Behavior of a Virulent Strain Derived from *Agrobacterium radiobacter* Strain K84 After Spontaneous Ti Plasmid Acquisition

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ABSTRACT

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The behavior of the virulent transconjugant K84N6 derived from *Agrobacterium radiobacter* strain K84 after spontaneous Ti plasmid transfer in crown gall tissue in a biocontrol experiment was studied and compared with the behavior of the wild-type *A. tumefaciens* donor of the Ti plasmid. The main difference between the strains was a greatly reduced ability of the transconjugant to catabolize nopaline. Host range, ability to induce tumors in several fruit trees, and stability of the pathogenic determinants in isolates from tumors did not differ between the strains. Nevertheless, in a biocontrol experiment, the transconjugant was not controlled by strain K84 or K1026 in peach × almond hybrids and survived in the plant rhizosphere for 9 months with larger population densities than the wild strain. The appearance and persistence in soil of strains harboring a Ti plasmid in the K84 chromosomal background could represent a risk in the medium term, if they show good competitive ability.

Agrobacterium tumefaciens is the causal agent of crown gall, a neoplastic disease of plants. Virulent bacteria contain a tumor-inducing Ti plasmid that is responsible for plant cell transformation via the introduction of T-DNA into the plant chromosome. Many functions involved in pathogenicity are encoded on the Ti plasmid including the synthesis of auxins and cytokinins, opine production, and opine utilization (12,29). Several authors demonstrated that host-range specificity can be determined by genes located in the T-DNA region and in the *vir* region of the Ti plasmid (2,39).

The Ti plasmid is a conjugative plasmid whose transfer to other bacterial recipients is induced by several opines (30). Transfer of the Ti plasmid to avirulent strains of *Agrobacterium* was reported in several in vitro experiments using the promiscuous plasmid RP4 (3) or conjugal opines as transfer inducers (7,30) and also was detected in planta (13).

A. radiobacter strain K84 is a very efficient biocontrol agent of crown gall (21). Strain K84 harbors three plasmids: pAgK434, pNoc, and pAgK84. Plasmid pAgK84 encodes the production of agrocin 84, an antibiotic involved in the biocontrol of crown gall (14). Transfer of pAgK84 from K84 to strains of *A. tumefaciens* has been reported (26,37,42,43). Strain K1026, a Tra^- deletion mutant, was constructed to eliminate the possibility of this transfer (11) and was proposed as a safe substitute for K84 (10,41,43).

Several authors previously suggested the possibility that strain K84 could acquire the Ti plasmid by conjugal transfer (21). However, it was assumed that strain K84 was protected against Ti-plasmid acquisition because of the presence of pNoc, a conjugative nononcogenic plasmid-encoding nopaline catabolism that belongs to the same incompatibility group as the Ti plasmid (8). Therefore, both plasmids are unable to replicate in the same cell. Nevertheless, it was demonstrated in our laboratory that Ti-plasmid transfer from *A. tumefaciens* to strain K84 can spontaneously occur in planta under semi-natural conditions (42). This transfer event was detected only in one tumor in a biocontrol experiment using strain K84 against a strain of *A. tumefaciens* biovar 1, which utilized nopaline and was sensitive to agrocin 84. The transconjugants induced tumors, produced agrocin 84, and contained a Ti plasmid with the same size as that of the donor strain. Further analysis demonstrated that the transconjugant strain K84N6 harbored a functional Ti plasmid that differed from that of the donor strain 325-4 (42), suggesting a recombination event between the incompatible plasmids pNoc and pTi.

The frequency at which the Ti plasmid can be transferred from pathogenic strains to strain K84 in the field is not yet known, as well as the repercussion on biocontrol effectiveness. Nevertheless, such pTi transfer could be a potential threat to the use of biocontrol strains K84 and K1026, because the transconjugant K84N6 causes crown gall, produces agrocin 84, and is immune to the antibiotic. For this reason, in this study, we evaluated the behavior of this transconjugant strain, comparing it with that of the virulent Ti-plasmid donor. The ability of K84N6 to induce tumors, survive in the rhizosphere, and retain virulence in the soil and in the tumor environment were studied. The efficacy of biocontrol strains K84 and K1026 against this new strain also was analyzed in order to evaluate the potential threat of this transconjugant to the commercial use of these biocontrol strains.

MATERIALS AND METHODS

Bacterial strains. The characteristics of the strains used are summarized in Table 1. *A. tumefaciens* K84N6 was obtained after spontaneous Ti-plasmid transfer from the wild strain 325-4 (donor) to strain K84 (recipient). It was isolated from a tumor of a peach × almond GF677 hybrid (*Persica vulgaris* Mill. × *Prunus dulcis* Weeb) in a biological control experiment conducted under greenhouse conditions in pots with a substrate inoculated with strain 325-4 (42). Strains K84 and K1026 of *A. radiobacter* were used in the biological control experiments. *A. radiobacter* strain K1026 was

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supplied by G. C. Bullard (Bio-care Technology, Woy-Woy, Australia). *A. tumefaciens* strain C58 was used as the indicator strain in assays of agrocin 84 production. Bacteria were grown in nonselective peptone yeast glycerol agar (PYGA) medium (5 g of bactopeptone per liter [Oxoid Ltd., Basingstoke, England], 3 g of yeast extract per liter [Oxoid Ltd.], 10 ml of glycerol per liter, and 20 g of agar per liter) for all the experiments (19).

Opine utilization. Nopaline and octopine utilization was measured after growing strains 325-4, K84N6, and K84 individually for 72 h at 26°C in minimal medium S (17) containing either nopaline or octopine as the sole carbon and nitrogen source at a final concentration of 100 μg/ml. Utilization of nopaline and octopine was studied by determining the amount of the substrates at 24, 48, and 72 h after incubation. This experiment was conducted twice. Presence of the two opines was measured according to the method of Lippincott et al. (16), with some modifications (17), as follows: 1 ml of the culture supernatant was supplemented with 1 ml of 0.9 M NaOH and 1 ml of α-naphthol-diacetyl (17). After shaking, samples were allowed to settle for 30 min at room temperature and their optical density was measured in a Titertek Multiskan (Flow Laboratories, Irvine, Scotland) at 450 nm. At least four replicates were used for each strain and opine, and each replicate consisted of four wells. Standard curves for octopine and nopaline were constructed.

Host range. Host-range assays were performed on greenhouse-grown plants by inoculation with *A. tumefaciens* strain 325-4 or the transconjugant strain K84N6. Bacterial suspensions prepared with 48-h cultures were used to inoculate the stems of the plants after they were wounded with a scalpel. Eight herbaceous species and two woody species were used as host plants. The inoculated hosts were tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), sunflower (*Helianthus annuus* L.), datura (*Datura stramonium* L.), bean (*Phaseolus vulgaris* L.), eggplant (*Solanum melongena* L.), watermelon (*Citrullus lanatus* (Thub.) Mansfed), vinca (*Vinca rosea* L.), rose (*Rosa* subsp. L. Hort indica), and grapevine (*Vitis vinifera* L.). Every plant was inoculated at two or three different points with a dose of 2×10^7 CFU per wound. The experiment was performed twice. The number of inoculated plants of every host is indicated in Table 2. Plants inoculated with every strain were randomly assigned, and tumor formation was recorded 2 months later.

Virulence assays. Virulence assays were performed on 1-year-old seedlings of cherry (*Prunus avium* L.) cv. Santa Lucía, apple (*Malus domestica* Borkh.) cv. Pajam 1, pear (*Pyrus communis* L.) cv. Kirchensaller, and peach × almond hybrids (*Persica vulgaris* Mill. × *Prunus dulcis* Weeb) GF677 and Adafuel. Before planting, strain 325-4 or K84N6 was introduced individually into a sterile potting mix containing 50% peat and 50% sand, according to the methods described by Vicedo et al. (43), to give a final concentration of 10^7 CFU/g of substrate. Prior to planting, the roots were pruned and the crowns were superficially wounded with a sterile scalpel. A total of 10 to 25 plants of every species was inoculated with each strain. The plants were arranged in a completely randomized design in order to minimize the environmental effects in the greenhouse. After 9 months, plants were dug up and evaluated for crown gall on the basis of the number of diseased plants and number and size of tumors per infected plant. A similar experiment was performed twice using 3-week-old tomato plants. In all, 60 tomato plants were inoculated with every strain. Tumor formation on roots was observed and analyzed after 4 months.

Pathogenicity stability. Stability of genes encoding pathogenicity was studied in two experiments in agrobacteria isolated from tumors obtained 40 days after inoculation of tomato stems with strain 325-4 or K84N6 (4×10^6 CFU per wound). Ten tumors obtained with every strain were selected to analyze the *Agrobacterium* isolates. Each tumor was comminuted in sterile water and appropriate dilutions were plated on semiselective media of biovar 1, described by Schroth et al. (36), or biovar 2, described by New and Kerr (23), to select strains 325-4 and K84N6, respectively. The *Agrobacterium* spp.-like colonies were counted, and 30 colonies per tumor were purified, assessed for *Agrobacterium* spp. characteristics, and tested for pathogenicity on tomato plants.

Biological control of crown gall. One-year-old plants of the GF677 hybrid were used for biocontrol assays performed under controlled conditions in the greenhouse with 60 to 90% relative humidity and temperatures between 20 and 26°C. Just before planting, plants were superficially wounded in the crown with a sterile scalpel and dipped into suspensions of peat preparations of K84 or the genetically modified strain K1026 (peat/water, 1:1, wt/vol; 10^9 CFU/ ml), prepared

TABLE 2. Host range of *Agrobacterium tumefaciens* strains 325-4 and K84N6[u]

Plant	Tumor induced[v]		Experiment 1[w]		Experiment 2	
	325-4	K84N6	325-4	K84N6	325-4	K84N6
Tomato	+	+	30/30	29/29	25/25	23/25
Tobacco	+	+	10/10	10/10	24/24	22/24
Sunflower	+	+	9/9	8/8	25/27	24/25
Datura	+	+	9/10	10/10	19/29	20/29
Bean	+	+	8/9	9/10	ND[x]	ND
Eggplant	d+[y]	d+	10/10	9/9	ND	ND
Watermelon	+	+	7/10	9/10	19/30	20/29
Vinca	+	+	8/8	6/9[z]	26/26	24/28[z]
Rose	+	+	1/5	1/5	ND	ND
Grapevine	+	+	5/5	5/6	ND	ND

[u] Results of two different experiments are shown.
[v] Plants were inoculated at the stem with *A. tumefaciens* strains 325-4 or K84N6 with a dose of 2×10^7 CFU per wound. Each plant was inoculated at two or three different points on the stem. Tumor formation was recorded 2 months later.
[w] Number of galled plants per inoculated plant. Number of galled plants produced by strains 325-4 and K84N6 were compared within each host by comparison of proportions using the approximate *Z* test.
[x] ND = not determined.
[y] d+ indicates small proliferations at the inoculation site.
[z] Indicates a significant difference ($P < 0.05$) between strains 325-4 and K84N6.

TABLE 1. Bacterial strains, characteristics, and plasmids of *Agrobacterium* spp.

Strain	Biovar	Sensitivity to agrocin 84[w]	Opine utilization[x]	Plasmid size (kb)[y]	Description
A. tumefaciens					
325-4	1	S	Nop, Oct[z]	pTi 325-4 (196), pAt 325-4 (151)	From a peach tumor in Spain, donor of Ti plasmid
K84N6	2	R	Analyzed in this study	pAgK434 (>300), pTi K84N6 (190), pAgK84 (47.7)	Transconjugant obtained after Ti-plasmid transfer from strain 325-4 to *A. radiobacter* strain K84
C58	1	S	Nop	pAtC58 (410), pTiC58 (195)	Indicator of agrocin 84 sensitivity
A. radiobacter					
K84	2	R	Nop, Oct[z]	pAgK434 (>300), pNoc (173), pAgK84 (47.7)	Biocontrol agent of crown gall
K1026	2	R	Nop, Oct[z]	pAgK434 (>300), pNoc (173), pAgK1026 (41.8)	Strain K84 with Tra$^-$ agrocin 84 plasmid (11), biocontrol agent of crown gall

[w] R = resistant and S = sensitive. Sensitivity to agrocin 84 was determined by method of Stonier (38).
[x] Nop = nopaline and Oct = octopine. Opine utilization was determined by method of López et al. (17).
[y] Characteristics of plasmids were described by Vicedo et al. (42).
[z] Octopine is slowly degraded by these strains.

as previously described (19). One hundred plants were used per treatment in individual pots and were arranged in a completely randomized design. Before planting in pots, the sterile substrate (50% peat, 50% sand) was inoculated with strain 325-4 or strain K84N6 of *A. tumefaciens* as described above, to a final concentration of 10^7 CFU/g of substrate. Plants were grown for 9 months in a contained greenhouse and, after harvest, evaluated for the number of diseased plants and number and weight of tumors per plant.

Root colonization by *Agrobacterium*. Population sizes of *A. tumefaciens* and *A. radiobacter* in the rhizosphere of plants from the biocontrol experiment described above were determined 9 months after inoculations. For each strain and treatment, six plants were randomly selected after harvest. Roots (10 g per plant) were soaked in 0.25× Ringer solution (35) with 0.05% Tween 20 and shaken for 45 min at 200 rpm on an Orbit Environ Shaker (Lab-Line Instruments, Inc., Melrose Park, IL). The number of bacteria from the roots was counted after plating dilutions of washings on semiselective media. Strain 325-4 was isolated on a medium described by Schroth et al. (36) for biovar 1 strains. Strains K84N6, K84, and K1026 were isolated on a semiselective medium for biovar 2 (23). A total of 20 to 30 *Agrobacterium* spp.-like colonies per plate were purified and analyzed. Several tests were used to distinguish among the different strains (Table 1). Specific biovar tests were conducted according to Moore et al. (20) to distinguish and confirm biovar 1 (strain 325-4) or 2 (strains K84 and K84N6). Agrocin 84 production by strains K84 and K84N6 was assayed according to the Stonier procedure (38). Pathogenicity on tomato plants was used to distinguish between pathogenic (325-4 and K84N6) and nonpathogenic (K84 and K1026) strains. Plasmids profiles of selected colonies were analyzed as described by Vicedo et al. (43).

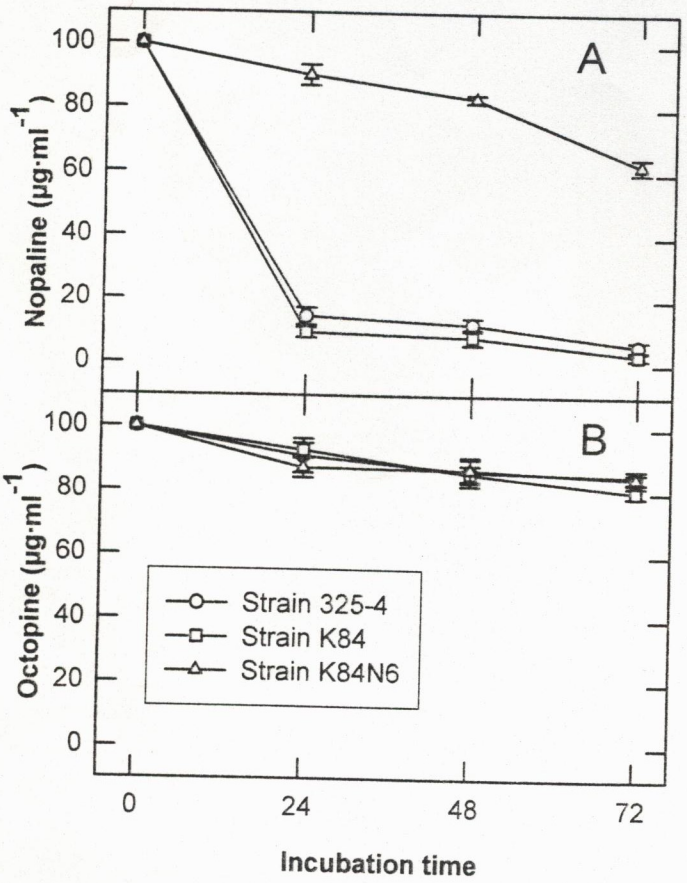

Fig. 1. Utilization of nopaline and octopine by *Agrobacterium radiobacter* strains 325-4 and K84 and the transconjugant strain K84N6 after growing for 72 h in a minimal medium supplemented with nopaline and octopine as a sole carbon and nitrogen source. Opine concentration was measured in the bacterial supernatant after 24, 48, and 72 h of incubation, according to López et al. (17) in two experiments. Data are mean of four replicates consisting in four wells each. Error bars represent the standard error of the mean. At 24, 48, and 72 h, nopaline levels were reduced significantly less ($P < 0.05$) by strain K84N6 than by strains 325-4 and K84; strains 325-4 and K84 did not differ significantly. There were no differences among strains in octopine utilization.

Statistical analysis. Statistical analysis of data was performed using the statistical analysis package SPSS 6.0 for Windows (SPSS Inc., Chicago). Opine utilization was compared among strains by analysis of variance (ANOVA), and means were separated by the least significant difference (LSD) test. To compare host range and virulence of strains K84N6 and 325-4, data of number of infected plants were analyzed by comparison of proportions using the approximate *Z* test (44). Quantitative data (number and size of tumors) were analyzed using Student's *t* test. In biocontrol and root colonization assays, a two-way ANOVA (two factors: strain and treatment) was used to analyze data of number of infected plants, number and weight of tumors per plant, and population sizes. When significant interactions were found, the LSD procedure was used to compare means.

RESULTS

Octopine and nopaline utilization. After incubation for 24 h, strain 325-4 reduced the nopaline level from 100 to 14.6 µg/ml, and strain K84 reduced the nopaline level to 8.4 µg/ml. No significant differences ($P > 0.05$) were observed between these two strains. Strain K84N6 decreased nopaline levels very slightly (91.6, 83.2, and 62.6 µg/ml after incubation for 24, 48, and 72 h, respectively), and the population size of this strain did not increase during the assay (data not shown). At 24, 48, and 72 h, nopaline levels were reduced significantly less ($P < 0.05$) by K84N6 than by strains 325-4 and K84. There were no significant differences among strains K84N6, 325-4, and K84 in octopine utilization, and octopine levels decreased slightly during the incubation (Fig. 1).

Host range. Strains 325-4 and K84N6 were able to induce tumors in tomato, tobacco, sunflower, grapevine, datura, bean, watermelon, and vinca, but only small excrescences were observed in eggplants. Except on vinca, strains 325-4 and K84N6 did not differ significantly in virulence as measured by the number of diseased plants (Table 2).

Virulence assays. The virulence of strains 325-4 and K84N6 was tested on several fruit trees considered typical host plants for *A. tumefaciens*. The incidence of crown gall was high (80 to 100% galled plants) when the most susceptible hosts, peach × almond hybrids Adafuel and GF677, were grown in potting mix amended with strain 325-4. Less susceptible hosts (pear and apple) showed much lower disease incidence (4 to 8% galled plants). Similar results were obtained when the transconjugant strain K84N6 was used. No statistically significant differences ($P > 0.05$) in the number of infected plants nor in the number and size of tumors per infected plant were observed when using strains 325-4 and K84N6 (Table 3). In the experiments with tomato plants, the transconjugant strain K84N6 resulted in significantly fewer galled plants than strain 325-4 (Table 3).

Pathogenicity stability. The transconjugant strain was detected in tumors in significantly ($P < 0.05$) higher populations (1.1×10^7 CFU per tumor) than the wild type (2.1×10^6 CFU per tumor) after 40 days. However, when the pathogenicity of the recovered isolates was tested, there were no significant differences in the percentage of pathogenic colonies (96.8 and 97.8% from strains 325-4 and K84N6, respectively). These results demonstrated that the pathogenic determinants of strain K84N6 remained stable in tomato tumors at least to the same extent as the wild-type strain 325-4.

Biological control. *A. tumefaciens* strains 325-4 and K84N6 did not differ practically in ability to cause crown gall. The factorial ANOVA analysis revealed significant *A. tumefaciens* strain × treatment interactions in the number of infected plants and the number of tumors. The LSD test revealed that strains K84 and K1026 were very efficient in controlling galls induced by strain

325-4, because they significantly ($P < 0.05$) reduced the percentage of infected plants (4.0 and 7.0%, respectively) as compared with the nontreated control (91.0%) (Table 4). There was no difference between crown gall suppression by K84 and K1026. In contrast, in potting mix with the transconjugant strain K84N6, a statistically significant but biologically ineffective reduction on crown gall incidence occurred in K84- or K1026-treated plants (84.3 and 81.7% of infected plants, respectively) as compared with the nontreated plants (97.5%). No significant differences were observed between treatments with K84 or K1026 against K84N6 (Table 4).

The weight of the tumors caused by strains 325-4 and K84N6 on K84- or K1026-treated plants was significantly less than that on nontreated controls ($P < 0.05$). The transconjugant strain induced more galls per plant than strain 325-4 ($P < 0.05$) in nontreated and in K84- or K1026-treated plants. The number of tumors per plant differed significantly between nontreated and treated plants growing in soil inoculated with strain K84N6 (Table 4).

Root colonization by *Agrobacterium* strains. At 9 months after soil inoculation, populations of the *Agrobacterium* strains in the rhizosphere were measured in the biocontrol experiment described above (Fig. 2). A factorial ANOVA test was used to analyze in two separate analyses the population sizes of *A. tumefaciens* (strains 325-4 and K84N6) and *A. radiobacter* (strains K84 and K1026). In both cases, the analysis revealed significant *A. tumefaciens* strain × treatment interactions. For *A. tumefaciens* populations, the LSD analysis showed that the population size of strain 325-4 was significantly greater ($P < 0.05$) in the rhizosphere of nontreated plants (2×10^5 CFU/g of root) than in the rhizosphere of K84- or K1026-treated plants (8.6×10^3 and 1.3×10^4 CFU/g of root, respectively) (Fig. 2A). After 9 months of incubation, the population size of strain K84N6 on nontreated plants was 3.6×10^6 CFU/g of root and was 3.2 and 2.1×10^6 CFU/g of root on plants treated with strains K84 and K1026, respectively. The population size of strain K84N6 did not differ significantly between nontreated plants and plants treated with strains K84 or K1026 (Fig. 2B). In every case, the population size of transconjugant strain K84N6 was significantly greater ($P < 0.05$) than that of donor strain 325-4.

After 9 months in a substrate inoculated with strain 325-4, strains K84 and K1026 maintained very high population sizes. Population sizes of strains K84 and K1026 were 3.5×10^6 and 9.0×10^5 CFU/g of root, respectively (Fig. 2A). In contrast, when the soil was amended with strain K84N6, the population sizes of strains K84 and K1026 were 1.8 and 1.7×10^2 CFU/g of root, respectively (Fig. 2B). No significant differences were observed between population sizes of strains K84 and K1026 (Fig. 2B). Population sizes of strains K84 and K1026 were significantly lower ($P < 0.05$) in the substrate inoculated with strain K84N6 than in the substrate inoculated with strain 325-4 (Fig. 2A and B).

TABLE 3. Incidence and severity of grown gall on hosts grown in potting mix amended with *Agrobacterium tumefaciens* strains 325-4 or K84N6

Host	Strain[t]	No. of plants analyzed	Infected plants (%)[u]	No. of tumors/ infected plant[v]	Size of tumors (cm)/ infected plant
Cherry	325-4	10	50.00	1.00 ± 0.0	4.20 ± 0.6
	K84N6	10	80.00	1.62 ± 0.4	3.02 ± 0.6
Pear	325-4	25	4.00	1.00[w]	4.00[w]
	K84N6	24	4.15	1.00[w]	4.00[w]
Apple	325-4	23	8.69	1.50 ± 0.5	1.96 ± 0.5
	K84N6	26	3.84	1.00[w]	1.50[w]
Adafuel[x]	325-4	6	100.00	1.33 ± 0.2	2.65 ± 0.4
	K84N6	5	80.00	2.25 ± 0.9	3.55 ± 0.3
GF677[x]	325-4	21	95.23	3.35 ± 0,5	1.88 ± 0.1
	K84N6	22	100.00	3.59 ± 0,4	1.46 ± 0.1
Tomato	325-4	59	86.44	4.90 ± 0.5	0.04 ± 0.0
	K84N6	63	44.44[y]	2.03 ± 0.3[y]	0.71 ± 0.01[y]
	325-4	34	97.06	3.84 ± 0.4	ND[z]
	K84N6	32	87.50[y]	2.68 ± 0.2[y]	ND

[t] One-year-old seedlings of different fruit trees and 3-week-old tomato plants were planted in sterile soil amended with *A. tumefaciens* (325-4 or K84N6 to a final concentration of 10^7 CFU/g of substrate. Tumor formation in the roots and crown was recorded 9 months later for fruit trees and 4 months later for tomato plants.

[u] Number of infected plants produced by strains 325-4 and K84N6 were compared within each host by comparison of proportions using the approximate *Z* test. No comparison was made between different hosts.

[v] Number and size of tumors (mean ± standard error) produced by strains 325-4 and K84N6 were compared using Student's *t* test. No comparison was made between different hosts.

[w] Only one tumor was obtained.

[x] Peach × almond hybrid.

[y] Indicates a significant difference ($P < 0.05$) between strains 325-4 and K84N6.

[z] ND = not determined.

DISCUSSION

Plasmid transfer between different *Agrobacterium* strains or related species can result in transconjugants with characteristics and behaviors different from those of the donor and the recipient strains (15,24,32) or in transconjugants with characteristics similar to their parental strains (37). In this paper, the behavior of the pathogenic transconjugant strain K84N6, derived from strain K84 of *A. radiobacter* after spontaneous Ti-plasmid acquisition from strain 325-4 of *A. tumefaciens* (42), is described.

The opine-utilization assays demonstrated that transconjugant strain K84N6 metabolized nopaline slightly, whereas its parental strains, 325-4 and K84, utilized this opine very efficiently. As we previously described (42), the Ti plasmid of the transconjugant was modified as a consequence of the plasmid transfer event, possibly due to a recombination with the resident pNoc plasmid of strain K84. The new Ti plasmid of K84N6 appeared to have a deletion in the left part of the nopaline catabolic region (42), which contains the *ocd*, *arc*, and *nox A* genes implicated in nopaline degradation (34,46). This fact could explain the result that the new strain was greatly reduced in its ability to utilize nopaline. According to the opine concept (29), strains having the ability to catabolize an opine should be more competitive in the tumor environment than those lacking this trait. Thus, this could imply a disadvantage for

TABLE 4. Effects of treatments with the strains K84 and K1026 on the infection caused by *Agrobacterium tumefaciens* strain 325-4 and the transconjugant strain K84N6

Strain[x]	Treatment[y]	No. of plants analyzed[z]	Infected plants (%)	Weight of tumors/ infected plant	No. of tumors/ infected plant
325-4	Nontreated	61	91.07 a	22.33 ± 2.9 a	2.65 ± 0.2 a
	K84	50	4.0 b	0.70 ± 0.02 b	1.00 ± 0.0 a
	K1026	57	7.00 b	0.78 ± 0.47 b	1.00 ± 0.0 a
K84N6	Nontreated	80	97.50 c	29.73 ± 2.7 a	8.34 ± 0.6 b
	K84	70	84.37 d	13.37 ± 2.2 b	3.80 ± 0.3 a
	K1026	77	81.69 d	14.78 ± 2.6 b	2.89 ± 0.3 a

[x] *A. tumefaciens* strain 325-4 or K84N6 was introduced into the soil by irrigation with a bacterial suspension to obtain a final concentration of 10^7 CFU/g of substrate.

[y] Plants of peach × almond hybrid GF677 were treated by dipping the roots in a suspension of strain K84 or K1026 peat preparations before being planted in a substrate inoculated individually with each *A. tumefaciens* strain. Nontreated plants were dipped in water.

[z] Tumor formation was recorded 9 months after the treatments. The percentage of infected plants and the number and weight of tumors (mean ± standard error) per infected plant were determined. A factorial analysis of variance test (two factors: strain and treatment) was used to analyze individually the data obtained. A significant *A. tumefaciens* strain × treatment interaction was found in the percentage of infected plants, as well as in the number of tumors per infected plant. In these cases, the least significant difference procedure was used to compared means. The weight of the tumors in plants treated with strains K84 and K1026 was not significantly different in plants inoculated with 325-4 and with K84N6, because the sample size in 325-4 was very small. Means within the same treatment followed by different letters are significantly different at $P < 0.05$.

the transconjugant, at least in the tumor environment. The fact that this new Ti plasmid confers oncogenicity, but neither nopaline nor octopine utilization, supports the Ti-plasmid evolution hypothesis previously proposed (25). According to it, related nopaline-type Ti plasmids were derived from a single ancestral nopaline-type Ti plasmid that has been altered by insertion, deletion, or recombination with other endogenous *Agrobacterium* plasmids.

Tumor formation was observed in most of the host plants that were inoculated with K84N6, suggesting that the host range of the transconjugant is very similar to that of the parental strain. Thus, although some modifications were found in the genetic structure of the transferred Ti plasmid (42), none of them seems to affect strongly the genes located in the T-DNA and in the *vir* A and C regions that are involved in host specificity (2,39). In additional studies of virulence on fruit trees, strains K84N6 and 325-4 did not differ in the ability to induce crown gall under field conditions when they were inoculated in the soil. The incidence of crown gall on common hosts of *A. tumefaciens* (cherry and peach × almond hybrids GF677 and Adafuel) was very high (50 to 90%) and similar for both strains. The pathogenic determinants also were stable in *Agrobacterium* colonies isolated from tumors produced by strain 325-4 or K84N6. All these results suggest that the Ti plasmid-encoded functions for pathogenicity can be expressed in the chromosomal background of strain K84 and do not seem to be incompatible with the new genetic background. Other workers (22,24,32), however, obtained transconjugants from other bacterial species in which the plasmid-encoded functions have changed (i.e., host specificity) compared with the plasmid-donor strains. These authors suggested that the plasmid-encoded functions may be expressed in another genetic background, although some of them may undergo modifications. These changes may be the consequence of a different regulation of expression of plasmid-borne genes in the parental chromosomal background than in the transconjugant (22), of modifications undergone by these genes when transferred to a different bacterium (31), or the consequence of a functional or structural incompatibility of the transferred plasmid with the new chromosomal background (24).

The results of the biological control experiment demonstrated that the wild type, but not the transconjugant, was controlled by strain K84 or K1026. This assay was not repeated because of the difficulties and restrictions of working with genetically modified organisms. However, the high number of plants used for the experiments lends reliability to the results obtained. When strain 325-4 was used as the pathogen and the plants were treated with strains K84 and K1026, only 4 and 7%, respectively, of the plants developed galls. In contrast, when strain K84N6 was the pathogen, 84.3 and 81.6% of the plants treated with strains K84 and K1026, respectively, developed galls. Strain K84N6 harbors pAgK84, a plasmid encoding agrocin 84 production and immunity to it (33). The production of agrocin 84 is one of the main factors involved in the biocontrol by strains K84 and K1026 (14). This could explain why strain K84N6 was not effectively controlled by any of

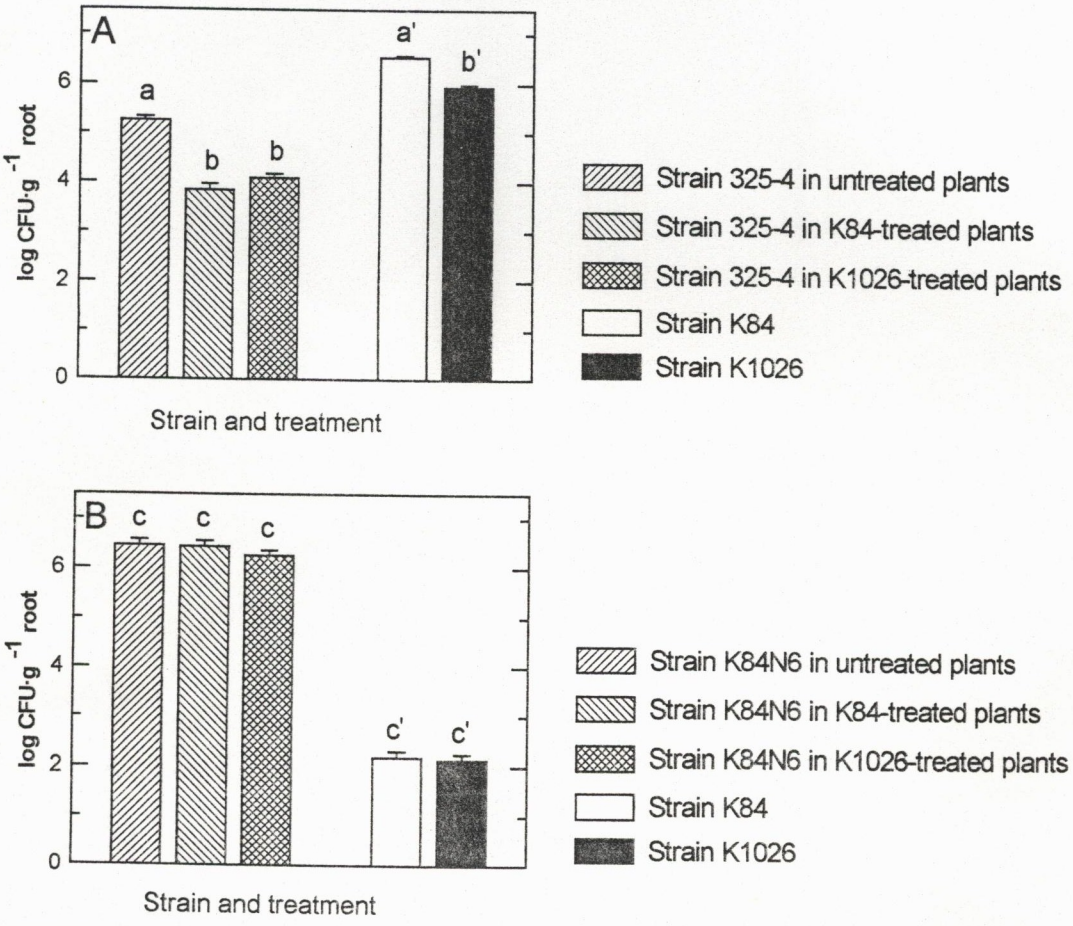

Fig. 2. Population sizes of *Agrobacterium tumefaciens* and *A. radiobacter* in the rhizosphere of the plants from the biocontrol experiment. At 9 months after inoculations, the population sizes of *A. tumefaciens* strains **A**, 325-4 and **B**, K84N6 in nontreated, K84-treated, and K1026-treated plants were determined, as well as the populations of *A. radiobacter* strains K84 and K1026 in K84- or K1026-treated plants. The number of bacteria was counted after plating suitable dilutions of roots extracts on semiselective media and subsequent identification of the obtained isolates. Error bars represent the standard error of the means. In two separate tests, a factorial analysis of variance test was used to analyze the population sizes of *A. tumefaciens* and *A. radiobacter*. In both cases, a significant *A. tumefaciens* strain × treatment interaction was found. Across all treatments, population sizes of *A. tumefaciens* strain K84N6 were significantly higher ($P < 0.05$) than those of strain 325-4, as revealed by a least significant difference test. However, population size of *A. radiobacter* strain K84 or K1026 was significantly lower ($P < 0.05$) when soil was inoculated with strain K84N6 than when it was inoculated with strain 325-4. No comparison was made between population sizes of *A. tumefaciens* and *A. radiobacter*. Bars with a different letter are significantly different.

them, unlike the parental strain 325-4, which was sensitive to agrocin 84. However, biocontrol activity of strain K84 or K1026 against some agrocin 84-resistant strains has been reported (4,18, 43). In addition to agrocin 84 production, other mechanisms appear to be involved in crown gall suppression including root colonization and production of other antibiotic-like substances (6,27, 28). In our experiment, such mechanisms seem to be not as effective, because the transconjugant K84N6 was as good a root colonizer as strain K84 and probably produces the other antibiotic-like substances described for this strain. Nevertheless, strains K84 and K1026 still provide a slight, but significant, reduction of crown gall induced by strain K84N6, probably as a consequence of competition for nutrients or habitat or of production of other unknown mechanisms.

Microbial competition is an important factor for establishing bacteria on roots and in the rhizosphere (1,32), and better knowledge of the interactions between bacterial populations is essential for biocontrol. Survival on roots and in the rhizosphere of plasmid-containing strains was described for other bacterial species as being dependent on the host bacterium that harbors the plasmid (5). However, other authors reported only slight differences in survival between the transconjugant and the plasmid-donor strain (40,45). Our results demonstrated that the population size of the transconjugant was greater than that of the donor strain 325-4 in the rhizosphere of untreated and K84- or K1026-treated plants. Previous treatment of roots with agrocin 84-producing strains K84 or K1026 may favor root colonization by the transconjugant strain K84N6, resistant to this bacteriocin, as compared with the susceptible donor strain 325-4. It has been previously suggested that the inability of the transconjugant to metabolize nopaline could be a disadvantage for the strain. However, strain K84N6 reached higher population sizes in the rhizosphere than the donor strain, even on nontreated plants. Although it was greatly reduced in its ability to utilize nopaline, it may contain other opine-utilization traits, like agrocinopines. Furthermore, opine-utilization traits may be more important for survival in the tumor environment than in the rhizosphere. This was verified by comparing bacterial populations in opine-producing transformed plants (9). Our results indicate a better adaptation of the transconjugant to the rhizosphere habitat. Although previous studies demonstrated that strains K84 and K1026 survive in the rhizosphere in high populations (27,41), in our case, the biocontrol agents were recovered in very low populations from the rhizosphere when the soil was inoculated with the transconjugant. This supports the hypothesis that strain K84N6 is more competitive than strains K84 and K1026, and the acquisition of the Ti plasmid by K84 background could be the origin of such ability. The horizontal transfer of Ti plasmid in nature is not well documented (8), and the biological relevance of the pTi transfer to strain K84 could be related to its frequency, which seems to be very low (M. J. López-López, *unpublished data*). The studies of features of the transconjugant strain K84N6 show that even though in some characteristics it does not differ from the donor strain (host range, ability to induce tumors, stability of the pathogenicity), it does differ in the inability to use nopaline and its better survival in roots. Because this kind of pathogenic transconjugants derived from strain K84 cannot be controlled with the available biological methods of crown gall control, the appearance of these transconjugants and their possible dissemination can entail a risk that should be further evaluated.

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