MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 4, pp. 259-267, 1990

Localization and Characterization of the Region Encoding Catabolism of Mannopinic Acid from the Octopine-Type Ti Plasmid pTi15955

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Received 19 January 1990. Accepted 8 March 1990.

The region of the octopine-type tumor-inducing (Ti) plasmid pTi15955 encoding catabolism of mannopinic acid was localized to an 18-kilobase (kb) segment mapping between Ti plasmid coordinates 70 and 88. A subclone containing only this region normally did not allow utilization of any other mannityl opine. However, spontaneous mutants of the plasmid were isolated that conferred catabolism of agropinic acid. While the mutants remained regulated for mannopinic acid utilization, induction by this opine resulted in increased activity associated with agropinic acid catabolism. Respirometric studies and results from growth assays on mannopinic acid analogues indicated that the genes encoded on the 18-kb subclone were regulated in a wild-type fashion. By means of these analogues, spontaneous constitutive mutants were isolated. In several cases, the mutant phenotype was associated with an insertion event mapping within a 300-base pair region of the subclone insert. Although constitutive for catabolism of mannopinic acid, these mutants remained unable to catabolize any of the other mannityl opines. Segregation studies and genetic reconstitution experiments showed that one of the subclones isolated directly in *Agrobacterium* was actually composed of two complementing recombinant plasmids contained in the same cell. This indicated that the genes encoding mannopinic acid catabolism were organized into at least two complementation groups. These results point to a high degree of complexity in the organization and regulation of Ti plasmid genes encoding catabolism of a relatively simple carbon source.

Crown galls and hairy roots induced by members of the genus *Agrobacterium* harboring tumor-inducing (Ti) or root-inducing (Ri) plasmids exhibit, as one of their distinguishing characteristics, the production of novel, low molecular weight carbon compounds called opines (Tempé and Petit 1983). These substances, which are often imine conjugates of amino acids with a keto acid or a sugar, are virtually unique to these plant neoplasias. There are 10 to 12 known opine classes, but a given tumor or hairy root generally produces no more than two or three types. The types of opines produced by these transformed plant tissues are specified by the strain of *Agrobacterium* that induced the tumor or hairy root, with the genes for opine production being encoded on the T-DNA transferred from the Ti or Ri plasmid of the bacterium to the plant during the transformation event. Almost without exception, the inciting strain of *Agrobacterium* is able to utilize as a sole carbon source the specific opines produced by the neoplasia it induced. Genes encoding these traits are also contained on the Ti and Ri plasmids, but at locations outside of the T-DNA. However, such strains are usually unable to catabolize opines of other classes. The mechanisms by which virulent agrobacteria induce these plant neoplasias are well-understood and have been the subjects of several recent reviews (Binns and Thomashow 1988; Zambryski 1988; Ream 1989)

Opines are not required for either induction or maintenance of transformed plant tissues (Montoya *et al.* 1977). However, because they are specifically catabolized by the agrobacteria, it has been suggested that they play a role in the natural ecology of this organism. In a model presented by Tempé and Petit (1983), opines function as selective nutritional sources for the propagation of opine-utilizing *Agrobacterium* populations associated with crown gall or hairy root growths. The finding that certain opines act as specific inducers of Ti plasmid conjugal transfer (Petit *et al.* 1978; Ellis *et al.* 1982) and as potentiators of *vir* gene induction (Veluthambi *et al.* 1989) imparts effector roles to these compounds and emphasizes their importance in the interactions between *Agrobacterium* and its host plants.

The mannityl opines are a family of four such compounds containing mannose conjugated with either glutamate or glutamine. Mannopine (MOP) and mannopinic acid (MOA) are unmodified conjugates while agropine (AGR) and agropinic acid (AGA) are cyclized derivatives of MOP (Firmin and Fenwick 1978; Dahl *et al.* 1983; Tate *et al.* 1982). The genes for biosynthesis and catabolism of these opines were first found to be associated with octopine- and AGR-type Ti plasmids as well as with *A. rhizogenes* AGR- and MOP-type Ri plasmids (Guyon *et al.* 1980; Petit *et al.* 1983). Recently, Lopez *et al.* (1988) described tumorigenic strains able to catabolize MOA and nopaline, an opine combination not previously reported. Thus, the mannityl opine traits seem to be associated with a wide variety of *Agrobacterium* isolates.

Genes for the catabolism of the four mannityl opines span a contiguous 45-kilobase (kb) segment of the octopine-

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type Ti plasmid pTi15955 (Dessaux *et al.* 1987). This represents a large amount of coding capacity for catabolism of a relatively simple group of carbon compounds. In fact, we have shown that this region, called *moc*, encodes at least two pathways for catabolism of both MOA and MOP (Dessaux *et al.* 1987, 1988). One of the MOA pathways may correspond to one of the MOP pathways (Dessaux *et al.* 1988). Furthermore, genetic and physiological analyses suggest that the catabolism of AGA and MOA may share common components (Chilton and Chilton 1984; Dessaux *et al.* 1987).

We have initiated studies to more closely define the genetic and physiological characteristics of these catabolic traits. Physiological studies of the MOA-specific catabolic pathway indicate that this opine is taken up and degraded to mannose by a Ti plasmid-dependent system (Dessaux *et al.* 1988). The mannose is subsequently converted to fructose by a Ti plasmid-independent activity present in agrobacteria. Nothing is known about the pathways for catabolism of the other three mannityl opines. However, MOA also can be utilized via one of the MOP-specific pathways, but only if the genes encoding this pathway are derepressed (Dessaux *et al.* 1988).

In this study we show that genes encoding the MOA-specific catabolic pathway are confined to a region of the Ti plasmid with a maximum size of about 18 kb which is organized as at least two separable complementation groups. This region does not encode catabolism of MOP or AGR, but mutants can be obtained that grow with AGA as the sole carbon source. A regulatory function that responds to MOA but not AGA is contained within this region. The data suggest that the pathway encoded by this region allows degradation of both MOA and AGA, but not of MOP or AGR. The specificity for MOA is not associated with the manner in which expression of the genes is regulated.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains NT-1 and 15955 of *A. tumefaciens* have been described previously (Dessaux *et al.* 1987), as has strain 1231 of *Escherichia coli* (Pischl and Farrand 1984). Plasmid pTi15955 is an octopine-type Ti plasmid and also encodes catabolism of the mannityl opines. The IncQ vector pKT231 was described by Bagdasarian *et al.* (1981). Cosmid clones pYDH105, pYDH303, pYDH304, and pYDH306 contain inserts from the *moc* region of pTi15955 DNA in the IncP1 vector pCP13 (Dessaux *et al.* 1987). Plasmid pJS4159K1 represents *Kpn*I fragment 1 of pTi15955 cloned into pJS400, a vector based on the agrocinogenic plasmid pAgK84 (S. K. Farrand, unpublished). Plasmid pRK2013 (Figurski *et al.* 1979) in *E. coli* 1231 was used as a conjugal helper. Cultures of *Agrobacterium* were incubated at 28° C, while those of *E. coli* were grown at 37° C. All liquid cultures were incubated with shaking to ensure aeration.

Media and chemicals. Nutrient agar (NA; Difco Laboratories, Detroit, MI) and L broth (low salt, Gibco Laboratories, Grand Island, NY) were used as the rich solid and liquid media. AT medium (Petit and Tempé 1978) containing 0.15% $(NH_4)_2SO_4$ was used as the minimal medium for *Agrobacterium*. The four mannityl opines and the galactose and glucose analogues were synthesized as described previously (Petit *et al.* 1983; Dessaux *et al.* 1986). Carbon sources were included at final concentrations of 10 mM for opines and 0.2% for glucose or mannose unless otherwise stated. AT minimal medium containing opines or opine analogues as sole carbon source was solidified with Agar Purified or Noble Agar, both from Difco. Otherwise, all media were solidified with Bacto Agar (Difco). Antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO) and added to various media at the following final concentrations: tetracycline, 10 μg/ml for *E. coli* or 1.5 μg/ml for *Agrobacterium*; rifampicin, 50 μg/ml; and streptomycin, 250 μg/ml for *Agrobacterium* or 7.5 μg/ml when selecting pKT231 transformants of *E. coli* 1231.

Genetic techniques. *Agrobacterium* was transformed with plasmid DNA by the freeze-thaw technique described by Holsters *et al.* (1978). *E. coli* was transformed essentially as described by Maniatis *et al.* (1982). Triparental matings to transfer pCP13 or pKT231 derivatives into strains of *Agrobacterium* were performed using *E. coli* 1231(pRK2013) as the helper (Ditta *et al.* 1980). To isolate spontaneous *Agrobacterium* mutants able to utilize AGA or galactopinic acid (GOA), approximately 10^8 cells were spread over the surface of AT minimal plates containing the opine or the MOA analogue as the sole carbon source. The plates were sealed with Parafilm and incubated at 28° C for up to 5 wk. Potential mutants were single-colony purified on nonselective medium. Several isolated colonies were retested for the mutant phenotype on minimal medium containing the selective opine or analogue.

DNA analysis and molecular techniques. Plasmid isolations from *Agrobacterium* and *E. coli*, restriction endonuclease digestions, and agarose gel electrophoretic analyses were performed as described previously (Slota and Farrand 1982; Farrand *et al.* 1985). Partial *Hin*dIII digests of pJS4159K1 were produced and cloned into the *Hin*dIII site of pKT231 by means of the techniques described by Maniatis *et al.* (1982).

Opine utilization assays. The ability to utilize mannityl opines or their analogues as sole carbon sources was assessed visually on solid or in liquid AT minimal media supplemented with ammonium sulfate. Generally, the cultures were observed daily for a 3- to 5-day period. Growth is expressed on a scale from (−) indicating no growth to (++++) indicating excellent growth, based on comparisons with appropriate positive and negative control cultures.

Opine or analogue utilization was confirmed by determining the disappearance of the compound from liquid culture supernatants using high-voltage paper electrophoresis in formic/acetic acid buffer, pH 1.9 (Dessaux *et al.* 1987). Compounds separated by electrophoresis were visualized with the alkaline silver nitrate reagent of Trevelyan *et al.* (1950).

Oxygen uptake assays. Oxygen uptake rates were determined manometrically in a Warburg respirometer as previously described (Dessaux *et al.* 1988). Cells were pregrown in AT minimal medium supplemented with nitrogen and either mannose or one of the mannityl opines

as the sole carbon source. Oxygen uptake rates were measured for cells resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing the carbon source to be tested at a final concentration of 10 mM. Rates are expressed as microliters of O_2 taken up per hour per milligram of protein.

RESULTS

Opine catabolic phenotypes conferred by recombinant clones. The recombinant cosmid clone pYDH304 confers utilization of MOA, AGA, and MOP on strain NT-1 (Dessaux *et al.* 1987). Plasmid pJS4159K1, containing *Kpn*I fragment 1 of the same Ti plasmid, confers catabolism of MOA only (Table 1). The inserts of pJS4159K1 and pYDH304 contain in common a region encompassing *Hin*dIII fragments 11, 12, and 20 and most of 7 (Fig. 1). The map positions of several other cosmid clones encoding mannityl opine catabolism (Dessaux *et al.* 1987) are also shown in Figure 1.

Table 1. Utilization of mannityl opines encoded by clones and subclones of pTi15955

Plasmid[a]	Growth with[b]			
	MOA	AGA	MOP	AGR
pTi15955	++++	++++	++++	++++
pYDH105[c]	−	++++	++	+/−
pYDH303[c]	++++	++++	+/−	+/−
pYDH304[c]	++++	++++	++	+/−
pYDH306[c]	++++	−[d]	−	−
pJS4159K1	++++	−(M)[e]	−	−
pMix1	++++	−(M)	−	−
pHP526	++++	−(M)	−	−
pJS4159K1AGA$^+$	++++	++++	−	−
pHP526AGA$^+$	++++	++++	−	−

[a] All plasmids were tested in the strain NT-1 chromosomal background.
[b] Growth was assessed after 5 days of incubation on AT minimal medium supplemented with the indicated opines (MOA, mannopinic acid; AGA, agropinic acid; MOP, mannopine; and AGR, agropine) as sole carbon sources as described in the text. (++++) indicates wild-type growth; (++), good growth but less than wild-type; (+/−), very poor growth; and (−), no growth.
[c] Data for strains harboring these plasmids were taken from Dessaux *et al.* (1987).
[d] We would expect AGA$^+$ mutants to appear following prolonged incubation of strain NT-1(pYDH306) on AGA-containing medium. However, such cultures were not incubated for periods long enough to allow such mutants to appear.
[e] (M) indicates that mutants able to utilize AGA appeared after 3 to 5 wk of incubation. See text.

After prolonged incubation, colonies of strain NT-1(pJS4159K1) appeared on plates containing AGA as the sole carbon source. Several of these colonies were purified and retested for utilization of the four mannityl opines. Each utilized both MOA and AGA, but could not grow with MOP or AGR (Table 1). Analysis of compounds present in culture supernatants at the end of growth experiments in liquid minimal media verified these utilization patterns. When colony growth was seen on solid media containing a given opine, that opine disappeared from analogous liquid culture supernatants (Fig. 2). Conversely, when no growth was observed on solid media, the opine remained in the supernatants of liquid cultures (Fig. 2 and data not shown).

Following prolonged incubation, a few colonies also appeared on solid media containing MOP and AGR as sole carbon sources. However, these isolates were identified by high-voltage paper electrophoresis analysis as being AGA$^+$ mutants and were probably selected for by AGA formed spontaneously from MOP or AGR in the medium over the long incubation periods (Tate *et al.* 1982).

Growth on opine analogues. Analogues of mannityl opines containing alternate sugars are generally not catabolized by strains of *Agrobacterium* containing Ti or Ri plasmids (Chilton and Chilton 1984). However, addition to the medium of inducing amounts of the natural opine allows the cells to utilize these analogues (Chilton and Chilton 1984; Y. Dessaux, J. Tempé, and S. K. Farrand, unpublished observations). Furthermore, spontaneous variants able to grow on such analogues as sole carbon and energy sources can be readily isolated, and most such isolates show the properties of regulatory mutants (Chilton and Chilton 1984; Y. Dessaux and S. K. Farrand, unpublished observations; see below). This indicates that such analogues are utilizable but noninducing substrates for the systems associated with transport and catabolism of the opines (Chilton and Chilton 1984).

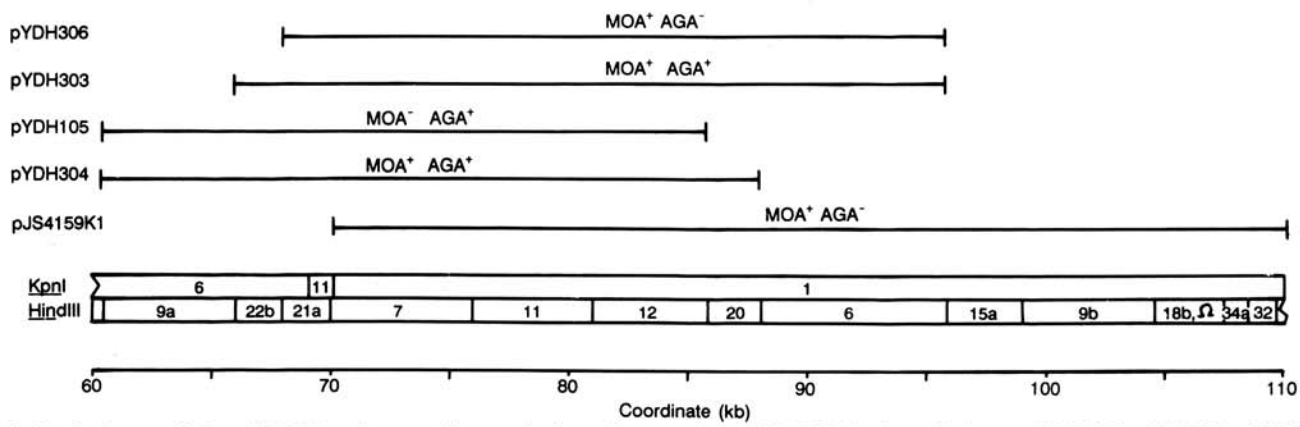

Fig. 1. Physical map of the pTi15955 region encoding catabolism of mannopinic acid (MOA). Cosmid clones pYDH105, pYDH303, pYDH304, and pYDH306 have been described by Dessaux *et al.* (1987). Clone pJS4159K1 represents *Kpn*I fragment 1 cloned in *Agrobacterium* vector pJS400 as described in the text. The MOA and agropinic acid (AGA) catabolic phenotypes conferred on strain NT-1 by each clone are indicated by (+), good growth, or (−), no growth. Map and coordinates are from De Vos *et al.* (1981).

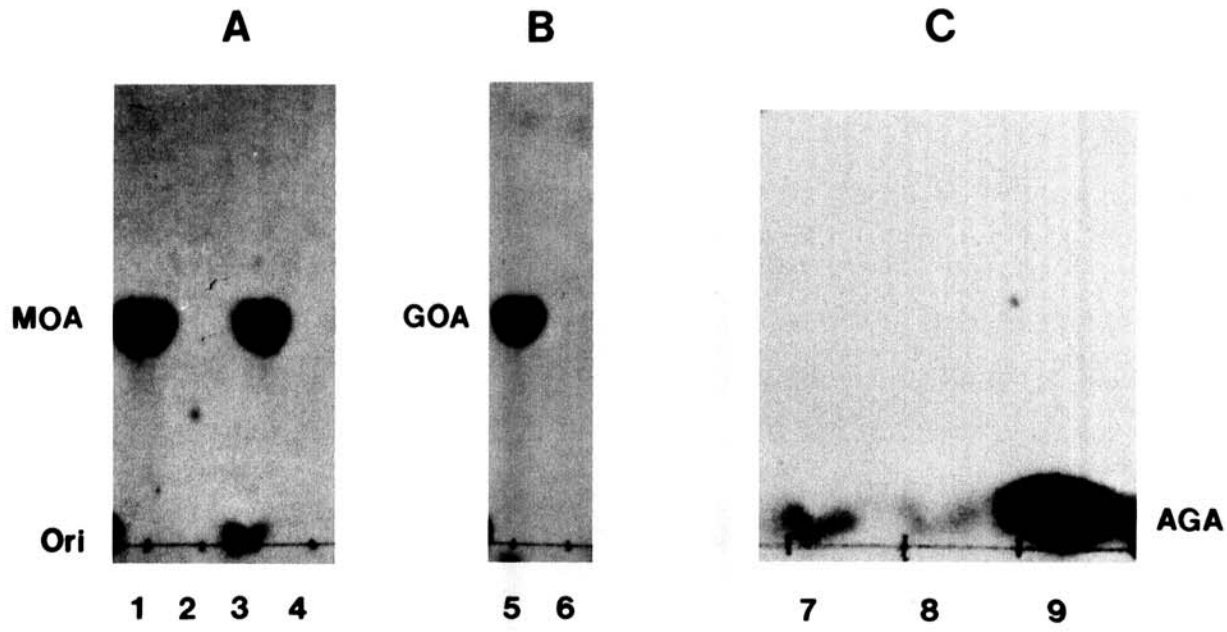

Fig. 2. Analysis of opines present in culture supernatants following growth of strain NT-1 containing various catabolic plasmids. Cells were grown in AT liquid minimal medium containing (**A**) mannopinic acid (MOA), (**B**) galactopinic acid (GOA), or (**C**) agropinic acid (AGA) as the sole carbon source, and culture supernatants were analyzed by high-voltage paper electrophoresis as described in the text. Lanes contain culture supernatants following incubation with: (1) no bacteria; (2 and 7) NT-1(pTi15955); (3) NT-1; (4, 5, and 9) NT-1(pJS4159K1); (6) NT-1(pJS4159K1GOA$^+$); and (8) NT-1(pJS4159K1AGA$^+$). Ori designates the origin of electrophoresis and is the position at which the culture supernatants were spotted. The direction of electrophoresis was toward the anode at the top of the figure. Labels to the sides of each electrophoretogram show the positions to which the authentic compounds migrate.

Strain NT-1(pJS4159K1) was unable to grow on AT plates containing the MOA analogue, GOA, as the sole carbon source (Table 2). However, addition to the medium of inducing amounts of MOA allowed growth on GOA. Actual utilization was verified by monitoring the disappearance of the analogue from culture supernatants as a function of growth in liquid minimal medium (data not shown).

Following prolonged incubation, strain NT-1(pJS4159K1) gave rise to discrete, rapidly growing colonies on AT minimal plates containing GOA (Table 2). Several of these colonies were purified and retested. Each grew on minimal medium containing GOA as the sole carbon source, but remained unable to utilize AGA, MOP, or AGR (Table 2 and data not shown). Electrophoretic analysis of liquid culture supernatants confirmed that the cells were growing at the expense of the opine analogue (Fig. 2B). The GOA$^+$ mutants were also able to catabolize glucopinic acid (GLA), a second noninducing MOA analogue (Table 2).

Oxygen uptake studies. Opine inducibility of the catabolic functions can be shown by oxygen uptake studies (Dessaux *et al.* 1988). Cells able to utilize a given mannityl opine show virtually no oxygen uptake in the presence of that opine if pregrown with a sugar such as mannose as the sole carbon source. However, pregrowth with the opine yields cells showing high O_2 uptake rates when incubated with that opine as the sole carbon source (see Table 3).

Oxygen uptake rates for strain NT-1(pJS4159K1) and an analogue-utilizing mutant were determined in the presence of mannose or one of the mannityl opines as the sole carbon source. As expected, strain NT-1(pJS4159K1), pregrown with mannose, showed no significant oxygen uptake when incubated with any of the opines (Table 3). Oxygen consumption with MOA is induced in strain NT-1(pJS4159K1) by pregrowth with this opine. However, unlike strain 15955, growth of strain NT-1(pJS4159K1) on MOA does not induce O_2 uptake in the presence of AGA. The AGA$^+$ mutant of NT-1(pJS4159K1), pregrown with mannose, showed only slightly increased levels of O_2 consumption when incubated with AGA (Table 3). Oxygen uptake rates with MOA, MOP, and AGR remained at endogenous levels. However, pregrowth with MOA yielded

Table 2. Utilization of opine analogues encoded by wild-type and mutant plasmids and clones[a]

	Growth with				
Plasmid	**MOA**	**AGA**	**GOA**	**GLA**	**GOA+MOA[b]**
pTi15955	++++	++++	−(M)	NT	++++
pJS4159K1	++++	−(M)	−(M)	−(M)	++++
pMix1	++++	−(M)	−(M)	NT	++++
pHP526	++++	−(M)	−(M)	NT	++++
pJS4159K1GOA$^+$	++++	−(M)	++++	++++	NT
pHP526GOA$^+$	++++	−(M)	++++	++++	NT

[a]Strain backgrounds, growth assays, abbreviations, and symbols are as indicated in Table 1: GOA, galactopinic acid; GLA, glucopinic acid; and NT, not tested.
[b]GOA was supplied at a final concentration of 5 mM; MOA at an inducing level of 0.1 mM.

high O_2 uptake rates when the mutant was incubated with either MOA or AGA (Table 3). Contrasting with these results, the GOA^+ mutants, pregrown with mannose, showed high levels of O_2 uptake when incubated with MOA (data not shown).

Subcloning the MOA catabolic region. To localize the genes encoding MOA utilization, a partial *Hin*dIII digest of pJS4159K1 was prepared and ligated to *Hin*dIII-cleaved pKT231. A portion of this mixture was transformed into *A. tumefaciens* NT-1. Six transformants able to utilize MOA were obtained by direct selection on AT medium containing the opine as the sole carbon source. A second portion of the ligation mixture was transformed into *E. coli* 1231 with selection for progeny resistant to streptomycin encoded by the vector. Selection for antibiotic resistance could not be imposed in the *Agrobacterium* transformation since the spontaneous mutation rate to streptomycin resistance far exceeds the transformation frequency.

Restriction endonuclease analysis showed that all six MOA-utilizing NT-1 transformants harbored plasmid DNA containing inserts corresponding to *Hin*dIII fragments Δ7, 11, 12, and 20 of pJS4159K1. One of these plasmids, designated pMix1 (Fig. 3), was retained for further study. *E. coli* 1231 transformants harbored plasmids containing various insertions. Those corresponding to the region common to pYDH304 and pJS4159K1 were retained (Fig. 3). One plasmid, pHP526, like pMix1, contained only *Hin*dIII fragments Δ7, 11, 12, and 20.

Each of the subclones shown in Figure 3 was mobilized from *E. coli* into strain NT-1, selecting for resistance to streptomycin. Transconjugants were verified by restriction endonuclease analysis of plasmid DNA and tested for their ability to catabolize MOA. Only *Agrobacterium* progeny containing pHP526 were able to utilize this opine for growth (Table 2, Figs. 2 and 3). As with cosmid clones such as pYDH304 (Dessaux *et al.* 1987), pHP526 did not confer MOA utilization on *E. coli* hosts (data not shown).

Opine catabolic phenotypes conferred by the subclones. Strain NT-1 containing either pMix1 or pHP526 utilized MOA but not AGA, MOP, or AGR as the sole carbon source (Table 1). As with pJS4159K1, strains harboring

Table 3. Oxygen consumption in the presence of opines by strains of *Agrobacterium* containing wild-type and mutant plasmids[a]

Plasmid[b]	C source in preculture[c]	Oxygen uptake (μl O_2/hr/mg protein) with[d] MAN	MOA	AGA	MOP	AGR
pTi15955[e]	MAN	108	6	2	18	4
	MOA	92	118	104	8	8
	AGA	94	140	140	10	16
pJS4159K1[e]	MAN	62	4	20	4	4
	MOA	ND[f]	96	28	24	2
$pJS4159K1AGA^+$	MAN	ND	6	40	6	2
	MOA	ND	98	94	24	22

[a] Abbreviations are as given in Table 1: MAN, mannose.
[b] Plasmids and clones are as described in the text and Tables 1 and 2. All assays were performed in the NT-1 chromosomal background.
[c] Cells were pregrown in AT minimal medium containing mannose at 0.2% or the opine at 10 mM as described in the text.
[d] Determined as described in the text. Values of 20 or less are considered insignificant; values above 40 indicate that the substrate is a good energy source (Dessaux *et al.* 1986).
[e] Values for strains containing pTi15955 and pJS4159K1 are taken from Dessaux *et al.* (1988).
[f] ND is not determined.

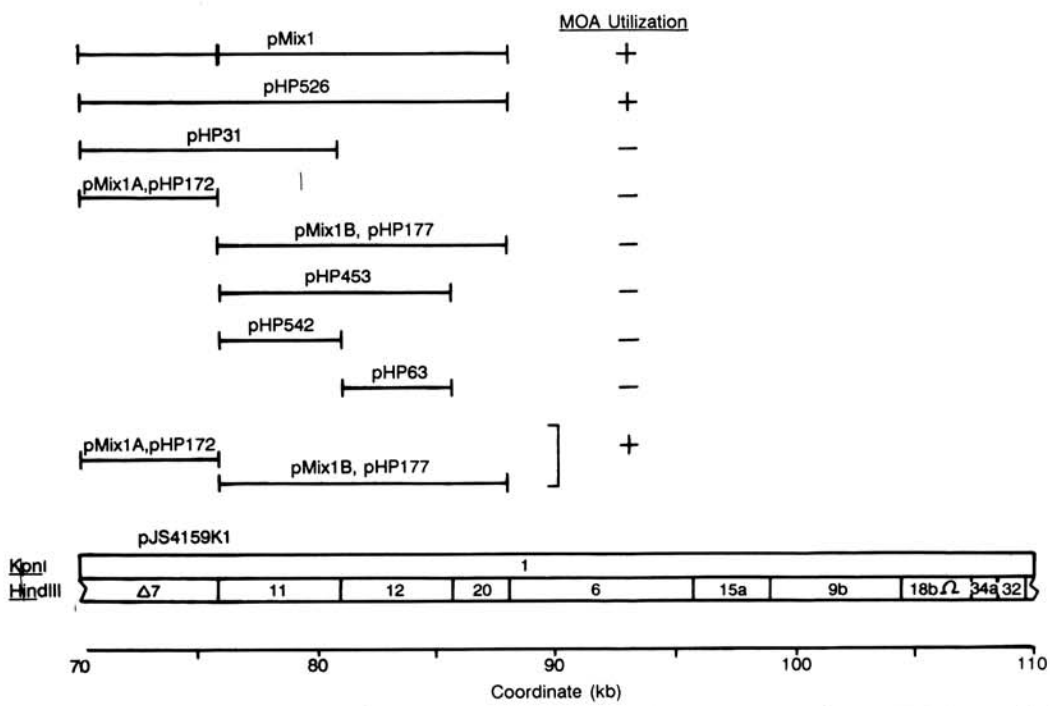

Fig. 3. Map positions and opine catabolic phenotypes conferred by *Hin*dIII subclones of pJS4159K1. Partial *Hin*dIII digests of pJS4159K1 were cloned in pKT231 and analyzed as described in the text. The capacity to utilize mannopinic acid (MOA) was determined in strain NT-1. The bracket enclosing the lowest set of subclones indicates phenotypes of NT-1 constructs harboring both classes of plasmids (see text). Map and coordinates are as described in Figure 1.

these two subclones gave rise to mutant colonies able to grow on medium containing AGA, but not MOP or AGR as the sole carbon source (data not shown). Similarly, strain NT-1 containing either of the two recombinant clones yielded a few fast growing colonies when incubated on plates containing GOA. These isolates showed properties similar to those shown by the AGA^+ and GOA^+ mutants of NT-1(pJS4159K1) (data not shown).

Restriction endonuclease analysis. Restriction endonuclease analysis of plasmid DNA isolated from four independent GOA-utilizing mutants of NT-1(pHP526) yielded two types of patterns when compared to the parent plasmid. One isolate contained a plasmid giving *Hin*dIII and *Bam*HI restriction patterns indistinguishable from pHP526 (Fig. 4). Three isolates contained plasmids in which *Hin*dIII fragment 11 and *Bam*HI fragment 19 showed mass increases of approximately 1.3 kb (Fig. 4). Digestion with *Eco*RI showed that an unaltered fragment 25 is present in each of the insertion derivatives (data not shown).

pMix1 is two plasmids. Plasmid pMix1 was stable in strain NT-1 when propagated on minimal medium with MOA as the sole carbon source. However, growth on NA containing streptomycin (NAS) yielded MOA^+ and MOA^- segregants. The MOA^+ isolates contained plasmids having restriction endonuclease patterns indistinguishable from pMix1 (data not shown). Analysis of several streptomycin-resistant MOA^- isolates showed that they contained one of two different plasmids. One, called pMix1A, was indistinguishable from pHP172 (see Fig. 3) and contained *Hin*dIII fragment Δ7, while the other, called pMix1B, resembled pHP177 (see Fig. 3) and contained *Hin*dIII fragments 11, 12, and 20. No MOA^- segregant harbored a plasmid containing all of the *Hin*dIII fragments present in pMix1. This suggested that pMix1 is actually two different recombinant clones contained in the same cell.

To test this, derivatives of strain NT-1 were constructed that contained either pHP172 or pHP177. Neither could utilize MOA (Fig. 3). Each derivative was transformed with the second plasmid. When plated on minimal medium containing MOA, opine-utilizing transformants were readily obtained (Fig. 3). Restriction endonuclease analysis showed that each contained *Hin*dIII fragments indistinguishable from pMix1 (data not shown). Like strain NT-1(pMix1), these transformants yielded MOA^-, streptomycin-resistant segregants when grown on NAS medium. In a similar set of experiments, derivatives of strain NT-1 containing pMix1A or pMix1B could be transformed to MOA^+ with the complementing pMix1 or pHP plasmid (data not shown).

Plasmid pHP526 yielded a *Hin*dIII restriction pattern indistinguishable from pMix1 (data not shown). However, strain NT-1(pHP526) did not produce MOA^- segregants when grown on NAS. All streptomycin-resistant colonies utilized MOA when patched to opine tester plates (data not shown).

DISCUSSION

Analysis of pJS4159K1 and comparison of this clone with cosmid clones previously described (Dessaux *et al.* 1987) indicate that the genes encoding catabolism of MOA are contained within an 18-kb region of pTi15955 spanning *Hin*dIII fragments 11, 12, 20 and a portion of 7 (Fig. 1). This interpretation is confirmed by analysis of the pHP subclones derived from pJS4159K1. Only pMix1 and pHP526 (Fig. 3) confer utilization of MOA (Table 1). The requirement for fragment 20 cannot be inferred from an analysis of these subclones since a construct containing only *Hin*dIII fragments Δ7, 11, and 12 was not isolated. However, analysis of overlapping cosmid clones clearly shows that fragment 20 is required for MOA catabolism. Cosmid pYDH304, which contains fragment 20, confers MOA utilization while pYDH105, which differs only by the absence of this fragment, does not (Fig. 1). This placement is also consistent with the results of Dessaux *et al.* (1988). They identified two Tn*5* insertions in the closely related octopine-type plasmid, pTiAch5, that abolished catabolism of MOA. One insertion mapped to *Hin*dIII fragment 11 while the other is located in *Hin*dIII fragment 12. Furthermore, they showed that the Ti plasmid from the wild-type octopine strain 89.10 had sustained a deletion removing these two *Hin*dIII fragments. Strain 89.10, although able to catabolize AGA, MOP, and AGR, is characterized by its inability to utilize MOA. A detailed map of the MOA catabolic region is shown in Figure 5.

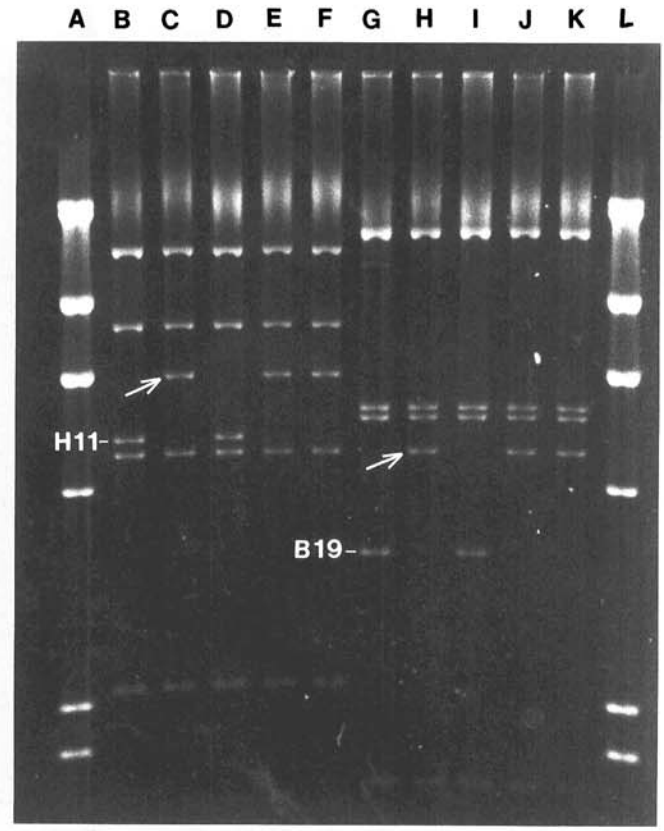

Fig. 4. Restriction endonuclease analysis of pHP526 and its mutants that confer catabolism of mannopinic acid analogues. Plasmid samples were isolated and digested with (**B–F**) *Hin*dIII or (**G–K**) *Bam*HI; the fragments were separated by electrophoresis on 0.7% agarose gels and visualized by staining with ethidium bromide. Lanes contain the following: (**A** and **L**) λ DNA digested with *Hin*dIII; (**B** and **G**) pHP526; and (**C–F** and **H–K**) pHP526GOA$^+$ derivatives. The arrows mark the new positions of the altered *Hin*dIII and *Bam*HI fragments.

Two lines of evidence indicate that the MOA catabolic system encoded by pJS4159K1 and pHP526 is regulated in a wild-type fashion. First, respirometric studies with strain NT-1 harboring these clones show that oxygen uptake in the presence of MOA requires that the cells be previously cultured with the opine (Table 3). No O_2 uptake with MOA was seen when these strains were pregrown with mannose. In this respect, the requirements for MOA utilization, as measured by oxygen consumption, are identical to those seen for strains with the intact Ti plasmid (Dessaux *et al.* 1988). Second, NT-1 derivatives harboring these clones are unable to utilize the MOA analogue, GOA (Table 2). However, GOA-utilizing mutants arise spontaneously, and these isolates utilize MOA in a constitutive fashion as determined by respirometry (data not shown). In these respects, strains harboring the recombinant clones behave exactly as do those containing the parental Ti plasmid. Chilton and Chilton (1984) observed that strains of *A. tumefaciens* containing the octopine-type Ti plasmids pTi15955 or pTiA6 did not utilize GOA, but produced spontaneous mutants able to catabolize this analogue. Such mutants concomitantly acquired the ability to utilize all the sugar analogues of MOA tested. Our observation that GOA^+ derivatives of NT-1(pJS4159K1) also utilize GLA (Table 2) is entirely consistent with the hypothesis that the spontaneous mutations have resulted in constitutive expression of genes encoding the MOA catabolic pathway.

These results also indicate that the pathway encoded by pHP526 is specific for MOA and does not accept MOP as a substrate. The specificity may be at the level of transport or degradation, but is not a function of regulation; pHP526 mutants constitutive for MOA catabolism still fail to utilize MOP (data not shown). This contrasts with the pathway for MOP catabolism examined by Dessaux *et al.* (1988). This pathway, expression of which is under the control of MOP, will accept MOA as a legitimate substrate, but only if preinduced with MOP.

Recombinant plasmids from several of the GOA^+ mutants of pHP526 appeared to have sustained insertions associated with the derepressed phenotype. Identical alterations were observed in some GOA^+ derivatives of pJS4159K1 (data not shown). We speculate that these mutations arose by the transposition of an indigenous strain C58 insertion sequence (IS) element into a regulatory locus on the recombinant clones. Since in each case the alteration was associated with *Hin*dIII fragment 11 and *Bam*HI fragment 19 but not with *Eco*RI fragment 25, the insertion must map to an approximately 300-base pair (bp) region just to the right of the *Bam*HI 13-19 junction (De Vos *et al.* 1981; see Fig. 5). The area to the left of *Eco*RI 25 is composed of a large number of very small unordered *Eco*RI fragments, making more precise mapping with this enzyme impossible. It is probable that the IS element responsible for these mutations is similar if not identical to IS*426* identified in strain C58 (Vanderleyden *et al.* 1986). Like the insertion in our clones, this element is about 1.3-kb in size and contains no *Hin*dIII or *Bam*HI sites. Furthermore, IS*426* is known to transpose with high frequency in the C58 chromosomal background (Vanderleyden *et al.* 1986). As shown in Figure 5, the site at which the IS element was inserted is contained, within our mapping accuracy, in the 900-bp region at which a Tn*5* element, resulting in a MOA^- phenotype, was inserted in pTiAch5 (Dessaux *et al.* 1988). Why one element confers a constitutive phenotype while the other results in a null mutation is unknown. It is possible that this 900-bp region overlaps sequences coding for two products, one involved in regulation and the other in catabolism.

The finding that pMix1 is actually two pKT231 recombinant clones has several interesting implications. First, it indicates that the genes encoding catabolism of MOA are organized into at least two expression units. One unit must be contained within *Hin*dIII fragment Δ7 while the other is encoded by *Hin*dIII fragments 11, 12, and 20. Neither unit alone encodes all of the information required for catabolism of MOA (Fig. 3). If the locus were organized as a single expression unit, the *Hin*dIII site separating fragments Δ7 and 11 would have to fall in an

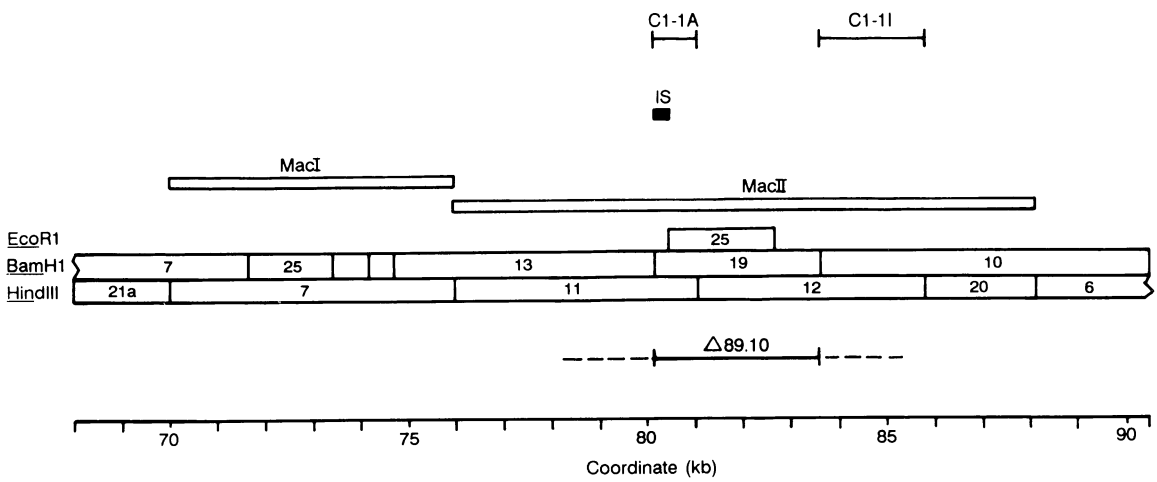

Fig. 5. Physicogenetic map of the pTi15955 region encoding catabolism of mannopinic acid (MOA). MacI and MacII represent the regions defined by pMix1A and pMix1B. C1-1A and C1-1I correspond to the two MOA^- Tn*5* insertions in pTiAch5 described by Dessaux *et al.* (1988) while Δ89.10 shows the approximate position of the deletion in the MOA^- octopine-type Ti plasmid, pTi89.10. The solid box labeled IS shows the map position of the spontaneous insertion events in pHP526 associated with derepression of the MOA catabolic pathway. Map and coordinates are as described in Figure 1.

intercistronic region. Furthermore, the downstream portion of the unit would then have to be expressed from a vector promoter; a set of conditions that together would seem to be unlikely.

Second, the two separate recombinants of the same vector apparently can be maintained in the same bacterial cell. The possibility that the two plasmids are in different cells which cross-feed each other is ruled out by analysis of clones grown on nonselective media. If the latter were true, only two types of segregants would be detected; those with a plasmid containing fragment Δ7 and those with a plasmid containing fragments 11, 12, and 20. However, an additional type was detected that contained all four fragments. Such isolates utilized MOA, yet themselves segregated the two plasmids when recultured on nonselective medium. It is probable that the original *Agrobacterium* isolate containing pMix1 represents a strain which was simultaneously transformed with both plasmids. Direct selection for growth with the opine as the sole carbon source constitutes a strong selective pressure and most probably is responsible for the isolation of such a double transformant. The high copy number character of pKT231 (Bagdasarian *et al.* 1981) is most likely responsible for the ability of the cell to maintain the two distinct plasmids. Thus, although the two distinct pKT231 recombinants are of the same incompatibility group, nonstringent copy number control allows the cell to maintain several editions of the replicon. Selection for growth on MOA ensures that copies of each type are retained in a single cell. Removal from these selective conditions, however, allows segregation by random partitioning and results in the loss of one or the other plasmid type from a given cell.

Sufficient genetic information to allow catabolism of AGA is also encoded in the region of pTi15955 represented by pHP526. This is not fully consistent with the work of Dessaux *et al.* (1987). In their analysis of overlapping cosmid clones, *Hin*dIII fragments 21a and 22b, which map to the left of fragment 7, are required for utilization of AGA (see Fig. 1 and Table 1). Furthermore, information encoded in fragments 12 and 20, which is required for MOA utilization, is not needed for growth on AGA. We have no explanation for this discrepancy. However, clones such as pJS4159K1 and pHP526 must first undergo some mutational event before cells containing them express the AGA catabolic phenotype. This suggests that the wild-type clones fail to express some function, perhaps regulatory in nature, which is necessary for AGA catabolism. Alternatively, the mutation may have altered the specificity of some component of MOA catabolism such that it now recognizes AGA as a legitimate substrate.

In this respect, mutations in AGA utilization do not affect regulation of MOA catabolism. The AGA^+ mutants remain repressed for MOA utilization, but are partially derepressed for AGA catabolism (Table 3). However, respirometric studies indicate that pregrowth with MOA results in increased O_2 uptake by these mutants in the presence of AGA (Table 3). This indicates that on pJS4159K1 some functions associated with MOA catabolism are required for utilization of the lactam.

These results suggest that the pathways for catabolism of MOA and AGA overlap to some extent. Chilton and Chilton (1984) demonstrated that mutants showing constitutive expression of MOA were also derepressed for AGA catabolism, indicating that the two pathways are coordinately regulated. Two sets of experiments presented here would seem to be inconsistent with their results. First, wild-type pJS4159K1 encodes an MOA-inducible MOA catabolic pathway. However, pregrowth with MOA does not result in increased rates of O_2 consumption in the presence of AGA (Table 3). Second, GOA-selected mutants of pJS4159K1 derepressed for MOA utilization do not show increased rates of oxygen assimilation with AGA (data not shown). This apparent discrepancy can be explained by the fact that while Chilton and Chilton worked with intact Ti and Ri plasmids, our studies were performed with subclones. Furthermore, the pJS4159K1 derivatives used in our study were not mutated for AGA utilization. Thus, whatever the nature of the mutation in the AGA^+ variants, it does not appear to affect the central regulatory apparatus that controls expression of these two pathways.

These results demonstrate the complexity of the organization and regulation associated with opine catabolic determinants encoded by *Agrobacterium* virulence plasmids. Although compounds such as the mannityl opines are relatively simple, a considerable amount of genetic information and a complex regulatory system are devoted to their catabolism. Dessaux *et al.* (1988) identified two enzymatic activities associated with MOA catabolism; one that yielded mannose from the opine, the other that converted mannose to fructose. Only the former is encoded by the Ti plasmid. Further studies are required to identify other MOA-specific activities, including transport functions encoded by the 18 kb of DNA represented in a clone such as pHP526.

ACKNOWLEDGMENTS

We thank Pierre Guyon (Orsay) for helpful technical assistance and for preparation of opines and their analogues.

This work was supported in part by grants to S. K. Farrand from the National Cancer Institute (RO1-CA44051) and the Illinois Soybean Program Operating Board (86-12-77-3), and to J. Tempé by the Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, and the European Economic Community (contracts GBI-4-018-F and BAP-0015-F).

LITERATURE CITED

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C. H., Bagdasarian, M. M., Frey, J., and Timmis, K. N. 1981. Specific-purpose plasmid cloning vectors II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene 16:237-247.

Binns, A. N., and Thomashow, M. F. 1988. Cell biology of *Agrobacterium* infection and transformation of plants. Annu. Rev. Microbiol. 42:575-606.

Chilton, W. S., and Chilton, M.-D. 1984. Mannityl opine analogs allow isolation of catabolic pathway regulatory mutants. J. Bacteriol. 158:650-658.

Dahl, G. A., Guyon, P., Petit, A., and Tempé, J. 1983. Silver nitrate-positive opines in crown-gall tumors. Plant Sci. Lett. 32:193-203.

Dessaux, Y., Tempé, J., and Farrand, S. K. 1987. Genetic analysis of mannityl opine catabolism in octopine-type *Agrobacterium tumefaciens* strain 15955. Mol. Gen. Genet. 208:301-308.

Dessaux, Y., Guyon, P., Farrand, S. K., Petit, A., and Tempé, J. 1986. *Agrobacterium* Ti and Ri plasmids specify enzymic lactonization of mannopine to agropine. J. Gen. Microbiol. 132:2549-2559.

Dessaux, Y., Petit, A., Tempé, J., Demarez, M., Legrain, C., and Wiame, J.-M. 1986. Arginine catabolism in *Agrobacterium* strains: Role of

the Ti plasmid. J. Bacteriol. 166:44-50.
Dessaux, Y., Guyon, P., Petit, A., Tempé, J., Demarez, M., Legrain, C., Tate, M. E., and Farrand, S. K. 1988. Opine utilization by *Agrobacterium* spp.: Octopine-type Ti plasmids encode two pathways for mannopinic acid degradation. J. Bacteriol. 170:2939-2946.
De Vos, G., De Beuckeleer, M., van Montagu, M., and Schell, J. 1981. Restriction endonuclease mapping of the octopine tumor-inducing plasmid pTiAch5 of *Agrobacterium tumefaciens*. Plasmid 6:249-253.
Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.
Ellis, J. G., Kerr, A., Petit, A., and Tempé, J. 1982. Conjugal transfer of nopaline and agropine Ti plasmids - The role of agrocinopines. Mol. Gen. Genet. 186:269-274.
Farrand, S. K., Slota, J. E., Shim, J.-S., and Kerr, A. 1985. Tn*5* insertions in the agrocin 84 plasmid: The conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin production. Plasmid 13:106-117.
Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided *in trans*. Proc. Natl. Acad. Sci. USA 76:1648-1652.
Firmin, J. L., and Fenwick, G. R. 1978. Agropine - A major new plasmid-determined metabolite in crown gall tumors. Nature (London) 276:842-844.
Guyon, P., Chilton, M.-D., Petit, A., and Tempé, J. 1980. Agropine in "null-type" crown gall tumors: Evidence for the generality of the opine concept. Proc. Natl. Acad. Sci. USA 77:2693-2697.
Holsters, M., DeWaele, D., Depicker, A., Messens, E., van Montagu, M., and Schell, J. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. Mol. Gen. Genet. 163:181-187.
Lopez, M. M., Gorris, M. T., and Montojo, A. M. 1988. Opine utilization by Spanish isolates of *Agrobacterium tumefaciens*. Plant Pathol. 37:565-572.
Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Montoya, A., Chilton, M.-D., Gordon, M. P., Sciaky, D., and Nester, E. W. 1977. Octopine and nopaline metabolism in *Agrobacterium tumefaciens* and crown gall tumor cells: Role of plasmid genes. J. Bacteriol. 129:101-107.
Petit, A., and Tempé, J. 1978. Isolation of *Agrobacterium* Ti plasmid regulatory mutants. Mol. Gen. Genet. 167:147-155.
Petit, A., Tempé, J., Kerr, A., Holsters, M., van Montagu, M., and Schell, J. 1978. Substrate induction of conjugative activity of *Agrobacterium tumefaciens* Ti plasmids. Nature (London) 271:570-572.
Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F., and Tempé, J. 1983. Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol. Gen. Genet. 190:204-214.
Pischl, D. L., and Farrand, S. K. 1984. Characterization of transposon Tn*5*-facilitated donor strains and development of a chromosomal linkage map for *Agrobacterium tumefaciens*. J. Bacteriol. 159:1-8.
Ream, L. W. 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Annu. Rev. Phytopathol. 27:583-618.
Slota, J. E., and Farrand, S. K. 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin production. Plasmid 8:175-186.
Tate, M. E., Ellis, J. G., Kerr, A., Tempé, J., Murray, K. E., and Shaw, K. J. 1982. Agropine: A revised structure. Carbohydr. Res. 104:105-120.
Tempé, J., and Petit, A. 1983. La piste des opines. Pages 14-32 in: Molecular Genetics of the Bacteria-Plant Interaction. A. Pühler, ed. Springer-Verlag, Berlin.
Trevelyan, W. E., Procter, D. P., and Harrisson, J. P. 1950. Detection of sugars on paper chromatograms. Nature (London) 166:444-445.
Vanderleyden, J., Desair, J., De Meirsman, C., Michiels, K., Van Gool, A. P., Chilton, M.-D., and Jen, G. C. 1986. Nucleotide sequence of an insertion sequence (IS) element identified in the T-DNA of a spontaneous variant of the Ti plasmid pTiT37. Nucleic Acid Res. 14:6699-6709.
Veluthambi, K., Krishnan, M., Gould, J. H., Smith, R. J., and Gelvin, S. B. 1989. Opines stimulate induction of the *vir* genes of *Agrobacterium tumefaciens* Ti plasmid. J. Bacteriol. 171:3696-3703.
Zambryski, P. 1988. Basic processes underlying *Agrobacterium*-mediated DNA transfer to plant cells. Annu. Rev. Genet. 22:1-30.