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Arginine Catabolism in *Agrobacterium* Strains: Role of the Ti Plasmid

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We present a study of the enzymatic activities involved in the pathway for arginine catabolism by *Agrobacterium tumefaciens*. Nitrogen from arginine is recovered through the arginase-urease pathway; the genes for these two activities are probably chromosomally born. Arginase was found to be inducible during growth in the presence of arginine or ornithine. Urease was constitutively expressed. Ornithine, resulting from the action of arginase on arginine, could be used as a nitrogen source via transamination to Δ^1-pyrroline-5-carboxylate and reduction of the latter compound to proline by a reductase (both enzymatic activities are probably chromosomally encoded). Ornithine could also be used as a carbon source. Thus, we identified an ornithine cyclase activity that was responsible for direct conversion of ornithine to proline. This activity was found to be Ti plasmid encoded and inducible by growth in medium containing octopine or nopaline. The same activity was also chromosomally encoded in some *Agrobacterium* strains. In such strains, this activity was inducible during growth in arginine-containing medium.

Crown-gall tumors, incited by oncogenic *Agrobacterium* strains, produce specific compounds, generally N^2-substituted L-amino acids, called opines (19, 27). In *Agrobacterium tumefaciens* strains, the pathogenic functions are carried by large plasmids called Ti plasmids, a segment of which, the transferred DNA (T-DNA), is transferred into the genome of crown-gall tumor cells (for a review, see reference 12). T-DNA expression in these cells is responsible for their tumorous character and for opine synthesis (29, 30, 32).

In addition to these functions, Ti plasmids also carry genes that are responsible for the utilization of opines as specific growth substrates (carbon and nitrogen sources) (3, 4, 7, 10, 11, 13, 21). *Agrobacterium* strains and Ti plasmids are classified into five types, according to the nature of the opines synthesized in plant cells and degraded by the bacteria (these five types are octopine, nopaline, agropine, succinamopine, and grapevine [22]).

Octopine [N^2-(1-D-carboxyethyl)-L-arginine] and nopaline [N^2-(1-D-dicarboxypropyl)-L-arginine] are degraded by *Agrobacterium* strains carrying octopine or nopaline Ti plasmid, respectively, to arginine and pyruvic acid or to arginine and 2-ketoglutaric acid (16, 19; G. H. Bomhoff, Ph.D. Thesis, University of Leiden, Leiden, The Netherlands, 1974). Lysopine [N^2-(1-D-carboxyethyl)-L-lysine] is utilized by the octopine-type strains as well as octopine (26; M. F. Jubier, Ph.D. thesis, University of Paris XI, Paris, France, 1975). Ellis et al. (8) have found that arginine can be utilized as a nitrogen source by all *Agrobacterium* strains and that most of them can also utilize it as a sole carbon source. However, two strains of *A. tumefaciens* cannot grow on arginine as sole carbon source. These are strain C58 (which belongs to the nopaline class) and its Ti plasmid-cured derivative C58C1. Moreover, C58C1-derived strains, which harbor a wild-type Ti plasmid do not grow on arginine as the sole carbon source, except when it is induced or constitutive for opine catabolism (8). This clearly demonstrates the involvement of Ti plasmid genes in arginine catabolism and leads to the conclusion that opine and arginine catabolism are under the control of the same regulatory gene(s).

Preliminary investigations have been undertaken to determine the pathway and to identify the products of arginine catabolism (19). It appears to proceed via ornithine to glutamic acid, but the steps involved in this conversion remain unknown.

We present here the results of a biochemical study of arginine catabolism, in which cyclization of ornithine to proline was found to be a Ti plasmid-encoded step.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study are listed in Table 1. Organisms were grown following the conditions described by Petit and Tempé (21). Carbon and nitrogen sources are specified in each experiment. Unless otherwise specified, carbon sources were added to media at a 20 mM final concentration, and nitrogen sources were added at a 2 mM final concentration.

Chemicals. Chemicals used in this study were of the highest commercially available grades. Octopine, nopaline, and lysopine were synthesized by J. Tempé (26). L-[U-^{14}C]arginine and L-[U-^{14}C]ornithine were purchased from Amersham Corp., Arlington Heights, Ill. *o*-Aminobenzaldehyde was obtained from Fluka, and agmatine sulfate was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Testing of substrates as nitrogen and carbon sources. The testing of substrates as nitrogen and carbon sources was performed as described previously (8). Noble agar (Difco Laboratories, Detroit, Mich.) was used to solidify the media. These experiments were carried out in the absence and in the presence of octopine (2 mM) to induce arginine catabolism (8). All the strains were also plated on minimal medium with

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TABLE 1. Bacterial strains used in this study

Strain	Pathogenic plasmid	Observations	Source
C58	pTiC58	Wild-type nopaline strain	J. Schell
C58C1RS		Ti plasmid-cured derivative of C58; resistant to rifampin at 100 μg/ml and streptomycin at 500 μg/ml	J. Schell
R10	pTiR10	Wild-type octopine strain	OC[a]
R10c21	pTiR10c21	Mutant of R10 constitutive for octopine utilization (and for Ti conjugative transfer)	OC
G30	pTiR10	R10 Ti plasmid transconjugant in C58C1RS	OC
GV4R10c21	pTiR10c21	R10c21 Ti plasmid transconjugant in C58C1RS	OC

[a] OC, Our collection.

2 mM octopine alone as a control. A second control was run, in which glucose (2 g/liter) was the carbon source, and no nitrogen was added. When substrates were assayed as nitrogen source, mannitol (2 g/liter) was the carbon source.

Respirometry. Measurements of O_2 consumption by *Agrobacterium* strains which oxidized various substrates were performed on a Braun Warburg respirometer at 30°C by the method described by Umbreit et al. (28). The bacterial suspensions were obtained from cultures harvested in exponential phase, washed twice in 0.9% (wt/vol) NaCl and suspended in 50 mM Tris hydrochloride buffer (pH 7.5). The substrates were added to the reaction vessel at a final concentration of 5 mM.

Preparation of cell extracts. Cells from exponential-phase cultures (about 5×10^8 to 10^9 cells ml^{-1}) were harvested by centrifugation (10 min, 7,000 × *g*) and washed twice in 0.9% (wt/vol) NaCl. Cells were suspended in potassium phosphate (pH 7.5, 20 mM)–dithiothreitol (1 mM)–ethylene glycol, (5% [vol/vol]) buffer and disrupted by sonication for 4 min in a Mullard sonic oscillator (100 W, 20 kHz). After sonication, the resulting suspension was centrifuged (15 min, 20,000 × *g*). All these operations were carried out at 0 to 4°C. Unless otherwise specified, the supernatant was used for enzyme assays.

Separation of products of arginine and ornithine catabolism. Cell extracts were incubated for 1 h at 30°C in the presence of labeled arginine or ornithine. The incubation mixture (1.0 ml) consisted of cell extract (about 2.5 mg of protein), 100 μmol of Tris hydrochloride buffer (pH 8.0), 5 μmol of L-[U-^{14}C]arginine or L-[U-^{14}C]ornithine (0.4 μCi/μmol), 0.1 μmol of pyridoxal-5-phosphate, 4 μmol of $MgCl_2$, and 0.25 μmol of $MnCl_2$. At the end of the incubation period, 0.1 ml of 40% (wt/vol) trichloroacetic acid was added. The precipitate was eliminated by centrifugation, and the supernatant was filtered through a Millipore membrane (0.45-μm pore size). The reaction products were separated and identified by automated ion-exchange analysis on a Beckmann 120C amino acid analyzer. Fractions (2 ml) were collected from the columns, and the radioactivity was determined in a scintillation counter.

Enzyme assays. All incubations were performed at 30°C. Each assay was derived from an existing technique and was first improved with regard to substrate and effector concentrations, buffer, and pH. Linearity with both incubation time and protein concentration was verified for each enzyme. A unit of enzyme activity was defined as 1 nmol of product formed per min per mg of protein.

Arginase. Arginase (EC 3.5.3.1) activity determinations were performed by measuring the urea formed in a 2.0-ml reaction mixture containing 200 μmol of Tris hydrochloride buffer (pH 8.0), 50 μmol of arginine, and 0.5 μmol of $MnCl_2$. The reaction was initiated by the addition of arginine and stopped by the addition of 2.0 ml of 1 M HCl. Urea was measured by the method of Archibald (1).

Urease. The urease (EC 3.5.1.5) assay reaction mixture consisted of 50 μmol of potassium phosphate buffer (pH 7.5) and 10 μmol of urea in a final volume of 1.0 ml. The reaction was initiated by the addition of urea and stopped by the addition of 0.5 ml of Nessler reagent at 0°C. The absorbance was read at 430 nm (28).

Ornithine transaminase. The reaction mixture for ornithine transaminase (EC 2.6.1.13) contained, in a total volume of 2.0 ml, 100 μmol of Tris hydrochloride buffer (pH 9.0), 100 μmol of ornithine, 40 μmol of pyruvate, and 1 μmol of pyridoxal-5-phosphate. The reaction was initiated by the addition of ornithine and stopped by the addition of 2.0 ml of 15% (wt/vol) trichloroacetic acid. The precipitate was eliminated by centrifugation. Δ^1-pyrroline-5-carboxylate (P5C) was measured after reaction with *o*-aminobenzaldehyde (0.2 ml of 0.5% [wt/vol] *o*-aminobenzaldehyde in ethanol); the absorbance of the complex was read at 440 nm (33).

Proline dehydrogenase. Cell debris was not removed from the crude extract for the proline dehydrogenase (EC 1.5.99.8) assay. The reaction mixture contained, in a total volume of 3.0 ml, 0.6 mmol of potassium phosphate buffer (pH 6.0), 1.3 mmol of proline, and 4.0 μmol of *o*-aminobenzaldehyde. The reaction was initiated by the addition of the bacterial extract and stopped by the addition of 0.4 ml of 20% (wt/vol) trichloroacetic acid. The precipitate was eliminated by centrifugation, and the absorbance was read at 440 nm (33).

Ornithine cyclase. Ornithine cyclase (ornithine cyclodeaminase; EC 4.3.1.12) activity was assayed by measuring the proline or the ammonium formed.

(i) Proline measurement. Proline measurement was a modification of the method of Costilow and Laycock (5). The reaction mixture contained, in a 0.2-ml final volume, 8.5 μmol of potassium phosphate buffer (pH 7.5) and 2.0 μmol of L-[U-^{14}C]ornithine (0.75 μCi/μmol). The reaction was started by the addition of bacterial extract. At 1, 2, and 5 min, 50-μl samples were removed from the reaction mixture, rapidly added to 10 μl of 1 M acetic acid, and blended in a Vortex mixer. Ornithine and proline in the reaction mixture were separated by high-voltage paper electrophoresis in water-formic acid-acetic acid buffer (910, 30, and 60 ml, respectively). A 10-μl fraction was spotted along with 5 μl of carrier L-proline (10 mM) on Whatman 3MM chromatography paper, and the amino acids were separated by electrophoresis at 100 V/cm for 10 min. The positions of ornithine and proline on the electrophoretograms were deduced from those of reference standards containing these amino acids, after they were sprayed with ninhydrin reagent. Spots corresponding to ornithine and proline were cut out, and the radioactivity was determined in a scintillation counter.

(ii) Ammonium ion measurement. The reaction mixture contained (in a total volume of 0.5 ml) 25 μmol of potassium phosphate buffer (pH 7.5) and 5 μmol of L-ornithine. The reaction was initiated by the addition of the bacterial extract and stopped by the addition of 0.5 ml of 50% (wt/vol) trichloroacetic acid. After centrifugation the ammonium ion produced was estimated by using the glutamate dehydroge-

nase method (2), in a 0.5-ml fraction of the supernatant neutralized with 1 M Tris base.

Assays were made immediately after sonication, without centrifugation, because of the great lability of ornithine cyclase.

Protein measurements. Proteins were determined by the method of Lowry et al. (15), using bovine serum albumin as a standard.

RESULTS

Utilization of arginine-related compounds by *Agrobacterium* strains. Nutritional tests were performed on a strain which does not contain a Ti plasmid (C58C1) and on a transconjugant of this strain that harbors a Ti plasmid (G30). We also studied the behavior of the donor strain (R10) and of a mutant of this strain constitutive for octopine catabolism (R10c21) (Table 1). A number of potential products of arginine catabolism were analyzed for their utilization as nitrogen and nitrogen-carbon sources (Table 2).

Strains harboring an octopine Ti plasmid showed poor growth on the medium containing only 2 mM octopine. Very poor growth was observed on the control that did not contain a nitrogen source. The resuts given in Table 2 demonstrate that (i) the utilization of arginine and ornithine as carbon source depends on the presence of the Ti plasmid and is inducible by octopine in strain G30, (ii) the presence of the Ti plasmid is not necessary for growth on proline or glutamate, and (iii) none of the other compounds tested can be used as sole carbon source even in the presence of octopine.

TABLE 2. Utilization of arginine-related compounds by various *Agrobacterium* strains

N and C-N source	Utilization by the following *Agrobacterium* strains[a]:			
	C58C1RS	R10	R10c21	G30
Octopine				
N	m	+++	+++	+++
C-N	−	+++	+++	+++
Arginine				
N	+++	+++	+++	+++
C-N	−	+++	+++	m
C-N-octopine (2 mM)	−	+++	+++	+++
Ornithine				
N	+++	+++	+++	+++
C-N	−	+++	+++	m
C-N-octopine (2 mM)	−	+++	+++	+++
Proline				
N	+++	+++	+++	+++
C-N	+++	+++	+++	+++
Glutamate				
N	+++	+++	+++	+++
C-N	+++	+++	+++	+++
Agmatine				
N	+++	+++	+++	+++
C-N[b]	−	−	−	−
Putrescine				
N	+++	+++	+++	+++
C-N[b]	−	−	−	−
Carbomyl putrescine				
N	+	+	+	+
C-N[b]	−	−	−	−
Citrulline				
N	+	+	+	+
C-N[b]	−	−	−	−

[a] Abbreviations and symbols: m, No growth on these media, but utilizing mutants appeared; +++, very good growth; +, growth; −, nó growth.

[b] Addition of octopine (2 mM) to these media did not promote growth of all strains at the expense of these substrates.

Bacterial respiration in the presence of possible metabolites of arginine degradation. The technique described by Stanier (25) to elucidate a metabolic pathway was particularly valuable in our study since the utilization of arginine as the sole carbon and energy source by strain G30 strictly depended on octopine induction. By this method, oxygen consumption is taken as an indicator of metabolic activity in the presence of a given substrate. Oxygen uptake was measured manometrically after addition of the substrate to a suspension of cells grown in the presence or in the absence of the presumed inducer.

The results shown in Table 3 indicate that glutamate dissimilation is probably constitutive for all the strains tested. Oxygen uptake in the presence of proline requires induction. In strains C58C1 and G30, oxygen uptake in the presence of octopine, arginine, and ornithine depended on the presence of the Ti plasmid. In strain G30, dissimilation of these three latter substrates was induced by octopine. Octopine and arginine dissimilation occurred in strain GV4R10c21 (mutant constitutive for octopine degradation) without induction by octopine, but required induction by arginine (to induce the arginase activity; see Table 5). Oxygen uptake in the presence of ornithine only depended on induction of proline degradation. In strain R10, arginine and ornithine dissimilation were not dependent on induction by octopine (Table 3).

In vitro analysis of the degradation products of arginine and ornithine. Extracts of strain G30, grown on mannitol–arginine, or on mannitol–arginine–2 mM octopine to induce arginine utilization as carbon source, were incubated with L-[U-^{14}C]arginine. Cell debris was not removed from the extracts in this experiment. Cell extracts, prepared from cells grown in mannitol (2 g/liter)–arginine (2 mM), were incubated with 5,000 nmol of L-[U-^{14}C]arginine as described above. After incubation, 275 nmol of arginine was recovered, with 4,400 nmol of ornithine being formed from arginine. No glutamate, no P5C, and no proline were detected. Similarly, cell extracts prepared from bacteria grown in the presence of mannitol (2 g/liter)–arginine (2 mM)–octopine (2 mM) were incubated with 5,000 nmol of labeled arginine (see above). After incubation, 217 nmol of residual arginine was recovered; 3,390 nmol of ornithine, 176 nmol of proline, 98 nmol of P5C, and trace amount of glutamate were formed from arginine. A similar experiment was carried out under the same conditions with L-[U-^{14}C]ornithine instead of L-[U-^{14}C]arginine, which also demonstrated that conversion of ornithine to proline and P5C depends on the induction by octopine (data not shown).

P5C could result from ornithine transamination and then be reduced to proline, or ornithine could be directly transformed into proline followed by proline oxidation to P5C. To elucidate the sequence of appearance of P5C and proline, we performed trap experiments. We incubated cell extracts with labeled ornithine, alone (see above) or with an exces of cold P5C or cold proline (Table 4). The results presented in Table 4 and the experiments presented below confirmed that ornithine is first converted to proline, which in turn gives P5C. (i) Labeled P5C was not trapped when an excess of

TABLE 3. Testing substrates as carbon and energy source: measurements of O_2 uptake in various *Agrobacterium* strains[a]

Strain	Growth medium[b]	Uptake (μl of O_2 consumed/h per mg of protein) in the following substrates[c]:				
		Octopine	Arginine	Ornithine	Proline	Glutamate
C58C1RS	Man, NH_4	ND[d]	0	6	10	56
	Man, arg	ND	2	2	1	156
	Man, pro	ND	0	18	128	96
G30	Man, NH_4	6	0	7	11	40
	Man, arg	1	5	4	14	72
	Man, orn	ND	5	ND	13	ND
	Man, pro	3	8	16	65	ND
	Man, oct	93	92	90	80	ND
	Arg, oct	176	148	123	100	60
R10	Man, NH_4	1	0	4	15	160
	Man, arg	12	149	78	94	67
	Man, orn	11	140	128	146	ND
	Man, pro	9	16	62	166	ND
	Man, oct	100	106	104	77	ND
GV4R10c21	Man, NH_4	10	0	1	4	65
	Man, arg	72	79	66	34	25
	Man, orn	70	62	48	34	25
	Man, pro	21	4	84	34	59

[a] Measurements were made in a Warburg respirometer.
[b] Abbreviations: man, mannitol; arg, arginine; orn, ornithine; pro, proline; glu, glutamate; oct, octopine; the first designation is the carbon source; the second is the nitrogen source. Mannitol was added at 2 g/liter; the other carbon sources were added at 20 mM, and the nitrogen sources were added at 2 mM.
[c] Values below 10 are not considered to be significantly different than 0. Values above 40 indicate that the assayed substrate was a good energy source. Average relative errors are estimated to 20%; i.e., a value of 50 should be read 50 ± 10.
[d] ND, Not done.

unlabeled P5C was added during the incubation of a centrifuged extract with L-[U-^{14}C]ornithine (Table 4). (ii) Conversion of proline to P5C by *Agrobacterium* extracts has already been shown (Y. Dessaux, M. Demarez, and C. Legrain, manuscript in preparation). This activity seemed to be membrane bound. The results presented in Table 4 show that no P5C was detected when the extracts were cleared out of cell debris by centrifugation. (iii) Mutants which lost the proline dehydrogenase activity have been obtained (Dessaux et al., in preparation). No P5C production was detected when cell extracts obtained from these mutants were incubated with ornithine, while proline accumulation was observed (data not shown).

In vitro assay of enzymes activities. Various enzymes that might be involved in arginine degradation were assayed in Ti plasmid-containing strains (R10 and G30) and in the Ti plasmid-free derivative C58C1. Enzyme activities were measured under different growth conditions (Table 5).

Arginase activity was detected in octopine-, arginine-, and ornithine-grown cells and did not depend on the presence of the Ti plasmid. In strains containing an octopine Ti plasmid, arginase activity was induced when cells were grown in presence of octopine, but not in presence of lysopine (Table 5).

Urease and ornithine transaminase activities were constitutive and were not related to the presence of the Ti plasmid. Weak ornithine transaminase activity was measured (Table 5).

Proline dehydrogenase activity was detected only when (i) the cells were grown in the presence of proline or (ii) when the utilization of arginine or ornithine as a carbon source was induced. This activity was also independent of the Ti plasmid.

Ti plasmid-encoded step: proline-forming activity. The previous results indicated that the Ti plasmid-encoded conversion of ornithine to proline did not proceed via a transaminase reaction. Experiments were performed to identify the product of ornithine degradation. We incubated 10 μmol of ornithine with 4.9 mg of protein produced by a cell extract prepared from strain G30 grown in arginine (20 mM) and octopine (2 mM). At the end of the incubation (30 min), the amounts of proline, ammonium ion, and residual ornithine were determined by various techniques. Measurements performed by automated amino acid analysis gave the following results: unreacted ornithine, 8.1 μmol; proline, 2.3 μmol; NH_4^+, 2.3 μmol. Measurements performed with labeled ornithine and assay of the ammonium ion by the glutamate dehydrogenase method gave the following results: unreacted ornithine, 7.9 μmol; proline, 2.5 μmol; ammonium ion, 2.2 μmol. Since equimolar amounts of proline and ammonia were formed during incubation of ornithine with extracts obtained from strain G30 grown on arginine-octopine, proline must be formed from ornithine via a deamination reaction; therefore, formation of proline appears to be catalyzed by an enzyme system analogous to the ornithine cyclase (deaminating) from *Clostridium sporogenes* (6).

TABLE 4. Analysis of products from degradation of ornithine by cell extracts

Substrate contained in the incubation media	Metabolites recovered	Amt (nmol) of metabolites formed from ornithine on the following extracts of strain G30[a]:	
		20,000 × *g* supernatant of crude extract	Crude extract
Labeled ornithine alone (5,000 nmol)	Proline, P5C	580	522
		0	105
Labeled ornithine (5,000 nmol) and cold proline (4,000 nmol)	Proline, P5C	525	ND[b]
		0	ND
Labeled ornithine (5,000 nmol) and cold P5C (4,000 nmol)	Proline P5C	250	ND
		0	ND

[a] Grown on mannitol (2 g/liter) and arginine and octopine (2 mM each).
[b] ND, Not done.

Preliminary in vitro assays were performed with crude extracts from strain G30; the following observations were made. The proline-forming activity was very unstable. Its half-life was about 200 min at 0°C and 10 min at 30°C. Attempts to stabilize the activity by the addition of ethylene glycol, glycerol, and substrates and potential cofactors like NAD and NADP remained unsuccessful. *Clostridium* ornithine cyclase was reported to be very sensitive to oxygen (6); however, *Agrobacterium* cyclase in vitro activity was not higher when extraction and incubation were performed under an argon atmosphere. Ornithine cyclase activity was detected in vitro from pH 6.0 to 9.0; the highest activity was observed at pH 7.5. The K_m for ornithine was found to be 5 mM in crude extracts (determined either by proline formation or ammonia production). Filtration of crude extracts on a Sephadex G-25 column resulted in a 40% loss of activity. This result was not improved by the addition of NAD, NADP, or FAD to the assay mixture. A search for effectors that could affect the cyclase activity only revealed that the enzyme was inhibited by proline (30% inhibition with 10 mM proline).

Ornithine cyclase activity was next assayed under different growth conditions in Ti plasmid-containing strains and in the Ti plasmid-free derivative C58C1 (Table 6). Whatever the growth conditions were, no ornithine cyclase activity was detected in strain C58C1. In strain G30 (pTi octopine), the presence of cyclase activity required induction by octopine (or by lysopine; data not shown), while nopaline was the inducer in strain C58 (pTi nopaline). Cyclase activity was present under all conditions tested in a mutant constitutive for octopine degradation (strain GV4R10c21). In this strain, the lower activity observed in the presence of mannitol could be the result of a possible catabolite repression. In addition to the pTi-born cyclase activity which was inducible by both octopine and lysopine, strain R10 also had a proline-forming activity when the cells were grown in the presence of arginine (Table 6). This result indicates that strain R10 could also possess another gene for the cyclase which would not be under the control of octopine.

TABLE 5. In vitro enzyme assays

Strain	Growth medium[a]	Activities (nmol of product formed/min per mg of protein) of the following enzymes:			
		Arginase	Urease	OTAse[b]	Proline dehydrogenase
C58C1RS	Man, NH_4	8.3[c]	208	14	0.1[d]
	Man, arg	4,867	168	13	0.1[d]
	Man, pro	18.3[c]	200	14	18
	Man, arg, oct[e]	4,716	158	3	0.1[d]
R10	Man, NH_4	1.6	258	13	0.1[d]
	Man, oct	1,883	333	13	12
	Man, arg	3,416	383	12	13
	Man, orn	3,133	342	12	ND[f]
	Man, pro	3[c]	900	11	17
	Man, lyso	3[c]	458	15	ND
	Arg	7,450	550	9	50
	Pro	7[c]	567	11	48
	Glu	92	500	6	0.1
G30	Man, NH_4	7[c]	433	17.5	0.1[d]
	Man, oct	2,333	233	11	ND
	Man, arg	4,567	467	12	0.1[d]
	Man, orn	1,450	150	5	0.1[d]
	Man, pro	3[c]	300	9	13
	Man, lyso	7[c]	466	17	ND
	Arg, oct	5,366	367	15	13

[a] Abbreviations: man, mannitol; arg, arginine; orn, ornithine; pro, proline; glu, glutamate; oct, octopine; lyso, lysopine; the first designation is the carbon source; the second is the nitrogen source. Mannitol and NH_4 were added to the media at 2 g/liter; the other carbon sources were added at 20 mM, and the nitrogen source was added at 2 mM.
[b] OTAse, Ornithine transaminase.
[c] Because of endogenous urease activity in the extract, these values were underestimated.
[d] Activities correspond to the sensitivity limit of the assay.
[e] Octopine was added at 2 mM.
[f] ND, Not done.

TABLE 6. In vitro assay for ornithine cyclase activity

Culture medium[a]	Activities (nmol of proline formed/min per mg of protein) for the following strains:				
	C58C1RS	R10	G30	GV4R10c21	C58
Mannitol (2 g/liter), NH_4 (2 g/liter)	0.1	0.1	0.1	8.3	0.1
Mannitol (2 g/liter), arginine (2 mM)	0.1	16.7	0.1	6.7	0.1
Octopine (10 mM)	NG[b]	66.7	33.3	43.3	NG
Arginine (20 mM)	NG	56.7	NG	28.3	NG
Proline (20 mM)	0.1	0.7	0.5	25	0.1
Arginine (20 mM), octopine (1 mM)	NG	58.5	21.7	33.3	NG
Arginine (20 mM), nopaline (1 mM)	NG	ND[c]	ND	ND	10

[a] Concentrations of substrates are indicated in parentheses. The first designation is the carbon source; the second is the nitrogen source.
[b] NG, No growth of the strain on the medium.
[c] ND, Not determined.

DISCUSSION

All *Agrobacterium* strains we studied utilize arginine as nitrogen source, independently of the presence of a Ti plasmid. This was achieved by the arginase-urease pathway (Fig. 1). The presence of arginase activity in gram-negative bacteria seems to be restricted to a few species. It has only been reported for the genus *Proteus* (23) and the *Agrobacterium* and *Rhizobium* group (31; L. Wu and L. Unger, Book of Abstracts, Noodwijkerhout, N.L., European Molecular Biology Organization Workshop on Plant Tumour Research, 1978). Ornithine utilization as nitrogen source by strains devoid of cyclase activity probably proceeds via the constitutive ornithine-δ-transaminase activity (Fig. 1). The weak activity of ornithine-δ-transaminase measured in vitro agrees with the doubling time of about 4 h observed when ornithine is the only nitrogen source. Ornithine decarboxylase activity, which degrades ornithine to putrescine, has also been detected in extracts of all the strains tested (unpublished data).

The results obtained by Ellis et al. (8) and by Petit et al. (20) demonstrate that arginine and ornithine utilization as carbon source are two functions borne by nopaline or octopine Ti plasmids; in addition, in strains containing an octopine Ti plasmid, octopine induces arginine and ornithine degradation. Our results show that ornithine degradation is indeed the function encoded by the Ti plasmid. Utilization of ornithine as carbon source proceeds via cyclization of ornithine into proline. Proline is then converted into glutamic acid, probably by proline dehydrogenase and P5C dehydrogenase activities (Fig. 1). Mutants of strains G30 lacking proline dehydrogenase activity have been obtained (Dessaux et al., in preparation). They are unable to grow on arginine or on ornithine as sole carbon source, confirming the role of proline as an intermediate in this catabolic pathway.

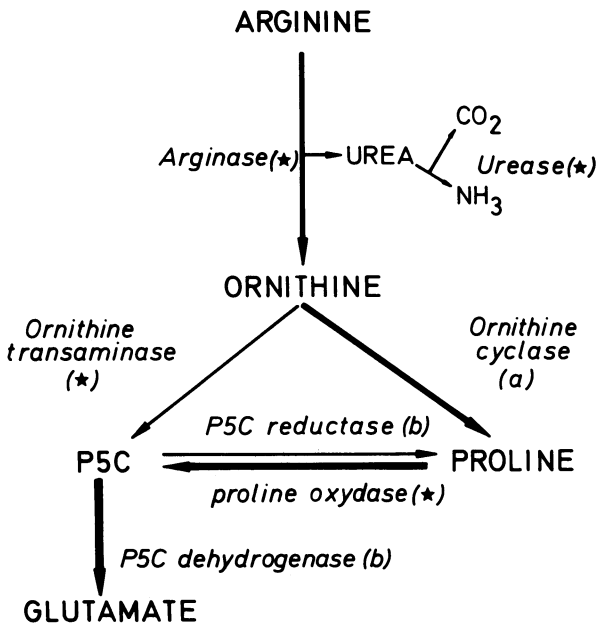

FIG. 1. Arginine catabolic pathway in *A. tumefaciens* strains. Symbols and abbreviations: *, chromosomal genes probably determine these enzymes; (a), ornithine cyclase activity is a Ti plasmid-borne function (strains R10, G30, C58); in strain R10, chromosomal genes probably determine also this activity; (b), mentioned only in this figure; data concerning proline catabolism will be published later; ⟶, pathway of assimilation as carbon and nitrogen source; →, pathway of assimilation as nitrogen source only.

The measurement of ornithine cyclase levels in several *A. tumefaciens* strains (strains inducible or constitutive for opine and arginine degradation and strains devoid of pTi plasmid), as a function of the growth conditions, confirms the role of this activity in arginine and ornithine catabolism and its genetic localization on the Ti plasmid. It should be mentioned that several *Agrobacterium* strains, e.g., strain R10, possess a second gene which is probably chromosomal and independent of the opine control and which codes for ornithine cyclase activity. Some octopine-type strains have been cured of their Ti plasmid. Consistent with the last observation, the cured derivative was always found to be able to grow on arginine as the sole carbon source.

During the course of this work, a study was published that reported the presence of ornithine dehydrogenase activity in strain C58 (24). We were unable to measure any ornithine dehydrogenase activity in all the strains we tested, including strain C58, even under the conditions described by Schardl and Kado (24). Moreover, our strain C58 was unable to grow on arginine or ornithine as the sole carbon source without induction by nopaline. The reason for this discrepancy remains unclear.

To our knowledge, ornithine cyclase activity has only been reported in two anaerobic organisms: *Clostridium sporogenes* (6, 17, 18) and *Treponema denticola* (14). Recently, this activity was also detected in some of the *Pseudomonas* species (V. Stalon, personal communication) which are closely related to the *Agrobacterium* and *Rhizobium* group (9).

A further characterization of the ornithine cyclase activity of *Agrobacterium* strains requires stabilization of the activity to allow purification of the enzyme or the multienzyme complex.

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