Coordinated Regulation of Octopine Degradation and Conjugative Transfer of Ti Plasmids in Agrobacterium tumefaciens: Evidence for a Common Regulatory Gene and Separate Operons

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By using the analog noroctopine, mutants of Agrobacterium tumefaciens were isolated with altered regulation patterns for the Ti plasmid-borne octopine utilization genes. These could be divided into three classes: (i) strains with a constitutive level of octopine enzymes and a high degree of spontaneous Ti transfer; (ii) one strain with constitutive octopine enzymes but no spontaneous Ti transfer; and (iii) strains with an altered inducibility in which, contrary to the wild-type Ti plasmid, conjugation and octopine utilization were induced by noroctopine. These results are best explained by the activity of a common regulatory gene. In a second step, using homo-octopine, mutants were isolated with lesions preventing the utilization of octopine. All mutations were plasmid borne and did not prevent the induction of tumors. Plasmids of two isolates were characterized by large deletions resulting in a decreased virulence and the absence of octopine in the tumor. With a plasmid carrying an inserted transposon Tn1, a significant number of strains were isolated which were unable both to utilize octopine and to transfer the Ti plasmid. This suggests that there may be another common factor—presumably positive—between these traits. Transfer-negative mutants were still virulent. This seems to exclude a role for the conjugative transfer during the process of plant tumor induction. A way to test octopine oxidase by the use of permeable cells is described.

The Ti plasmids of Agrobacterium tumefaciens have been shown to be involved in the process of crown gall tumor formation on dicotyledonous plants (2, 34, 37). These large plasmids (molecular weight above 10^9) determine (2) whether a tumor will contain octopine [N^2-(D-1-carboxyethyl)-L-arginine; Fig. 1; 22] or nopaline [N^2-(1,3-dicarboxypropyl)-L-arginine; 8]. The presence of a part of the Ti DNA, called the T DNA region, and its transcription in tumor tissue has been demonstrated (4, 6). At present there is no conclusive evidence showing whether this T DNA region contains structural genes for an octopine-synthesizing dehydrogenase or if it only triggers the synthesis of such an enzyme coded by a gene from plant origin.

Ti plasmids, which control octopine production in the tumor, confer upon their bacterial host the ability to degrade octopine to arginine and pyruvate and thereby allow utilization of this compound as a sole source of nitrogen (20, 26). Evidence both from biochemical (12, 19; G. H. Bomhoff, Ph.D. Thesis, University of Leiden, 1974) and genetic experiments (16, 23) suggests that octopine synthesis in the tumor and octopine degradation in the bacterium are reactions catalyzed by different enzymes whose synthesis is controlled by distinct plasmid genes. The enzymes involved in octopine utilization are inducible by the substrate (12, 17). The conjugative transfer of Ti plasmids in planta (13, 14, 35) and ex planta (2, 7, 14) has been reported extensively. In the latter circumstances transfer is highly stimulated by the presence of octopine. This suggests that the transfer of the Ti plasmid by conjugation is controlled coordinately with the degradation of octopine. Noroctopine (Fig. 1; 27), an analog of octopine, was used to isolate regulatory mutants (A. Petit and J. Tempé, unpublished data). Petit et al. (28) reported that some of these mutants have indeed an increased level of transfer.

In a previous report (17) we postulated that the utilization of octopine consists of four components: (i) a regulatory system, (ii) a specific permease, (iii) an oxidase responsible for the conversion of octopine into arginine, and (iv) degradation of arginine by catabolic enzymes. It is likely that the first three components are coded for by Ti plasmid-borne genes. This has
been demonstrated for the permease gene (uadB) and the regulatory gene (uadR) (17). Because plasmidless strains can degrade arginine, the fourth component is coded for by chromosomal genes. It cannot be excluded, however, that the Ti plasmid codes for an additional arginine-degrading system.

The main objective of this work was (i) to establish whether the utilization of octopine is genetically related to the conjugal Ti transfer by the functioning of a common regulatory system and (ii) to test whether there is any relationship between the genes involved in octopine metabolism and tumor induction. The work was based on the analysis and isolation of mutant Ti plasmids with lesions either in regulatory functions or in structural genes.

A method has been developed to test octopine oxidase in permeable cells. Together with the existing octopine permease test (17), this oxidase test enabled us to distinguish for the first time between the different types of Uad⁻ mutants.

Formerly the induction of mutations, resulting in the loss of the capability to utilize octopine, implicated the abolishment of the sole selectable marker. The isolation of Ti plasmids carrying inserted transposons (P. J. J. Hooykaas et al., unpublished data) overcame this difficulty. Such a plasmid carrying transposon Tn1 (9) was used in this study to see if mutations in octopine genes would affect conjugal transfer. Ti plasmids were isolated that were unable to promote their own transfer to other bacteria. Virulence was tested to see if the conjugation mechanism may also be involved in the transfer of the Ti DNA to the plant cell during tumor induction, as was assumed by Tempé et al. (33).

**MATERIALS AND METHODS**

**Bacterial strains.** In this study we used mainly the Ti plasmid carried by *A. tumefaciens* Ach5. This strain was received in 1974 from R. H. Hamilton, Pennsylvania State University. Based on restriction endonuclease fingerprints, the plasmid seems to be identical to the Ti plasmid of strain B6S3 (31). The plasmid pAL657 was derived from Ti-B6S3 by P. J. J. Hooykaas in our laboratory by a procedure to be described. It is constitutive for transfer as well as octopine degradation and carries Tn1 (9) inserted in Sma restriction fragment 5 without any known phenotypic traits affected. During its history this plasmid has been treated with UV, mitomycin (36), and nitrosoquainidine (2), and may thus carry hidden mutations. All strains and plasmids are listed in Table 1. All incubations were carried out at 29°C.

**Isolation of cointegrate plasmid RP4:** Ti. Following the procedure of Schell et al. (31), LBA57 was mated on rich medium (beef extract) with LBA101. After 18 h the bacteria were resuspended, washed with 0.9% (wt/vol) NaCl, and plated on synthetic medium with octopine as the sole source of nitrogen to select for the Ti plasmid and 1 mg of tetracycline per liter to select for RP4. Of 100 Uad⁻ Tc⁻ recipient colonies tested, 9 were positive for 100% cotransfer of both plasmids to LBA112. Sedimentation in sucrose gradients revealed that these strains harbored a plasmid cointegrate. They were virulent. One of the cointegrate plasmids was transferred to LBA67. The resulting strain (LBA89) was used as a donor in the experiments described here and could be counterselected by omitting its amino acid requirements. Segregation into the two separate plasmids was less than 1%.

**Conjugation conditions.** Logarithmically growing cultures were concentrated to $E_{\text{opt}} = 0.8 (2 \times 10^9$ organisms per ml) and mixed 1:1. A 0.1-ml sample was pipetted onto a membrane filter (0.45-µm pore size, Millipore Corp.) placed on the conjugation medium. With cointegrate plasmids the mating time was 20 to 24 h on LC medium; with Ti plasmids it was 46 to 48 h on SM medium. In the latter conditions it was necessary to add 1 mg of MnSO₄ per liter. In most Ti conjugations, LBA4011 (Rif⁻) was the recipient. When the donor was Rif⁺ as with LBA4014 and its derivatives, LBA4009 (Nal’ Str’) was used. When octopine was added to induce Ti transfer, the concentration was 2 g/liter. After mating, the filter was removed from...
the plate, and the bacteria were resuspended in 5 ml of 0.9% (wt/vol) NaCl and, after appropriate dilution, plated on selective media.

**Media.** The composition of the synthetic medium (SM) has been described (15). When this medium was used to select octopine-utilizing bacteria, \((\text{NH}_4)_2\text{SO}_4\) was omitted and replaced by 0.1 g of octopine per liter, the phosphate concentration was increased five times, and plates were solidified by the addition of 1.8% (wt/vol) agar (Difco) that had been washed with 50 times its weight of redistilled water. Selection of pAL657 and its derivatives was on SM with 10 mg of carbenicillin per liter. LC was used as a rich medium (16).

**Isolation of mutants.** When selecting noroctopine-utilizing bacteria, this compound was added as the carbon source (2.5 g/liter) to SM instead of glucose; 5 \(\times\) 10\(^6\) bacteria were spread per plate. After 3 weeks or longer, colonies were picked and purified. The isolation of mutants containing plasmids with lesions preventing octopine utilization was possible (Tempé, unpublished data) by the spreading of 10\(^8\) cells of a sensitive constitutive strain on an SM plate with 2.0 g of homo-octopine per liter. Resistant colonies were suspended and streaked on SM with developing colonies tested for octopine utilization. Nonutilizers were purified and rechecked. When mutants were derived from pAL657, purification was on SM with 10 mg of carbenicillin per liter.

**Testing of octopine permease and oxidase.** Determination of permease activity has been described (17). Octopine oxidase was tested after tolune treatment (24) as follows: the bacteria (induced with 0.1 g of the substrate per liter, when necessary) were cultured in SM, cooled quickly, harvested by centrifugation at 3,000 \(\times\) g for 20 min (4°C), and washed twice with cold 0.02 M KH\(_2\)PO\(_4\) (pH 7.2). After resuspension in cold 0.2 M KH\(_2\)PO\(_4\) (pH 7.2) at 10\(^{10}\) bacteria per ml, MgSO\(_4\) was added to a final concentration of 2 \(\times\) 10\(^{-3}\) M.

A tolune suspension was prepared by sonic disruption for 30 s of 1 ml of 0.2 M phosphate buffer at 4°C to which 20 \(\mu\)l of tolune (spectroscopic grade) had been added. When the tolune suspension formed an emulsion, 1 ml of the bacteria was added, and the mixture was gently swirled for 5 min. The suspension was subsequently diluted with 18 ml of buffer at 4°C and centrifuged at 2,000 \(\times\) g in the cold. The supernatant was carefully decanted, and the walls of the tube were cleaned with a tissue to remove residual tolune. The pellet was resuspended in 0.4 ml of 0.05 M glycine buffer (pH 9.4). For the final test, 5 \(\mu\)l of a \([^{3}H]\) octopine stock (8.5 \(\times\) \(10^{-4}\) M) was added to 25 \(\mu\)l of tolune-treated cells. After 8 min of incubation at 25°C, the reaction was stopped by the addition of 3 \(\mu\)l of concentrated acetic acid.

A 5-\(\mu\)l sample of the test was spotted on MN214 electrophoresis paper (Mackery, Nagel & Co.) and subjected to electrophoresis in formic acid-acetic acid-water (11:30:159, vol/vol) for 30 min at 15 V/cm (Bomhoff, Thesis). Cold tolune and arginine were included as references together with 1 \(\mu\)l of a solution of methyl green as a visible marker. After the run the paper was air dried and cut into strips of 2 cm parallel to the direction of electrophoresis. Radioactive strips were cut into square pieces of 0.5 cm. The paper pieces were eluted with 1 ml of water for 2 h, then 8 ml of a scintillation cocktail (Hydrolum; Lumac) was added to determine the \(^{3}H\) counts. Strips with reference standards were stained with phenanthrenequinone reagent (38). Fluorescent spots were correlated with the labeled samples.

**Determination of octopine and noroctopine in medium.** The utilization of octopine or noroctopine was tested in several experiments by determining the
concentration of these compounds in the medium after incubation of the bacteria. After 1-ml samples were centrifuged for 2 min in an Eppendorf table centrifuge, 0.5 ml of the supernatant was stained with α-naphthol diacetyl reagent (29). Extinction at 535 nm was measured with a Zeiss spectrophotometer. In every experiment a reference line was made with standard dilutions of octopine. Sometimes samples were stored at −20°C after removal of the bacteria by centrifugation.

Virulence tests. Virulence tests were performed mainly on Kalanchee daigremontiana (32), because the reaction of Helianthus annuus with Aeh5 and derivatives was rather weak and irreproducible.

Determination of octopine in tumors. Tumors were extracted as described by Schilperoort and Bomhoff (32). Dithioerytrol (10⁻¹ M) was added to prevent oxidation of polyphenols. Paper electrophoresis was carried out as described by Bomhoff (Thesis).

Isolation of octopine-utilizing revertants. Octopine-utilizing revertants were isolated by plating 2 × 10⁶ washed bacteria on SM medium containing 0.1 g of octopine per liter as the sole source of nitrogen. Incubation was for at least 7 days.

Chemicals. All salts were of standard analytical grade. Part of the noroctopine was a generous gift of J. Tempé, Versailles, France. Homo-octopine was synthesized from L-homoarginine (Koch-Light) and 2-hromopropionic acid (Merck, zur synthese) following the procedure of Bomhoff (Thesis). The stereoisomers were not separated. The synthesis of [³H]octopine from L-[¹⁴C]arginine has been described (16). O-[¹⁺⁺]Octopine was obtained from Sigma. Oxytetracycline was from Mycofar, Delft. Carbenicillin (Pyopen) was from Beecham. Rifampin was a gift of Ciba-Geigy. Toluene was Uvasol (spectroscopic) from Merck.

Symbols. Uad' is used to designate strains that are unable to utilize the amino acid derivative octopine but are unaffected in the utilization of arginine. These mutants are unable to degrade lysopine and octopinic acid (16). Tra' is used to describe a strain that is unable to promote the transfer of its Ti plasmid even under appropriate conditions. Uad' refers to constitutive octopine degradation. Uad' inducible degradation. Tra' designates constitutive conjugative transfer, Tra' inducibility. Arg', Met', and Lle' indicate requirement for arginine, methionine, and isoleucine, respectively. Rif' means resistance against 20 mg of rifampin per liter, Nal' resistance against 50 mg of nalidixic acid per liter, and Str' resistance against 500 mg of streptomycin per liter.

RESULTS

Testing of octopine oxidase activity. The membrane-bound octopine oxidase (12) of A. tumefaciens plays an essential role in the degradation of octopine by the oxidation of this compound into arginine and pyruvic acid. Studies of the regulation of the octopine genes require a simple and reliable system to test the oxidase activity of a great number of strains. The data obtained in this way may complete the results from permease activity tests. Jubier and Lejeune (12) have described a test system based on the incubation of membrane fractions with an artificial electron acceptor and lysopine as the substrate. Lysopine is not commercially available, and octopine showed a reaction independent of added enzyme preparations. In addition to these problems, the method is laborious, and preparations lacking any activity were frequently obtained. Therefore we developed the procedure described in Materials and Methods. The measured conversion of [³H]octopine to [³H]arginine was dependent on induction and on the presence of the Ti plasmid (Fig. 2). In a mutant which is constitutive for permease activity, LBA4017, oxidase activity was present without previous incubation in octopine medium. The permeability of the cells was shown by (i) a drop in the viable count of the tolueene-treated bacteria, (ii) the inability to fix any [³H]octopine in the permease test, and (iii) the fact that mutants that lack permease activity converted octopine as quickly as wild types when similarly treated. The optimal activity was at pH 9.0 to 9.5 (Fig. 3). At pH 9.4 the Kₘ was about 7 × 10⁻⁴ M. The formation of arginine was linear for a least 10 min.

In control experiments with cold octopine, we could detect pyruvate as the other reaction product. The incubation mixture was heated for 10 min to inactivate enzymes; after the addition of NADH and lactate dehydrogenase, the presence of pyruvate was detected by a significant decrease in the absorption of the cofactor at 340 nm.

The measured oxidase activity was not only induced in Aeh5 by octopine (Fig. 2) but also by lysopine and octopinic acid, related compounds that are also found in the tumor (1; A. Goldmann-Ménage, Thesis, Université de Paris, 1970). The latter compounds were able to compete with [³H]octopine conversion (data not shown). This was expected because it has already been shown that these compounds share a common degradation system with octopine (16, 17).

Isolation of noroctopine-utilizing mutants. To study the effect of regulatory mutations on Ti transfer, noroctopine (Fig. 1) was used to isolate mutants constitutive for the octopine enzymes.

If noroctopine was supplied as the carbon source, instead of glucose, growth was extremely slow, but on plates with strain Aeh5 small colonies were detectable after incubation for several weeks. Frequency was less than 10⁻⁶. Of 14 colonies tested, 6 were found positive for constitutive synthesis of octopine permease and octopine oxidase.

The eight remaining strains were not consti-
REGULATION OF Ti PLASMIDS IN A. TUMEFACIENS

Fig. 2. Determination of octopine oxidase activity in toluene-treated cells (see the text). (a) LBA4011, a plasmidless strain; (b) Ach5 without previous induction by growth with octopine; (c) Ach5 with induction; and (d) LBA4017, a constitutive mutant without induction. The arrows indicate the reference samples of octopine (O) and arginine (A).

Constitutive but were, contrary to the wild type, inducible by noroctopine (Fig. 4). In some isolates homo-octopine also acted as an inducer. The strains that were induced by homo-octopine were sensitive to this compound. A regulatory gene product with an altered substrate specificity seemed to be present. This was confirmed by the induction of Ti transfer by noroctopine in this type of mutant (Table 2). The transfer was not stimulated by homo-octopine, as expected from the toxic effect on the donor bacteria.

When noroctopine was supplied as the nitrogen source with Ach5 and B6S3 and as the arginine source with Arg- mutants, all utilizing isolates were of the noroctopine-inducible type.

The plasmidless strain Ach5-C3 was never able to utilize noroctopine under any conditions. **Constitutive mutants.** Six strains isolated from plates with noroctopine as the carbon source were constitutive for the octopine degradation enzymes. The activity of the permease and the oxidase (Fig. 2) was about three times higher than in wild-type strains induced with 0.1 g of octopine per liter. This suggests that in induced cultures the expression of the involved genes was only one-third of the maximal possible level. The high activity in constitutive strains was probably the reason for the increased sensitivity to homo-octopine. At the same time this facilitated growth with noroctopine and octopine as the carbon source, because under these conditions maximal expression is needed to supply the cell with carbon and energy. The mutation causing the derepressed state of the octopine enzymes was Ti plasmid borne, as was shown by transfer experiments.
These six strains were tested for spontaneous transfer of the Ti plasmid. Five out of this set were able to transfer the plasmid in the absence of octopine at a frequency of $10^{-1}$ to $10^{-2}$. The other strain, LBA4017, behaved like the wild-type Ach5: in the absence of inducer transfer was lower than $10^{-7}$, whereas in the presence of octopine (2 g/liter) it was as high as $10^{-1}$. These classes of mutant plasmids must be comparable to those found by Petit et al. (28). Schell and van Montagu (30) and Hooykaas (unpublished data) have isolated mutants constitutive for transfer without being constitutive for the octopine enzymes. All these mutants fit well in a regulation model consisting of two separate operons, a $uad$ operon with genes for octopine permease and octopine oxidase (17) and a tra operon containing all the genes coding for transfer functions like that described for the F plasmid of Escherichia coli (10), both controlled by a common regulatory gene. In this way it is possible to explain the constitutiveness of a single trait by an operator mutation and the constitutiveness of both traits by a regulator mutation. This model has recently been proposed also by another group (28).

**Isolation of mutants unable to utilize octopine.** Homo-octopine had a toxic effect on A. tumefaciens strains constitutive for octopine-degradative enzymes. This effect must be due to the presence of octopine oxidase, because uninduced cultures are resistant (Fig. 5), as are oxidaseless mutants. The fact that A. tumefaciens is not sensitive to homo-arginine might be explained by the assumption that the uptake of homo-arginine is very low. With constitutive

![Graph](image)

**Fig. 3.** Determination of optimal pH value for octopine oxidase activity in toluene-treated cells of strain LBA4012. The incubation was with different buffers: ○, 0.02 M phosphate; ●, 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride; □, 0.05 M glycine-NaOH.

![Graph](image)

**Fig. 4.** Determination of the induction of octopine permease activity by different substrates. (a) Strain Ach5; (b) LBA4016, a strain with altered inducibility. Symbols: ○, no substrate added; ●, arginine; □, octopine; ■, noroctopine; △, homo-octopine.

**Table 2.** Induction of transfer of the Ti plasmid with a wild-type donor (Ach5) and a mutant with altered inducibility (LBA4016).  

<table>
<thead>
<tr>
<th>Added inducer</th>
<th>Transfer frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LBA4011 x Ach5</td>
</tr>
<tr>
<td>None</td>
<td>$5.5 \times 10^{-5}$</td>
</tr>
<tr>
<td>Arginine</td>
<td>$2.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>Octopine</td>
<td>$2.9 \times 10^{-5}$</td>
</tr>
<tr>
<td>Noroctopine</td>
<td>$3.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>Homo-octopine</td>
<td>$1.5 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

*Conjugation was allowed for 44 h. The concentration of the added inducer was 0.5 g/liter.
mutants homo-octopine can be used to select for Uad\(^-\) bacteria (Tempé, unpublished data). If octopine permease as well as the oxidase is essential for both octopine utilization and sensitivity to homo-octopine, it is conceivable that homo-octopine-resistant Uad\(^-\) mutants would have lost one or both of these functions. The isolation of resistant mutants that are Uad\(^+\) is conceivable from (i) reversion to the repressed state, (ii) resistance due to an overproduction of arginine, and (iii) resistance caused by a mutation modifying the cellular site where homo-arginine exerts its effects. The latter two possibilities appear tenable from data on canavanine-resistant mutants of *E. coli* (21).

LBA4017 and LBA4012 were used to isolate homo-octopine-resistant mutants. With LBA4017 most resistant colonies were indeed Uad\(^+\), but with LBA4012 many were Uad\(^-\). This might be due to a higher reversion to Uad\(^+\) of LBA4012. All 65 Uad\(^-\) strains tested grew well on arginine as the nitrogen source. The strains still exhibited the Ti-coded property of the exclusion of phage AP1 (35). This is remarkable, since it is known that Ach5 can lose its plasmid by growth at an elevated temperature. Nevertheless, loss of the Ti plasmid at 29\(^\circ\)C may be so low that even by this selection technique no plasmidless strains were isolated.

After introduction of the co-integrate plasmid RP4: Ti by conjugation with LBA89, all strains were again Uad\(^+\). The only possible conclusion is that all Uad\(^-\) mutants isolated up to now, including those reported (16), carry mutations on the Ti plasmid. Only 9 of the 65 strains took up \(^{[\text{H}]\text{octopine}} in the permease test. These must have been mutants with a lesion preventing octopine oxidase activity. This was a rather low frequency, because among the mutants isolated formerly (16) the majority were oxidase mutants.

It was possible to prove that oxidase and permease are essential for octopine utilization (Fig. 6 and 7). Mutant LBA4059, missing oxidase, was Uad\(^-\), whereas LBA4031, missing permease, behaved in an identical manner. Both mutants were isolated primarily as homo-octopine-resistant colonies. This means that selection on homo-octopine is not exclusive for one of both types. About 40% of the mutants were able to revert to Uad\(^+\), indicating that they carried point mutations. All strains were virulent, showing a decoupling of both characters. When the tumors were extracted, in all except two tumors octopine was found. These two exceptions LBA4018 and LBA4019 were among three bacterial strains that reproducibly induced weak tumors on *K. daigremontiana* (Fig. 8). This was in contradiction to previous reports (16, 23) demonstrating that the loss of the capacity to utilize octopine did not result in the absence of octopine in the tumor. At that time these findings provided the reason to conclude that octopine synthesis is coded by genes other than the *aad* genes. This hypothesis could only be maintained by the assumption that the two Ti plasmid strains that induced tumors without octopine had suffered a deletion resulting in the loss of the *aad* genes as well as the genes responsible for octopine synthesis in the plant.

![Graph](image1.png)

**Fig. 5.** Growth of wild-type strain Ach5 and a constitutive derivative LBA4012 in the presence of 2 g of homo-octopine per liter. ○, Ach5 without homo-octopine; ●, Ach5 with homo-octopine; □, LBA4012 without homo-octopine; ■, LBA4012, with homo-octopine.

![Graph](image2.png)

**Fig. 6.** Growth of diverse mutants with octopine (0.2 g/liter) as the sole nitrogen source. ○, LBA4014, a constitutive mutant; ●, LBA4011, a plasmidless strain; □, LBA4031, a mutant strain lacking permease activity; ■, LBA4059, a mutant strain lacking oxidase activity.
Work to be published separately in detail (B. Koekman, G. Ooms, P. M. Klapwijk, and R. A. Schilperoort) demonstrated that this was in fact the case. In plasmids of both strains, LBA4018 and LBA4019, a 35 to 40% deletion is present (Fig. 9). By using several restriction endonucleases it could be shown that these plasmids lost a piece of DNA to the right of the T region (5) and even a piece of the T region itself. So it is possible that the genes that control octopine synthesis are located in the right of the T region.

**Mutant plasmids derived from pAL657.** While using LBA4012 and LBA4017 it was not possible to test what the influence of an Uad phenotype should be on the transfer properties of Ti plasmids. Octopine utilization was the only selectable trait coded by the plasmid. Recently, Hooymaas (submitted for publication) succeeded in the isolation of a Ti plasmid Uad' Tra' carrying a transposon, Tn1, conferring it with carbenicillin resistance (9). We isolated Uad' mutants from strain LBA4014 harboring this plasmid, pAL657. The strains isolated via homo-octopine selection were analyzed with respect to reversion to Uad−, character of the Uad mutation, transfer properties, and virulence. These properties are summarized in Table 3.

It was found that a certain number were avirulent. This was surprising in view of the results described in the preceding paragraph. This might be a consequence of the induction of deletions by Tn1, a well-known property of transposons (18, 25). An analysis of the DNA of these avirulent mutant plasmids by restriction endonuclease treatment showed that they carried deletions starting in that piece of the plasmid where Tn1 had been localized (Koekman et al., submitted for publication). The remaining strains were of interest because the main part of them did not show Ti transfer in spite of extensive trials. For nonreverting strains, such behavior was not strange because deletions could be present showing the loss of both properties. As long as the structural genes coding for transfer and Uad functions are not exactly localized, such deletions cannot be excluded. With revertible strains that were not able to accomplish Ti transfer, the situation was different, because here one mutation was preventing both the activity of the octopine enzymes and the transfer of the Ti plasmid.
Fig. 9. Agarose gel electrophoresis of plasmid DNA from strains LBA4018 and LBA4019 digested with restriction endonuclease Sma from Serratia marcescens. Control was the plasmid DNA of strain Ach5 (Koekman et al., submitted for publication).

Table 3. Properties of Uad− mutants derived from LBA4014 harboring plasmid pAL657

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Independent isolates</th>
<th>Reversion to Uad+</th>
<th>Permease activity</th>
<th>Oxidase activity</th>
<th>Ti transfer</th>
<th>Virulence</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>Deletion, T region losta</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Deletion, T region conserveda</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>No visible deletiona</td>
</tr>
<tr>
<td>21</td>
<td>8</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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</table>

a Details to be published separately (Koekman et al.).

The nature of this mutation remains an intriguing question. A mechanism like superrepression—a decreased affinity of a repressor for the substrate—might be functioning. It is important, however, to remember that the mutants were derived from a strain that was already Uad−Tra+ and presumably had no active repressor. To explain this type of mutation by superrepression, one has to assume two independent mutations involving the repressor gene occurring at the same time, one affecting the operator binding site and the other affecting the substrate binding site. Furthermore, one would expect that by use of higher concentrations of octopine the superrepression would be overcome. In six independent strains tested for induction of octopine permease activity in the presence of 2 g of octopine per liter, no induction was observed. Induction of octopine permease in wild types is already visible after growth with 0.01 g/liter (17). Increase of the concentration of the inducing substrate by factor of 200 did not have any effect, making superrepression improbable. The mutation was not induced by reinsertion of TnI because Uad− revertants were still Cb−.

Discussion

The octopine oxidase activity test using permeable cells treated with toluene has allowed us to distinguish between the different types of Uad mutants. Because mutants devoid of octopine permease activity degrade octopine as quickly as the wild-type bacteria when tested in this way, the assay appears to be independent of bacterial transport processes. The result of this novel test reflects therefore only the presence of octopine oxidase.

With the homo-octopine selection method, both permease and oxidase mutants were isolated. This shows that both functions are essential to maintain the Uad+ phenotype. It also demonstrates that the technique is not selective for one type of mutant. The results presented in a previous paper (16) showed that by using a replica-plating method mainly oxidase mutants were isolated.

All the uad mutations isolated could be localized on the Ti plasmid. This strengthens the evidence that all genes that govern the bacterial conversion of octopine into arginine are located on the Ti plasmid.

The results described, especially those with regulation mutants, are all in agreement with a model invoking two Ti plasmid-located operons, one (uad) coding for the octopine-degrading enzymes permease and oxidase and the other (tra) coding for the transfer functions. These appear to be coordinately controlled by a common regulatory gene. The mutant Ti plasmids with altered inducibility demonstrated the value of this model, which has also been proposed by Petit et al. (28).

It remains to be proven that the product of the regulatory gene is a repressor, a negative
control factor. Nevertheless, this is the simplest method to explain the appearance of mutant plasmids constitutive for two separate operons. The mutants with only one of both functions constitutive may be operator mutants. Since incompatibility phenomena prevent the construction of strains diploid for the uad genes, dominance patterns cannot be determined. We are currently developing methods to transpose the uad genes to a replicon compatible with Ti to make such complementation analysis possible. In addition, it is important to determine the nature of the mutation that prevented the transfer as well as the octopine degradation. It is possible that there is another common factor needed for transcription of both operons. This may be a common operator-promotor region as present in the argECBH cluster of E. coli (11) or a gene product. Another possibility is that the regulatory gene product exerts both positive and negative control, like the araC gene product in E. coli (3).

It is conceivable that regulation is not only exerted on the uad and tra genes but also on unknown Ti gene functions influencing virulence. In this consideration the regulatory gene behaves as a master gene controlling the transcription of nearly the complete plasmid. This mechanism is supported by recent experiments in our laboratory where bacteria with derepressed plasmids (Uad+ Tra-) appear much more efficient in transforming plant cells in vitro than wild-type bacteria (Marton et al., Nature, in press).

Our results confirm former data (16, 23) indicating that the genes coding for octopine degradation are not essential for the process of tumorigenesis. Even large deletions resulting in the loss of all Uad functions did not prevent tumor induction. Since tumors induced by bacteria harboring these deleted plasmids contained octopine, the genes controlling octopine synthesis were thus located outside the missing region. This is supported by the physical mapping of deletion mutations (Koekman et al., submitted for publication), which showed that the octopine utilization genes are located to the right of the T region (5), whereas the octopine synthesis genes must be located in the right part of the T region.

It is remarkable that all isolated Tra- mutants with an intact T region (Table 3) were virulent. This indicates that the functioning of the conjugative mechanism responsible for the transfer of the Ti plasmid from bacterium to bacterium may not be responsible for the transfer of the plasmid to the plant cell during tumor induction as was suggested by Tempe and co-workers (33).

The mechanism whereby the bacterium introduces the T-DNA into the plant cell remains unclear.

Schell and van Montagu (30) proposed that the piece of Ti DNA that is present in transformed plant cells behaves like a transposon. One characteristic of a transposon is its discrete structure with its ends involved in the transposition process (18). It is conceivable that the insertion of the T region is governed by a mechanism comparable to the jumping of transposons. The deleted plasmids carried by LBA4018 and LBA4019 do not fit into this hypothesis. The tumor induced by these bacteria do not contain octopine and are missing a part of the T region (Fig. 9) (Koekman et al., submitted for publication). This means that there are no genes essential to tumorigenesis in the lost piece of DNA. The suggestion that a transposon-like structure is improbable is further supported by biochemical evidence that the right end of the T region may vary (Ledeboer, unpublished data).

The two deleted-plasmid mutants reported here make clear that octopine synthesis plays no essential role in tumor formation by octopine type Ti plasmids. There are reports of some atypical natural isolates of A. tumefaciens which induce tumors without octopine or nopaline (23, 26), but it is conceivable that these tumors contain other unknown tumor-specific compounds.

The growth of tumors induced by LBA4018 and LBA4019 was rather weak. It remains to be seen whether this decrease was due to the absence of octopine or to the abolishment of other genes in the T region.

Whole regions of the Ti plasmid are not essential for tumor induction. This might be as much as 40% (LBA4018) to the right of the T region. We are currently developing methods to select for deletions on the left side to monitor the effect on virulence.

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LITERATURE CITED
