline (NOP); octopine (OCT).

[b] Opine content in extracts from lab tumors incited by a particular pathogenic strain of *Agrobacterium*, as shown in third column, when inoculated to tomato (T) and/or sunflower (SF) seedlings and analyzed electrophoretically for the presence of agropine (AGR), mannopine (MOP), octopine (OCT), and nopaline (NOP) in the same manner as described for parent field tumors. The number preceding the host abbreviation is the number of tumors yielding the opine(s). None means no opines were detectable.

[c] Control strains were rhizogenic strain A4 and tumorigenic strain B6.

yielded 41 nopaline-catabolizing agrobacteria, three of which were pathogenic (Table 4). Only four of 15 fluorescent pseudomonad strains catabolized opines.

Lab tumors. There was good agreement between the kind of opine synthesized in lab tumors initiated by pathogenic *Agrobacterium* strains isolated from plum and cherry field tumors and the opine present in the field tumor (Table 5 and 6). However, these pathogenic *Agrobacterium* isolates did not always incite lab tumors with the same opine as the parent field tumor, and some strains incited lab tumors which synthesized opines even though they were isolated from field tumors containing no detectable opines (Table 4). Diversity was most apparent among agrobacteria from IDA-B type field tumors

TABLE 5. Opine content in laboratory tumors incited by *Agrobacterium* strains originally isolated from plum field tumors

Field tumor	Field tumor opines	Pathogenic *Agrobacterium* isolate	Bacterial opine catabolism[a]	Lab tumor[b]	
				Hosts	Opines
A-1	NOP	A1/85	NOP[c]	3 T, 2 SF	NOP
		A2/85	NOP[c]	2 T, 2 SF	NOP
A-2	NOP	A82/85	NOP+MOP	6 SF	NOP
		A84/85	NOP+MOP	1 SF	NOP
		A103/85	NOP[c]+MOP	3 T, 2 SF	NOP
		A113/85	NOP+MOP	8 SF	NOP
		A132/85	NOP[c]+MOP	3 SF	NOP
		A134/85	NOP[c]+MOP	3 T, 4 SF	NOP
		A144/85	NOP[c]+MOP	2 T, 2 SF	NOP
A-5	NOP	A259/85	NOP[c]+MOP	1 SF	None
		A4 (control)[d]	AGR+MOP	2 SF	AGR+MOP
		B6 (control)[d]	OCT	3 T	OCT

[a] *Agrobacterium* strains were tested for ability to catabolize opines (5 mM) as the sole source of carbon and nitogen in liquid medium. Opine abbreviations: agropine (AGR), mannopine (MOP), nopaline (NOP); octopine (OCT).

[b] Opine content in extracts from lab tumors incited by a particular pathogenic strain of *Agrobacterium*, as shown in third column, when inoculated to tomato (T) and/or sunflower (SF) seedlings and analyzed electrophoretically for the presence of agropine, mannopine, octopine, nopaline, DL-succinamopine, and LL-leucinopine in the same manner as described for parent field tumors. The number in front of T and SF is the number of tumors analyzed which yielded opine. None means no opines were detectable.

[c] Octopine also was catabolized in the presence of NOP.

[d] Control strains were rhizogenic strain A4 and tumorigenic strain B6.

TABLE 6. Opine content in laboratory tumors incited by *Agrobacterium* strains originally isolated from cherry field tumors

Field tumor	Field tumor opines	Pathogenic *Agrobacterium* isolate	Bacterial opine catabolism[a]	Lab tumor[b]	
				Hosts	Opines
I-1	NOP	I6/85	OCT	1 SF	None
		I9/85	NOP	3 SF	NOP
I-2	NOP	I22/85	NOP	2 SF	None
		I36/85	NOP	4 SF	NOP
		I40/85	MOP+NOP	3 T, 4 SF	AGR+MOP
I-3	NOP	I44/85	NOP	3 T, 2 SF	NOP
		I45/85	NT	3 T	NOP
				1 SF	NOP
				1 SF	None
		I51/85	NOP	3 T, 3 SF	NOP
		I53/85	NOP	3 T, 4 SF	NOP
I-4	NOP	I66/85	NOP	3 T, 2 SF	NOP
		I68/85	NOP	2 SF	NOP
		I73/85	NOP	3 T, 2 SF	NOP
I-6	AGR+MOP	None isolated			
		A4 (control)[c]	AGR+MOP	2 SF	AGR+MOP
		B6 (control)[c]	OCT	3 T	OCT

[a] *Agrobacterium* strains were tested for the ability to catabolize opines (5 mM) as the sole source of carbon and nitogen in liquid medium. Opine abbreviations: agropine (AGR), mannopine (MOP), nopaline (NOP); octopine (OCT).

[b] Opine content in extracts from lab tumors incited by a particular pathogenic strain of *Agrobacterium*, as shown in third column, when inoculated to tomato (T) and/or sunflower (SF) seedlings and analyzed electrophoretically for the presence of agropine, mannopine, octopine, nopaline, DL-succinamopine, LL-leucinopine, and an unidentified leucinopine-like iminodiacid in the same manner as described for parent field tumors. The number preceding the host abbreviation is the number of tumors yielding the opine(s). None means no opines were detectable.

[c] Control strains were rhizogenic strain A4 and tumorigenic strain B6.

(Table 3) and blueberry (Table 4). Some lab tumors contained no recognizable opine (Table 5, 6, and 7), and several strains catabolized mannopine in addition to nopaline, even though mannopine was not detected in tumors of tomato or sunflower that were incited by these strains (Table 5).

Comparison of the data from plum, cherry, and blackberry lab tumors shows that at least two different types of mannopine/nopaline-catabolizing agrobacteria were isolated in this study: (i) those which confer only nopaline synthesis on the plant tumor and (ii) those conferring agropine/mannopine synthesis only (Table 5). None of the 11 mannopine-nopaline-catabolizing strains tested in 44 tumors on three hosts conferred the ability to synthesize both nopaline and mannityl opines.

DISCUSSION

One of the striking findings from this work was the remarkable diversity of *Agrobacterium* isolates from an individual tumor or group of tumors in the same field or location and the lack of correspondence between bacterial opine catabolism and the opine content of the tumor of isolation. This finding has significant etiological and epidemiological implications since it is theoretically possible that all of the field tumors in a collection area might be incited by the progeny of a single pathogenic *Agrobacterium* strain (clonal origin). If true, all the tumors would contain the same opine. Such clonal origin was indicated in only one (plum) of the four testable collections containing at least one recognizable opine. There were two different tumor opine types present in the collections of blackberry (nopaline and IDA-B) and cherry field tumors (nopaline and agropine), while grape tumors were a mixture of nopaline tumors and tumors lacking a detectable opine. Thus, in the majority of the collections containing at least some recognizable opine, the field tumors in a small area were incited by more than one type of Ti plasmid. This result is not totally unexpected because field tumors were collected some months after initiation of the tumor, and the diversity of bacteria in these tumors could have resulted, in part at least, from colonization of the tumor by bacteria other than the initiating pathogen which also were present in the habitat.

We found frequent occurrence of novel mannopine-nopaline type agrobacteria in field tumors from cherry, plum, and blackberry. These novel nopaline-mannopine catabolic types induced tumors containing either mannopine (plus agropine) or nopaline, but not both. *Agrobacterium* strains that induce tumors producing both mannopine and nopaline are at present unknown, nor have wild-type strains that catabolize both mannopine and nopaline been reported previously. The previously described agropine strains of *A. tumefaciens* catabolize agropine and mannopine, but not nopaline (29, 31). Mannopine strains can catabolize mannopine, but not agropine or nopaline (49).

Opine catabolic patterns for octopine, mannopine and nopaline were similar for all strains whether the analysis was done by observing growth of the bacteria (increase in cell mass) in liquid opine media or by disappearance of opines from a cocktail of opines after a 52-h incubation. Additional opines were utilized from the cocktail after a much longer incubation, but this slower growth could have been from regulatory mutants as described for other bacteria (17) or the result of inefficient catabolism of other opines in the cocktail once the opine catabolic genes for the correct opine were induced.

Agrobacteria that catabolize both mannopine and nopaline raise a variety of questions about the combined utilization of

TABLE 7. Characteristics of bacterial isolates from apple field tumors that yielded no opines

Field tumor[a]	*Agrobacterium*		*Pseudomonas*
	Pathogenic[b]	Opine utilization[c]	Opine utilization[c]
Below ground			
1	0/5	ND	1 OCT
2	0/8	ND	ND
3	0/3	2 OCT	3 OCT
4	0/5	1 MOP; 1 OCT	ND
5	0/5	2 MOP	1 OCT
Above ground			
1	1/15[d]	1 MOP+NOP	ND
2	0/2	ND	2 OCT
3	1/15	ND	ND
4	6/9[e]	7 MOP 1 MOP+OCT 1 NOP	ND
5	0/8	ND	ND

[a] One field tumor from each plant was removed and processed to obtain bacterial isolates. Five samples from one site were from tumors below ground (crown), and five from the other site were aerial tumors on the lower trunk.

[b] Randomly selected isolates were purified and assayed for pathogenicity on tomato seedlings except for those from above-ground tumor 1, which also were assayed on apple seedlings. The numerator is the number of pathogenic strains. The denominator is the total number of strains tested.

[c] Each strain was tested individually in liquid medium containing a single opine as the sole source of carbon and nitrogen. Opine abbreviations: MOP, mannopine; NOP, nopaline; OCT, octopine; ND, no opine catabolism detected.

[d] Strain G11b used opine but was avirulent on tomato. It was virulent on apple.

[e] Strain G56b was virulent on tomato (not tested on apple); G61b and 62b were virulent on apple and tomato; G63 and G68b were virulent only on apple.

these two opines. Are mannopine and nopaline catabolic genes on a single virulence plasmid? If so, why didn't our lab tumors induced by such strains synthesize both nopaline and mannopine? Are they on two different plasmids? Or is there a conventional Ti plasmid present along with catabolic genes for the other opine on the chromosome? Laboratory mutation to mannopine catabolism has been observed for two nopaline strains, C58 and T37 (39). In addition, two new strains have recently been reported (8, 51) which induce tumors containing nopaline and the agropine analog, chrysopine (22), and deoxyfructosylglutamine, a probable precursor (28) of mannopine, agropine, and chrysopine in plant tumors.

The mannopine-nopaline strains found in this study raise the possibility that multiple-opine strains may exist in further unrecognized combinations and that there may be mechanisms and opportunities for exchange of both anabolic and catabolic opine genes between strains involving either the Ti-plasmid or nononcogenic plasmids or both. The reason for retention of the ability to catabolize an opine not present in the tumor incited by the strain is not clear, although ability to colonize tumors incited by other agrobacteria might be an evolutionary advantage for long-term survival and expansion of the gene pool through plasmid exchange.

Inability to detect any opine in so many of the field tumors in this study, plus the presence of avirulent *Agrobacterium* isolates in these tumors, is difficult to explain. One possibility is that those tumors lacking detectable opines are not the result of oncogenic transformation by *Agrobacterium* T-DNA, even though they were visually identical to opine-yielding tumors. Other possibilities are that some of these isolates which were not tested on the host from which they were originally isolated are narrow-host-range strains (25) or that they induce tumors containing an unrecognized opine, or even no opine. It has been shown that *A. tumefaciens* strains isolated from apple tumors can mutate to avirulence after initiating a tumor but retain the ability to catabolize opines (4). On the other hand, a fully functional opine synthase gene may not always be transferred to the host plant, or the host may interfere with expression of the gene. Octopine/agropine-type Ti-plasmids are known to contain two T-DNA regions, one of which (TR) encodes tumor-forming functions and octopine synthase, the other (TL) carrying mannityl opine biosynthetic genes (38). While transfer of TL is required for tumorigenesis, transfer of TR and mannityl opine biosynthesis does not always occur. The parent field tumors without detectable opines may be examples of this type of failure to transfer opine synthesis genes.

How well do the results from this field study agree with the opine concept? The opine concept proposes that T-DNA genes permit the tumor to sequester photosynthate in a form (opine) catabolizable only by enzymes encoded on the nontransferred region of the Ti plasmid of the tumor-inciting *Agrobacterium* strain, thus providing an exclusive ecological niche for the inciting strain (46). However, there have been no studies to determine whether this hypothesis accurately describes naturally occurring tumors in the field. The study reported here shows agreement with the basic hypothesis in some hosts, but not in others. Opine-catabolizing *Agrobacterium* and fluorescent *Pseudomonas* bacteria were isolated from all naturally occurring tumors regardless of whether the tumors synthesized an opine or were lacking detectable opines. To our knowledge, this is the first report of such cohabitation of natural tumors by different opine-catabolizing genera, although cohabitation was not totally unexpected since Nautiyal and Dion had reported that coryneforms, pseudomonads, and agrobacteria were frequent colonizers of potato tuber tissue (42). However, in the work reported herein, the pattern of opine catabolism by both *Agrobacterium* and *Pseudomonas* (data not shown) was typically variable and often different than expected based on the opine present in the parent field tumor.

Other inconsistencies with the opine concept were noted. Strain B42a was isolated from a nopaline-type blackberry field tumor but induced an agropine-mannopine type laboratory tumor. Some opine-catabolizing *Agrobacterium* isolates induced a single analyzable tumor containing no recognizable opine (A259, B212, i65, and i22). Other cases were observed in which virulent *Agrobacterium* strains (D50 and D100) induced mixed collections of tumors, some with the appropriate opine and others with no detectable opine, a phenomenon that might be due to gene silencing or incomplete T-DNA transfer.

The heterogeneity of bacterial strains we observed from a single below-ground tumor also may have resulted from colonization of the developing tumor by a variety of opine-catabolizing microorganisms potentially present in soil, some of which show a preference for a tumor habitat (42). The ultimate population level of a given opine-catabolizing bacterial strain would depend in part upon its competitive ability to catabolize opines (5, 6) or on intergeneric and/or intrageneric transfer of opine-catabolizing genes. Mannopine catabolic genes have been transferred *in vitro* by conjugation to one *P. fluorescens* and one *P. putida* strain, but only the *P. fluorescens* transconjugant could utilize mannopine (26). The latter phenomenon has not been demonstrated in planta under field conditions.

Several conclusions can be drawn from this work relative to the role of opines in the ecology of *Agrobacterium* and non-*Agrobacterium* microorganisms associated with naturally occurring field tumors. The validity of the opine concept in natural field tumors was demonstrated for some plant hosts, but diversity of opine synthesis and utilization was observed among other tumors and associated bacteria. This was particularly evident among tumors collected from wild blackberry (11), some of which synthesized a new opine and were populated by what appears to be a new *Agrobacterium* species (14). Tumors on host plants from the same field or location did not always synthesize identical opines, indicating that the Ti plasmid in the *Agrobacterium* population was not common to all strains. This was substantiated by the observation that pathogenic agrobacteria from the same field tumor induced laboratory tumors which synthesized a variety of opines. Even though the number of pathogenic agrobacteria isolated from field tumors yielding no identifiable opines was low, many of these isolates induced laboratory tumors that synthesized opines. The opine produced by a field tumor does not dictate the population type of *Agrobacterium* and *Pseudomonas* bacteria which colonize that tumor. Furthermore, since opine-catabolizing pseudomonads as well as agrobacteria were isolated from nearly every field tumor, it appears that opine-utilizing pathogenic agrobacteria did not enjoy a competitive edge. Several possibilities exist which could contribute to the diversity reported in this study. (i) Both *Agrobacterium* and non-*Agrobacterium* microorganisms may inhabit soil or plant roots near a developing tumor, and their colonization of the tumor could contribute to the population diversity observed. (ii) The tumor habitat is a complex nutritional environment of which opines are but one component. The opine component would not determine whether a particular group of microorganisms prevail in the tumor, but it could confer a selective advantage on opine-catabolizing cells once a particular microbial type became established (32, 50). (iii) Conjugal transfer among these populations likely contributes additionally to the diversity. However, that question was not addressed in this study.

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