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Transport of Nonmetabolizable Opines by *Agrobacterium tumefaciens*

M. KRISHNAN,[1] JOHN W. BURGNER,[1] W. SCOTT CHILTON,[2] AND STANTON B. GELVIN[1]*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Department of Botany, North Carolina State University, Raleigh, North Carolina 27695[2]

Received 28 June 1990/Accepted 12 November 1990

We have examined the uptake of [^{14}C]octopine and [^{14}C]nopaline by *Agrobacterium tumefaciens* strains containing the C58 chromosomal background in medium suitable for the induction of *vir* genes. All strains tested could transport both of these opines, regardless of the presence or type of Ti plasmid (octopine or nopaline) present in the bacterium. The transport of these opines required active cellular metabolism. Nonradioactive octopine, nopaline, and arginine competed effectively with [^{14}C]octopine and [^{14}C]nopaline for transport into *A. tumefaciens* A136, suggesting that the transport of these opines occurs via an arginine transport pathway not encoded by the Ti plasmid.

The gram-negative phytopathogenic bacterium *Agrobacterium tumefaciens* causes the disease crown gall on a wide variety of dicotyledonous plants and some monocotyledonous plants. Tumorigenesis initiates with the perception by the bacterium of a number of phenolic compounds, such as acetosyringone, synthesized by wounded plant cells (1, 12). Induction of the bacterium by these compounds results in the activation of the Ti-plasmid-encoded *vir* (virulence) genes, resulting in the excision of T-DNA (transferred DNA) from the Ti (tumor-inducing) plasmid and its transfer to the plant cell (13, 14, 18). Following integration of the T-DNA into the plant nuclear genome, the T-DNA genes are transcribed and translated into enzymes involved in the biosynthesis of phytohormones auxin and cytokinin and in the biosynthesis of unusual amino acid and sugar derivatives termed opines. These opines are secreted into the tumor where they can be utilized by the inciting *Agrobacterium* strain as carbon sources and sometimes nitrogen sources (15, 16).

We have recently described a possible third function for opines in the *Agrobacterium* infection cycle. Certain opines, such as octopine, nopaline, leucinopine, and succinamopine, can potentiate the induction of *vir* genes by acetosyringone. The opines by themselves have no major effect upon *vir* gene induction. Unexpectedly, several opines that could not be metabolized by the bacterium (such as nopaline, leucinopine, and succinamopine in the case of the octopine-type *Agrobacterium tumefaciens* A348) stimulated acetosyringone induction of the *vir* genes to a greater extent than did the metabolizable opine octopine. These results led us to investigate and confirm the transport of the nonmetabolizable opine nopaline into *A. tumefaciens* A348 (17). In this report, we demonstrate the transport of the nonmetabolizable opines octopine and nopaline into various *A. tumefaciens* strains, including strain A136 which lacks a Ti plasmid.

All *A. tumefaciens* strains used in this study contained the C58 chromosomal background. *A. tumefaciens* A136 (lacking a Ti plasmid), A348 (harboring the octopine-type Ti plasmid pTiA6), or A208 (harboring the nopaline-type Ti plasmid pTiT37) were grown to a concentration of 10^9 cells per ml, harvested by centrifugation at 10,400 × *g* for 8 min, and suspended in K3 medium supplemented with 5 mM MES–NaOH, pH 5.6, (17) at a concentration of 2×10^8 cells per ml. The cells were incubated at 22°C with [^{14}C]octopine (50,000 cpm/ml; 270 mCi/mmol) plus 1 μM nonradioactive octopine or with [^{14}C]nopaline (50,000 cpm/ml; 270 mCi/mmol) plus 1 μM nonradioactive nopaline. Radioactive opines were synthesized from [^{14}C]arginine (270 mCi/mmol) and pyruvic acid (octopine) or [^{14}C]arginine and α-ketoglutaric acid (nopaline) essentially by the method of Jensen et al. (4). The reaction was monitored by high-voltage electrophoresis at pH 1.8, using phenanthrenequinone detection of nonradioactive opines and arginine standards and scintillation counting of 1-cm strips of those channels containing the radioactive preparations. No [^{14}C]arginine was detectable in the opine preparations (at the 1% confidence level). When used as competitors, nonradioactive octopine, nopaline, or arginine were present at concentrations of 0.5 to 50 μM. Transport was initiated by the addition of cells to the incubation mixture. At the end of the incubation time, 1-ml samples were withdrawn and filtered through nitrocellulose membrane filters (0.2-μm pore size; GSTF; Millipore Corp., Bedford, Mass). The filters were washed twice with 3 ml of K3 medium and counted in a liquid scintillation counter. Three repetitions of each experiment were conducted.

Figure 1A shows the rate of uptake of [^{14}C]octopine and [^{14}C]nopaline by *A. tumefaciens* A136; similar plots (not shown) were obtained with strains A348 and A208. Because we wanted to correlate these results with our previous observations that octopine and nopaline could stimulate the acetosyringone induction of *vir* genes, we conducted these transport experiments in K3 medium supplemented with 5 mM MES buffer, pH 5.6. This was the same medium used to demonstrate the potentiation of *vir* gene induction by opines (17), and the bacterial cells were not growing under these conditions. The linearity of uptake of octopine and nopaline by all of these strains extends for an incubation period of at least 20 min (data not shown). Although the octopine-type *A. tumefaciens* A348 took up octopine at a slightly higher rate, *A. tumefaciens* A208 and A136 transported octopine at nearly equivalent rates (within a factor of 2). Both octopine and nopaline transport require active cellular metabolism because preincubation of the bacteria for 5 min with 10 mM

* Corresponding author.

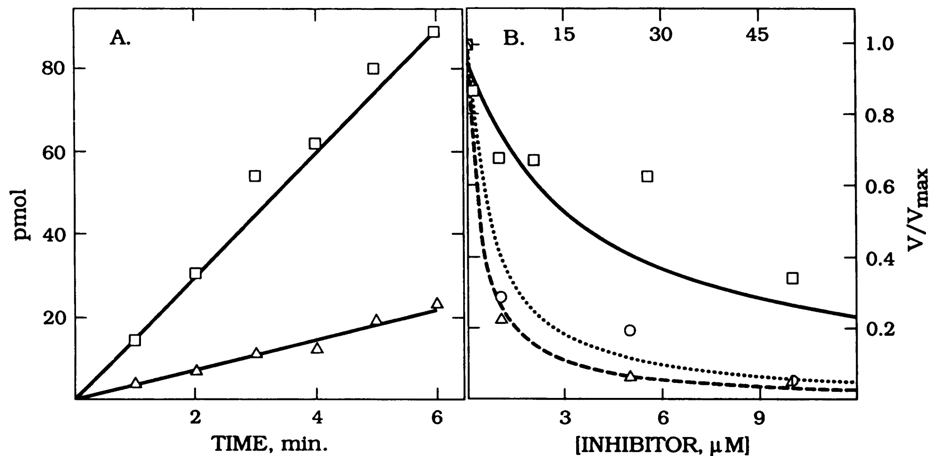

FIG. 1. (A) Octopine (□) and nopaline (△) transport by *A. tumefaciens* A136. The initial concentration of opines was 1 μM in both cases. (B) Plots of relative rate of uptake against concentration of inhibitor: unlabeled nopaline versus [^{14}C]octopine (□——□, upper scale), unlabeled octopine versus [^{14}C]nopaline (△- - -△, lower scale), and unlabeled arginine versus [^{14}C]octopine (○···○, lower scale). The initial concentration of labeled opine was 1 μM. Both labeled and unlabeled compounds were added simultaneously. The calculation of the lines is described in the text.

sodium azide completely inhibits octopine and nopaline uptake (17; also data not shown).

The transport of octopine and nopaline into *A. tumefaciens* A136 saturates at high opine concentration, and its mechanism probably involves equal and independent binding sites. Transport rates for these opines were obtained as a function of opine concentration, and these rates were fitted by nonlinear least squares to the equation for a simple rectangular hyperbola. The calculated K_m values for octopine and nopaline uptake are 0.28 ± 0.09 and 4.3 ± 1 μM, respectively, and the calculated V_{max} values are 32 ± 2 and 75 ± 10 pmol/min/2 × 10^8 bacterial cells, respectively. The transport of arginine was sufficiently rapid that plots of uptake against time were decidedly curved at low arginine concentrations (<5.0 μM), even when the rate of transport was measured at 30 s (the fastest time at which we could filter the bacteria). We estimated graphically that the K_m value is no larger than 0.5 μM and the V_{max} value is about 190 pmol/min/2 × 10^8 bacterial cells for this bacterial strain.

In order to obtain information regarding the mechanism of octopine and nopaline transport by *A. tumefaciens* A136, we incubated the bacteria with either radioactive octopine or nopaline in the presence of various unlabeled compounds. The data in Fig. 1B show that the uptake of [^{14}C]octopine and [^{14}C]nopaline are inhibited proportionally by increasing concentrations of nonradioactive nopaline (□) and octopine (△). These data also show that arginine (○) is a potent inhibitor of octopine uptake. In Fig. 1B, the lines were calculated assuming that the inhibition by the unlabeled opine is competitive and that rate/V_{max} equals [opine]/$\{1 + K_m(1 + [I]/K_I)\}$ where [opine] and $[I]$ are the concentrations of the labeled and unlabeled compounds, respectively, while K_I is the apparent inhibitor constant for the unlabeled compounds. The values for K_m and V_{max} are given above. A value for the K_I of the unlabeled compound was obtained by assuming that its K_m and K_I are the same. This is a reasonable assumption if the binding step is at equilibrium (i.e., if transport is relatively slow compared with binding steps). The lines are a reasonable fit to the data; hence, we conclude that the same transport system is used for each compound. Because the transport of [^{14}C]arginine by *A. tumefaciens* A136 under these experimental conditions was too rapid to measure using this assay (see above), we did not attempt to assess quantitatively the ability of octopine or nopaline to compete with arginine transport.

Because, to our knowledge, the uptake of octopine or nopaline into *A. tumefaciens* cells lacking a Ti plasmid has not previously been reported, we determined that the radioactive material transported into *A. tumefaciens* A136 was indeed the opine and not arginine derived from the extracellular degradation of the opine. Following 2 h of incubation of *A. tumefaciens* A136 with [^{14}C]nopaline, the bacterial cells were washed three times with K3 medium (30 ml per wash), pelleted by centrifugation at 10,400 × *g* for 10 min and lysed by repeated freezing and thawing cycles. We recovered greater than 73% of the radioactivity associated with the bacteria in the supernatant solution after centrifugation of the lysate. High-voltage paper electrophoresis of this supernatant solution (9) followed by autoradiography indicated that all detectable radioactivity remained with a compound having a mobility identical to that of nopaline. We detected no radioactivity associated with any compounds that migrated as arginine (Fig. 2). These results further indicate that *A. tumefaciens* A136 can transport nopaline. In addition, these results demonstrate that the radioactive nopaline associated with the bacterial cells was taken up by the bacteria and was not merely bound to the bacterial membranes.

In general, the ability of *A. tumefaciens* to transport and catabolize opines is encoded by genes on the Ti plasmid (2, 7). Reports from a number of laboratories have indicated that the uptake and use of octopine and nopaline are under the control of genes inducible by the metabolizable opine (3, 5, 6, 11). Several exceptions to this mode of regulation exist, however. Petit and Tempe (10) have shown that octopine can be catabolized by nopaline oxidase encoded by nopaline-type Ti plasmids, and Hooykaas et al. (3) have demonstrated that the Ti-plasmid-encoded nopaline permease can facilitate the transport of octopine into *A. tumefaciens*. These oxidase and permease activities are induced by nopaline. In addition, Montoya et al. (8) have shown that most biotype II strains of *A. tumefaciens* and *A. radiobacter* that utilize nopaline could also degrade octopine. In these strains, octopine catabolic activity was constitutive and most likely not encoded by the Ti plasmid.

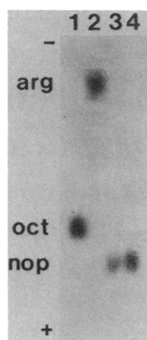

FIG. 2. High-voltage paper electrophoresis of soluble radioactive compounds from *A. tumefaciens* A136 following incubation with [^{14}C]nopaline. Transport, isolation, and electrophoresis conditions were as described in the text. Lane 1, [^{14}C]octopine standard; lane 2, [^{14}C]arginine standard; lane 3, [^{14}C]nopaline standard; lane 4, ^{14}C compounds isolated from *A. tumefaciens* A136 following incubation with [^{14}C]nopaline.

Our finding that *A. tumefaciens* strains containing the C58 chromosomal background transport both octopine and nopaline independent of the presence of a Ti plasmid justifies in part the ability of nonmetabolizable opines to enhance the acetosyringone induction of *vir* genes. Future research will address the mechanism by which such opines stimulate this induction.

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