Spontaneous Transfer of the Ti Plasmid of *Agrobacterium tumefaciens* and the Nopaline Catabolism Plasmid of *A. radiobacter* Strain K84 in Crown Gall Tissue

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ABSTRACT

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Spontaneous transfer of the Ti plasmid from *Agrobacterium tumefaciens* to strain K84 of *A. radiobacter* was observed and studied for the first time in an experiment on biological control of crown gall. This transfer was detected in a tumor from a K84-treated plant grown in soil inoculated with a nopaline strain of *A. tumefaciens* biovar 1 sensitive to agrocin 84. The transconjugant strain was virulent and produced agrocin 84. Southern blot hybridization analysis with several probes (T-DNA and right adjacent regions and *vir* genes) showed important changes at the Ti plasmid, suggesting that recombination between Ti plasmid and pAtK84b in K84 could have happened, resulting in a new Ti plasmid. Transfer of both plasmids of strain K84, pAtK84b and pAgK84, responsible for nopaline catabolism and agrocin 84 production, respectively, to *A. tumefaciens* also was detected in isolates from the same tumor. Southern blot hybridization of plasmids from one of these avirulent isolates with a nopaline plasmid-specific probe of strain K84 indicated there was a replacement of Ti plasmid by pAtK84b in *A. tumefaciens*, explaining its avirulence. These results show that plasmid exchanges can occur spontaneously between *A. tumefaciens* and *A. radiobacter*. This kind of transfer generates genetic diversity in *Agrobacterium* and may influence the biocontrol efficiency of *A. radiobacter*.

The Ti plasmid of *Agrobacterium tumefaciens* is responsible for induction of crown gall, a neoplastic disease in plants. During infection, a specific region of Ti plasmid, the T-DNA, is transferred to plant cells and integrated into plant nuclear DNA. Transfer of T-DNA is due to the activity of plant-inducible virulence genes (*vir*) located in the virulence region of Ti plasmid (23). The subsequent expression of T-DNA genes in a plant leads to the formation of tumors (through synthesis of auxin and cytokinins) and to the production of opines, low molecular weight metabolites that can be degraded by the inducing agrobacteria. Genes responsible for opine uptake and catabolism are located on Ti plasmid (32). Several opines, called conjugal opines, induce catabolism, as well as the conjugal transfer of Ti plasmid to bacterial recipients (13). Conjugation of nopaline-type Ti plasmids is induced by the sugar phosphate opines agrocinopines A and B (8), whereas octopine induces transfer of octopine-type Ti plasmid (22).

Transfer of Ti plasmid to strains of *A. radiobacter* was achieved in planta by coinoculating tomato plants with donor and recipient strains (18). In vitro experiments with *Agrobacterium* donors failed to transfer Ti plasmid at appreciable frequencies (12). However, transfer frequencies increased with either RP4, a promiscuous plasmid for mobilization of Ti plasmid (3), or by preincubation of the donor with the appropriate conjugal opines (8,21,33).

Plasmids largely homologous to Ti plasmid have been found in several nonpathogenic *Agrobacterium* strains, such as *A. radiobacter* strain K84. This strain contains plasmid pAtK84b (39), which has large areas of homology (over 50%) with the Ti plasmid of *A. tumefaciens* strain C58 (5). Plasmid pAtK84b may be a deletion product of a pTiC58-type plasmid that has been disarmed in the T-DNA and *vir* regions (5), leading to a loss of oncogenicity; pAtK84b belongs to the same incompatibility group as the nopaline-type Ti plasmids (5,20). This incompatibility group, Inc Rh1, includes the octopine/mannityl opine-type Ti plasmids, the nopaline/agrocinopine-type Ti plasmids, and some nononcogenic opine catabolic plasmids (9). pAtK84b is a conjugative plasmid whose transfer is inducible by agrocinopines and nopaline (8). Recently, Farrand (9) suggested that chromosomal genes also may be involved in regulating opine-inducible conjugal transfer functions of pAtK84b.

A. radiobacter K84 has been used commercially for over 20 years for biological control of crown gall in stone fruit trees, roses, and other ornamentals (4,26). One of the mechanisms involved in control is the biosynthesis and secretion of an antibiotic substance, agrocin 84, encoded by a small plasmid, pAgK84. pAgK84 is a conjugative plasmid (10) that is transferable to *A. tumefaciens* and confers immunity to agrocin 84 (30). To prevent this transfer, a stable deletion mutant has been constructed by recombinant DNA technology (17). The resultant strain, K1026, is as effective as K84 in biological control (44) and has been proposed as a replacement for K84 in several countries.

The presence of pAtK84b in K84 and K1026 strains has been thought to safeguard biological control by preventing acquisition of Ti plasmid (5,20) because incompatible plasmids are unable to replicate in the same cell (20,28). However, such protection may not be as effective as once thought. Stockwell (40) found that

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a transposon-tagged Ti plasmid had transferred to strain K84 after coinoculation of *A. tumefaciens* and K84 in tomato plants in a growth chamber. No characterization of the transferred plasmid was performed.

The main objective of the present paper was to study spontaneous transfers of plasmids between *A. tumefaciens* and *A. radiobacter* under seminatural conditions. Analyses were performed in tumors caused by *A. tumefaciens* in fruit trees treated with strains K84 or K1026 of *A. radiobacter*. Once these transfers were detected, a genetic characterization of the transconjugants and transferred plasmids was performed to determine what, if any, change had occurred.

MATERIALS AND METHODS

Bacterial strains. Table 1 shows the bacterial strains used and their characteristics and plasmids. Strain K1026 was supplied by G. C. Bullard (Bio-Care Technology, Woy-Woy, Australia). *Escherichia coli* strains 1231 (pBR322::*Bam*HI fragment C), DH1 (pTHE17), and DH1 (pTHE7) were supplied by S. K. Farrand (University of Illinois, Urbana), and strain HB101 (pVK102) was supplied by B. Clare (University of Adelaide, Australia).

Biocontrol experiment. A biocontrol experiment was performed in a greenhouse in 28-cm-diameter pots with sterile substrate containing 50% peat and 50% sand. The minimum and maximum temperatures were 20 and 26°C, respectively, and the relative humidity fluctuated between 60 and 90%. Plants of 1 year-old peach × almond GF677 hybrid were used. The plants were fertilized every 15 days with a solution 15-10-5 (N-P_2O_5-K_2O) with a final concentration of 1.4 g of fertilizer per liter. Every month the plants were irrigated with a 25% solution of Fe chelate.

Strain 325-4 of *A. tumefaciens* was grown on nonselective PGYA medium (24) (5 g of Bacto-peptone, 10 µl of glycerol, 5 g of yeast extract, and 20 g of agar per liter), and a bacterial suspension was inoculated into the substrate before planting, reaching a final concentration of about 10^7 CFU/g of substrate.

One control and two treatments (K84 or K1026) were used in this experiment, with 100 GF677 plants per treatment. Plants were wounded superficially and treated by dipping in a suspension of peat preparations of strain K84 or K1026 (peat/water, 1:1, wt/vol) just before planting (44). Control plants were dipped in water. Strains K84 and K1026 of *A. radiobacter* were prepared as peat inoculum (25) and supplied by F. Temprano and R. Orive (Servicio de Investigación Agraria, Sevilla, Spain). The concentration of bacteria in peat inoculum was about 10^9 CFU/ml. After growing for 9 months, the plants were analyzed to record the number of plants with tumors and the number and weight of tumors per diseased plant.

The two treatments and the control were randomly assigned to 100 pots each. Quantitative variables from the biocontrol experiment were analyzed by the following model: $Y_{ij} = \mu + T_i + \varepsilon_{j(i)}$, where Y_{ij} is the observed value of the variable in the jth pot (j = 1, 100(i)) belonging to the ith treatment (i = 1,3). The least significant difference procedure was used for a priori mean separation (treated versus untreated and between treatments).

Proportions were compared by the approximate Z test according to the formula:

$$Z = \frac{\hat{p}_1 - \hat{p}_2}{\sqrt{\hat{p}(1-\hat{p})\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}}$$

where $\hat{p}_i$ is the estimated proportion of the ith treatment and $\hat{p}$ is the weighed average of the proportions for both treatments of size n_1 and n_2.

Characterization of recovered isolates. Isolation and characterization of *Agrobacterium* isolates from tumors were performed as described by Vicedo et al. (44). Biovar, agrocin 84 production, and pathogenicity on tomato were determined for all isolates from tumors of plants treated with strain K84 or K1026. The method described by López et al. (24) was used to evaluate opine utilization. API20 NE and ATB G antibiograms (API System S.A.,

TABLE 1. Bacterial strains used, characteristics and plasmids

Bacteria Strain	Biovar	Sensitivity to agrocin 84[w]	Opine utilization[x]	Plasmid size[y] (kb)	Description
Agrobacterium radiobacter					
K84	2	R	Nop, Oct	pAtK84a (>300), pAtK84b (173), pAgK84 (47.7)	Produces agrocin 84. Used in biological control.
K1026	2	R	Nop, Oct	pAtK84a (>300), pAtK84b (173), pAgK1026 (41.8)	Derived from K84 with Tra⁻ agrocin 84 plasmid (17). Used in biological control.
A. tumefaciens					
C58	1	S	Nop	pAtC58 (410), pTi C58 (195)	Indicator strain for agrocin 84 sensitivity and molecular weight marker.
325-4	1	S	Nop, Oct[z]	pTi 325-4 (196), pAt 325-4 (151)	Isolated from a peach tumor in Spain and used to inoculate soil.
Escherichia coli					
1231 (pBR322::*Bam*HI fragment C)				pBR322::*Bam*HI fragment C	*Bam*HI fragment C of pAgK84 cloned into pBR322. Strain 1231 is described by Pischl and Farrand (34).
DH1 (pTHE17)				pTHE17	Contains the entire T-DNA region of pTiC58 cloned into pCP13 (S. K. Farrand, *personal communication*).
DH1 (pTHE7)				pTHE7	Contains the clockwise half of the pTiC58 *vir* region cloned into pCP13 (S. K. Farrand, *personal communication*).
HB101				pVK102	*Hin*dIII fragment 9 of pAtK84b, cloned in the cosmid vector pVK102. Contains *Hin*dIII fragment 9, nonhomologous with any region of pTiC58 (5).

[w] R = resistant; S = sensitive. Sensitivity to agrocin 84 was determined by the Stonier method (42) with Stonier medium (41).
[x] Nop = nopaline; Oct = octopine. Opine utilization was determined by the method of López et al. (24).
[y] Size and number of plasmids were evaluated by the Eckhart method (7) as described by Albiach and López (2).
[z] Octopine was slowly degraded by this strain.

Montalieu-Vercieu, France) were used to compare the biochemical and physiological characteristics and antibiotic resistance patterns of strains K84 and 325-4 with those of selected isolates (putative transconjugants) obtained from tumors.

Molecular characterization of plasmids from recovered isolates. Plasmid profiles of strains K84 and 325-4 and transconjugant isolates were analyzed by agarose gel electrophoresis by the Eckhart method (7) as modified by Albiach and López (2). The plasmids were purified by the large-scale extraction method with CsCl-EtBr gradient described by Hirsch et al. (14). Plasmids were digested with *Sma*I, *Bam*HI, and *Acc*65I restriction endonucleases. The resulting fragments were separated by agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 5 V/cm. Molecular sizes of the plasmids and their restriction fragments were estimated by means of a BASIC program based on the model of Plikaytis et al. (35). Southern transfer was performed on Hybond N nylon membranes (Amersham International, Buckinghamshire, England). Nonradioactive probes were prepared according to the method described by Vicedo et al. (44) with digoxygenin d-UTP (Boehringer GmbH, Mannheim, Germany) as the labeled nucleotide:

(i) pAgK84 probe: from plasmid pBR322::*Bam*HI fragment C; *Bam*HI fragment C of pAgK84 contains the genes for synthesis and secretion of agrocin 84 (37).

(ii) T-DNA probe: *Sma*I fragment 7 of pTiC58 (in cosmid pTHE17; S. K. Farrand, *personal communication*) corresponds to an inner sequence of the T-DNA region, including *onc* genes 1, 4, and 6a and most of 2 (16).

(iii) *virB* probe: contains *Eco*RI fragment 19 of pTiC58, which corresponds to a portion of the *virB* region (36) and was obtained by digestion of pTHE7 with *Eco*RI (S. K. Farrand, *personal communication*).

(iv) *noc* probe: *Sma*I fragment 5 of pTiC58 (in pTHE17) contains a small portion of the right border of T-DNA and the left part of the nopaline catabolism region of pTiC58 that encodes the enzymes involved in nopaline degradation (*ocd*, *arc*, and *noxA*) (15,38,45). It was used as a probe because the portion outside the T-DNA could present homology with plasmids of avirulent strains.

(v) *vir* probe: *Eco*RI fragments 15, 17, and 19 of pTiC58 (in pTHE7) contain *virB*, *virH*, and *virE* genes (36).

(vi) pAtK84b probe: *Hin*dIII fragment 9 of pAtK84b, cloned into pVK102, was used to prepare a specific probe for the nopaline catabolic plasmid of strain K84 because this fragment shows no homology with the nopaline-type plasmid pTiC58 (5).

Hybridizations, washings, and chemiluminescent detection were done following the instructions of the supplier (Boehringer).

In vitro and in planta conjugal transfer of *Agrobacterium* Ti plasmid. To test the ability of *A. tumefaciens* strain 325-4 to transfer Ti plasmid to *A. radiobacter* strain K84 in vitro, the filter-mating method was used. The minimal medium described by Petit et al. (33), supplemented with tumor extract (60 µl/ml) or nopaline as the carbon and nitrogen source, was used as the preculture and mating medium. To obtain tumor extracts, 1-month-old tomato plants were inoculated with strain 325-4. After 20 days, tumors were removed and macerated to extract opines according to the method described by Dessaux et al. (6).

Transfer of Ti plasmid also was studied in in planta assays by coinoculating the donor strain (*A. tumefaciens* 325-4) and recipient strain (*A. radiobacter* K84) in tomato plants according to the method described by Kerr (19). Another experiment was performed with strain K1026 as the recipient strain. In all assays, biovar 2 isolates were selected by plating appropriate dilutions in a selective medium (27). To identify transconjugants, purified, selected colonies were analyzed for agrocin 84 production and pathogenicity.

RESULTS

Biocontrol experiment. Biocontrol efficiency of strains K84 and K1026 on GF677 peach × almond hybrid plants is shown in Table 2. Strains K84 and K1026 were very effective in controlling crown gall in these tests. Significant differences ($P < 0.05$) were observed between treated and untreated plants in percentage of infected plants and weight of galls per diseased plant. All tumors on the treated plants were located on roots and were younger and smaller compared to those present on untreated plants.

Characterization of recovered isolates. The characteristics of *Agrobacterium* isolates from tumors are listed in Table 3. All tumors produced in treated plants were analyzed. Thirty-three isolates from tumors of K1026-treated plants were studied; all were nonpathogenic agrocin producers and belonged to biovar 2.

In one tumor of a K84-treated plant, seven biovar 2 isolates were pathogenic on tomato. These isolates produced a bacteriocin in Stonier medium (42) that, like agrocin 84, inhibited the growth of *A. tumefaciens* strain C58. Twelve biovar 1 isolates from the same tumor produced agrocin 84 and were nonpathogenic. Pathogenic isolates of biovar 1 were not found in the tumors of any treated plants. Results of API20 NE and ATB G antibiograms showed no differences between virulent biovar 2 isolates and strain K84 nor between avirulent biovar 1 agrocin 84 producers and strain 325-4 (data not shown), indicating similar chromosomal genotypes.

In vitro and in planta conjugal transfer of *Agrobacterium* Ti plasmid. Results of in vitro conjugal transfer experiments showed that, after analysis of 490 isolates of biovar 2 and producers of agrocin 84, there was no transfer of Ti plasmid after preincubation and mating on tumor extract (transfer frequency $<5 \times 10^{-2}$ per recipient). No transconjugants were detected among 270 isolates of K84 or 310 isolates of K1026 from in planta coinoculation assays (transfer frequency $<3 \times 10^{-2}$ per recipient).

Molecular characterization of plasmids from recovered isolates. Plasmid profile analyses by the Eckhart method showed that virulent isolates of biovar 2 had a plasmid profile similar to that of strain K84 (Fig. 1). One plasmid with the same mobility as pAtK84b hybridized with T-DNA and *virB* probes. The plasmid of these isolates, which exhibited the same electrophoretic mobility as pAgK84, hybridized with the pAgK84 probe (agrocin 84 synthesis and secretion region). Plasmid patterns, hybridizations,

TABLE 2. Biological control of agrocin 84-sensitive strain 325-4 of *Agrobacterium tumefaciens* with strains K84 and K1026 of *A. radiobacter*

Treatment[y]	No. of plants analyzed	% plants diseased[z]	Mean fresh weight (g) of galls/diseased plant[z]
Control	78	69.2 b	21.1 b
K84	85	5.8 c	8.0 c
K1026	99	1.0 d	1.2 d

[y] One-year-old peach × almond hybrid GF677 plants were planted in soil inoculated with strain 325-4 after dipping in water or in peat-water preparations (1:1, wt/vol) of strains K84 and K1026.

[z] Different letters correspond to significant differences ($P < 0.05$).

TABLE 3. Characteristics of isolates from tumors obtained in a biocontrol assay of *Agrobacterium tumefaciens* strain 325-4

Treatment	No. of tumors[w]	No. of isolates	Biovar 1[x]		Biovar 2[x]	
			Path.	Nonpath.	Path.	Nonpath.
Control	10	61	13	48	0	0
K84	6	110	0	12[y]	7[z]	91
K1026	2	33	0	0	0	33

[w] All the tumors obtained in plants treated with K84 and K1026 were analyzed.

[x] Path. = pathogenic; Nonpath. = nonpathogenic.

[y] These isolates were able to synthesize agrocin 84.

[z] These isolates were found in the same tumor as the nonpathogenic biovar 1 isolates.

and analysis of metabolic characteristics suggest that these isolates were mostly similar to strain K84 but had acquired virulence. One of these isolates, K84N6, was chosen for further studies.

Avirulent agrocinogenic biovar 1 isolates contained two plasmids with sizes similar to those of strain 325-4 (151 and 196 kb) and two additional plasmids (Fig. 1). One of the additional plasmids exhibited a similar electrophoretic mobility to that of pAgK84 (47.7 kb), and the second migrated very close to the Ti plasmid. Hybridization of Southern blots with the pAgK84 probe showed that pAgK84 had been transferred from K84 to strain 325-4 (data not shown). Avirulence of these biovar 1 isolates, as determined by tests on tomato plants, was confirmed by the lack of hybridization signal of the Ti-like plasmid with the T-DNA probe (Fig. 1). One of these avirulent isolates, 325-4S19, was chosen for further studies.

The results suggest that in one tumor some cells of strain K84 became virulent, whereas others of strain 325-4 lost pathogenicity. To study the genetic similarity of the isolates obtained, plasmids of the virulent biovar 2 isolate K84N6 and the avirulent biovar 1 isolate 325-4S19 were compared with plasmids of strains K84 and 325-4.

Restriction fragment length polymorphism (RFLP) analysis of plasmids from strains 325-4, K84, K84N6, and 325-4S19 with T-DNA, *noc*, *vir*, and pAtK84b probes (Fig. 2) showed that the virulent biovar 2 isolate (K84N6) had hybridization patterns similar to those of strain 325-4 and very different from those of strain K84. Using the T-DNA probe (Fig. 2A), similarities in hybridization between strains 325-4 and K84N6 were observed, whereas avirulent strains (K84 and 325-4S19) did not hybridize with this probe. Thus, hybridization of *Sma*I and *Acc*65I digestions of plasmids from both strains (325-4 and K84N6) showed no differences with the T-DNA probe. However, hybridization of *Bam*HI restriction fragments revealed two RFLPs containing 11.6- and 5.3-kb fragments of strain 325-4 and 13.1- and 9.4-kb fragments of K84N6 (Fig. 2A). Using the *noc* probe (Fig. 2B), very different hybridization patterns were observed between the plasmid digestions from strains 325-4 and K84N6. It is interesting that pTi325-4, but not pTiK84N6, exhibits high homology to this probe. Using the *vir* probe (Fig. 2C), no differences were found between the plasmid digestions from 325-4 and K84N6 with *Sma*I or *Bam*HI. Nevertheless, with *Acc*65I digestion, differences were detected. For 325-4, two hybridization signals appeared that corresponded to 8.8 and 3.5 kb, whereas for K84N6 we obtained a different band of 11.2 kb. Using the pAtK84b probe (Fig. 2D), no hybridization was observed for either pathogenic strain, 325-4 and K84N6.

The agrocinogenic avirulent biovar 1 isolate (325-4S19) showed no differences from strain K84 with any of the four probes nor with any restriction digestion, suggesting a close genetic similarity between the plasmids of both strains. No hybridization signal was obtained with the T-DNA probe for the plasmid digestions from these two strains (Fig. 2A). Identical hybridization signals for both nonpathogenic strains (325-4S19 and K84) were obtained with the *noc* and *vir* probes (Fig. 2B and C). Further analysis with the pAtK84b probe resulted in identical signals for both strains (Fig. 2D). Hybridization of this probe with *Sma*I and *Bam*HI plasmid digestions from both strains revealed two fragments of about 8.5 and 9 kb, respectively. For the *Acc*65I digestions, two bands, corresponding to fragments of about 21.2 and 14.6 kb, hybridized. The pAtK84b probe did not hybridize with the plasmid digestions of the tumorogenic strain, 325-4. These results indicate that one of the two largest plasmids present in strain 325-4S19 is indistinguishable from the nopaline catabolic plasmid of K84.

DISCUSSION

Three plasmid transfers between an agrocin 84-sensitive strain of *A. tumefaciens* (325-4) and strain K84 of *A. radiobacter* were detected in one of six tumors, although 110 isolates from all the tumors obtained were studied. It is the first time that spontaneous conjugal transfer of Ti plasmid to *A. radiobacter* strain K84 has been detected under seminatural conditions and that transferred plasmids have been studied. Our results show that the nopaline-type Ti plasmid was transferred to strain K84 and that recombination may have taken place between this plasmid and the nopaline catabolism plasmid of K84, converting this biocontrol strain to virulence. Other transfers took place in the opposite direction, from strain K84 to strain 325-4. One transfer involved pAtK84b, which entered into the recipient strain (325-4) and very likely replaced the resident Ti plasmid. A second transfer involved pAgK84, which was transferred to the same cell. As a result, some virulent bacteria from strain 325-4 became avirulent and produced agrocin 84.

Nopaline-type Ti plasmids and pAtK84b belong to the same incompatibility group (20). Incompatibility between two plasmids implies that both plasmids can stay only transiently in the same cell (20,28). In the meantime, however, important changes, such as recombination, can take place between both plasmids, or one may dislodge the other. Our results show that both events can take place in the transconjugants that formed in a single tumor. As the pAtK84b present in recipient strain K84 can be activated for con-

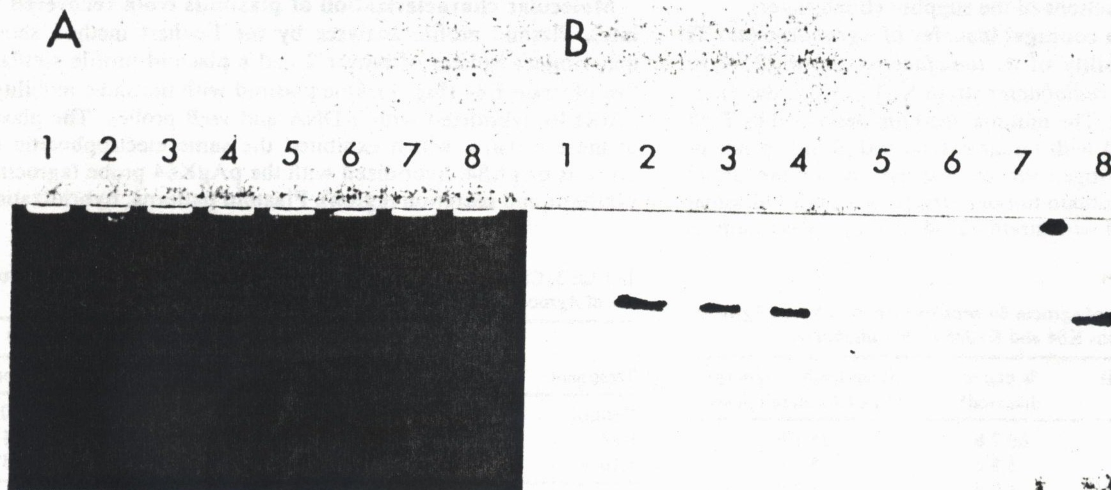

Fig. 1. Plasmid profiles and Southern hybridization of *Agrobacterium* isolates obtained from a tumor in a biocontrol experiment. **A,** Plasmids were separated by agarose gel electrophoresis by the Eckhart method (7) and stained with ethidium bromide. **B,** Southern analysis by hybridization with the T-DNA probe from pTiC58. Lane 1, *A. radiobacter* strain K84; lanes 2 through 4, virulent biovar 2 isolates; lanes 5 through 7, avirulent, agrocin 84-producing biovar 1 isolates; and lane 8, *A. tumefaciens* strain 325-4.

jugation by the same opine that activates the Ti plasmid in the donor, the recipient can exhibit entry exclusion, preventing acquisition of the donor Ti plasmid at a high frequency. (S. K. Farrand, *personal communication*). This could explain why so few Ti plasmid transfer events were detected.

Seven biovar 2 pathogenic isolates from a tumor presented many characteristics similar to those of strain K84 (biovar, biochemical, and physiological characteristics, as well as antibiotic resistance and agrocin 84 production), indicating that some K84 cells had acquired the genes responsible for tumor formation. This hypothesis was supported by plasmid profiles and hybridization of Southern blots with specific DNA probes from regions encoding pathogenicity.

RFLP analysis of the transferred plasmids was performed to distinguish between replacement and recombination between the incompatible plasmids or other genetic changes. Three RFLPs between the plasmids of virulent strain 325-4 and isolate K84N6 at two distant regions of the Ti plasmid (T-DNA and *vir* region) were detected. In addition to these differences, a deletion at the left part of the nopaline catabolism region and the right border of the T-DNA seems to have happened given the lack of hybridization signal with the *noc* probe. In fact, analysis of nopaline utilization showed that transconjugant K84N6 metabolized nopaline more slowly than strains 325-4 and K84 (data not shown). In spite of these important changes, which could have arisen as a consequence of recombination between the Ti and pAtK84b plasmids, the plant cell transformation ability of the new Ti plasmid harbored by K84N6 was not disturbed.

There is little information about in vitro transfer of Ti plasmid to strain K84 of *A. radiobacter*, and one experiment was performed to compare transfer to strains K84 and K1026. Ellis et al. (8) described the transfer of Ti plasmid in vitro with strain C58C1 as

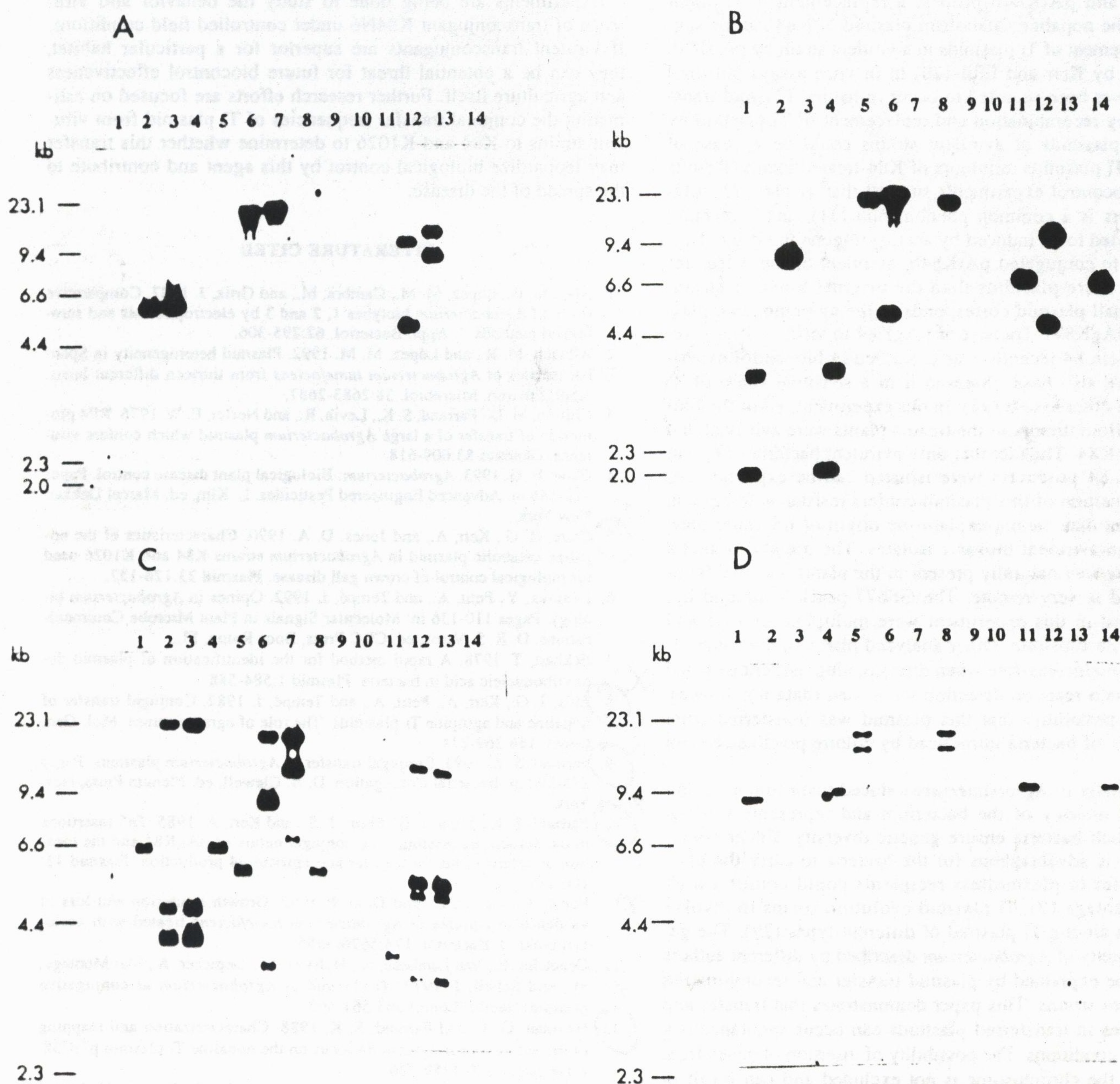

Fig. 2. Southern analysis and hybridization to specific probes of digested plasmids from *Agrobacterium* strains K84 and 325-4 and transconjugants K84N6 and 325-4S19. Plasmids were digested with restriction enzymes *Sma*I (lanes 1 through 4), *Acc*65I (lanes 5 through 8), and *Bam*HI (lanes 11 through 14) and hybridized to A, T-DNA, B, *noc*, and C, *vir* probes of pTiC58 and D, pAtK84b probe of strain K84. Lanes 1, 5, and 11, plasmids of avirulent 325-4S19 isolate; lanes 2, 6, and 12, plasmids of *A. tumefaciens* strain 325-4; lanes 3, 7, and 13, plasmids of virulent isolate K84N6; lanes 4, 8, and 14, plasmids of *A. radiobacter* strain K84. Lanes 9 and 10, molecular weight markers.

the recipient cell that harbored pAtK84b and pAgK84 plasmids. Conjugation of Ti plasmid to strain K84 was detected in planta by Stockwell et al. (40) in 1 of 25 galls by coinoculation of the pathogen and strain K84 in tomato plants. Transconjugants were not detected in our in vitro or in planta mating experiments with the same strain as in vivo. This seems to indicate that the frequency of Ti plasmid transfer can change depending on the conditions.

Avirulent biovar 1 agrocinogenic isolates resulting from strain 325-4 also were studied. Plasmid electrophoresis by the Eckhart method and hybridization with T-DNA and *virB* probes showed that plasmids of these isolates lack both regions necessary to induce tumors. Furthermore, the pAtK84b probe, which showed no homology with Ti plasmid of strain C58 (5), hybridized only with plasmids of both avirulent strains (325-4S19 and K84). It is possible that either pTi325-4 had suffered deletions in such regions or pAtK84b entered into the virulent strain, recombining with resident Ti plasmid or replacing it. Given that no RFLP was obtained between the plasmids from strain K84 and isolate 325-4S19 with the *noc*, *vir*, and pAtK84b probes, a replacement of resident pTi325-4 by the nopaline catabolism plasmid of K84 can be suggested. Replacement of Ti plasmids in a virulent strain by pAtK84b was observed by Kerr and Ellis (20) in in vitro assays but until now it had never been reported to occur in nature. Plasmid transfer followed by recombination and replacement of Ti plasmid by incompatible plasmids of avirulent strains could be a cause of instability of Ti plasmids in tumors of K84-treated plants. Results from other biocontrol experiments suggest that Ti plasmid instability in tumors is a common phenomenon (31), and it recently has been reported to be induced by acetosyringone in vitro (11).

In addition to conjugated pAtK84b, avirulent biovar 1 isolates harbored two more plasmids than the original biovar 1 strain, 325-4. The small plasmid corresponds to the agrocinogenic plasmid of K84 (pAgK84). Transfer of pAgK84 to virulent strains resistant to agrocin 84 recently was described in biocontrol experiments (44). We also have observed it in a sensitive strain of *A. tumefaciens* in other assays (43). In our experiment, all of the biovar 1 isolates from tumors of the treated plants were avirulent and contained pAgK84. The fact that only avirulent bacteria of biovar 1 and agrocin 84 producers were isolated can be explained because the acquisition of this plasmid confers resistance to agrocin 84. Our present data cannot explain the origin of the other plasmid detected in avirulent biovar 1 isolates. The possibility that a third *Agrobacterium* naturally present in the plants was the donor of this plasmid is very remote. The GF677 peach × almond hybrid plants used in this experiment were multiplied in vitro and planted in sterile substrate. Other analyzed plants of the same origin were *A. tumefaciens*-free when direct plating, enrichment, and polymerase chain reaction detection were used (data not shown). However, the possibility that this plasmid was transferred from another species of bacteria introduced by culture practices cannot be excluded.

Plasmid transfer in *Agrobacterium*-induced plant tumors is important to the biology of the bacterium and represents a major avenue by which bacteria ensure genetic diversity. Under conditions where it is advantageous for the bacteria to carry the plasmid, its transfer to plasmidless recipients could confer a high selective advantage (9). Ti plasmid evolution seems to involve recombination among Ti plasmid of different types (29). The genetic heterogeneity of *Agrobacterium* described by different authors (2,29) could be explained by plasmid transfer and recombination among different strains. This paper demonstrates that transfer and genetic changes in transferred plasmids can occur spontaneously in seminatural conditions. The possibility of insertion of genes from plasmids into the chromosome is not excluded and can result in heterogeneity of serological reactions (1), protein profiles, or other phenotypic features.

The frequency at which Ti plasmid is conjugatively transferred to strain K84 in the field is not yet known nor are its repercussions on biocontrol effectiveness. Biological control was very efficient in our experiment when transfer of Ti plasmid to K84 took place. Given that plasmid transfer occurred in both directions in the same tumor, it could have contributed to a certain balance between pathogenic and nonpathogenic populations. The tumor in which conjugation has been detected was young, and there probably was not enough time for the pathogenic transconjugants to spread and infect new plants. The transfer of pAtK84b and replacement of Ti plasmid was proposed by Kerr and Ellis (20) as a biocontrol mechanism. Our results partially support this hypothesis.

Ti plasmid conjugation to strain K1026 was not observed in tumors of K1026-treated plants. However, this strain probably would behave as K84 does, since there is no reason to believe there is any interaction between the Ti plasmid and pAgK84, at least with respect to the Ti plasmid transfer (S. K. Farrand, *personal communication*). Transfer of Ti plasmid to K1026 has never been detected in experiments performed with different strains of *A. tumefaciens* (data not shown).

Experiments are being done to study the behavior and virulence of transconjugant K84N6 under controlled field conditions. If virulent transconjugants are superior for a particular habitat, they can be a potential threat for future biocontrol effectiveness and agriculture itself. Further research efforts are focused on estimating the conjugal transfer frequencies of Ti plasmid from virulent strains to K84 and K1026 to determine whether this transfer may jeopardize biological control by this agent and contribute to the spread of the disease.

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