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Ti Plasmid and Chromosomal Ornithine Catabolism Genes of *Agrobacterium tumefaciens* C58

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The pTiC58 plasmid *noc* genes of *Agrobacterium tumefaciens* C58 code for nopaline oxidase (*nocC*), nopaline permease (*nocP*), the inducible periplasmic protein *n*1 (*nocB*), and a function(s) required for ornithine catabolism (*nocA*). In addition, strains C58 and Ach-5 of *A. tumefaciens* have chromosomal ornithine catabolism genes. The chromosomal *orc* gene codes for ornithine dehydrogenase. Strain C58 is normally *orc*, but *orc*$^+$ mutants can be selected. We have characterized both chromosomal *orc* and pTiC58 *nocA* plasmid genes. Complementation of most chromosomal *orc* mutants by pTiC58 restored growth on both nopaline and L-ornithine but did not restore ornithine dehydrogenase activity. We conclude that ornithine is an intermediate of nopaline degradation and that the Ti plasmid and chromosome both code for ornithine-degradative enzymes. A model for nopaline catabolism is presented.

Agrobacterium tumefaciens C58 induces crown gall tumors that produce nopaline [N^2-(1,3-dicarboxypropyl arginine)] regardless of the host plant infected (3, 24, 25; G. H. Bomhoff, Ph.D. thesis, University of Leiden, The Netherlands, 1974). The genes required for virulence and the induction of these tumors are located on a large Ti plasmid (28, 29, 31). Besides these genes, a specific segment of the TiC58 plasmid, called the T region, is transferred to the plant cell and encodes the synthesis of nopaline (7, 8, 19). The TiC58 plasmid also contains catabolic genes that allow the bacterium to use nopaline as a substrate for nitrogen and carbon (3, 24). These nopaline catabolism (*noc*) genes are located on a 14.4-kilobase segment of DNA (map position, 3.9 to 18.3 kilobases) on pTiC58 and include genes for nopaline oxidase, nopaline uptake, the inducible periplasmic protein *n*1 (C. L. Schardl and C. I. Kado, Mol. Gen. Genet., in press), and ornithine catabolism (5, 26). We have recently identified the pTiC58 ornithine catabolism locus (*nocA*) at the left-hand end of the *noc* region adjacent to the T region (Schardl and Kado, in press).

Based on the work of Petit et al. (25, 26), Ellis et al. (5) have postulated that ornithine is an intermediate of octopine and nopaline degradation and that ornithine is degraded to glutamic semialdehyde, which in turn is converted to glutamate, thus providing a carbon source for the bacterium. Various *Agrobacterium* strains also contain chromosomal ornithine catabolism genes (Schardl and Kado, in press; C. L. Schardl, unpublished data).

Here we report that *A. tumefaciens* degrades ornithine by two separate pathways. One of these pathways is coded on the pTiC58 plasmid and the other on the chromosome. The chromosome codes for ornithine dehydrogenase, an enzyme that has not been reported previously in *A. tumefaciens*.

MATERIALS AND METHODS

Chemicals. Nopaline was prepared by the method of Jensen et al. (10) with minor modifications (Schardl and Kado, in press). L-Ornithine (hereafter referred to as ornithine), *o*-aminobenzaldehyde, spectinomycin (Sp), streptomycin (Sm), rifamycin SV (Rf), and nitrosoguanidine were obtained from Sigma Chemical Co., St. Louis, Mo. Nutrient broth and Noble agar were obtained from Difco Laboratories, Detroit, Mich. L-[^{3}H]ornithine (specific activity, 15.2 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

Growth media. Growth tests were performed as described previously (Schardl and Kado, in press) on 2% Noble agar or broth containing SK medium, which contains (per liter): K_2HPO_4 (10.25 g), KH_2PO_4 (7.25 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.15 g), $CaCl_2$ (0.1 g), and $FeSO_4$ (0.0025 g) with ornithine (2 mg/ml), nopaline (2 mg/ml), ornithine (2 mg/ml) plus nopaline (0.2 mg/ml), or sucrose (0.5%) plus ammonium chloride (0.1%) included as carbon and nitrogen sources. SM medium has been described by Klapwijk et al. (15) and was supplemented in the same way as SK medium. For ornithine dehydrogenase assays, *A. tumefaciens* strains were grown in NNO broth, which contained: 10

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TABLE 1. *A. tumefaciens* strains[a]

Strain	Ti plasmid	Chromosome background	Tn*5* insertion site (kilobases from *Sma*I fragment 5 junction)	Ti plasmid locus	Phenotype	Ornithine utilization	Source or reference
C58	pTiC58	Wild type	NA	NA	Vir$^+$ Noc$^+$	+	(20)
NT1	None	C58	NA	NA	Vir$^-$ Noc$^-$	−	Cured of Ti plasmid at 37°C (29)
NT1SS	None	C58	NA	NA	Vir$^-$ Noc$^-$ Sp^r Sm^r	−	Cured of Ti plasmid at 37°C; this study
CS11	None	C58	NA	NA	Vir$^-$ Noc$^-$ Sp^r Sm^r	−	Spontaneous Sp^r and Sm^r mutant; this study
1D1145	pTiC58	C58	None	NA	Vir$^+$ Noc$^+$	+	This study
1D1499	None	CS11	None	NA	Vir$^-$ Noc$^-$ Sp^r Sm^r	+	Spontaneous Orc$^+$ mutant; this study
1D1500	None	NT1SS	None	NA	Vir$^-$ Noc$^-$ Sp^r Sm^r	−	Schardl and Kado, in press
1D1500 (pJK133)	pTiC58::Tn*5*	NT1SS	3	*nocA*	Vir$^+$ Noc$^+$ Sp^r Sm^r Km^r	−	Tn*5* insertion in *nocA* gene of nopaline operon; this study
1D1500 (pJK270)	pTiC58::Tn*5*	NT1SS	183	T region	Vir$^+$ Noc$^+$ Sp^r Sm^r Km^r	+	Tn*5* insertion in *Kpn*I fragment 3 of the T region; this study
LBA4301	None	Ach-5-C3	NA	NA	Vir$^-$ Noc$^-$ Rec$^-$ Rf^r	+	(18)

[a] Abbreviations: Vir, virulence; Noc, nopaline catabolism; Rec, recombination; NA, not applicable.

mM potassium phosphate (pH 7.0), 10% nutrient broth, 1 mM ornithine, and 0.5 mM nopaline. Medium 523 (12) and medium LB (22) have been described previously.

Bacterial strains. *A. tumefaciens* strains and their properties are listed in Table 1.

Matings and plasmid analysis. *A. tumefaciens* matings were performed as previously described (14). Plasmids were analyzed by a rapid miniscreen procedure (13).

Mutagenesis. Cells were grown in 523 broth to late-log phase and then diluted to 2×10^8 cells per ml. Nitrosoguanidine was added to 200 μg/ml. Cells were aerated for 15 min at 30°C, harvested by centrifugation, and washed three times with sterile distilled water. Mutagenized cells were diluted for single colonies and plated on SK agar containing ornithine (2 mg/ml). After 10 days of incubation at 30°C, small colonies were tested for ketolactose production (2) and saved. Nitrous acid mutagenesis was performed by the method of Schwartz and Beckwith (27). Insertional mutagenesis with Tn*5* has been described previously (14).

Enzyme assays. Ornithine dehydrogenase activity was measured by using a modification of the lysine dehydrogenase assay of Misono and Nagasaki (23). Cells were grown in 100 ml of NNO medium to mid-log phase (Klett reading, 100 to 200; green filter), harvested by centrifugation at 5,000 × *g* at 4°C for 10 min, and washed with 50 mM Tris-hydrochloride (pH 8) in 10% glycerol. Cells were resuspended in 2 to 4 ml of the same buffer, and dithiothreitol was added to 1 mM. The cells were broken in a French pressure cell at a pressure of 1 metric ton/cm^2, and the debris was removed by centrifugation at 13,000 × *g* at 4°C for 15 min. Protein was assayed by the method of Bradford (4). A 500-μg amount of protein was assayed for dehydrogenase in 100 mM sodium carbonate (pH 9.5)–600 mM ornithine (pH 8.2)–10 mM oxidized NAD–5 mM *o*-aminobenzaldehyde in a final volume of 1 ml. The final reaction mixture had a pH of 8.4. After 15 min the reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid, and color was developed for 5 min at 23°C. For pH and ornithine concentration dependence of ornithine dehydrogenase activity in cell-free extracts, assays were made with 500 μg of protein in 5 mM *o*-aminobenzaldehyde in 100 mM Tris-hydrochloride–100 mM L-ornithine hydrochloride (adjusted to pH with KOH)–10 mM NAD in a final volume of 0.5 ml. Glutamic semialdehyde production was assayed in a Beckman UV 5230 recording spectrophotometer at 443 nm (9).

Ornithine uptake was assayed by a modification of the nopaline uptake assay (Schardl and Kado, in press). CSOR55 (see below) and 1D1499 cells were grown in SK containing 1 mM ornithine, 1 mM arginine, and 0.5% sucrose, harvested by centrifugation, and washed with 10 mM potassium phosphate (pH 7.0). Ornithine uptake was measured in the same buffer containing 5×10^8 cells, 1 mM ornithine, and 20,000 cpm of L-[^{3}H]ornithine in a final volume of 0.2 ml. The mixture was incubated for 15 and 30 min at room temperature. Cells were harvested, washed with 10 mM potassium phosphate (pH 7.0)–100 mM ornithine, and counted as previously described (Schardl and Kado, in press). No difference in uptake was observed between these strains.

TABLE 2. Nopaline and ornithine catabolism of *A. tumefaciens* C58 derivatives[a]

Mutant	pTi locus	Chromosomal locus	Mutagen	L-Ornithine + nopaline	L-Ornithine	Glutamic acid	Nopaline	Ornithine dehydrogenase activity[b]
1D1499	None	Orc$^+$	Spon	+	+	+	−	100
1D1499(pJK270)	*nocA*$^+$	Orc$^+$		+	+	+	+	NT
1D1500	None	Orc$^-$		−	−	+	−	47 ± 8
1D1500(pJK270)	*nocA*$^+$	Orc$^-$		+	−	+	+	23 ± 6
1D1500(pJK133)	*nocA*	Orc$^-$	Tn*5*	−	−	NT	±	NT
CSOR4	None	Orc$^-$	NTG	−	−	+	−	41 ± 8
CSOR4(pJK270)	*nocA*$^+$	Orc$^-$		−	−	NT	±	25 ± 8
CSOR12	None	Orc$^-$	NTG	±	±	+	−	45 ± 8
CSOR12(pJK270)	*nocA*$^+$	Orc$^-$		+	±	NT	+	26 ± 6
CSOR55	None	Orc$^-$	NTG	−	−	+	−	NT
CSOR55(pJK270)	*nocA*$^+$	Orc$^-$		+	−	NT	+	NT
CSOR56	None	Orc$^-$	NA	−	−	+	−	NT
CSOR56(pJK270)	*nocA*$^+$	Orc$^-$		−	−	NT	±	NT

[a] Abbreviations: Spon, spontaneous mutant; Tn*5*, kanamycin resistance transposon Tn*5*; NTG, nitrosoguanidine; NA, nitrous acid; NT, not tested.

[b] Ornithine dehydrogenase activity is given as a percentage of the control, 1D1499, from which its extract contained an average of 0.212 absorbance unit at 443 nm per 250 μg of protein after 15 min under the assay conditions described in the text.

Cell fractionation. *A. tumefaciens* cells were grown in NNO, and cell-free extracts were prepared as described above. Membranes were pelleted by centrifugation at 100,000 × *g* for 2 h, and the soluble and insoluble fractions were assayed for ornithine dehydrogenase as described above.

RESULTS

Ornithine dehydrogenase. The pTiC58 plasmid-containing *A. tumefaciens* strain 1D1145 catabolizes ornithine as well as the Ti plasmid-free derivative 1D1499. Thus, ornithine catabolism is presumably a chromosomal function. A search for various enzymes that might degrade ornithine revealed the presence of ornithine dehydrogenase activity. This enzymatic activity was measured in crude extracts of strain 1D1499 in which the enzyme had been induced with nopaline and ornithine. This enzyme was active at pH 7.4 to 8.6, with an optimum at pH 8.4, and had a K_m for ornithine of 11 mM in crude extracts. The enzyme was active when either NAD or NADP was used as a cofactor. No activity was seen either in the absence of cell extract or when reduced pyridine nucleotides were substituted for NAD.

Crude extracts of strain 1D1499 were centrifuged at 100,000 × *g* at 4°C for 2 h, and the soluble and insoluble fractions were assayed for dehydrogenase activity. Ornithine dehydrogenase activity was localized in the soluble fraction and was not removed by high-speed centrifugation.

Ornithine catabolism mutants. It was reported previously that the TiC58 plasmid-free strain C58C1 was naturally unable to utilize ornithine and that this deficiency could be complemented by introducing either wild-type pTiC58 or pTiAch5 into this strain (5). We obtained similar positive complementations by the insertion of pJK270 into TiC58 plasmid-free strain 1D1500, which, like C58C1, is unable to utilize ornithine naturally (Table 2). The inability of 1D1500 to utilize ornithine is hereafter designated Orc$^-$. When a TiC58 plasmid (pJK133) containing a Tn*5* insertion in the *nocA* locus was introduced into 1D1500 by conjugation, the resulting exconjugants remained Orc$^-$ and grew poorly on nopaline as a carbon and nitrogen source, namely, 1D1500(pJK133) (Table 2). Thus, pJK133 with a mutation in the *nocA* locus was unable to complement strain 1D1500, which is naturally Orc$^-$. Unlike strains C58C1 and 1D1500, we observed that the Ti plasmid-free strain LBA4301, a derivative of the octopine strain Ach-5, naturally utilizes ornithine as a sole carbon and nitrogen source. Attempts were therefore made to construct chromosomal Orc$^+$ mutants of Ti plasmid-free derivatives of C58.

Four independent Orc$^+$ spontaneously derived mutants were obtained by first curing C58 of its Ti plasmid at 37°C and then by direct selection on SK plates containing ornithine. One of these mutants, 1D1499 (Table 1), was used in the following studies.

To determine whether ornithine dehydrogenase is required for ornithine catabolism, we prepared stable Orc$^-$ mutants by nitrosoguanidine or nitrous acid mutagenesis of 1D1499. Cells were plated on SK agar containing 1% LB broth and ornithine and grown for 10 days at 30°C. Slow-growing colonies were selected and tested for growth on SM medium containing either ornithine or sucrose and ammonium chlo-

ride (see text). Production of 3-ketolactose and growth on SK agar containing glutamate (2 mg/ml) as a carbon source were also tested. All mutants were 3-ketolactose positive. Mutants CSOR4 and CSOR12, including strain 1D1500, were deficient in ornithine dehydrogenase activity (Table 2). The mutants, including CSOR55 and CSOR56, were unable to grow on L-ornithine despite the presence of ornithine dehydrogenase activity (23 to 47% of control) (Table 2). It is possible that these mutants produce a defective enzyme and that the in vivo level of ornithine dehydrogenase activity is low, perhaps due to a reduced affinity for ornithine. Alternatively, ornithine may be at concentrations that are detrimental in these mutants.

Ti plasmid complementation of chromosomal mutants. The TiC58 plasmid *nocA* gene may code for either a regulator of the expression of the ornithine catabolic gene(s) on the chromosome or for enzymes of an ornithine-degradative pathway. Complementation analysis of the chromosomal mutants by a TiC58 plasmid was undertaken to distinguish these possibilities. pJK270 was introduced into 1D1499 and into the above mutants by mating with LBA4301(pJK270). The transconjugants were selected for kanamycin resistance (Km^r) (150 μg/ml), Sp^r (150 μg/ml), Sm^r (500 μg/ml), and Rf^s (100 μg/ml) on SM agar containing these antibiotics. The presence of pJK270 in 1D1499 and in the mutants was verified by agarose gel electrophoresis. These transconjugants were tested for ornithine catabolism on SK agar containing either ornithine and nopaline or nopaline alone. The phenotypes are shown in Table 2. The TiC58 plasmid pJK270 conferred ornithine catabolism on two of the mutants, CSOR12 and CSOR55, but did not complement mutants CSOR4 and CSOR56. Contrary to our original expectations, pJK270 did not confer increased levels of ornithine dehydrogenase activity; in fact, the plasmid seems to lower its activity in these particular mutants and in 1D1500, suggesting that a repressor affecting the efficient expression of Orc genes may be coded by the Ti plasmid. Also, no ornithine δ-transaminase or ornithine oxidase activities were detected in the mutants containing pJK270 (data not shown). It therefore appears that the pTiC58 plasmid does not encode ornithine dehydrogenase or these latter enzymes but codes for an unidentified ornithine-degradative enzyme.

Complementation of *nocA* of pTiC58. Strain 1D1500(pJK133), containing a pTiC58 plasmid with a Tn*5* insert in the *nocA* gene (pJK133), does not catabolize ornithine and grows very slowly on SK agar containing nopaline, although production of nopaline permease and nopaline oxidase remains unaffected by the Tn*5* insertion

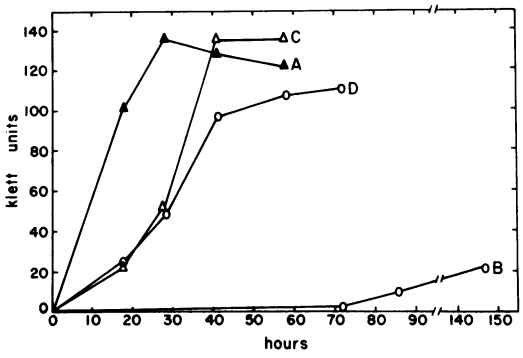

FIG. 1. Kinetics of growth in SK broth containing nopaline as a sole carbon and nitrogen source. A, 1D1500(pJK270); B, 1D1500(pJK133); C, LBA4301(pJK270); D, LBA4301(pJK133).

(Schardl and Kado, in press). These results support the hypothesis that ornithine is an intermediate of nopaline catabolism (5, 25, 26). Thus, we predicted that the ornithine catabolic chromosomal gene(s) would complement the *nocA* mutation and restore growth on nopaline. When pJK133 was introduced into the Orc^+ pTi plasmid-free strain LBA4301 by conjugation, the LBA4301 chromosome did indeed complement *nocA* (Fig. 1). This result demonstrated that chromosomal ornithine catabolic gene product(s) can function in nopaline breakdown. Thus, we conclude that ornithine is an intermediate of the nopaline degradative pathway, which is shown in Fig. 2.

DISCUSSION

Based on the work presented here and on recent work in this laboratory (Schardl and Kado, in press), we postulate a model of the nopaline catabolism pathway (Fig. 2). Our model is analogous to the octopine catabolism model proposed by Ellis et al. (5) and involves enzymatic steps similar to the octopine-degradative enzymes previously described (11, 15–17; G. H. Bomhoff, Ph.D. thesis, University of Leiden, The Netherlands, 1974; M.-F. Jubier, Ph.D. thesis, Universite de Paris-Sud, France, 1975; P. M. Klapwijk, Ph.D. thesis, University of Leiden, The Netherlands, 1979; W. T. Lu and L. Unger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, K16, p. 129), including nopaline permease, nopaline oxidase, arginase, and both chromosomal- and pTiC58-coded enzymes that catalyze the degradation of ornithine.

In the present study, we present evidence for L-ornithine dehydrogenase activities in *A. tumefaciens*. So far, an L-lysine ε-dehydrogenase activity in *A. tumefaciens* is the only similar dehydrogenase characterized (23). We have shown that mutants unable to grow on L-orni-

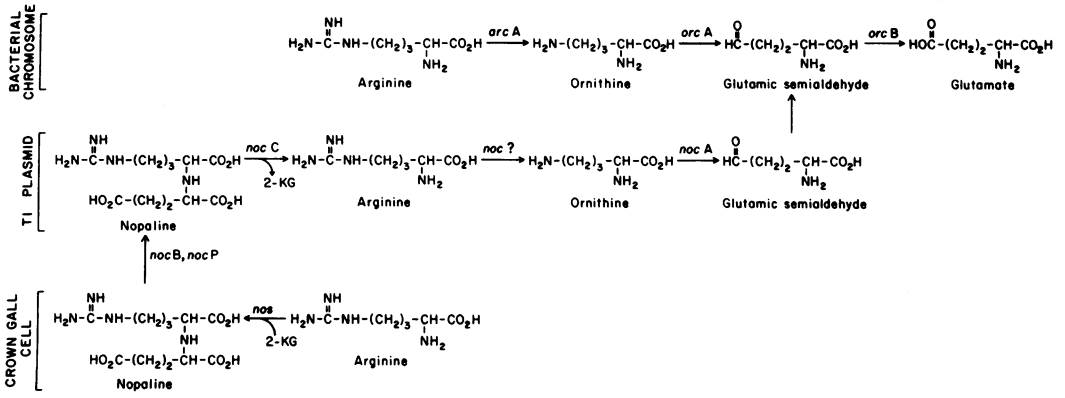

FIG. 2. A model for nopaline degradation in *A. tumefaciens* C58. Genes coding for each step are indicated. Glutamic semialdehyde is indicated as a possible intermediate of both the pTiC58-coded and chromosomal ornithine-degradative pathways. The following genes are abbreviated: *arcA*, arginase; *orcA*, ornithine dehydrogenase; *orcB*, glutamate semialdehyde oxidase; *nocC*, nopaline oxidase; *nocA*, uncharacterized ornithine degradative enzyme; *nocB*, nopaline-binding protein *n*1; *nocP*, nopaline permease; *nos*, nopaline synthase. 2-KG, 2-Ketoglutaric acid.

thine are concomitantly deficient in ornithine dehydrogenase activity. This suggests that ornithine dehydrogenase is a catabolic enzyme. Since the TiC58 plasmid complemented some of these ornithine catabolism mutants, it is likely that this plasmid encodes an enzyme or enzymes for ornithine degradation. This plasmid did not, however, confer higher levels of ornithine dehydrogenase and did not confer other enzymes, such as ornithine δ-transaminase or ornithine oxidase, that degrade ornithine to glutamic semialdehyde. However, we cannot rule out completely the presence of these enzymes, which may require different extraction or unique assay procedures for detection of activity. Alternatively, the TiC58 plasmid may code for ornithine decarboxylase. This enzyme, which degrades ornithine to putrescine, is required for ornithine catabolism in *Escherichia coli* (1), *Lactobacillus* sp. 30a (6), and *Rocholimaea quintana* (30).

In all cases we have found that a functional ornithine catabolism gene, whether on the chromosome or on the TiC58 plasmid, is required for nopaline catabolism. Thus, ornithine seems to be an intermediate of the nopaline degradation pathway. This is verified by the fact that a Tn*5* insertion in the *nocA* locus of the pTiC58 plasmid (pJK133) not only eliminated pTi-conferred ornithine catabolism but greatly reduced the ability of the Orc$^-$ strain harboring pJK133 to grow on nopaline as a carbon source. Since this mutant was shown to produce nopaline permease and nopaline oxidase (Schardl and Kado, in press), the nopaline catabolism deficiency was presumably due to its inability to break down ornithine. The chromosomal ornithine catabolism gene of strain LBA4301 complemented the *nocA* mutation on the Ti plasmid, so that LBA4301(pTi*nocA*) was capable of growth on nopaline as well as ornithine. We conclude that the lesion in the pTi*nocA* locus did not affect the degradation of nopaline to ornithine but blocked a catabolic step after ornithine formation.

Degradation of a molecule of nopaline by nopaline oxidase should yield a molecule of 2-ketoglutarate, thus liberating 50% of the available carbon for bacterial growth. It is remarkable that strain 1D1500(pJK133) showed almost no growth on nopaline, in contrast to the nopaline-utilizing derivative 1D1500(pJK270) (Fig. 1). It is unlikely that such a difference in growth rates is solely due to inability of the strain to use half of the nopaline molecule.

If a high intracellular level of ornithine reduces transcription of the *noc* genes, we expect to see concomitant reductions in the nopaline-binding protein *n*1 (Schardl and Kado, in press; manuscript submitted) and nopaline oxidase levels attributable to the *nocA* mutation in pTiC58. However, we have reported that the amount of these proteins produced by strain 1D1500(pJK133) is similar to the *noc*$^+$ control (Schardl and Kado, in press). Thus, it is likely that ornithine is an inhibitor of nopaline oxidase rather than a regulator of all *noc* genes.

pTi-coded arginine and ornithine catabolism genes have been reported in several *A. tumefaciens* strains, including the octopine-type strains Ach-5 and B6 (5, 26). Since the pTi-free strain LBA4301 catabolized ornithine, the pTiAch-5 plasmid in its parental strain Ach-5 is not required for growth on this substrate in the laboratory; however, it is unclear what selective advantage might be conferred on the bacterium by

the maintenance of two ornithine catabolism genes in nature. It is important to note that the Ti plasmid *nocA* gene appears to be coded on an operon along with other *noc* genes (*nocB* and *nocC*) and is induced by nopaline rather than ornithine (Schardl and Kado, in press). It follows that a *nocA*$^+$ bacterium growing on nopaline would not require a high intracellular level of ornithine to induce ornithine degradation. The time required to accumulate enough ornithine to induce the chromosomal ornithine catabolism genes can thus be circumvented, and the bacterium would begin to grow on this substrate more quickly than other soil bacteria that lack this genetic arrangement.

Mutants such as CSOR4 were not complemented by the Ti plasmid. CSOR4 was deficient in ornithine dehydrogenase, and its mutation might have had a polar effect on other ornithine catabolic functions. Clearly, chromosomal functions are required for TiC58-coded ornithine degradation. If both the TiC58 plasmid and chromosomal pathways generate glutamic semialdehyde as an intermediate, a chromosomal dehydrogenase (or oxidase) gene may be necessary for further degradation of this compound. Alternatively, the chromosome-specific gene may code for a coenzyme or cytochrome required for both ornithine-degradative pathways. Nonetheless, we have identified the chromosomally encoded enzyme ornithine dehydrogenase and have shown that it is required for ornithine catabolism by the TiC58-free strain 1D1499. That the *noc* genes of pTiC58 code for a second ornithine catabolism pathway was also demonstrated. Since *A. tumefaciens* lives in the soil and in the crown gall of plants, each of the ornithine catabolism pathways may have evolved in such a way as to allow the bacterium to adapt to and take advantage of its specific environment. A comparison of these two pathways and the properties of the enzymes involved may provide clues as to the environment inside the crown gall tumor.

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