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Octopine and Nopaline Metabolism in *Agrobacterium tumefaciens* and Crown Gall Tumor Cells: Role of Plasmid Genes

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Crown gall tumors produced octopine or nopaline or neither compound, depending on the bacterial strain that incited the tumor. The genes specifying production of octopine or nopaline by the tumor were transferred to recipient bacterial strains when the large plasmid associated with virulence was transferred by either conjugation or deoxyribonucleic acid-mediated transformation. Our results, which confirm the work of others (Bomhoff et al., 1976; Goldman et al., 1968; Petit et al., 1970), indicate that, in general, the strains that utilize octopine induce tumors that synthesize octopine, and those that utilize nopaline induce tumors that synthesize nopaline. However, there were several notable exceptions. One class utilized both octopine and nopaline, but the tumors induced by these strains produced only nopaline. Another class utilized nopaline, but their tumors synthesized neither nopaline nor octopine. Mutants were isolated from a number of either octopine- or nopaline-utilizing strains that no longer could utilize the relevant guanido amino acid. These strains, which were mutant in the gene specifying octopine or nopaline oxidase, still retained the permease for these amino acids as well as virulence. Tumors induced by these mutants still synthesized approximately the same levels of octopine and nopaline as tumors induced by their parents. These results suggest that the plasmid gene that determines production of octopine or nopaline by the tumor is distinct from the plasmid gene that determines their catabolism by the bacteria.

Crown gall tumors, incited by *Agrobacterium tumefaciens* in dicotyledonous plants, contain the unusual guanido amino acids octopine N^2-(D-1-carboxyethyl)-L-arginine (15) and nopaline N^2-(1,3-dicarboxypropyl)-L-arginine (3, 4). Production of octopine or nopaline by tumors is not dependent upon what host plant is used, but is determined by which bacterial strain incites the tumor (G. H. Bomhoff, thesis, University of Leiden, Leiden, The Netherlands, 1974; 3, 16). In general, nopaline-containing tumors are incited by bacterial strains that catabolize nopaline, whereas octopine-containing tumors result from infection by strains able to catabolize octopine (M. P. Gordon et al., *in* I. Rubenstein [ed.], *Proceedings of a Symposium on the Molecular Biology of Plants*, in press; 1, 16). Tumors in many cases continue to produce high levels of octopine and nopaline when cultivated in tissue culture free from any viable *A. tumefaciens* (3). Thus production of unusual guanido amino acids in high levels is a characteristic of many, but not all, established lines of crown gall tumors (Bomhoff, thesis, 1974; Gordon et al., in press; 16).

It is now clear that large *A. tumefaciens* plasmids, first discovered by Zaenen et al. (21), are indeed associated with virulence as suggested by these investigators. Loss of virulence upon incubation of certain strains at elevated temperature (6) is due to elimination of a large plasmid (19). Acquisition of virulence upon conjugation in planta (5, 9, 10) is associated with acquisition of the plasmid (19). Acquisition of plasmid upon conjugation in vitro results in the acquisition of virulence (M.-D. Chilton et al., Genetics, in press). Most plasmids which have been demonstrated to carry tumor-inducing genes have thus far been found to carry either nopaline catabolism or octopine catabolism genes (Chilton et al., in press; 17, 18). This genetic linkage presumably accounts for the close correlation between virulence and octopine/nopaline utilization in *Agrobacterium* strains (11). Octopine or nopaline production as well as other characteristics of the tumor might reasonably be determined by bacterial plasmid genes replicating in the transformed plant cell.

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Thus, the mechanism determining octopine/nopaline production is of singular interest because it bears on the question of gene transfer from *A. tumefaciens* to the plant cell.

Studies of the bacterial octopine-catabolizing enzyme and the tumor octopine-synthesizing enzyme fail thus far to support the view that the tumor contains the bacterial enzyme. Although the bacterial octopine oxidase is a membrane-bound enzyme that is cytochrome linked (Bomhoff, thesis, 1974; 12), the tumor octopine dehydrogenase is a soluble enzyme that requires nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide as cofactor (Bomhoff, thesis, 1974). However, since neither enzyme has been purified to homogeneity, no definitive conclusions can yet be drawn.

The experiments reported here provide new insight into the problem of how virulent strains of *A. tumefaciens* determine what unusual guanido amino acid will be produced in crown gall tumors. They confirm and extend previous reports that the guanido amino acid synthesized by the tumor is specified by genes on the plasmid in the tumor-inducing strain (1; Gordon et al., in press; E. W. Nester et al., *in 10th Annual Miles Symposium*, in press). They also support the biochemical data which suggest that the octopine- and nopaline-synthesizing enzymes in the tumor are not coded by the same genes that code for octopine and nopaline degradation on the plasmid.

MATERIALS AND METHODS

Organisms. The bacterial strains used were strains either isolated originally from natural sources or derived by conjugation of virulent donor strains with avirulent recipient strain A136 in planta as previously described (19) or with avirulent recipient strain A200 (Chilton et al., in press). The identification of exconjugants from crosses in planta in most cases was confirmed by deoxyribonucleic acid (DNA) hybridization studies of the type reported previously (19). In particular, the exconjugant strain was demonstrated to have chromosomal DNA that was completely homologous to labeled recipient DNA. Further, labeled plasmid DNA from the exconjugant was shown to have complete homology with unlabeled donor plasmid DNA sequences.

DNA isolation. Bacterial DNA was isolated as described previously (19). Plasmid DNA was isolated by the procedure described elsewhere (T. C. Currier, and E. W. Nester, Anal. Biochem., in press) except that the $CHCl_3$ extraction step was omitted, and the plasmid DNA was further purified by an additional rebanding in a CsCl/ethidium bromide gradient. Labeled plasmid was isolated from bacteria grown with [^{3}H]thymidine as described previously (19).

Analysis of octopine/nopaline production by tumors. Tumors were induced on immature leaves of young kalanchoe plants by streaking bacterial colonies down the leaf on each side of the midrib with a sterile toothpick, producing a longitudinal wound site. This technique yielded a large tumor in the shortest possible time. After 2 to 4 weeks, tumor tissue was excised and ground in a mortar with an equal weight of 95% ethanol, and the extract was clarified by centrifugation (12,000 × *g*, 10 min, Sorvall SS34 rotor). A 50-μl aliquot of the extract was spotted on Whatman 3MM paper together with relevant standard compounds and electrophoresed, using a Gilson high-voltage electrophoresis apparatus at 2,000 V for 1 to 1.5 h (pH 3.5 buffer system previously described [19]). The dried paper was first examined for intrinsically fluorescent compounds by illumination with a short-wavelength blacklight. The paper was next dipped in phenanthrene-quinone reagent (20) and observed for 30 min. Guanidines at first gradually develop a yellow fluorescent spot which slowly changes to blue-white fluorescence. Reference standards for electrophoresis were octopine and nopaline.

Isolation and characterization of octopine and nopaline nonutilizing strains. For isolation of nopaline auxotrophs, arginine auxotrophs treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (2) were plated on AB minimal agar (19) supplemented with 0.5 μg of L-arginine per ml plus 50 μg of nopaline per ml. Small colonies were picked with sterile toothpicks and streaked onto AB minimal medium supplemented with either arginine (500 μg/ml) or nopaline (50 μg/ml). Mutants unable to degrade nopaline grew very poorly on the nopaline-supplemented medium but well on the arginine-supplemented medium. However, mutants were only considered for further study if whole cells could take up but not incorporate [^{3}H]nopaline into acid-insoluble protein. Arginine revertants were isolated (Chilton et al., Genetics, in press) from all Nop$^-$ mutants before they were inoculated into plants. These revertants resembled the original mutant strains in their inability to utilize nopaline.

Isolation of octopine nonutilizing mutants followed the same technique except that octopine was substituted for nopaline.

Measurement of uptake of labeled nopaline and octopine. Bacteria in mannitol-glutamate medium in the exponential phase of growth (28°C, with shaking) were supplemented with [^{3}H]nopaline or [^{3}H]octopine, and 0.1-ml aliquots were withdrawn at intervals for analysis of radioactivity. Total radioactivity incorporated was assayed by trapping cells on membrane filters (Millipore Corp.) and washing them with 5 ml of mannitol-glutamate medium. The radioactivity incorporated into macromolecules was determined by collecting the trichloroacetic acid-precipitated material on glass fiber filters (Schleicher & Schuell), washing with 5 ml of 5% trichloroacetic acid, rinsing with 95% ethanol, and drying. The counting efficiency of the two procedures was normalized by comparing the radioactivity obtained by each method in samples that had essentially all label in trichloroacetic acid-insoluble form.

Preparation of cell-free octopine/nopaline oxidase activity. A 1-ml culture was inoculated into 200 ml of AB minimal medium supplemented with 0.5%

glucose, and the culture was grown overnight with shaking at 30°C. Sterile octopine or nopaline was then added to the log-phase culture to a final concentration of 20 μg/ml. After 1.5 to 2.5 h of shaking at 30°C, the cells were harvested at 4°C by centrifugation at 8,000 × g for 10 min and were washed with 0.1 M potassium phosphate buffer, pH 7.0. All subsequent steps were carried out at 4°C. The final pellet was resuspended in 1 ml of 0.1 M potassium phosphate buffer, pH 7.0. The cells were disrupted by treatment with an MSE sonic oscillator, set at the maximum power setting, for a total of 2 min in 30-s bursts. Insoluble debris was removed by centrifugation at 17,000 × g for 15 min, following which the supernate was centrifuged at 105,000 × g for 2 h. The pellet was suspended in 0.5 ml of water, and 0.1-ml aliquots were quick-frozen in a dry ice-ethanol bath and stored at −20°C.

Enzyme assays. The reaction mixture contained 4 to 40 μg of the pellet protein that sedimented at 105,000 × g, 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.5, and 1.2 μg of [^{3}H]octopine (8,000 cpm/μg) or [^{3}H]nopaline (8,000 cpm/μg) in a final volume of 25 μl. The reaction was carried out at 30°C, and the synthesis of arginine was linear with incubation times up to 2.5 h. The reaction was terminated by freezing the sample in a dry ice-ethanol bath. The samples were thawed, and 10- or 20-μl aliquots were spotted on Whatman 3MM filter paper. The paper was subjected to electrophoresis as described previously, using 1.2 M pyridine acetate buffer, pH 6.1. After drying, the electrophoretogram was cut into 1.5-cm strips and then into 1-cm pieces, which were placed in 1.0 ml of water in a plastic scintillation vial. The radioactivity was eluted from the paper fractions for 1 h, and then 10 ml of Triton X-100-toluene-Liquifluor (1:2) was added. Vials were counted in a Beckman LS-100 liquid scintillation counter. The specific activity (S.A.) of an enzyme preparation, expressed as milligrams of arginine formed per minute per milligram of protein, was determined by the following formula:

$$\text{S.A.} = \frac{\text{(radioactivity recovered as arginine/ total radioactivity recovered)} \times \text{mg of substrate}}{\text{mg of protein} \times \text{time}}$$

Protein was determined by the method of Lowry et al. (14).

Test for reversion of the nopaline and octopine trait. Approximately 3 g of kalanchoe tumor tissue was excised from a leaf, and 2 g was analyzed for the presence of nopaline or octopine as previously described. One gram of the tumor was surface sterilized for 1 min with 70% ethanol and then ground in 2 ml of AB minimal medium, using a sterile mortar and pestle. The supernatant solution was pipetted into a sterile tube, glycerol was added to a final concentration of 10%, and then the solution was titered for the concentration of bacteria on nutrient agar plates. It was then quick-frozen in a dry ice-ethanol bath and stored at −120°C. Then a rough estimate of the total number of cells in the solution was obtained (approximately 10^7 to 10^8 cells per g of tissue). The solution was thawed and plated to yield approximately 10^5 cells per plate. To test for nopaline reversion, nopaline was used as the sole source of nitrogen and carbon. To test for octopine reversion, octopine was used as the sole source of nitrogen. Reconstruction experiments indicated that octopine and nopaline revertants could be quantitatively recovered and readily identified in the background of mutants.

Source of chemicals. Unlabeled octopine was purchased from Sigma Chemical Co., and arginine was purchased from ICN Pharmaceuticals, Inc. Nopaline was isolated from a nopaline-producing tumor as previously described (19) and was shown to be chromatographically indistinguishable from an authentic sample of nopaline synthesized by W. Szydbak and W. S. Chilton of this university. Larger quantities were synthesized by W. S. Chilton by a procedure that will be reported in another publication. The ^{3}H-labeled octopine and nopaline were prepared as decribed previously (19).

RESULTS

Octopine/nopaline utilization by bacteria and production by tumors. We have observed that for a large number of *A. tumefaciens* strains, octopine-utilizing bacteria incite octopine-producing tumors whereas nopaline-utilizing bacteria incite nopaline-producing tumors, thus confirming the findings reported earlier (Nester et al., in press; 1, 3) (Table 1). Our previous report (7) that strain CGIC was an exceptional strain must now be viewed with suspicion. Repeated attempts in this laboratory have failed to reproduce the claimed induction of a nopaline-producing teratoma by strain CGIC. A third group of strains, represented by AT1, AT4, and 542, utilize neither octopine nor nopaline, and their tumors do not synthesize either compound.

Two groups of strains do not demonstrate this strict correlation. The first group is represented by strains AT181 and EU6, both of which utilize nopaline but incite tumors that do not produce a significant amount of either nopaline or octopine (Table 1). Our results with strain EU6 confirm an earlier report (16), whereas the data on strain AT181 as well as more recent data from Morel's group (private communication to J. Lippincott from G. Morel) correct previously published information (16). Another group, represented by strains 27, 223, and 2A, utilize both octopine and nopaline, whereas tumors that they induce synthesize high levels of nopaline but not octopine.

Evidence that plasmid-borne genes determine octopine/nopaline production by tumors. To confirm the expectation that the utilization of octopine and nopaline is coded by plasmid-borne genes, the plasmid was transferred from most of the strains in Table 1 into a plasmidless avirulent strain (A200) by either DNA-

mediated transformation (J. Schell, private communication), RP4-mediated conjugation (Chilton et al., in press), or conjugation in planta (9, 10).

Table 2 presents the octopine/nopaline production characteristics of tumors incited by these exconjugants. In every case, the exconjugant strain was identical to its donor with respect to which unusual guanidine, if any, the induced tumors produced. For example, exconjugants of strains B6-806, B6-V87, A6NC, 15955, and 140, all of which utilized octopine, incited octopine-producing tumors. Three of these exconjugants were selected by screening potential exconjugants for virulence, whereas the other two were selected for octopine utilization (Chilton et al., in press) by plating on octopine-containing media as a source of arginine. These results show that the plasmid in these five strains is associated with virulence, octopine utilization, and octopine production (by tumor) traits.

Similarly, exconjugants of strains C58 and T37 incited nopaline-producing tumors, just as did their virulent donor strains. Both exconjugants utilized nopaline. The three exconjugants, represented by strains A178, A203, and A507, represent a somewhat different situation.

TABLE 1. *Agrobacterium strains and their utilization patterns of octopine and nopaline*

Strain	Source	Utilization by bacteria	Production by tumor
A6NC	R. A. Schilperoort[a]	Octopine	Octopine
15955	ATCC[b]	Octopine	Octopine
CGIC	L. W. Moore	Octopine	Octopine
B_6-V87	L. W. Moore	Octopine	Octopine
$B_6$806	J. A. Lippincott	Octopine	Octopine
B_2A	C. Pootjes	Octopine	Octopine
140	A. Kerr	Octopine	Octopine
C58	R. H. Hamilton	Nopaline	Nopaline
T37	J. A. Lippincott	Nopaline	Nopaline
542	G. Melchers	None	None
AT1	J. A. Lippincott	None	None
AT4	J. A. Lippincott	None	None
EU6	A. C. Braun	Nopaline	None
AT181	J. A. Lippincott	Nopaline	None
27	A. Kerr	Nopaline and octopine	Nopaline
223	NCPPB[b]	Nopaline and octopine	Nopaline
2A	L. W. Moore	Nopaline and octopine	Nopaline

[a] See reference 3.

[b] ATCC, American Type Culture Collection, Rockville, Md.; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden England.

TABLE 2. *Bacterial utilization of octopine or nopaline and production of these compounds in tumors induced by strains containing only the virulence-associated plasmids*

Strain	Plasmid donor	Mode of transfer[a]	Utilization	Tumor production
A277	$B_6$806	1,3	Octopine	Octopine
A285	B_6-V87	3	Octopine	Octopine
A336	15955	1,2	Octopine	Octopine
A422	A6NC	1,2	Octopine	Octopine
A503	140	3	Octopine	Octopine
A174	C58	3	Nopaline	Nopaline
A208	T37	3	Nopaline	Nopaline
A289	EU6	1,3	Nopaline	None
A519	AT181	1	Nopaline	None
A281	542	3	None	None
A178	27	1,3	Nopaline	Nopaline
A203	223	1,3	Nopaline	Nopaline
A507	2A	1	Nopaline	Nopaline

[a] The plasmid from these donor strains was transferred into plasmidless strain A200 (*arg*$^-$ *rif nal*) when transfer was by DNA-mediated transformation or RP4-mediated conjugation. When the transfer was accomplished by conjugation in planta, the recipient was A136 (*rif nal*). In the first two transfer procedures, selection was made on the basis of guanido amino acid utilization and drug resistance; in the latter case, exconjugants were identified as drug-resistant, virulent cells. 1, DNA-mediated transformation (J. Schell, personal communication); 2, RP4-mediated conjugation (Chilton et al., in press); 3, in planta conjugation (9).

Although the donor strains in these crosses utilized both octopine and nopaline, the exconjugants uniformly utilized only nopaline. Furthermore, the tumors induced by the donors as well as the exconjugants produced only nopaline (Tables 1 and 2). The significance of these observations will be considered in a future publication.

Exconjugants of donor strains 542, EU6, and AT181 incited tumors that produced neither octopine nor nopaline. The exconjugant of strains 542, like its donor, utilized neither compound, and the exconjugants from strains EU6 and AT181, like their donors, utilized nopaline.

It is clear from these results that the plasmid associated with virulence in these strains also controls the production or nonproduction of octopine and nopaline in the tumors they induce. There is almost perfect correlation between the octopine/nopaline utilization trait borne by the virulence-associated plasmid and the octopine/nopaline production trait conferred upon the tumor. The exceptions lie in the virulence-associated plasmids of strains EU6 and AT181.

Evidence that the genes for octopine/nopaline utilization are distinct from the genes governing octopine/nopaline production by tumors. Two explanations are possible to account for the close correlation of octopine/nopaline utilization by the bacteria with octopine/nopaline production by tumors. Perhaps the simplest interpretation is that the degradative enzyme in the bacteria (octopine or nopaline oxidase) is the same enzyme that functions in the tumor (octopine or nopaline dehydrogenase) (3, 16). However, the gross characterization of both enzymes suggests that they differ in a number of properties (Bomhoff thesis, 1974; 14). The alternative explanation is that the utilization and production traits are coded by separate plasmid-borne genes. By this explanation, strains EU6 and AT181 contain plasmids with functional nopaline degradation genes, but lack functional genes that determine tumor synthesis of nopaline. One approach to distinguishing between these possibilities is to determine whether tumors induced by mutants that lack the ability to degrade octopine or nopaline can still synthesize the appropriate guanido amino acid.

Mutants that lacked octopine and nopaline oxidase activity were isolated in several different octopine- and nopaline-degrading strains, and the tumors they induced were checked qualitatively for their levels of octopine and nopaline. The results appear to be clear-cut. Six mutants were isolated from five different strains; two degraded octopine and three degraded nopaline (Table 3). In all cases, the level of either octopine or nopaline in the tumors induced by these mutants was approximately the same as the level formed in tumors induced by the wild-type parental cells. A fourfold difference in level of octopine or nopaline could be detected.

TABLE 3. *Octopine and nopaline oxidase activity in oct⁻ and nop⁻ mutants*

Strain	Original parent strain	Characteristics	Sp act[a]
C58		Wild type	134.5
A297	223	Nop⁻	<0.1
A443	C58	Nop⁻	<0.1
A517	27	Nop⁻	<0.1
A6		Wild type	7.8
A514	A6	Oct⁻	<0.1
A515	A6	Oct⁻	<0.1
A516	$B_6$806	Oct⁻	<0.1

[a] Defined as the nanograms of arginine formed per minute per milligram of protein.

Characterization of mutants. When bacteria from four tumors were reisolated and screened for their level of reversion to the wild-type phenotype, in no case did more than 1 in 10^6 colonies revert. Thus, the phenotype of the tumors must result from the mutants injected into the plant. Each of the mutants was identified as lacking either octopine or nopaline oxidase activity by the following criteria: (i) nutritional requirements—the arginine auxotrophs require arginine, and neither octopine nor nopaline will substitute; (ii) the cells are permeable to octopine or nopaline, as indicated by their ability to take up the [^{3}H]guanido amino acid into an acid-soluble pool (Fig. 1); (iii) cell-free extracts lack octopine or nopaline oxidase activity as measured by their inability to convert [^{3}H]octopine or [^{3}H]nopaline to [^{3}H]arginine (Table 3). Mixing of the mutant extracts with wild-type extracts did not decrease the octopine or nopaline oxidase activity of the wild-type extracts. This suggests that an inhibitor of oxidase activity is not present in the mutants.

DISCUSSION

The data presented in this paper and elsewhere (Nester et al., in press; 1) convincingly demonstrate that for a variety of strains, octopine and nopaline utilization traits are carried by the same large plasmid that confers virulence. However, two classes of naturally occurring isolates serve as exceptions to this general rule. In one class, the strains utilize both octopine and nopaline, but the tumors induced by these strains produce only nopaline. Three biotype II strains, 223, 27, and 2A, had this property. The octopine utilization trait cannot be

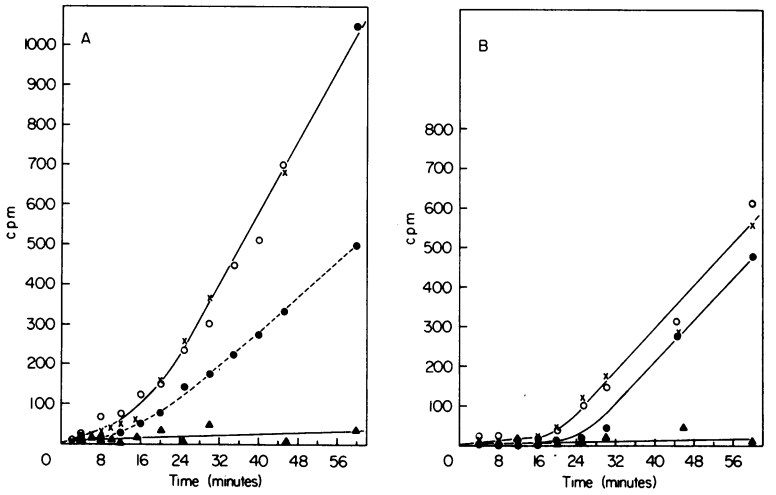

FIG. 1. *Uptake and incorporation of nopaline and octopine by growing bacteria. Every one of the Nop⁻ and Oct⁻ mutants gave essentially the same kinetics of uptake and incorporation. These curves represent a typical experiment. (A) [³H]nopaline uptake and incorporation. Symbols:* (×) *Wild-type (C58) total uptake* (●) *wild-type acid-insoluble incorporation;* (○) *Nop⁻ mutant total uptake;* (▲) *Nop⁻ mutant acid-insoluble incorporation. (B) [³H]octopine uptake and incorporation. Symbols:* (×) *Wild-type (A_6) total uptake* (●) *wild-type acid-insoluble incorporation;* (○) *Oct⁻ mutant total uptake;* (▲) *Oct⁻ mutant acid-insoluble incorporation.*

transferred to recipient cells by either plasmid DNA-mediated transformation or RP4-mediated conjugation. These data are most readily interpreted if the octopine utilization gene is not on a plasmid in these particular strains. Evidence supporting this interpretation will be detailed in a separate publication. The second exception to this rule is found in strains that utilize nopaline but induce tumors that synthesize neither nopaline nor octopine. In these two cases there is no question that the nopaline trait is located on the virulence-associated plasmid since this plasmid has been transferred to a plasmidless strain. The transformants simultaneously gain virulence and the ability to degrade nopaline. In our view, the most reasonable explanation for this latter observation is that the gene concerned with nopaline utilization in the bacterium is not the gene responsible for nopaline synthesis in the tumor. Our interpretation regarding these natural isolates is strongly supported by a study of mutants that have lost the ability to degrade either octopine or nopaline. These mutants induce tumors that synthesize about as much of guanido amino acids as do the tumors induced by the parent strains. These appear to be bona fide mutants in the gene specifying octopine or nopaline oxidase, since extracts of the mutants lack the enzyme activity and whole cells are permeable to octopine or nopaline. It is important to point out that the majority of presumed mutants selected as being able to grow on arginine but not octopine or nopaline as the sole source of nitrogen were not mutant in the oxidase gene. Most of these strains could still incorporate the labeled guanido amino acid into acid-insoluble material, presumably protein, when growing. These mutants were not characterized further. Although we cannot rule out the possibility that the mutants that lack oxidase activity are defective in a regulatory gene, it seems highly unlikely that all six mutants isolated from five different strains would be of a regulatory nature.

These data do not answer the intriguing question of whether the structural genes that code for octopine and nopaline dehydrogenase are located in the bacteria, in the plant, or both. We have not been able to demonstrate convincingly that [³H]arginine can be converted into [³H]nopaline or [³H]octopine in growing bacteria. Further, the fact that exogenous octopine and nopaline must be added to induce the permease for these compounds suggests that only very low levels, if any, could be present in bacterial cells. However, we have also been unable to demonstrate convincingly the presence of octopine and nopaline in uninfected plants. A previous report from this laboratory (7) that octopine is present in a variety of uninfected plants has not been repeatable in our laboratory. Thus, at this time, we have been able to detect these unusual amino acids unequivocally only in crown gall tumors. Several recent reports agree with these data (Bomhoff, thesis, 1974; 8).

The significance of octopine and nopaline to

crown gall tumor induction is open to speculation. It is clear that these compounds are not absolutely required, since not all tumors synthesize them. Lippincott et al. have presented data indicating that octopine and nopaline are tumor growth factors (13). These compounds can also be viewed as bacterial growth stimulators, which serve as a source of nitrogen and carbon to which only the inciting *Agrobacterium* strain can respond. The levels of the basic amino acids histidine, arginine, and lysine are elevated about 1,000-fold in the wound site of *Kalanchoe daigremontiana* (Gordon et al., in press). The association of the tumor-inducing bacteria with the plant results in the conversion of one or more of these amino acids into a sequestered source of carbon and nitrogen for the inciting bacterium. What precise roles bacterial and plant genes play in the synthesis of these unique amino acids remain to be elucidated.

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