Transformed Plants Producing Opines Specifically Promote Growth of Opine-Degrading Agrobacteria

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To investigate the effect of plant transformation on opine catabolic (and pathogenic) *Agrobacterium*, we designed an experimental model in which the changes of a bacterial population associated with the root system of plants could be easily monitored. The bacterial population was composed of opine catabolic and noncatabolic strains of *Agrobacterium*. Bacteria were cocultivated with either transformed or normal *Lotus* plants. The composition of the bacterial population was estimated by dilution and plating of the growth medium on various media. We demonstrated that growth of bacteria was stimulated when they were associated with transformed plants. Furthermore, growth of opine-utilizing bacteria was specifically favored when bacteria were associated with transformed plants, but not when bacteria were associated with normal plants. This work indicates that transformation of plant cells by agrobacteria indeed can favor growth of the catabolic strain. This finding may lead to creation of engineered plant-bacteria interactions in which both partners will benefit from their association.

Additional keywords: artificial rhizosphere, plant-bacteria association, Ri plasmid.

The *Agrobacterium*-plant interaction constitutes a unique example of naturally occurring genetic transformation of an eukaryotic organism by bacteria. By transferring a fragment of DNA, called the T-DNA, from its pathogenic plasmid to the nuclear genome of the plant cell, *Agrobacterium* modifies the developmental pattern of the host cell. The latter multiplies to form a tumor known as crown gall when transformed by *A. tumefaciens* T-DNA, or differentiates into roots (hence the name hairy root given to the disease) when transformed by *A. rhizogenes* T-DNA (Birot *et al.* 1987; Melchers and Hooykaas 1987; Binns and Thomashow 1988; Zambryski *et al.* 1989; Tempé and Casse-Delbart 1989; Tepfer 1989; Winans 1992). *Agrobacterium* also modifies the metabolic activity of the plant cell. Thus, crown gall or hairy root cells produce unusual compounds termed opines. Synthesized by enzymes encoded by T-DNA genes at the expense of the pool of metabolites of the plant cells, these low-molecular weight molecules pass through the plant cell membrane to diffuse within the intercellular space. There, opines probably serve as carbon and most often nitrogen source for the inciting *Agrobacterium*. Genes involved in opine degradation are borne on the pathogenic plasmid (Dessaux *et al.* 1992; Gelvin 1992). Furthermore, some of the opines induce the conjugative transfer of this plasmid from a pathogenic strain to an avirulent recipient (Kerr *et al.* 1977; Petit *et al.* 1978; Ellis *et al.* 1982; Dessaux *et al.* 1992).

The features described above led to the formulation of the opine concept (Tempé *et al.* 1979) and the genetic colonization theory (Schell *et al.* 1979). Both concepts propose that opines are essential to the *Agrobacterium*-plant interaction by favoring growth and dissemination of the pathogen. This assumption is supported by experimental results such as the generalized occurrence of opines in crown gall tumors and in hairy roots and by the demonstration of *in planta* transfer of the pathogenic plasmids of *Agrobacterium* (Kerr 1969; Kerr 1971; Kerr *et al.* 1977). Opinelike molecules have also been detected in nodules incited by several strains of *Rhizobium*. These molecules are specifically degraded by the bacterial strain that induced nodule formation (Murphy and Saint 1992).

That several plant-bacteria interactions are associated with opine production and opine degradation suggests that one may create or strenghten other microbe-plant interactions by engineering plants to produce opines and bacteria to degrade these molecules. If bacteria are potentially beneficial to plant growth, both partners could finally benefit from their association. Before initiating such studies, it is necessary to determine whether a tumorlike or a hairy rootlike plant environment indeed favors growth of pathogenic bacteria. Though opine structures, synthesis, and degradation have been studied for many years, there are only a limited number of investigations on their ecological role, and, surprisingly, the above assumption has never been clearly demonstrated. To address this question, an experiment was designed in which the changes of a bacterial population placed, or not, in a transformed, opine-producing environment could be monitored. This model involved *Lotus corniculatus* plantlets and strains of *Agrobacterium*. Normal and transformed plantlets producing the opines mannopine, mannopinic, and agropinic acids (Firmin and Fenwick 1978; Coxon *et al.* 1980; Tate *et al.* 1982; Petit *et al.* 1983) were propagated *in vitro*. Various mixtures of opine-utilizing, and opine nonutilizing, agrobacteria

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were inoculated to the growth medium of these plants. Bacteria and plants were cocultivated for several weeks. In this system, the ratio of catabolic cells to total *Agrobacterium* cells decreased when associated with normal plants. Interestingly, the total bacterial population was higher when bacteria were associated with transformed plants, and in these conditions, the growth of the catabolic bacteria was clearly stimulated.

RESULTS

Preliminary assays of plant-bacteria associations.

In the course of this study, various plant growth media were used to test the interaction between plants and bacteria. Of these media, CL14 was found to allow satisfactory growth of both the plant and the bacteria. Most bacterial growth media including the AT mineral base are buffered at pH 7.0, whereas CL14 plant growth media is buffered at pH 5.5. It was therefore necessary to cultivate bacterial cells on the CL14 medium, pH 5.5, before associating them with plants. Failure to do so resulted in drastic and immediate decrease of the number of bacterial cells (data not shown). Also, a simple and reliable enumeration system had to be established. Quantifying on CL14MOA and CL14MSY was found to be a convenient way to measure the bacterial population. Total free-living cells were measured on the nonselective CL14MSY medium. Several control experiments were performed on ATMS and ATR. The efficiency of recovery on CL14MSY was identical to that obtained on ATMS and ATR media (data not shown). On the CL14MOA medium, opine-utilizing bacteria grew well and yielded large colonies, while the nonutilizing cells multiplied poorly (residual growth in the presence of 100 mg/L of yeast extract) to form small ghost colonies. Solidifying this medium with Gelrite yielded a very clear culture medium, which greatly improved the accuracy of distinguishing between ghost colonies and true colonies.

Associated cultures of plants with pair of strains C58C1RS and C58C1RS(pRi8196).

In a first experiment, we inoculated two sets of six tubes containing normal *Lotus* plants and two sets of six other tubes containing opine-producing plants (all grown on CL14 medium) with a 1:1 mixture of strains C58C1RS and C58C1(pRi8196). Both experiments were therefore performed on 12 normal and on 12 transformed plantlets. Total number of bacteria added to the growth medium was about 10^3 cfu/ml. A second experiment was performed exactly as the first one except that the mixture contained a ratio of 9:1 C58C1RS to C58C1RS(pRi8196). Samples of the growth media were removed at the beginning of the experiment and after 1, 3, and 4 wk. The bacterial population was enumerated and analyzed as indicated in Materials in Methods. Results are presented in Figure 1A–C. Several conclusions could be drawn from these results. First the amount of bacteria added to the media and the composition of the inoculum were as expected: about 10^3 cells per milliliter and about 50% or 10% C58C1RS(pRi8196) cells. When cocultivated with normal plants, the population of bacterial cells sharply increased within 1 wk to reach a plateau at about 1.2×10^7 cells per milliliter (Fig. 1A). The population of catabolic, therefore pathogenic (p) bacteria also increased, however, much more slowly than did the population of noncatabolic, nonpathogenic (np) cells. This resulted in a decrease in the ratio of pathogenic/total cells (p/p+np), which shifted from 0.44 to 0.07 in the first experiment (Fig. 1B), and from 0.13 to 0.04 in the second experiment (Fig. 1C). When the bacteria were associated with transformed opine-pro-

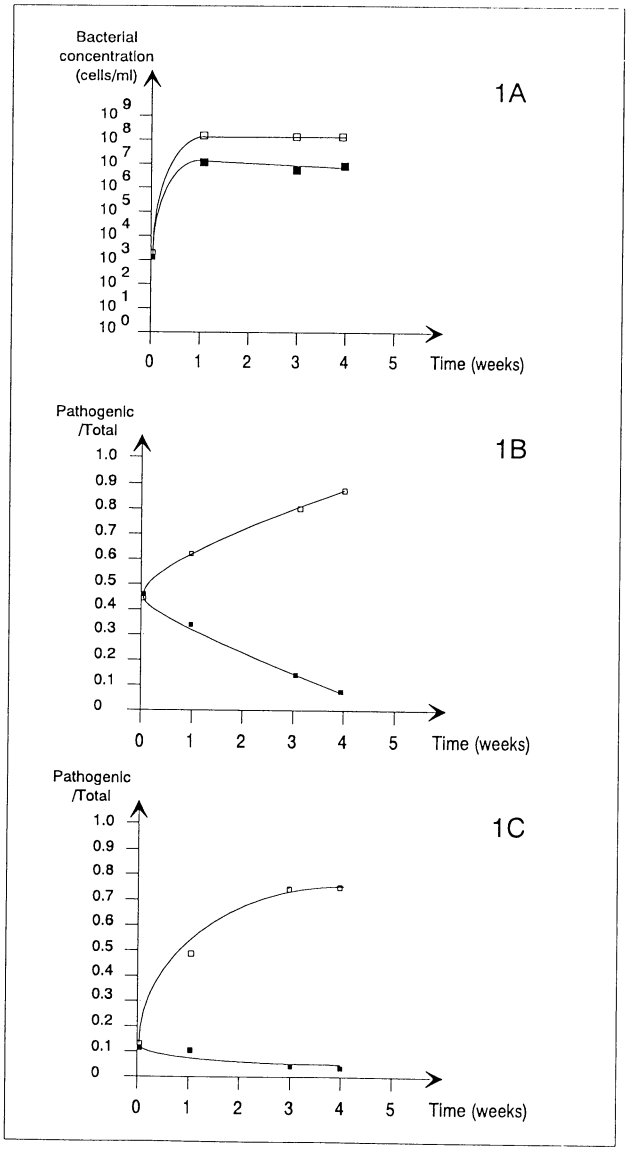

Fig. 1. Associated growth of strains C58C1RS and C58C1RS (pRi8196) with normal and transformed *Lotus* plants. **A,** Changes in the total bacterial population grown in the presence of transformed, opine-producing plants (□) and with normal plants (■). Mean errors are given by the size of the symbols in the graphs. **B,** Changes of the ratio of pathogenic cells (p) to pathogenic plus nonpathogenic (total) cells (p+np) when bacteria were grown in the presence of transformed, opine-producing plants (□) and in the presence of normal plants (■). At t_0, the ratio was about 0.5 (i.e., population was composed of 50% pathogenic cells). **C,** Changes of the ratio p/p+np when bacteria were grown in the presence of transformed, opine-producing plants (□) and in the presence of normal plants (■). At t_0, the ratio was about 0.1 (i.e. population was composed of 10% pathogenic cells).

ducing plants, the total population also sharply increased to reach a plateau at about 1.5 10^8 cells per milliliter (Fig. 1A). The increase in the population of opine-utilizing bacteria exceeded that of the C58C1RS population. Therefore the ratio p/p+np increased from 0.44 to 0.88 in the first experiment and from 0.13 to 0.75 in the second experiment (Fig. 1B,C).

Associated cultures of plants with pair of strains C58C1RS and C58C1RS(pArA4a) or C58C1RS and C58C1RS (pArA4a,b,c).

A similar experiment was undertaken with two other pairs of strains, which were as above. Results obtained with these two pairs of strains, however, cannot be expressed as were those obtained with strains C58C1RS and C58C1RS(pRi8196). In this latter strain, opine degradation and pathogenicity are physically linked functions since they are borne on the same plasmid. Enumerating opine-catabolizing bacteria was therefore equivalent to enumerating pathogenic strains. Oppositely, transconjugants harboring pArA4a are able to degrade opines, but remain nonpathogenic (Table 1). Therefore, in experiments performed with strains harboring pArA4a alone or associated with other plasmids, changes in the bacterial populations were assessed by evaluating the changes of, and expressed as a ratio of opine catabolic cells to total cells (catabolic/catabolic + noncatabolic, i.e. c/c+nc), and not of pathogenic cells to total cells.

A series of six tubes containing independenly growing normal plants, and a series of six tubes containing independently growing opine-producing plants, were inoculated with a mixture of two strains. The final concentration of bacteria was 10^3 cells per milliliter, of which 50% were C58C1RS. Here also, a second series of inoculations were performed on sets of six independently growing normal plants and six independently growing transformed plants, with a similar concentration of bacteria, of which 90% were C58C1RS. Samples from the media were removed at the beginning of the experiment and after 1, 3, and 5 wk. The changes of the bacterial population were analyzed as indicated in Materials and Methods. Results, given in Figures 2 and 3, resemble those obtained in the experiments performed with strain C58C1RS(pRi8196). First, the population of bacterial cells increased to reach a plateau. However, the maximum number of cells varied from one experiment to another (Figs. 2A and 3A). As also observed previously, the total population was highest when the bacteria were cocultivated with opine-producing plants (one order of magnitude). Most importantly, the ratio of catabolic to total cells (c/c+nc) (Figs. 2B,C and 3B,C) decreased when the bacteria were grown in association with normal plants and clearly increased when bacteria were associated with opine-producing plants (Figs. 2B,C, and 3B,C). Especially, in the experiments performed with strains C58C1RS and C58C1RS(pRiA4a,b,c,), the ratio c/c+nc shifted from 0.1 or 0.5 (according to the experiments) to about 0.99 (Figs. 3B,C).

Estimation of opine concentration in plants and in plant-bacteria cocultivation medium.

To evaluate the putative selective pressure brought by opine production, the amount of mannityl opine contained in transgenic plants and released into the cocultivation medium was estimated by a semiquantitative technique. This estimation was performed by spotting defined volumes of the medium onto an high-voltage paper electrophoregram along with samples of chemically synthesized mannityl opines of known concentrations. The intensity of the spots following silver nitrate staining and a simple calculation allowed us to evaluate the concentration of opine released into the cocultivation medium. Afer 1 wk of growth in the absence of bacteria, the concentration of

Table 1. Bacterial strains used in this study

Strains	Resident catabolic plasmid	Resident pathogenic plasmid	Description	Origin
C58C1RS	None	None	Ti plasmid-free *Agrobacterium* resistant to 100 μg/ml rifampicin and 500 μg/ml streptomycin	OC[a]
C58C1RS(pRi8196)	pRi8196	pRi8196	Transconjugant of C58C1RS harboring pRi8196. This plasmid encodes degradation of MOP, MOA and AGA[b]	OC Petit *et al.* 1983
C58C1RS(pArA4a)	pArA4a	None	Transconjugant of C58C1RS harboring pArA4a. This plasmid encodes degradation of MOP, MOA, and AGA	OC Petit *et al.* 1983
C58C1(pArA4a,b,c)	pArA4a pArA4b pArA4c	pArA4b pArA4c	Transconjugant of C58C1RS haboring pArA4a,b and c. Plasmid pArA4a encodes degradation of MOP, MOA, and AGA and plasmid pArA4b encodes degradation of AGR	OC Petit *et al.* 1983

[a] Our collection.
[b] MOP, mannopine; MOA, mannopinic acid; AGA, agropinic acid; AGR, agropine.

mannityl opines released into the medium was estimated at 1–2% (w/v), i.e., about 50 mM. In similar conditions, opines were estimated to accumulate to levels of 30 μg/mg dry weight of plant tissue. Interestingly, in the presence of bacterial cells, only trace amounts of opines were detected in the medium by high-voltage paper electrophoresis (data not shown).

DISCUSSION

This work aimed at providing a demonstration that a tumorlike, or a hairy rootlike plant environment, favors growth of pathogenic agrobacteria. To do so, an experimental model allowing easy monitoring of the association between bacteria and plants, had to be established. The *Lotus corniculatus* plants were chosen because they could easily be manipulated *in vitro*. For instance, opine-producing plants spontaneously regenerated from roots induced by *Agrobacterium rhizogenes* (Petit *et al.* 1987). Also, *Lotus* showed great tolerance to the growth conditions (media, temperature, photoperiod) and could be propagated easily from cuttings (A. Petit, personal communication). The experiments were performed *in vitro* because this technique allowed us to control the growth parameters of both plant and bacterial partners, and to conveniently monitor the changes of the bacterial population. This experimental model of an artificial rhizosphere worked well. Indeed, plants sustained growth of the bacterial populations from 10^3 cells per milliliter to over 10^8 cells per milliliter and withstood the presence of such high concentrations of bacteria without apparent disturbances.

The experiment performed with strains C58C1RS and C58C1RS(pRi8196) was repeated twice so each measurement resulted from the analysis of series of 12 inoculations. Results obtained with strain C58C1RS(pRi8196) are consistent with those obtained with strain C58C1RS(pArA4a)

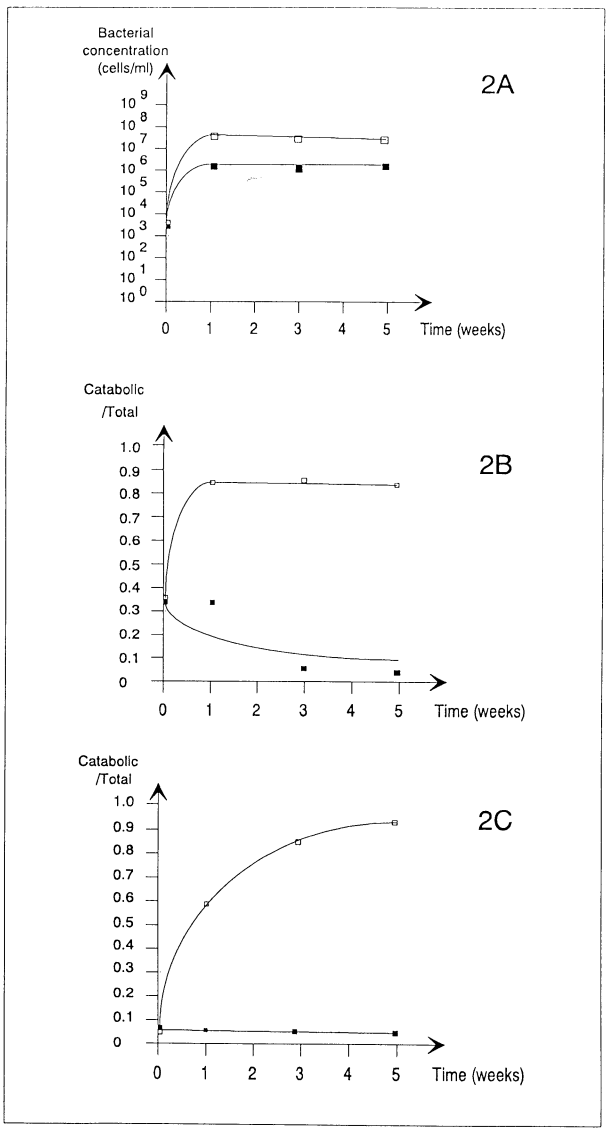

Fig. 2. Associated growth of strains C58C1RS and C58C1RS (pArA4a) with normal and transformed Lotus plants. Legends and symbols are as in Figure 1.

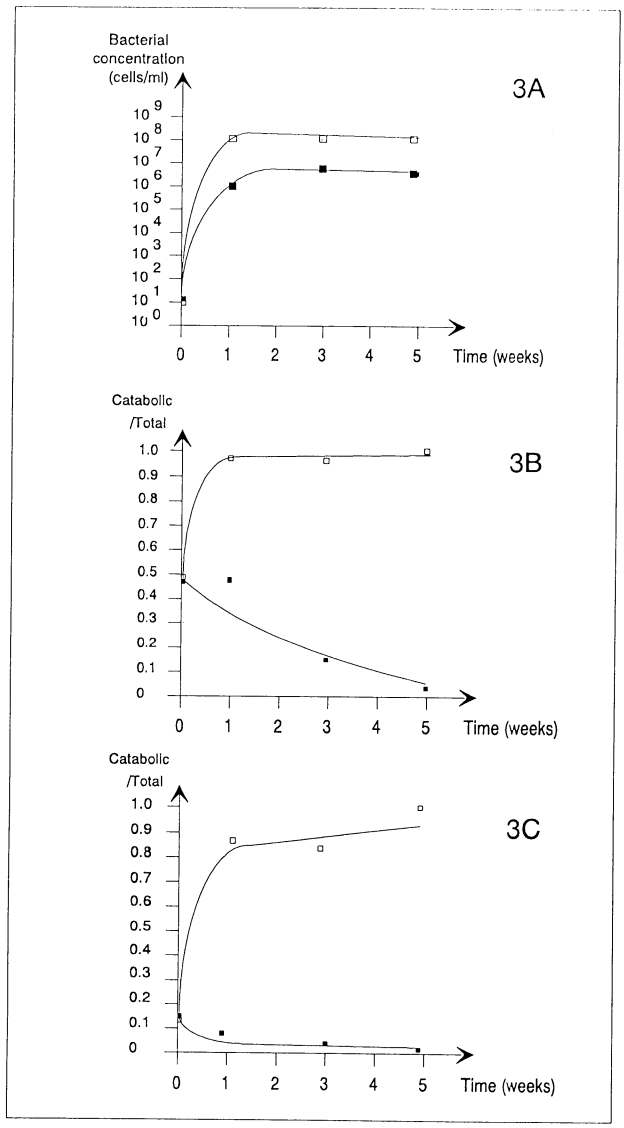

Fig. 3. Associated growth of strains C58C1RS and C58C1RS (pArA4a,b,c) with normal and transformed *Lotus* plants. Legends and symbols are as for Figure 1.

and C58C1RS(pArA4a,b,c,). Therefore, there is little doubt on the validity of the figures reported in this work, even though significant differences were observed in the number of cells counted (for example, approximately one order of magnitude between similar experiments performed with strains harboring pArA4a and pArA4a,b,c). These differences were not due to an increased bacterial growth in one out of the six or 12 tubes since the turbidities (appreciated by visual inspection) of the plant-bacteria growth media were always similar in each tube in a given experiment. Rather, since these experiments were done at different times, conditions may have been slightly different in the growth chambers (new light tubes, gradient of temperature in the room, etc.). However, because both catabolic and noncatabolic strains were inoculated to the same plants, any local perturbation due to the growth conditions (local temperature changes, local decrease of the light, etc.) affected both strains simultaneously. Therefore, the ratio of catabolic (c/c+nc) or pathogenic (p/p+np) to the total bacteria should not have been affected by these variations.

In this study on plant-bacteria associations, two phenomena were observed. First, the total bacterial populations were highest when the cells were grown in association with transformed *Lotus*-producing opines. The difference was about an order of magnitude, sometimes greater. The reason for this is unknown. However, in the experimental model used in this work, the carbon and energy sources came only from the root exudates of the plants. Therefore, this phenomenon must be related to the quantity and quality of these exudates. Although not investigated, transformed plants could produce more growth substrates for the agrobacteria. Consistent with this, the root system of opine-producing plants was always more developed than that of the normal plants.

The second observation was that transformed opine-producing plants stimulated growth of bacteria able to catabolize the opines. Especially, experiments performed with a mixture of strains composed initially of 10% catabolic agrobacteria showed that the proportion of these strains greatly increased to reach levels up to 99% after 4–5 wk of coculture with plants (Fig. 3B,C). Again, because both catabolic and noncatabolic strains were inoculated to the same plants, any local disturbances affected both strains simultaneously. Therefore the ratio c/c+nc should not have been affected by these variations. This result is of importance because it constitutes the first direct demonstration that a transformed, opine-producing plant environment specifically favors growth of catabolic agrobacteria. For all known *A. tumefaciens* strains, and for mannopine- and cucumopine-type *A. rhizogenes* strains, opine degradation and pathogenicity are physically linked functions, since they are both carried on the pathogenic plasmids. For these strains, the previous result therefore suggests that an opine-producing plant environment does specifically favor growth of pathogenic agrobacteria (Fig. 1A–C). Though obtained from experiments performed *in vitro*, our data support the biological description of crown gall tumors and hairy root formations as ecological niches promoting growth of virulent *Agrobacterium* strains.

Several hypotheses can be proposed to account for the specific growth stimulation of pathogenic agrobacteria. It could be suggested that pathogenic strains possess an increased capacity to colonize the rhizosphere of the plants by a mechanism independent of opine catabolism. Indeed, large parts of plasmids pRi8196 and pArA4 encode no known functions (Koplow *et al.* 1984; Huffman *et al.* 1984; Birot *et al.* 1987). This objection can be ruled out by the analysis of the results obtained with strain C58C1RS (pRi8196) which did not grow better than strain C58C1RS when both were associated with normal plants. The same is true for the two other catabolic strains used in this work.

It also may be proposed that transfer of Ri plasmids contributed to the increase of the population of pathogenic agrobacteria. Consistent with this, the conjugal transfer of the Ri plasmid from a pathogenic strain to a nonpathogenic recipient has been demonstrated (Petit *et al.* 1983). However, the magnitude of this contribution is difficult to estimate. Several arguments indicate, but do not prove, that it should be low. First, transfer of all these plasmids is inefficient *in vitro* (transfer frequency estimated as 10^{-6} per donor cell). Also, none of the mannityl opines produced by the transgenic *Lotus* (mannopine, mannopinic, and agropinic acids) induce the conjugal transfer of the plasmids used in this study. Possibly, these plants could produced opines from the agrocinopine family. However, the role of opines from this opine family in inducing the conjugal transfer of Ri plasmids has not been demonstrated. Consistent with this, experiments were performed to improve the transfer efficiency of plasmid pRiA4, in which the donor strain (A4) was pregrown in presence of hairy root extracts. No increase in the plasmid transfer efficiency was observed in these conditions (A. Petit, unpublished data). All of these facts suggest that plasmid transfer, although it may occur, cannot totally account for the increase of the ratio of catabolic cells observed when bacteria were grown in association with transformed plants.

Rather, opine production by plantlets and opine degradation by bacteria should create an environment favoring growth of catabolic bacteria. Mannityl opines were shown to accumulate at concentrations up to 30 mg/g dry weight of plant tissue and up to 50 mM in cocultivation medium (see Results). These results are consistent with those obtained in similar studies performed on carrot hairy root cultures (A. Petit, unpublished results) and on transgenic tobacco plants (Savka and Farrand 1992). That such a high concentration of opines is released in the medium supports the assumption that opine utilization plays a role in favoring specifically the growth of opine-degrading bacteria. Further support for this assumption comes from the analysis of the opine content of the growth medium in the presence of bacterial cells. In these conditions, only trace amounts of opines were detected. These results should be correlated with the frequent isolation of nonpathogenic, opine-utilizing isolates from various crown gall tumors (reviewed in Dessaux *et al.* 1992). As a consequence of the above results and discussion, there is little doubt that transformed opine-producing plants do specifically favor growth of opine-degrading bacteria.

To precisely evaluate the possibility to engineer other plant-bacteria associations, further studies should now focus on the role of opines in favoring growth of genetically

modified (opine-utilizing) rhizobacteria. Although opines are valuable chemical mediators, other molecules might be of interest for such studies. These include the callistegines, although genes responsible for synthesis of these molecules are not known (Tepfer *et al.* 1988). Opinelike compounds, such as 3-*O*-methyl scyllo-inosamine or rhizolotine detected in nitrogen-fixing nodules induced by various strains of *Rhizobium* (Murphy *et al.* 1987; Scott *et al.* 1987), could also be of interest, especially since genes involved in synthesis and degradation of 3-*O*-methyl scyllo-inosamine were precisely localized within the *Rhizobium* genome (Murphy *et al.* 1987; Murphy and Saint 1992).

MATERIALS AND METHODS

Bacterial strains and routine bacterial growth media and conditions.

Agrobacterium tumefaciens strain C58C1RS (devoid of a pathogenic plasmid) and transconjugants C58C1RS (pAr8196), C58C1RS(pArA4a), and C58C1RS(pArA4a,b,c) (Petit *et al.* 1983), are described in Table 1. ATMS medium is AT minimal medium (Petit and Tempé 1978) supplemented with mannitol (2 g/L) and ammonium sulphate (2 g/L). ATR is ATMS supplemented with 5 g/L of yeast extract (Difco, Detroit, MI). The selective medium ATMOA is AT minimal medium containing 8 mM mannopinic acid (MOA) and 2 g/L of ammonium sulphate. When necessary, these media were solidified using 16 g/L of Bacto Agar (Difco). Unless otherwise stated, all incubations were performed at 26–28° C.

Chemicals.

All chemicals were from commercial sources except the opines mannopine, mannopinic acid, and agropinic acid, which were synthesized in the laboratory from mannose and glutamate or glutamine according to previously published procedures (Petit *et al.* 1983; Dessaux *et al.* 1986).

Opine analysis.

The presence of opines in plants or in growth media was determined by high-voltage paper electrophoresis (HVPE) at pH 1.9, followed by silver nitrate staining, all as previously described (Trevelyan *et al.* 1950; Petit *et al.* 1983).

Plant material and plant growth conditions.

Lotus corniculatus 'Rodéo' plants (Petit *et al.* 1987) were grown *in vitro* on a modified Monnier medium consisting of half-strength Monnier salt solutions (Monnier 1976) and Morel and Westmore vitamin mixture (Morel and Westmore 1951). When indicated, this medium was supplemented with 20 g/L of sucrose and solidified using (per liter)1.0 of Gelrite and 0.75 g of Phytagel (Sigma Chemicals) or 7 g of agar. Plantlets were propagated *in vitro* as clonal material. They all derived from the same plant termed "Lotier A" originating from a *L. cornicultus* culture established by Shen *et al.* (1988). This plant was regenerated from a nontransformed root culture. The same "Lotier A" was inoculated with *A. rhizogenes* strain 8196 to produce transformed roots synthesizing the opines mannopine, mannopinic, and agropinic acids. Numerous opine-producing plants were regenerated from these roots as previously described (Petit *et al.* 1987). These plants were analyzed for their capacity to produce high concentrations of opines as indicated above, and for their growth and phenotypic properties (size and general shape of the plant, characteristics of the root system, etc.) by visual inspection. A plant resembling a nontransformed "Lotier A" but producing significant amounts of opines was chosen and propagated by cuttings on the modified Monnier medium supplemented with sucrose. Plant material was propagated at 22° C under a 16-hr light period.

Plant-bacteria associations.

One-centimeter-long cuttings of normal or opine-producing plants were placed in tubes (160 × 25 mm) containing 1-ml liquid Monnier medium supplemented with sucrose for about 15 days to allow stem elongation and root multiplication. Plants were washed briefly but thoroughly in distilled sterile water, and placed in 1 ml of Coïc-Lessaint (CL14) mineral medium (Coïc and Lesaint 1975). This medium, devoid of any carbon source, contained (per liter): 1 ml of macroelement solution, 1 ml of NaCl-microelement solution, and 1 ml of Fe-EDTA solution. The macroelement solution was made of (per liter): 184.5 g of $MgSO_4$, $7H_2O$; 492 g of $Ca(NO_3)_2$; 383.8 g of KNO_3; 160 g of NH_4NO_3; 5.5 g of $Mg(NO_3)_2$, 6 H_20, 136.1 g of KH_2PO_4; and 17.4 g of K_2HPO_4 (pH 5.5). The NaCl-microelement mix contained (per liter): 11.7 g of NaCl; 2 g of $MnSO_4$, $4H_2O$; 1.5 g of H_3BO_3; 0.25 g of $CuSO_4$, $7H_2O$; and 0.05 g $(NH_4)_6$ Mo_7 O_{24}, $4H_2O$. The Fe-EDTA was made of (per liter): 5.55 g of $FeSO_4$, $7H_2O$, and 7.45 g of Na_2-EDTA. Plantlets were left on this medium for 15 days before inoculation with the mixture of bacteria. Before inoculation, this medium was removed, and tubes and plants were washed briefly with sterile distilled water.

Bacteria were grown in liquid ATMOA (opine-utilizing strain) or on ATMS (C58C1RS strain). Small volumes (about 0.25 ml) of these cultures (10^8 cells per milliliter) were used to inoculate 10 ml of fresh CL14MSY medium, which consisted of CL14 mineral medium supplemented with (per liter) 2 g of mannitol, 2 g of ammonium sulphate, and 300 mg of yeast extract. Incubations were performed at room temperature. Exponential-phase cultures in CL14MSY medium were centrifuged (10 min; room temperature; 6,000 × *g*), and the cells were washed with and resuspended in fresh CL14 mineral medium to a concentration of 10^3 cells per milliliter. The root systems of the plants were immersed in 1-ml volumes of the bacterial suspension. Plants and bacteria were cocultivated in a growth chamber for up to 35 days at 22° C under a 16-hr photoperiod (without shaking). Evaporation of water was regularly compensated for by adding sterile distilled water to the growth medium.

Experiments were performed on series of six plants, each grown independently. Changes in the bacterial population were monitored by dilution and plating. To do so, 100 μl of plant-bacteria growth media was removed from each tube and immediately replaced by fresh sterile CL14 medium. The six samples were pooled and mixed to yield 600 μl of medium. Dilutions of this medium were performed in distilled sterile water. Volumes of appropriate

dilutions were spreaded on CL14MSY (see composition above) and CL14MOA plates which was CL14 mineral medium supplemented with 8 mM mannopinic acid, 1 g/L of ammonium sulphate, and 100 mg/L of yeast extract. All these media were solidified with 6.5 g/l of Gelrite. Bacterial population sizes were calculated by counting colonies and multiplying by the dilution factor.

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