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Opine Utilization by *Agrobacterium* spp.: Octopine-Type Ti Plasmids Encode Two Pathways for Mannopinic Acid Degradation

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Octopine-type strains of *Agrobacterium tumefaciens* degrade the opine mannopinic acid through a specific pathway which involves cleavage of the molecule at the C—N bond between the amino acid and the sugar moieties. Mannose was identified as a product of the reaction. This pathway was inducible by mannopinic and agropinic acids, but not by mannopine or agropine, the two other mannityl opines. The transport system for this pathway appeared to be specific for mannopinic acid. A second, nonspecific pathway for mannopinic acid degradation was also identified. This involved some of the catabolic functions associated with the metabolism of mannopine and agropine. This second pathway was inducible by mannopine and agropine but not by mannopinic or agropinic acids. The transport system for this pathway appeared to have a broad specificity. Transposon Tn*5 *insertion mutants affected in the specific catabolic pathway were isolated and analyzed. These mutants continued to catabolize mannopine and agropine. Both mapped to a region of the Ti plasmid previously shown to be associated with the catabolism of mannopinic acid. Restriction enzyme analysis of the Ti plasmid from strain 89.10, an octopine strain that is naturally unable to utilize mannopinic acid, showed a deletion in this same region encoding the specific mannopinic acid degradation pathway. Analysis of recombinant clones showed that the second, nonspecific pathway was encoded in a region of the Ti plasmid associated with mannopine and agropine catabolism. This region shared no structural overlap with the segment of the plasmid encoding the specific mannopinic acid degradative pathway.

Opines are specific compounds that are synthesized by crown gall tumors elicited by *Agrobacterium tumefaciens*. Pathogenicity in this bacterium is associated with a high-molecular-weight plasmid, called the Ti (tumor-inducing) plasmid, a segment of which, the T-DNA (transferred DNA) is integrated into and expressed by the transformed plant cells. T-DNA expression determines the phenotype of the tumor cells, including opine production (for reviews, see references 18, 20, 27, 33, and 44). Some 20 different opines that belong to several structural groups have been identified (3, 4, 6, 13, 16, 21; for reviews, see references 30 and 38). All opines are not present in any given tumor. The classes of opines synthesized depend on the particular Ti plasmid harbored by the inciting *Agrobacterium* strain (6, 13, 16, 21, 29). There is a strong correlation between the opine phenotypes of crown gall tumors and the *A. tumefaciens* strains, because every opine detected in a tumor is a growth substrate for the inciting strain. The opine degradative pathways are encoded on the Ti plasmids but at locations separate from the transferred regions (for reviews, see references 18, 20, 27, 31, 33, 38, and 44). Since crown gall cells are genetically modified by the bacterium to redirect their metabolic activities toward the production of nutrients specific for the pathogen, *A. tumefaciens* represents an interesting example of biotrophic parasitism in which the pathogen obtains its nutrients from the living cell of its host (17). Plant pathologists have generally paid little attention to the nature of the plant metabolites on which pathogens feed, and the concept that specific growth substrates can be produced by host cells has not been studied much. In the case of *Agrobacterium* spp., this concept (36, 40) appears be generally applicable. Indeed, the discovery of several new opines in crown gall tumors (16, 21) and *Agrobacterium rhizogenes*-induced hairy root proliferations (28) and the observation that some opines induce the conjugative transfer of Ti plasmids have given a broad basis to this concept (12, 22, 23, 31). The question as to whether similar nutritional relationships exist in other plant-bacterial interactions was answered when a new substance (41), 3-*O*-methyl-*scyllo*inosamine, was identified in alfalfa nodules incited by a particular *Rhizobium meliloti* strain. This strain is able to catabolize 3-*O*-methyl-*scyllo*inosamine, and the genetic determinants for both its production and utilization are encoded on the Sym plasmid (26). This observation suggests that such trophic relationships between plants and the bacteria with which they interact are not restricted to the genus *Agrobacterium*.

We are interested in the evolution of these systems and undertook studies on the utilization of the four mannityl opines (Fig. 1) agropine (AGR), mannopine (MOP), mannopinic acid (MOA), and agropinic acid (AGA). These compounds are abundant in crown gall tumors induced by octopine- or AGR-type *A. tumefaciens* strains (16, 21, 37, 39) and in hairy roots induced by MOP- and AGR-type *A. rhizogenes* strains (28). Determinants involved in mannityl opine synthesis have been precisely mapped to the T_R-DNA

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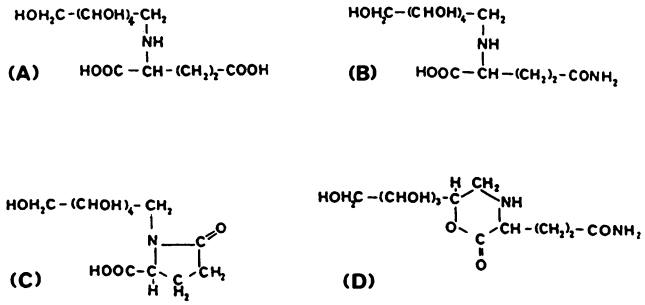

FIG. 1. Structural formulas of the mannityl opines. (A) MOA; (B) MOP; (C) AGA; (D) AGR.

of the octopine- and AGR-type Ti plasmids (14, 35). Studies on the biosynthetic pathway showed that synthesis of MOA and MOP is probably achieved in two steps by the same enzymes via deoxy-fructosyl derivatives (14). Agropine is a cyclization product of MOP, whereas AGA results from the spontaneous rearrangement of MOP or AGR (14, 35).

In contrast to the information available on mannityl opine biosynthesis, only limited data on the corresponding catabolic pathways are available. These functions have been localized to a 43-kilobase segment of the octopine Ti plasmid (7, 8, 10), part of which is actively transcribed in bacteria grown with AGR (19). The mannityl opine catabolic functions overlap one another (10), and some share common regulatory features. For example, it has been shown that preincubation with AGA induces the pathway for MOA catabolism as well as that for the inducing opine (5).

Here we report evidence for two catabolic pathways for MOA and detection of the enzymatic activities associated with one of them. We also supply additional data on the genetic location of the determinants encoding the catabolism of this opine.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1.

Chemicals. Opines were synthesized according to Petit et al. (28) and Dessaux et al. (8). Deoxy-fructosyl glutamate was synthesized as described by Ellis et al. (14). Radiolabeled MOA was synthesized by reductive condensation of L-[U-^{14}C]mannose (L-[U-^{14}C]MAN; Amersham, France) and unlabeled glutamic acid as described previously (28). All other chemicals were from commercial sources.

Growth media. LB medium (24) was used as the rich medium. Nitrogen-free minimal medium used to grow *Agrobacterium* spp. was described by Petit and Tempé (30). Unless stated otherwise, this medium was supplemented as follows: octopine or mannityl opines as sole carbon sources, 10 mM; sucrose, glucose, mannitol, or mannose (MAN), 2.0 g/liter; ammonium sulfate, 1.0 g/liter. When required, a mixture of neomycin (10 μg/ml) and kanamycin (40 μg/ml) was added to this medium. Bacto-Agar (Difco Laboratories, Detroit, Mich.) was incorporated at a final concentration of 1.6% to solidify the medium. For nutritional studies, Noble agar (final concentration, 1.5%; Difco) was used in place of Bacto-Agar. All cultures were incubated at 28°C, and liquid cultures were incubated with agitation to ensure sufficient aeration.

Isolation of mutants affected in mannityl opine catabolism. Tn*5* insertion mutants were obtained by using the suicide plasmid pJB4JI (1). Selection for the presence of Tn*5* in *Agrobacterium* spp. was performed on minimal medium supplemented with sucrose, ammonium sulfate, kanamycin, and neomycin; and resistant colonies were screened for their ability to grow on opine-containing media. Mutants affected in MOA catabolism were scored after 3 days of incubation.

Respirometry. Measurements of O_2 uptake by *Agrobacterium* strains were performed with a respirometer (Braun Warburg) essentially as described by Dessaux et al. (9). Substrates were added at a final concentration of 10 mM.

TABLE 1. Bacterial strains used in this study

Strain	Resident plasmid	Characteristics or comments	Growth on MOA as sole C source	Source
Wild-type *Agrobacterium* strains				
C58C1RS		Ti plasmid-less derivative of C58; resistant to rifampin and streptomycin	−	J. Schell
Ach5	pTiAch5	Wild-type octopine strain[a]	+	J. Schell
15955	pTi15955	Wild-type octopine strain[a]	+	OC[b]
89.10	pTi89.10	Wild-type octopine strain; unable to grow with MOA as sole C source	−	OC
Transconjugants in C58C1RS				
C58C1RS(pTiAch5)	pTiAch5	Selected for utilization of octopine	+	OC
C1-1A	pTiAch5::Tn*5*	Tn*5* insertion mutant	−	This study
C1-1I	pTiAch5::Tn*5*	Tn*5* insertion mutant	−	This study
Agrobacterium strains with cloned pTi15955 fragments				
NT-1(pJS4159K1)	pJS4159K1	*Kpn*I fragment 1 cloned in pJS400	+	S. K. Farrand
NT-1(pYDH208)	pYDH208	Cosmid subclone of pTi15955	−[c]	10
NT-1(pYDH402)	pYDH402	Cosmid subclone of pTi15955	−[c]	10
NT-1(pYDH299)	pYDH299	Cosmid subclone of pTi15955	−	10

[a] Ti plasmids pTiAch5 and pTi15955 were indistinguishable by restriction endonuclease analysis.
[b] OC, Our collection.
[c] These strains utilized MOA, but only on induction with MOP or AGR (see text).

MOA uptake. MOA uptake was assessed by using ^{14}C-labeled MOA, essentially as described by Miller (25), for lactose transport studies in *Escherichia coli*. Bacteria were grown in liquid medium (20 ml) to an optical density at 680 nm of about 1.0 (ca. 2×10^9 cells per ml). A 10-ml volume of the culture was centrifuged (10 min, 9,000 × *g*, room temperature), washed once with an equal volume of 0.9% NaCl, and centrifuged again under the same conditions. The cell pellet was suspended in 10 ml of minimal medium, chloramphenicol was added to a final concentration of 100 μg/ml, and the suspension was incubated for 5 min at 30°C. The cell titer was determined by measuring the optical density at 680 nm, and labeled MOA was added to the cell suspension to a final concentration of 1 mM (0.1 μCi). Samples of 1 ml were removed at 0, 2, 5, and 10 min following the addition of the labeled substrate and immediately filtered through membranes (diameter, 2.54 mm; pore size, 0.45 μm; HAWP; Millipore Corp., Bedford, Mass.). The filtered cells were washed with 10 ml of minimal medium, the membranes were dried, and the amount of trapped radioactivity was determined in a liquid scintillation counter. For competition experiments the competitor opine was added to the cell suspensions at a final concentration of 5 mM just prior to the addition of the ^{14}C-labeled MOA.

Preparation of cell extracts. Cells from exponentially growing cultures (about 3×10^8 to 8×10^8 cells per ml) were harvested by centrifugation (10 min, 7,000 × *g*) and washed twice with 0.9% NaCl. Cell pellets were suspended in a buffer consisting of 100 mM Tris hydrochloride, 2 mM dithiothreitol, and 980 mM ethylene glycol (pH 8.0) and disrupted by sonication on a sonic oscillator (20 kHz; 100 W; Measuring & Scientific Equipment, Ltd., London, England) on ice for two 4-min periods separated by a 1-min interval. Cell debris was removed by centrifugation (15 min, 20,000 × *g*, 4°C). The supernatants were used for all enzyme assays.

Enzyme assays. All incubations were performed at 37°C. MOA breakdown was assayed in a 500-μl reaction mixture containing 45 μmol of Tris hydrochloride (pH 8.0), 0.9 μmol of dithiothreitol, 440 μmol of ethylene glycol, 5 to 8 mg of protein, and 5 μmol of MOA. The reaction was initiated by adding the substrate. At various times 100-μl portions were removed and treated with 20 μl of 2 M acetic acid. After centrifugation (4 min, 11,600 × *g*), the supernatants were analyzed as described below. MAN-fructose isomerization was assayed in reactions as described above, except that MOA was replaced with 5 μmol of MAN. Protein concentrations were determined with a kit from Bio-Rad Laboratories (Richmond, Calif.) according to the instructions of the manufacturer.

Separation and detection of the mannityl opines and their metabolites. For thin-layer chromatography, Silica gel G 1500 thin-layer chromatography plates (20 by 20 cm; Schleicher & Schuell, Dassel, Federal Republic of Germany) were first soaked in 0.15 M NaH_2PO_4 solution for 1 min and then heated at 65 to 70°C until they were dry. Portions of 6 to 8 μl of supernatants from the reactions described above were spotted onto the plates, which were then developed in acetone–1-butanol–water (5:4:1; vol/vol/vol). Plates were dried in a stream of hot air, and sugars were visualized with the naphthoresorcinol reagent (45). For descending paper chromatography, 6 to 10 μl of the reaction supernatants were chromatographed as described by Dessaux et al. (8). Dried chromatograms were stained by using the silver nitrate reagent described by Trevelyan et al. (43), fixed, and washed as described by Petit et al. (28). For high voltage paper electrophoresis (HVPE), mannityl opines were separated by using acetic acid-formic acid (pH 1.9) buffer (28) or an ammonium carbonate (pH 9.2) buffer (14). Neutral sugars were separated by HVPE with 1% sodium borate buffer (34) or the ammonium borate (pH 9.2) buffer described by Ellis et al. (14). Electrophoretograms were stained with triphenyltetrazolium reagent (14), urea-phosphoric acid reagent (45), naphthoresorcinol reagent (45), or silver nitrate reagent (43). Before staining with silver nitrate, electrophoretograms from borate buffer electrophoreses were dipped in a 40 mM sulfuric acid–95% ethanol solution for 1 min, dried, and steamed over boiling water (M. E. Tate, unpublished results). After the labeled compounds were separated by HVPE, the dried paper was heated for an additional 45 min at 105°C. Strips of paper were cut, and radioactivity was determined in a liquid scintillation counter.

DNA isolation and analysis of Tn*5* insertions in the Ti plasmid. Supercoiled Ti plasmid DNA was extracted from wild-type and mutant strains (2), purified by two centrifugations to equilibrium in CsCl-ethidium bromide gradients (15), and submitted to restriction endonuclease digestion (24). Restriction enzyme digestion patterns of Ti plasmids from wild-type and mutant strains were compared after electrophoresis in 0.7% horizontal agarose gels (24).

RESULTS

Isolation and properties of Tn*5* insertion mutants. To locate the region of the Ti plasmid encoding MOA catabolism, pTiAch5 was subjected to mutagenesis with Tn*5*. This plasmid was indistinguishable from pTi15955, as assessed by digestion with *Hin*dIII. Of more than 4,000 random insertions that were analyzed, 2 were found to abolish the catabolism of MOA. The Ti plasmids from these two mutants were conjugated into C58C1RS (32) by selecting for growth with octopine as the sole carbon source. The transconjugants, called C1-1A and C1-1I, grew with MOP and AGR but not with MOA as the sole carbon source. Utilization of AGA was also affected in both mutants; growth of C1-1A and C1-1I on solid medium was comparable to that of wild-type Ach5 or of C58C1RS(pTiAch5), whereas growth in liquid medium began after a prolonged lag phase (data not shown).

Oxygen uptake studies. Pathways for the catabolism of MOA and AGA share some common regulatory features (5). To better understand the regulation of the pathways for catabolism of the four mannityl opines, we determined oxygen uptake rates in wild-type and mutant strains in the presence of MAN or one of the mannityl opines as the sole source of carbon and energy. The results (Table 2) indicated that oxygen uptake in the presence of MOA is induced in wild-type strain 15955 by preincubation with MOA, MOP, AGR, or AGA. However, such preincubation with MOA induced utilization of that opine and AGA but not that of MOP or AGR. In the two Tn*5* mutants that were unable to degrade MOA, O_2 uptake in the presence of MOA was induced only when the bacteria were preincubated with MOP or AGR. These results suggest that two pathways exist for the catabolism of MOA.

Catabolic pathway for MOA. The transport of MOA was quantified by measuring the uptake of ^{14}C-labeled MOA by resting cells. Transport in the wild-type strain was inducible by each of the four mannityl opines (Fig. 2a and b). In contrast, transport of MOA by the two Tn*5* mutants occurred only when the strains were preincubated with AGR or MOP (Fig. 2d and e). However, when pregrown with MAN and MOA together, we observed that strain C1-1I failed to

TABLE 2. Oxygen consumption by bacterial suspensions of strain 15955 and Tn5 insertion mutants

Strain	C source in preculture[a]	Oxygen uptake (μl of $O_2 \times h^{-1} \times$ mg of protein) with[b]:				
		MAN	MOA	MOP	AGR	AGA
15955	MAN	108	6	18	4	2
	MOA	92	118	8	8	108
	MOP	124	170	168	198	148
	AGR	72	98	148	142	42
	AGA	94	140	10	16	140
C1-1A	MAN	152	1	12	4	10
	MOP	178	158	158	168	126
	AGR	108	76	134	138	16
	AGA	150	16	ND[c]	ND	75
C1-1I	MAN	115	15	16	18	75
	MOP	154	74	168	170	29
	AGR	88	115	157	159	13
	AGA	136	26	ND	ND	136

[a] In all cases ammonium sulfate was supplied as the nitrogen source.
[b] Values below 20 are not considered significantly different from 0. Values above 40 indicate that the assayed substrate is a good energy source (9). The average relative error of each determination is estimated to be 20% (9).
[c] ND, Not determined.

accumulate any radiolabeled substrate (Fig. 2e), while strain C1-1A showed altered uptake kinetics (Fig. 2d).

The results presented in Fig. 2f and i show that in wild-type strain Ach5 preincubated with MOA or AGA, MOA transport was not significantly affected by the presence of competing amounts of the other mannityl opines. However, when the strain was preincubated with MOP or AGR, transport of the radiolabeled MOA was strongly suppressed in the presence of 5 mM MOP or AGR (Fig. 2g and h).

Cell extracts were examined for activities associated with the degradation of MOA. When assayed by HVPE in ammonium borate buffer, extracts from strain Ach5 showed a time-dependent disappearance of MOA and the concomitant appearance of two new spots, one that comigrated with MAN and the other that comigrated with fructose (Fig. 3). The identities of these compounds were confirmed by HVPE in 1% sodium borate buffer by descending paper chromatography and thin-layer chromatography as described above (data not shown). Identical results were obtained with extracts from strain 15955.

The sequential order of MAN and fructose formation and the possible involvement of deoxy-fructosyl glutamate, an intermediate in the tumor-associated biosynthetic pathway for MOA (14), were determined by metabolic trapping ex-

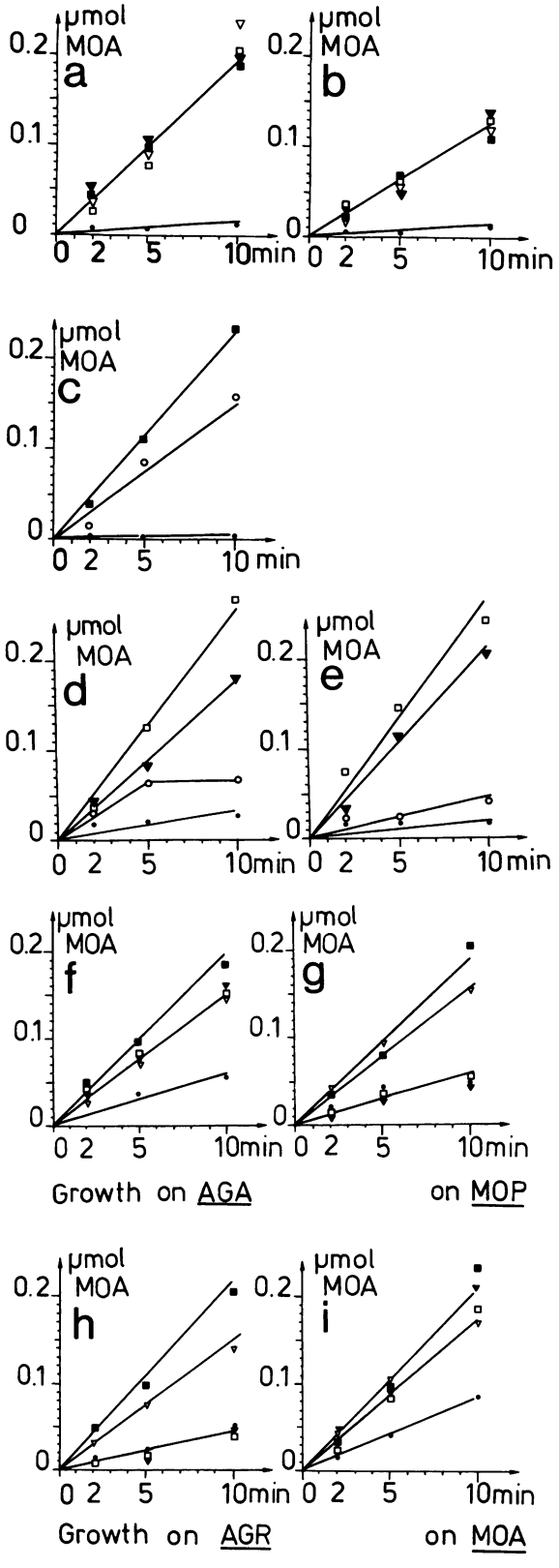

FIG. 2. Uptake of ^{14}C-labeled MOA by strains pregrown with various carbon sources. *Agrobacterium* strains were grown in minimal medium supplemented with various carbon sources. The cultures were harvested and the cells were assayed for ^{14}C-MOA uptake as described in the text. The following strains were assayed: 15955 (a and b), NT-1(pJS4159K1) (c), C1-1A (d), and C1-1I (e). For panels a, c, d, and e, carbon sources were MAN (●), MAN and MOA (○), MOA (■), MOP (▼), AGA (▽), and AGR (□). For panel b, carbon sources were MAN (●), MAN and MOA (■), MAN and MOP (▼), MAN and AGA (▽), and MAN and AGR (□). (f to i) Strain Ach5 was grown on minimal medium with the opine indicated below each panel as the sole source of carbon. Uptake of labeled MOA was assayed in competition with unlabeled AGA (▽), MOP (▼), AGR (□), or MOA (●). In each panel closed boxes represent MOA uptake in the absence of competitor.

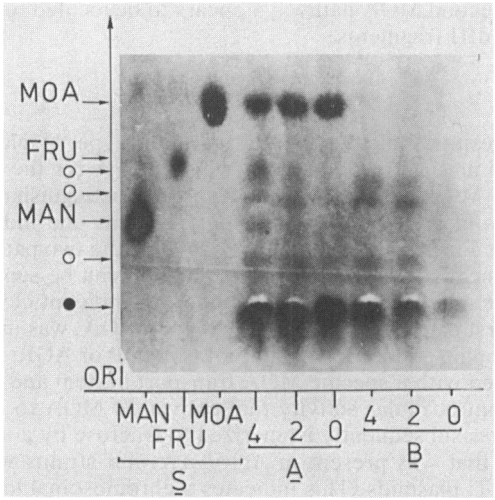

FIG. 3. MOA degradation in cell extracts. Extracts from *A. tumefaciens* Ach5 grown with MOA as the sole carbon source were prepared as described in the text. The extracts were incubated with MOA (A) or with no additional substrate (B). Samples were removed at the indicated times (in hours), treated with 2 M acetic acid, and analyzed by HVPE in borate buffer (pH 9.75), as described in the text. Abbreviations: ORI, origin; S, standards (MAN, fructose [FRU], MOA). Symbols: ○, unidentified silver nitrate-staining compounds appearing in all extracts; ●, Tris buffer.

periments. Cell extracts prepared from strain Ach5 grown with MOA as the sole carbon source were incubated with ^{14}C-labeled MOA (33 μCi; final concentration, 16.5 mM) and 20 mM unlabeled deoxy-fructosyl glutamate or 250 mM unlabeled mannose. In controls, incubation of such extracts with labeled opine but without competitor showed that 16% of the converted [^{14}C]MOA was recovered as MAN and 84% was recovered as fructose. With the addition of deoxy-fructosyl glutamate, no demonstrable radioactivity was trapped in this biosynthetic intermediate (data not shown). However, when unlabeled MAN was added to the incubation medium, 88% of the converted label was recovered as MAN and 12% was recovered as fructose (data not shown). This suggests that MAN is formed before fructose and that the former is converted to fructose during incubation. This was confirmed by incubating MAN with the cell extracts. The sugar was rapidly converted to fructose by a thermolabile activity (data not shown).

Occurrence and inducibility of enzymatic activities involved in MOA degradation. The inducibility of the MOA catabolic activities was determined by assaying cell extracts from cells grown under a variety of conditions. MOA-degrading activity was found only in strains that harbored a wild-type mannityl opine-type Ti plasmid (Table 3). This activity was present when such cells were pregrown with MOA or AGA and appeared to be inducible. No activity was detected in extracts from cells grown with glucose or MAN. Similarly, MOA-degrading activity could not be detected in extracts from cells pregrown with MOP or AGR, nor was activity detected in extracts prepared from the two Tn*5* insertion mutants, even when the mutants were pregrown with MOA.

The activity that isomerized MAN to fructose was detectable in cell extracts from all strains including the Ti plasmidless strain C58C1RS (Table 3). The presence of this activity was independent of the carbon source used to grow the cells.

Evidence for two pathways for MOA degradation. The evidence presented above suggests the existence of two pathways for MOA utilization, one of which is associated with induction by MOA and AGA and the other of which is induced by MOP or AGR. To confirm this, we examined the opine catabolic properties of strains containing cloned fragments of pTi15955. Strain NT-1(pYDH208) contains a recombinant plasmid encoding catabolism of MOP and AGR but not MOA or AGA (10), while the recombinant plasmid in strain NT-1(pJS4159K1) encodes utilization of MOA but not MOP or AGR (S. K. Farrand, J. E. Slota, J. Tempé, and Y. Dessaux, manuscript in preparation). Results from oxygen uptake studies showed that strain NT-1(pJS4159K1) utilized MOA when it was induced with that opine (Table 4), but that strain NT-1(pYDH208) did so only when it was pregrown with either MOP or AGR. Similar patterns were observed for the transport of [^{14}C]MOA. Uptake of MOA by strain NT-1(pJS4159K1) was induced by MOA (Fig. 2c) and was not modified by competition with any of the other mannityl opines, while that seen in strain NT-1(pYDH208) was induced by growth on agropine and was competed by unlabeled AGR and MOP (data not shown). Finally, strain NT-1(pJS4159K1) contained the MOA-inducible MOA-degrading activity (Table 3).

TABLE 3. Occurrence and inducibility of enzymatic activities associated with MOA catabolism

Strain	C source in culture[a]	MOA degradation activity[b]	MAN isomerase
C58C1RS	GLU	−	+
	MAN-MOA	−	+
C58C1RS(pTiAch5)[c]	GLU	−	+
	MAN	−	+
	MOA	+	+
	MOP	−	+
	AGA	+	+
	AGR	−	+
C1-1A	MAN-MOA	−	+
C1-1I	MAN-MOA	−	+
NT-1(pJS4159K1)	GLU	−	+
	MAN	−	+
	MOA	+	+

[a] In all cases ammonium sulfate was added as the nitrogen source. GLU, Glucose.
[b] +, Activity detected; −, activity not detected.
[c] Identical results were obtained with strains Ach5 and 15955.

TABLE 4. Oxygen consumption by strain NT-1 harboring various recombinant clones

Strain	C source in preculture[a]	Oxygen uptake (μl of $O_2 \times h^{-1} \times$ mg of protein) with:				
		MAN	MOA	MOP	AGR	AGA
NT-1(pJS4159K1)	MAN	62	4	4	4	20
	MOA	ND[b]	96	24	2	28
NT-1(pYDH208)[c]	MAN	ND	15	26	32	11
	MOP	ND	45	176	133	19
	AGR	ND	118	139	130	6

[a] Conditions were as described in footnotes *a* of Tables 2 and 3 and as in the text.
[b] ND, Not determined.
[c] This strain did not possess an MOA-inducible MOA catabolic pathway (see text and Table 1).

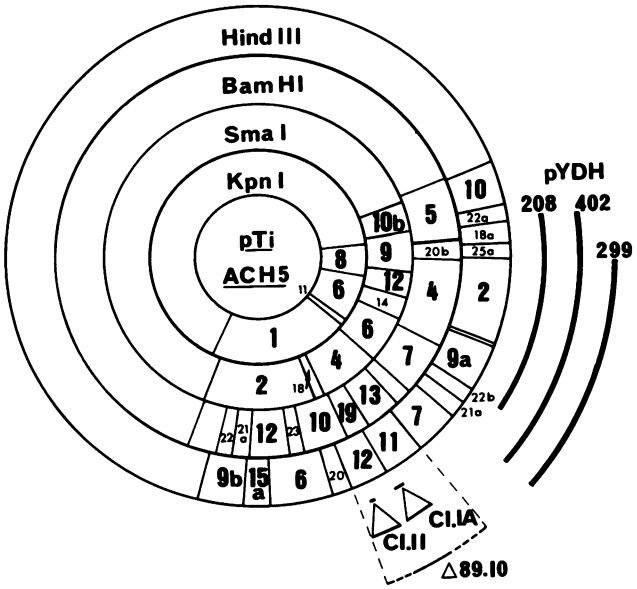

FIG. 4. Genetic locations of MOA catabolic determinants of pTiAch5. The map was constructed from previously published (10, 11) and unpublished (Farrand et al., in preparation) results and from the results of this study. The region encoding the specific MOA catabolic pathway is represented by *Kpn*I fragment 1 but could be restricted to *Hin*dIII fragments 7, 11, 12, and 20 (10). Positions of the Tn*5* insertions in the Ti plasmids of strains C1-1A and C1-1I are indicated, as is the deletion in pTi89.10. The dashed line represents the region of uncertainty for the extent of this deletion. Clones used to localize the nonspecific MOA catabolic pathway all contain the prefix pYDH and are shown as bold arcs.

Localization of genes encoding MOA-inducible MOA utilization. The location of the determinants for MOA-inducible MOA utilization was determined by examining strains that lack this pathway. First, the sites of the Tn*5* insertions in the two mutants lacking this pathway were determined by restriction endonuclease analysis and reference to the map of De Vos et al. (11). In strain C1-1I the insertion was located between base pair coordinates 8360 and 8580, while that in strain C1-1A mapped to a position between coordinates 8015 and 8105 (Fig. 4). We also examined the Ti plasmid that was present in strain 89.10. This strain cannot utilize MOA as the sole carbon source. Restriction enzyme digestion patterns showed that this plasmid was similar to pTiAch5 and pTi15955. However, pTi89.10 was missing *Bam*HI fragments 10, 13, and 19; *Sma*I fragment 4; and *Hin*dIII fragments 11 and 12 (data not shown). These fragments all mapped to the segment of the plasmid known to encode the catabolism of MOA (10; Farrand et al., in preparation), indicating that pTi89.10 contains a deletion in this region (Fig. 4).

Location of genes encoding MOP-inducible MOA utilization. To locate the genes involved in this second pathway, we followed the utilization of MOA from cultures of strain NT-1 harboring various subclones (Fig. 4) of pTi15955 (10). Clones such as pYDH208 and pYDH402 conferred on strain NT-1 the ability to utilize MOA, but only when they were grown in medium containing MOP or AGR. On the other hand, pYDH299, which encodes the catabolism of agropine only (10), did not allow strain NT-1 to utilize MOA, even when it was grown with all four mannityl opines (data not shown). Clone pYDH299 differed from the other two clones only in that it lacked *Hin*dIII fragments 18a and 22a (Fig. 4) (10). Thus, at least part of the genetic information necessary for the second MOA pathway appears to be located on these two *Hin*dIII fragments.

DISCUSSION

Our results indicate that the octopine-type Ti plasmids pTiAch5 and pTi15955 encode two pathways for the catabolism of MOA. These two pathways are distinguishable on the basis of their different transport systems and induction patterns. Furthermore, the genes encoding the two pathways are physically and genetically distinct and can be separated from one another on nonoverlapping recombinant clones.

The first pathway, which is specific for MOA, was induced by this opine and by AGA, but not by MOP or AGR. It was associated with a specific MOA transport system and with a soluble intracellular activity that converted MOA to MAN. MAN was subsequently isomerized to fructose by a soluble activity that was present in *Agrobacterium* strains with or without Ti plasmids. This indicates a chromosomal location for the gene encoding this activity but does not rule out the presence of a similar gene on the Ti plasmid.

The genetic determinants encoding the specific MOA pathway were localized by mapping Tn*5* insertion mutations to a region of the Ti plasmid known to encode the specific catabolism of MOA and AGA (Fig. 4) (10). Consistent with this placement, the two Tn*5* insertion mutants were also affected in their utilization of AGA (Table 2). This relationship between MOA and AGA utilization was consistent with the results of a study by Chilton and Chilton (5), who showed that catabolism of these two mannityl opines appears to be coordinantly regulated and inducible by AGA. However, the pathways for catabolism of these two opines must diverge since clones can be isolated which encode catabolism of one but not the other (10). In addition, results of the present study suggest that each of these two opines probably has its own transport system, since (i) AGA did not compete with MOA for the MOA-specific permease (Fig. 2) and (ii) the two Tn*5* mutants, while unable to utilize MOA, did show AGA-inducible oxygen uptake when AGA was added as the substrate (Table 2).

The second pathway did not appear to be specific for MOA. It was inducible by AGR and MOP, but not by MOA. In this pathway, MOA uptake was competed by AGR and MOP. Therefore, we assumed that this transport system is primarily for MOP or AGR and that MOA is a recognizable but noninducing substrate. Although we could not detect any MOA breakdown activity associated with this pathway in cell extracts, we suggest that MOA is degraded by enzymes belonging to the MOP or AGR pathways. However, the previously described MOP cyclase (8) is probably not involved in the nonspecific MOA pathway since it did not recognize this opine as a substrate.

Strain NT-1(pYDH402) grew on AGR, MOP, and on induction, MOA, whereas strain NT-1(pYDH299) grew only on AGR. From this result we infer that genes involved in the nonspecific utilization of MOA are located in *Hin*dIII fragments 18a and 25a (Fig. 4). This observation supports the idea that the nonspecific degradation of MOA takes place, at least in part, via the MOP catabolic system and suggests that fragments 18a, 25a, or both may encode a MOP transport system that can recognize MOA as a substrate.

Finally, the complexity of the catabolic systems by which the mannityl opines are degraded should be emphasized. The opine catabolic pathways that have been described to date are simple and straightforward. For example, the catabolism of octopine and nopaline by agrobacteria yields the same

molecules that have been identified in crown gall cells as precursors of these opines (for reviews, see references 33, 38, and 40). Thus, the pathways for catabolism appear to be functionally the reverse of those for biosynthesis. These catabolic systems are generally regulated by a single member of the opine family and are encoded on relatively small segments of Ti plasmid DNA. In contrast, MOA degradation occurs through two pathways with separate transport systems and different catabolic enzymes. Furthermore, at least for the specific MOA pathway, catabolism proceeds via a set of reactions that are different from those involved in the plant-associated biosynthesis of MOA (14, 35). At the genetic level, mannityl opine degradation involves large segments of Ti plasmid DNA (10) and complex interconnected systems of regulation (Fig. 2 and Tables 2 and 4) (5).

The origin of opine-related functions remains unknown. Opine catabolism functions have been found in bacteria other than *Agrobacterium* spp., including gram-positive coryneforms (42). However, when examined, these organisms showed no detectable DNA sequence homologies with corresponding opine catabolic determinants from Ti plasmids (P. Dion, personal communication; S. K. Farrand, unpublished data). Still, whether they have the same origin or result from independent evolution remains a matter of speculation. However, the potential importance of opines to the soil ecology in the vicinity of crown galls or hairy roots is emphasized by the existence of such nonagrobacterial catabolizers. Studies focused on these systems should further our knowledge concerning the complex interactions which take place between plants and their associated soil bacteria.

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LITERATURE CITED

1. **Beringer, J. E., J. L. Beynan, A. V. Buchanan-Wollaston, and A. W. B. Johnston.** 1978. Transfer of the drug-resistance transposon Tn*5* to *Rhizobium*. Nature (London) **276**:633–634.
2. **Casse, F., C. Boucher, J.-S. Julliot, M. Michel, and J. Dénarié.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**:229–242.
3. **Chang, C.-C., and C.-M. Chen.** 1983. Evidence for the presence of N^2-(1,3-dicarboxypropyl)-L-amino acids in crown gall tumors induced by *Agrobacterium tumefaciens* strains 181 and EU6. FEBS Lett. **162**:432–435.
4. **Chang, C.-C., C.-M. Chen, B. R. Adams, and B. M. Trost.** 1983. Leucinopine, a characteristic compound of some crown gall tumors. Proc. Natl. Acad. Sci. USA **80**:3573–3576.
5. **Chilton, W. S., and M.-D. Chilton.** 1984. Mannityl opine analogs allow isolation of catabolic pathway regulatory mutants. J. Bacteriol. **158**:650–658.
6. **Chilton, W. S., J. Tempé, M. Matzke, and M.-D. Chilton.** 1984. Succinamopine: a new crown gall opine. J. Bacteriol. **157**:357–362.
7. **De Greve, H., H. Decraemer, J. Seurinck, M. Van Monatgu, and J. Schell.** 1981. The functional organization of the octopine *Agrobacterium tumefaciens* plasmid, pTiB6s3. Plasmid **6**:235–248.
8. **Dessaux, Y., P. Guyon, S. K. Farrand, A. Petit, and J. Tempé.** 1986. *Agrobacterium* Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine. J. Gen. Microbiol. **132**:2549–2559.
9. **Dessaux, Y., A. Petit, J. Tempé, M. Demarez, C. Legrain, and J.-M. Wiame.** 1986. Arginine catabolism in *Agrobacterium* strains: role of the Ti plasmid. J. Bacteriol. **166**:44–50.
10. **Dessaux, Y., J. Tempé, and S. K. Farrand.** 1987. Genetic analysis of mannityl opine catabolism in octopine-type *Agrobacterium tumefaciens* strain 15955. Mol. Gen. Genet. **208**:301–308.
11. **De Vos, G., M. De Beuckeleer, M. Van Montagu, and J. Schell.** 1981. Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTiAch5 of *Agrobacterium tumefaciens*. Plasmid **6**:249–253.
12. **Ellis, J. G., A. Kerr, A. Petit, and J. Tempé.** 1982. Conjugal transfer of nopaline and agropine Ti plasmids—the role of agrocinopines. Mol. Gen. Genet. **186**:269–274.
13. **Ellis, J. G., and P. J. Murphy.** 1981. Four new opines from crown gall tumors—their detection and properties. Mol. Gen. Genet. **181**:36–43.
14. **Ellis, J. G., M. H. Ryder, and M. E. Tate.** 1984. *Agrobacterium tumefaciens* T_R-DNA encodes a pathway for agropine biosynthesis. Mol. Gen. Genet. **195**:466–473.
15. **Farrand, S. K., C. I. Kado, and C. R. Ireland.** 1981. Suppression of tumorigenicity by the IncW R plasmid pSa in *Agrobacterium tumefaciens*. Mol. Gen. Genet. **181**:44–51.
16. **Firmin, J. L., and G. R. Fenwick.** 1978. Agropine—a major new plasmid-determined metabolite in crown gall tumors. Nature (London) **276**:842–844.
17. **Fuchs, W. H.** 1976. History of physiological plant pathology, p. 1–26. *In* R. Heitefuss and P. H. Williams (ed.), Physiological plant pathology. Springer-Verlag, Berlin.
18. **Gelvin, S. B.** 1984. Plant tumorigenesis, p. 343–377. *In* T. Kosuge and E. W. Nester (ed.), Plant microbe interactions: molecular and genetic perspectives, vol. I. MacMillan Publishing Co., New York.
19. **Gelvin, S. B., M. P. Gordon, E. W. Nester, and A. I. Aronson.** 1981. Transcription of the *Agrobacterium tumefaciens* Ti plasmid in the bacterium and in crown gall tumors. Plasmid **6**:17–29.
20. **Gheysen, G., P. Dhaese, M. Van Montagu, and J. Schell.** 1985. DNA flux across genetic barriers: the crown gall phenomenon, p. 11–47. *In* B. Hohn and E. S. Dennis (ed.), Plant gene research: genetic flux in plants. Springer-Verlag, Vienna.
21. **Guyon, P., M.-D. Chilton, A. Petit, and J. Tempé.** 1980. Agropine in "null-type" tumors: evidence for generality of the opine concept. Proc. Natl. Acad. Sci. USA **77**:2693–2697.
22. **Kerr, A., P. Manigault, and J. Tempé.** 1977. Transfer of virulence in vivo and in vitro in *Agrobacterium*. Nature (London) **265**:560–561.
23. **Klapwijk, P. M., T. Scheulderman, and R. A. Schilperoort.** 1978. Coordinated regulation of octopine degradation and conjugative transfer of Ti plasmids in *Agrobacterium tumefaciens*: evidence for a common regulatory gene and separate operons. J. Bacteriol. **136**:775–785.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 360–362. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Murphy, P. J., N. Heycke, Z. Banfalvi, M. E. Tate, F. De Bruijn, A. Kondorosi, J. Tempé, and J. Schell.** 1987. Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. Proc. Natl. Acad. Sci. USA **84**:493–497.
27. **Nester, E. W., M. P. Gordon, R. M. Amasino, and M. F. Yanofski.** 1984. Crown gall: a molecular and physiological

analysis. Annu. Rev. Plant Physiol. **35**:387–413.
28. **Petit, A., C. David, G. A. Dahl, J. G. Ellis, P. Guyon, F. Casse-Delbart, and J. Tempé.** 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol. Gen. Genet. **190**:204–214.
29. **Petit, A., S. Delhaye, J. Tempé, and G. Morel.** 1970. Recherches sur les guanidines des tissus de crown-gall. Mise en évidence d'une relation biochimique specifique entre les souches d'*Agrobacterium tumefaciens* et les tumeurs qu'elles induisent. Physiol. Vég. **8**:205–213.
30. **Petit, A., and J. Tempé.** 1978. Isolation of *Agrobacterium* Ti plasmid regulatory mutants. Mol. Gen. Genet. **167**:147–155.
31. **Petit, A., and J. Tempé.** 1985. The function of T-DNA in nature, p. 625–636. *In* L. Van Vloten-Doting, G. S. P. Groot, and T. C. Hall (ed.), Molecular form and function of the plant genome. Plenum Publishing Corp., New York.
32. **Petit, A., J. Tempé, A. Kerr, M. Holsters, M. Van Montagu, and J. Schell.** 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature (London) **271**: 570–572.
33. **Puhler, A. (ed.).** 1983. Molecular genetics of the bacteria-plant interaction. Springer-Verlag, Berlin.
34. **Ribeiro, L. P., E. Mitidieri, and O. R. Affonson.** 1961. Paper electrophoresis: a review of methods and results, p. 292–302. Elsevier/North-Holland Publishing Co., Amsterdam.
35. **Salomon, R., R. Deblaere, J. Leemans, J.-P. Hernalsteens, M. Van Montagu, and J. Schell.** 1984. Genetic identification of functions of T_R-DNA transcripts in octopine crown galls. EMBO J. **3**:141–146.
36. **Schell, J., M. Van Montagu, M. De Beuckeleer, M. De Block, A. Depicker, M. De Wilde, G. Engler, C. Genetello, J.-P. Hernalsteens, M. Holsters, G. Seurinck, B. Silva, F. Van Vliet, and R. Villaroel.** 1979. Interaction and DNA transfer between *Agrobacterium tumefaciens*, the Ti plasmid, and the plant host. Proc. R. Soc. London Ser. B **204**:251–266.
37. **Tate, M. E., J. G. Ellis, A. Kerr, J. Tempé, K. E. Murray, and K. J. Shaw.** 1982. Agropine: a revised structure. Carbohydr. Res. **104**:105–120.
38. **Tempé, J., and A. Goldmann.** 1982. Occurrence and biosynthesis of opines, p. 427–449. *In* G. Kahl and J. S. Schell (ed.), Molecular biology of plant tumors. Academic Press, Inc., New York.
39. **Tempé, J., P. Guyon, A. Petit, J. G. Ellis, M. E. Tate, and A. Kerr.** 1980. Préparation et propriétés de nouveaux substrats cataboliques pour deux types de plasmides oncogènes d'*Agrobacterium tumefaciens*. C.R. Acad. Sci. Ser. D **290**:1173–1176.
40. **Tempé, J., P. Guyon, D. Tepfer, and A. Petit.** 1979. The role of opines in the ecology of the Ti plasmids of *Agrobacterium*, p. 353–363. *In* K. N. Timmis and A. Puhler (ed.), Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.
41. **Tempé, J., A. Petit, and H. Bannerot.** 1982. Présence de substances semblables à des opines dans les nodosités de luzerne. C.R. Acad. Sci. Ser. III **295**:413–416.
42. **Tremblay, G., R. Gagliardo, W. S. Chilton, and P. Dion.** 1987. Diversity among opine-utilizing bacteria: identification of coryneform isolates. Appl. Environ. Microbiol. **53**:1519–1524.
43. **Trevelyan, W. E., D. P. Procter, and J. P. Harrisson.** 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
44. **Verma, D. P. S., and T. Hohn (ed.).** 1984. Plant gene research: genes involved in microbe-plant interactions. Springer-Verlag, Vienna.
45. **Zweig, G., and J. Sherma (ed.).** 1972. Handbook of chromatography, vol. 2, p. 127. CRC Press, Inc., Cleveland.