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Multiple Genes Coding for Octopine-Degrading Enzymes in *Agrobacterium*

ALICE L. MONTOYA,[1] LARRY W. MOORE,[3] MILTON P. GORDON,[2] AND EUGENE W. NESTER[1]*

Departments of Microbiology and Immunology SC-42[1] and Biochemistry SJ-70,[2] University of Washington, Seattle, Washington 98195, and Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331[3]

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Most biotype 2 strains of *Agrobacterium tumefaciens* and *A. radiobacter* which utilize nopaline also degrade octopine. In all such strains studied, the ability to degrade octopine did not appear to be transferred to plasmidless recipient cells under conditions of plasmid transfer in which the ability to utilize nopaline was transferred. An octopine-degrading mutant was isolated in a strain cured of its plasmid, suggesting that genes of octopine degradation may have a chromosomal location in some strains. In strains in which octopine utilization is coded by plasmid genes, octopine degradation was always inducible, whereas in strains which degrade both octopine and nopaline, octopine utilization was constitutive although nopaline degradation was inducible. When plasmids coding for octopine-utilizing ability were transformed into a strain containing either a nopaline- or null-type plasmid, transformants able to degrade octopine were either not observed or were unstable upon purification. All of these data suggest that plasmids associated with virulence are incompatible with one another, and therefore imply that the major groups of plasmids associated with virulence have a common origin.

Crown gall tumors incited by *Agrobacterium tumefaciens* synthesize basic amino acid derivatives, principally octopine (21) and nopaline (8, 9), compounds not found in normal plant tissues (8, 25; G. H. Bomhoff, Ph.D. thesis, University of Leiden, Leiden, The Netherlands, 1974). Most oncogenic strains of *Agrobacterium* have the ability to convert either octopine or nopaline to arginine (19, 25), and most tumors have the ability to synthesize these same amino acids by a condensation of arginine with pyruvate or α-ketoglutarate, respectively (25). Other laboratories (2, 22) have confirmed the original observations of Petit et al. (25) that bacterial strains which degrade octopine induce tumors that synthesize octopine, and most strains that degrade nopaline induce tumors that synthesize nopaline. The few oncogenic strains that degrade neither octopine nor nopaline induce tumors that synthesize neither of these amino acids (22). These observations gained added significance when it was shown that the structural genes for octopine- and nopaline-catabolizing enzymes reside on Ti plasmids (2, 22, 29), a portion of which is incorporated into crown gall tumors (3, 23, 24).

Petit et al. proposed that the genes specifying octopine and nopaline degradation in the bacteria were transferred to the plant, where their products functioned in the reverse direction, resulting in the synthesis of octopine and nopaline (25). However, recent findings have rendered this interpretation unlikely. Several laboratories have now demonstrated that mutants which no longer catabolize octopine or nopaline still induce tumors which synthesize the usual levels of these amino acids (17, 22; J. Tempe, personal communication). The genes specifying degradation of octopine or nopaline are therefore not the same as the genes specifying production of these compounds in the tumor. At the present time, the location of the structural genes responsible for octopine and nopaline synthesis in the tumor has not been established. However, it is clear that information coded by the plasmid specifies which amino acid is synthesized by the tumor.

In an attempt to gain an insight into the biochemical basis of the correlation between octopine and nopaline degradation by bacteria and synthesis by tumors, we have examined bacterial strains that can catabolize both and yet induce tumors which synthesize only nopaline. In this paper, we present evidence that in these strains the ability to utilize octopine is conferred by a chromosomal gene which is regulated differently than the plasmid-borne gene. We also demonstrate that the Ti plasmids are

incompatible with one another, an observation which may explain why, apparently, two such plasmids are never found in the same strain.

MATERIALS AND METHODS

Identification of octopine- and nopaline-utilizing strains. The ability of a strain to utilize octopine and nopaline was determined in one or more of the following ways: by the ability of the strain to either grow on octopine and/or nopaline as a sole source of carbon and nitrogen, or as a sole source of nitrogen, or by the ability of the strain to incorporate the ^{3}H-labeled arginine moiety of octopine and/or nopaline into trichloroacetic acid-insoluble material (protein) in a growing liquid culture (22).

Identification of octopine or nopaline production by tumors. Octopine or nopaline was identified in tumor extracts by the procedure described previously (22). In addition, a more sensitive assay was employed in some tumors. Tumors produced by five of the strains which utilize both octopine and nopaline were tested for the presence of octopine by putting 100 μCi of [^{3}H]arginine onto a 4-week-old primary tumor, induced on a Kalanchoe plant, 14 h before it was extracted with ethanol. The extract was subjected to electrophoresis preparatively, and the radioactivity was eluted from the region of the electrophoretogram where octopine would be expected. This material was lyophilized, dissolved in 0.5 ml of water, and then tested as the substrate for a partially purified preparation of octopine dehydrogenase from squid (28). The reaction mixture was subjected to electrophoresis and liquid scintillation counting to detect any [^{3}H]arginine released as a product of the reaction. To rule out the possibility of an inhibitor being present in the preparation from the tumor, [1-^{14}C]pyruvate-labeled octopine was added to the reaction together with the ^{3}H counts from the tumor extract. In this case, ^{14}C-labeled pyruvate was liberated at the expected rate (N. Hasan, manuscript in preparation). Similarly, radioactivity was eluted from the region of the electrophoretogram where nopaline would be expected. This [^{3}H]nopaline was lyophilized, dissolved, and used as substrate for the nopaline-degrading enzyme from *A. tumefaciens*. The product of this reaction was identified as [^{3}H]arginine by electrophoresis. This control suggested that the inability to detect octopine in the tumor was not a result of its being degraded by the inciting strain of *Agrobacterium*.

Assay of octopine- and nopaline-degrading activity. Octopine- and nopaline-degrading activities were assayed by using [^{3}H]octopine and [^{3}H]nopaline as previously described (22). Arginine was identified as a reaction product of the enzyme activities by high-voltage electrophoresis (22). Octopine-degrading activity was also measured by using [1-^{14}C]pyruvate-labeled octopine in a procedure based on the release of radioactive pyruvate (N. Hasan, manuscript in preparation).

Gene transfer experiments. RP4-mediated conjugation was carried out as described by Chilton et al. (4). The procedure of Schell et al. (*in* I. Rubenstein, ed., *A Symposium on the Molecular Biology of Plants*, in press) was used for the transformation of *Agrobacterium*. In planta crosses were carried out as described by Kerr (15). All genetic transfer experiments were repeated from two to ten times.

Plasmid DNA isolation. Plasmid DNA was isolated by the procedure of Currier and Nester (6).

Chemicals. [^{3}H]octopine was prepared as described previously (22). Unlabeled octopine was synthesized by the procedure of Izumiya et al. (12). Nopaline and [^{3}H]nopaline were synthesized from arginine and α-ketoglutaric acid by the procedure of Jensen et al. (13). [^{14}C]octopine was synthesized enzymatically from arginine and [1-^{14}C]pyruvate, using octopine dehydrogenase prepared from squid muscle (28; N. Hasan, manuscript in preparation). Desmethyloctopine was prepared by R. Jensen as described by Herbst and Swart (10). The formulae for octopine, nopaline, and desmethyloctopine are shown in Fig. 1.

RESULTS

Strains surveyed. We have studied nine oncogenic and five nononcogenic strains of *Agrobacterium*, all of which utilize both octopine and nopaline (Table 1). With one exception, the oncogenic strains of *A. tumefaciens* that utilize both octopine and nopaline induce tumors that synthesize only nopaline. No octopine was detected in these tumors when [^{3}H]arginine was applied to the tumor as described in Materials

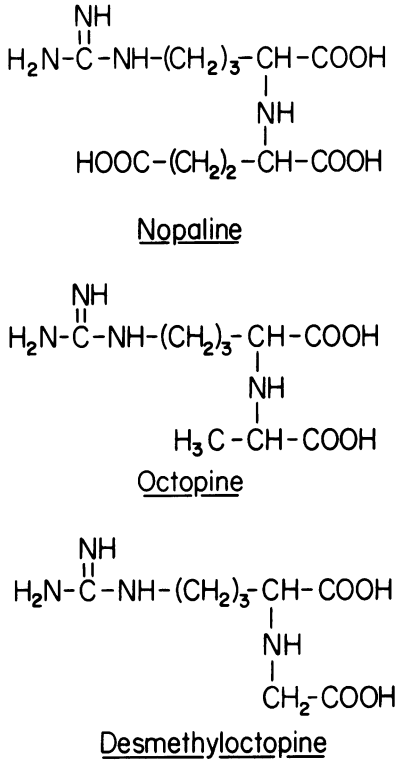

FIG. 1. *The chemical structures of nopaline, octopine, and desmethyloctopine.*

TABLE 1. *Bacterial strains which utilize both octopine and nopaline*

Strain	Received from[a]	Original source and site of isolation	Production by tumor
27	A. Kerr	Peach, South Australia	Nopaline
223	NCPPB	Unknown, New Zealand	Nopaline
K2a	L. W. Moore	*Prunus* species, Australia	Nopaline
K9/73	L. W. Moore	Willow, Oregon	Nopaline
B1/74	L. W. Moore	Almond, Oregon	Nopaline
B3/73	L. W. Moore	Norway Maple, Oregon	Nopaline
M3/73	L. W. Moore	Birch, Oregon	Nopaline
G1/73	L. W. Moore	Peach, California	Nopaline
T10/73	L. W. Moore	Rose, Missouri	None
84	A. Kerr	Soil under infected peach tree, Australia	Avirulent
G12/73	L. W. Moore	Peach, California	Avirulent
G6/73	L. W. Moore	Peach, California	Avirulent
B13/74	L. W. Moore	Cherry, Oregon	Avirulent
I8/75	L. W. Moore	Star roses, Pennsylvania	Avirulent

[a] A. Kerr, Waite Agricultural Station, South Australia; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England.

and Methods. [^{3}H]nopaline recovered from the same tumor extracts served as substrate for the nopaline-degrading enzyme from *A. tumefaciens* and yielded [^{3}H]arginine as a reaction product.

Gene transfer studies. A correlation has been established between the plasmid-coded trait for utilization of octopine or nopaline by an oncogenic strain of *Agrobacterium* and the ability of the tumor induced by that strain to synthesize either octopine or nopaline (2, 18, 19, 22, 24). However, since strains which could utilize both octopine and nopaline induced tumors that synthesized only nopaline, we considered the possibility that the gene for octopine utilization was present on the chromosome. To answer this question, we determined whether the ability to utilized octopine was transferred with the plasmid. Plasmids were isolated from a number of double-utilizing strains by a technique which results in the isolation of all size classes (6), and these preparations were used to transform plasmidless strains. All transformations were repeated from two to five times. Only the ability to utilize nopaline was transferred (Table 2). In the control experiment, a plasmid preparation from a strain which utilized only octopine (15955) (18) readily transferred the ability to utilize octopine. We have screened all but one of the double-utilizing strains available to us which were isolated from nature to see if the gene(s) concerned with octopine and nopaline utilization was transferable. In every case, only the ability to degrade nopaline was transferred.

Several other techniques of plasmid transfer were employed in an attempt to transfer octopine-degrading ability in the strains which utilize both octopine and nopaline. Neither conjugation in vitro nor in planta resulted in transfer of the octopine-utilizing ability, although in both cases the nopaline trait was transferred (Table 3).

TABLE 2. *Octopine and nopaline utilization after plasmid-mediated transformation*

Strain		No. of transformants/μg of DNA	
Donor	Recipient[a]	Nop$^+$	Oct$^+$
G12/73	A200	223	0
K2a	A200	115	0
T10/73	A200	185	0
27	A200	82	0
84	A200	30	0
223	A200	137	0
15955[a]	A200	0	246
K27	A592	233	0
K84	A592	64	0
G12/73	A592	68	0
T10/73	A592	107	0
B3/73	A592	32	0
B13/74	A592	96	0
G1/73	A592	1,076	0
I8/75	A592	1,610	0
K9/73	A592	1,738	0
M3/73	A592	36	0
15955	A592	0	140

[a] A200 is an arginine-requiring, plasmidless strain derived from heat-cured C58 (4). This strain is resistant to 5 μl of rifampin and 50 μl of nalidixic acid per ml. A592 is a plasmidless strain derived in our laboratory by David Garfinkel by heat curing ACH5 (4). This strain was resistant to 5 μl of rifampin, 50 μl of nalidixic acid, and 50 μl of chloramphenicol per ml. The plates were scored at intervals to 4 weeks after the transformation was performed. Strain 15955 carries an octopine-type plasmid and was used in this study as a control to show that the recipient cells could be transformed to Oct$^+$.

From the results of all of these plasmid transfer experiments, we tentatively conclude that the utilization of octopine is not coded by a plasmid-borne gene(s) in those strains of *A. tumefaciens* which utilize both octopine and nopaline.

TABLE 3. *Genetic transfer of octopine and nopaline utilization by conjugation and in planta transfer*

Method	Strain		Transconjugants	
	Donor	Recipient[a]	Nop$^+$	Oct$^+$
Conjugation	27 (RP4)	A200	213[b]	0[b]
In planta	27	A136	335[c]	0[c]
transfer	223	A136	297[c]	0[c]

[a] A136 is a plasmidless strain derived from heat-cured C58 which is resistant to 5 μl of rifampin and 50 μl of nalidixic acid per ml (4). A200 is an arginine-requiring strain of A136 derived by nitrosoguanidine treatment (4).

[b] Value represents number of transconjugants per 10^2 recipients.

[c] Value represents number of transconjugants per 10^3 recipients.

Isolation of octopine-utilizing mutants in plasmidless strains. The conclusion that octopine utilization may be coded by chromosomal genes is supported by the observation that it is possible to isolate octopine-utilizing mutants in plasmidless strains. When the plasmidless strain A136 was plated on octopine as the sole source of nitrogen, colonies arose after about 4 weeks of incubation at 28°C. These cells grew well on octopine as the sole source of nitrogen and converted octopine into arginine as measured by the incorporation of [^{3}H]octopine into trichloroacetic acid-insoluble material. Bacterial extracts were prepared which degraded octopine. Arginine and pyruvate were identified as products of the reaction. Arginine was identified by high-voltage electrophoresis (22), and pyruvate was identified as the 2,4-dinitrophenylhydrazone derivative (N. Hasan, manuscript in preparation). Thus, this enzyme carried out the same reaction as the octopine-degrading activity described by Petit et al. (25). Similar mutants were isolated when plasmidless strains were plated on an analog of octopine, desmethyloctopine, for 1 or 2 weeks. This compound can be used as a sole source of nitrogen or carbon and nitrogen by strains that can utilize octopine, but it does not induce the synthesis of enzymes involved in its utilization (A. Petit, Ph.D. thesis, Centre Nationale de la Recherche Scientifique, Paris, 1977; J. Tempé, personal communication).

Differential regulation of synthesis of octopine-degrading activity. If the gene controlling octopine-degrading activity is located on the chromosome in some strains and on a plasmid in others, it is conceivable that the regulation of synthesis of the enzyme might vary in the two situations. Therefore, the control of enzyme synthesis was studied in a strain which utilized both octopine and nopaline as well as in two other strains, one of which utilized only octopine and the other only nopaline. In strains with octopine-type Ti plasmids, an octopine-permease and an octopine-degrading activity are known to be synthesized (18, 22, 24). The uptake of labeled octopine, as well as its conversion to arginine and incorporation into trichloroacetic acid-precipitable material, exhibited a time lag that could be eliminated by growing the cells for 30 to 60 min in the presence of octopine before the addition of labeled octopine. If rifampin was added 1 min before the addition of octopine, octopine was not taken up (data not shown). Thus, the induction of the permease required de novo protein synthesis. In another experiment, rifampin was added after a 2-min induction period. In this case, octopine entered the cell but was not converted into arginine and incorporated into protein. This result suggests strongly that the synthesis of octopine-degrading enzyme also required de novo protein synthesis. We have examined five different strains containing octopine-type Ti plasmids, and they all follow this same pattern of enzyme induction.

The enzymes responsible for the uptake and conversion of nopaline to arginine (nopaline-degrading activity) are also inducible (26). Octopine induces the permease- and octopine-degrading activity of octopine-type plasmid strains (17). However, octopine cannot induce the synthesis of enzymes required for the conversion of nopaline to arginine and α-ketoglutarate; once these activities are induced by nopaline, octopine can be metabolized to arginine and pyruvate and subsequently incorporated into acid-insoluble material (26, 27).

We have examined the 14 strains in our collection which utilize both octopine and nopaline (Table 2). In 13 strains, the utilization of octopine occurred without any time lag, and preinduction with octopine did not alter these kinetics (data not shown). A representative strain is shown in Fig. 2. Octopine-degrading activity appears to be synthesized constitutively in these strains. The products of this reaction have been identified as described in Materials and Methods. However, the synthesis of nopaline permease- and nopaline-degrading activity followed the same pattern of regulation as found in the strains that utilized only nopaline with a time lag before uptake and utilization (Fig. 2). In one avirulent strain, G12/73, the uptake and conversion of octopine to arginine was inducible. In this strain, like all strains which utilize both octopine and nopaline, the octopine degradation trait was not transferred with the plasmid. All 14 strains utilized nopaline only after a period of induction. The mutant plasmidless strains which

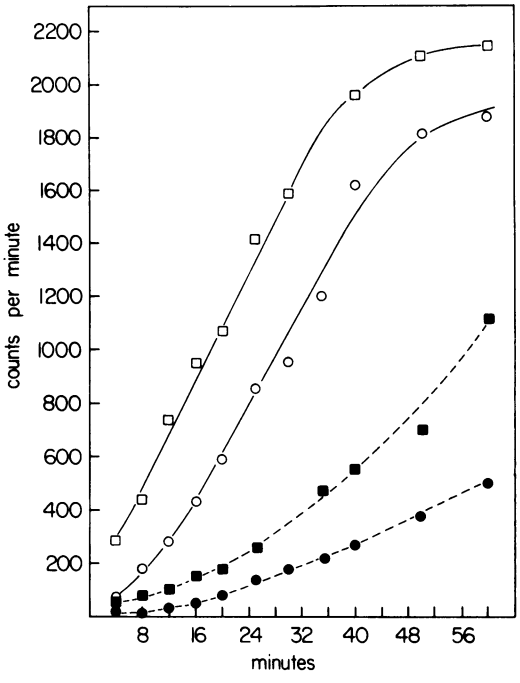

FIG. 2. *Kinetics of octopine and nopaline uptake and utilization of strain 27. Octopine: total counts uptake (□), trichloroacetic acid-precipitable counts (○); nopaline: total counts uptake (■), trichloroacetic acid-precipitable counts (●).*

were selected for octopine utilization also converted octopine to arginine without any time lag (data not shown).

Incompatibility of Ti plasmids. The data presented in this paper, together with data from another laboratory (16), are consistent with the notion that the octopine and nopaline plasmids may be incompatible. Of the 14 strains we examined, there is no evidence that octopine and nopaline plasmids exist in the same strain. Kerr and Roberts (16) screened over 70 natural isolates. They noted that biotype 1 strains can carry the octopine plasmid or the nopaline plasmid but not both.

To determine more directly whether this suggestion is correct, we attempted to transform a strain carrying an octopine plasmid with three different nopaline plasmids (Table 4). The capacity of each plasmid preparation for transformation was assessed by successfully transforming a plasmidless strain. The competence of the recipient octopine strain was measured by its ability to be transformed by the promiscuous plasmid RP4 (4). The data in Table 4 indicate that, although the recipient strains can be transformed by the plasmid RP4, they are not transformable by nopaline plasmids, although these

TABLE 4. *Plasmid-mediated transformation of octopine strains by nopaline and RP4 plasmids*

Strain		Contains Oct plasmid	Transformants/μg of DNA	
Donor	Recipient		Nop$^+$	Ap^r Km^r [a]
C58	A312	yes	0	—[b]
RP4	A312	yes	—	37
C58	A200	no	35	—
RP4	A200	no	—	56
27	A592	no	83	—
RP4	A592	no	—	66
27	ACH5	yes	0	—
RP4	ACH5	yes	—	20
A208	A592	no	23	—
A208	ACH5	yes	0	—

[a] Resistance to 100 μg of ampicillin (Ap) and 100 μg of kanomycin (Km) per ml of media.
[b] —, Not applicable.

latter plasmids are capable of transforming plasmidless derivatives of these same strains.

These data do not distinguish between surface exclusion of the entry of the nopaline plasmid and inability of the nopaline plasmid to replicate inside the recipient cell. Also, this experiment does not answer the intriguing question of whether the nopaline-type plasmid can exist in a cell with a virulence-associated plasmid which differs in gross base sequence homology. Naturally occurring plasmids associated with virulence exist which code for neither octopine nor nopaline degradation (22) and bear little base sequence homology with either the octopine or nopaline plasmids (5; M. H. Drummond and M. D. Chilton, submitted for publication). To gain some insight into these questions, plasmid was isolated from two single-utilizing strains, A277(pTiB6806) (22) for the octopine plasmid and A208(pTiT37) (22) for the nopaline plasmid. These preparations were used to transform a strain which contains a virulence-associated plasmid which is not homologous to either of the donor plasmids (Table 5).

The data indicate that transformants could be obtained when either an octopine-type plasmid or a nopaline-type plasmid was used as the donor and a strain containing a "null"-type plasmid was the recipient. Transformants were obtained with octopine or nopaline used as the sole source of nitrogen or as the sole source of carbon and nitrogen. However, upon purification, two colony types arose: a small, clear, watery colony, which looked like the original recipient, and a larger, opaque, white colony which looked like the original donor strain. When the small watery colony was restreaked, it gave only small watery colonies, whereas the large white colony gave rise to a few large white colonies and many more

TABLE 5. *Plasmid-mediated transformation of a strain containing a null-type plasmid by octopine and nopaline plasmids*

Donor plasmid	Plasmid type	Transformants/2 μg of DNA with:[a]	
		Plasmidless A592	Null-type plasmid A281
pTiB6806	Octopine	28	28
pTi-T37	Nopaline	68	64
RP4	—	80	62

[a] A592 is a plasmidless strain derived from ACH5, and A281 is an exconjugant obtained from an in planta cross of 542 with A136 (20).

small watery colonies. If the original transformants were streaked to nonselective media and back to selective media, few if any large white colonies were observed. Thus, it appeared that the octopine or nopaline plasmid was being lost even during the purification process on selective media. To test that the null-type plasmid was still present, large white colonies that had gone through two purification cycles on octopine- or nopaline-containing media were grown in the presence of octopine or nopaline, and plasmid DNA was prepared. Only the null-type plasmid (152×10^6 daltons) was detected, confirming the instability of the octopine or nopaline plasmid in the strains carrying a null-type virulence plasmid.

DISCUSSION

All biotype 2 *A. tumefaciens* and *A. radiobacter* strains examined in this study utilized both octopine and nopaline. The gene(s) conferring octopine-utilizing ability did not behave as though it were plasmid borne, whereas the genes conferring nopaline catabolism were always on a plasmid. The interpretation that the octopine trait in these strains is chromosomal would be strengthened considerably if a chromosome transfer system were available in this organism. Hopefully, the use of the plasmid R68-45, which mobilizes the chromosome of *Agrobacterium* (S. Farrand, personal communication), will permit us to localize the octopine-degrading activity on the chromosome.

The high correlation between the location of the octopine gene and its mode of regulation is intriguing but not understood. If the gene was located on the plasmid in naturally occurring isolates, then enzyme synthesis was inducible by octopine. If the gene was located on the chromosome, then its synthesis was usually constitutive. One exception to this correlation was noted. This exception was also the only biotype 1 strain found to utilize both octopine and nopaline. The plasmidless strains which could utilize octopine also appear to be constitutive for this utilization.

The results presented here are significant to the correlation between octopine and nopaline utilization by the bacteria and production by the tumor (25). Previous studies have strongly implicated plasmid genes as specifying whether octopine or nopaline is synthesized by the tumor (2, 19, 22, 25). These data support that conclusion. The data also indicate that the inciting organism does not always induce tumors which synthesize all the -opines that it can degrade. Thus, synthesis of octopine in tumors is correlated with a plasmid-borne gene in the inciting bacteria, and not merely the presence of a metabolite of octopine synthesis.

It is quite possible that octopine- and nopaline-utilizing stains could result in nature if a nopaline-utilizing strain were present in a tumor induced by an octopine-utilizing strain. Under these conditions, there should be selection for octopine-utilizing strains. One might expect a nopaline-utilizing strain to acquire the ability to utilize octopine by acquisition of the octopine plasmid. However, no such strains are found in natural isolates, suggesting that the octopine and nopaline plasmids are incompatible or mutually exclusive. The strains which do degrade both octopine and nopaline contain a nopaline plasmid and a nontransferable octopine trait. This suggests that the octopine degradation could have arisen by alteration of an existing chromosomal coded enzyme (dehydrogenase or oxidase) to utilize octopine as a substrate.

Although our inability to detect even unstable transformants, when Nop^+ strains are used as donors and Oct^+ strains as recipients could be interpreted as surface exclusion, this seems to be an unlikely explanation. We have recently been able to introduce the Oct^+ plasmid into a Nop^+ recipient when the Oct^+ plasmid was cointegrated with the promiscuous plasmid RP4 (11) (unpublished data). This cointegrate form of the Oct^+ Ti plasmid appears to be reasonably stable in strains containing a Nop^+ plasmid. These data coupled with the observations reported in this paper that Nop^+ plasmids and Oct^+ plasmids can form unstable transformants with the null-type plasmids make plasmid incompatibility the most reasonable explanation for these data.

The incompatibility of Ti plasmids is surprising in view of their lack of base sequence homology. As a general rule, plasmids of the same compatibility group have a significant proportion of their polynucleotide sequences in common (7). However, although the overall base composition is different in these three plasmid types, they do share regions of DNA homology which are spread throughout the plasmid (M.

Drummond and M. D. Chilton, submitted for publication). Presumably, one or more of these regions of homology is required for incompatibility.

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ADDENDUM IN PROOF

The entire complement of identifiable plasmids has been transferred from a strain of *Agrobacterium rhizogenes* able to utilize octopine to a plasmidless recipient strain. None of the transconjugants utilized octopine.

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