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Biological Control of *Agrobacterium tumefaciens*, Colonization, and pAgK84 Transfer with *Agrobacterium radiobacter* K84 and the Tra⁻ Mutant Strain K1026

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The efficacies of *Agrobacterium radiobacter* K84 and K1026 in root colonization, crown gall control, and plasmid transfer were compared. Levels of root colonization by K84 and K1026 of Montclar and Nemaguard peach seedlings were similar during the 21 days of the experiment. Four strains of *A. tumefaciens* bv. 1 were used for soil inoculations in biological control experiments on GF677 and Adafuel peach × almond rootstocks; two were sensitive and two were resistant to agrocin 84. Both strains K84 and K1026 were very efficient in controlling the sensitive strains, but some tumors appeared with both treatments. In the biocontrol of resistant strains, no galls were observed in K1026-treated plants, but some K84-treated plants had galls. Recovery of agrobacteria from galls in experiments with sensitive and resistant strains showed that all of the isolates from the controls or K1026-treated plants and most of the isolates from K84-treated plants had the same characteristics as the inoculated strains. Nine isolates from the K84-treated plants growing in soil inoculated with one resistant strain were virulent and produced agrocin 84. These isolates had a plasmid that hybridized with a probe prepared with the *Bam*HI C fragment from pAgK84. These results show the efficiency of K1026 in biocontrol of agrocin 84-sensitive and -resistant strains of *A. tumefaciens* and suggest the use of K1026 as a safer organism than K84 for biological control of crown gall.

Crown gall, which is caused by *Agrobacterium tumefaciens*, is distributed worldwide and is responsible for nursery and field losses among a large variety of plants, especially stone fruit trees (22, 23, 27, 29).

Biological control of crown gall with *A. radiobacter* K84 has been successful throughout the world for more than 10 years (11, 29, 34). The main mechanism involved in the biocontrol is the biosynthesis and secretion of agrocin 84 by K84 (18). Agrocin 84 is an antibiotic-like substance which specifically inhibits many tumorigenic *Agrobacterium* strains (19). This substance was identified as a disubstituted fraudulent analog of adenosine nucleoside (30). Production of agrocin 84 is encoded on a 47.7-kb plasmid, pAgK84 (9, 39). Genes encoding agrocin 84 biosynthesis are expressed in bacteria colonizing plant roots, and their expression is regulated by root exudate and by nopaline (44). López et al. (21) have demonstrated that other mechanisms must be implicated in this biocontrol because strain K84 cured of pAgK84 was as effective as its agrocinogenic parent in controlling *A. tumefaciens* strains resistant to agrocin 84. They suggested that mechanisms such as root colonization, blockage, and competition for infection sites could be involved in biological control. Farrand and Wang (13) discuss the role of the production of other agrocins in biocontrol by strain K84.

Also borne on pAgK84 are genes conferring immunity to agrocin 84 and conjugal transfer capacity (12, 35). Results obtained by Panagopoulos et al. (32) using one strain of *A. tumefaciens* sensitive to agrocin 84 suggested that conjugal transfer of pAgK84 is responsible for the breakdown of biocontrol of crown gall in one experiment. Other authors (40) observed transfer in field experiments after coinoculation of strain K84 and an *A. tumefaciens* strain resistant to agrocin 84. The transfer frequency in the field, in natural conditions, or after commercial use of strain K84 is unknown.

To overcome transfer of pAgK84 and to safeguard biological control of crown gall, a mutant of pAgK84 has been constructed (17). The resultant strain, K1026, is indistinguishable from the parent strain except that pAgK1026 is no longer capable of transfer to other bacteria because of a deletion of 5.9 kb overlapping the *tra* region of pAgK84. The use of a safer K84 is an objective for countries in which K84 is used. Strain K1026 has been proposed as a replacement for K84 to prolong the effective biological control of crown gall (16) and is being used for crown gall control in Australia. Only Jones and Kerr (16) have shown its effectiveness in comparison with K84 in a soil previously inoculated with a strain of *A. tumefaciens* sensitive to agrocin 84.

The effectiveness of K1026 against strains of *A. tumefaciens* resistant to agrocin 84 has not been studied. If K1026 can colonize roots and control agrocin 84-resistant strains of *A. tumefaciens* to the same extent as K84 can, then both K84 and K1026 must use other biocontrol mechanisms besides agrocin 84 production.

The present study was undertaken to determine the following: (i) the comparative colonization abilities of strains K84 and K1026 in roots of different stone fruit seedlings, (ii) the comparative efficacies of strains K84 and K1026 against *A. tumefaciens* strains sensitive and resistant to agrocin 84 in field conditions, and (iii) whether pAgK84 is transferred to tumorigenic agrobacteria in field experiments.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1.

(i) ***A. radiobacter*.** Strains K84, K1026, K434 and K84 Agr⁻ were used. The strains were kindly supplied by the following

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Biovar	Sensitivity to[a]: Agrocin 84	Sensitivity to[a]: New agrocin	Opine utilization[b]	Plasmid(s) (size [kb])[c]	Description
Agrobacterium radiobacter[d]						
K84	2	R	R	NOP, OCT	pAtK84b (186), pAgK84 (47.7)	Produces agrocin 84 (18) and a new agrocin (24)
K1026	2	R	R	NOP, OCT	pAtK84b (186), pAgK1026 (41.8)	Derived from K84 with Tra⁻ agrocin 84 plasmid (17)
K434	2	R	R	NOP, OCT	pAtK84b (186)	Spontaneous mutant of K84 lacking pAgK84 and producing agrocin 434 (6)
K84 Agr⁻	2	R	R	NOP, OCT	pAtK84b (186)	K84 cured of pAgK84 (4)
Agrobacterium tumefaciens						
C58	1	S	S	NOP	pAtC58 (410), pTiC58 (195)	Indicator strain for agrocin 84 production and molecular weight marker
804-42	1	S	S	NOP	19, 195, 238	
805-3	1	S	S	NOP	195, 265	
678-2	1	R	R	NOP	124, 154	
436-3	1	R	R	NOP	150, 180, 300	
Escherichia coli						
V517					53.7, 7.2, 5.5, 5.1, 3.9, 3.0, 2.7, 2.1	Molecular weight marker used to determine sizes of *A. tumefaciens* plasmids (26)
1231(pBR322::*Bam*HI C)					pBR322::*Bam*HI C	1231 described by Pischl and Farrand (33); *Bam*HI C fragment from pAgK84 cloned into pBR322; fragment responsible for production of agrocin 84 (35)

[a] R, resistant; S, sensitive. Sensitivity to agrocin 84 was determined by the Stonier method (42) with Stonier medium (41), a modification of Stonier medium (5), and mannitol glutamate medium (28). Sensitivity to new agrocin was determined by using strains K84 Agr⁻ and K434 in mannitol glutamate medium (28).
[b] NOP, nopaline; OCT, octopine. Opine utilization was determined by the method of López et al. (20).
[c] Size and number of plasmids were evaluated by the Eckhart method (8); *E. coli* V517 and *A. tumefaciens* C58 were used as molecular weight markers by the method of Albiach and López (1). Only the sizes are given in cases in which plasmid designations are not given.
[d] These *A. radiobacter* strains contain a large cryptic plasmid in addition to the plasmids indicated.

individuals: G. C. Bullard (Bio-care Technology, Woy-Woy, Australia), K1026; B. G. Clare (Waite Agricultural Institute, Glen Osmond, Australia), K434; and L. W. Moore (Oregon State University, Corvallis), K84 Agr⁻.

(ii) ***A. tumefaciens***. Strains 804-42, 805-3, 678-2, and 436-3 were used. They were isolated in Spain from peach rootstocks. Strains 804-42 and 805-3 were sensitive to agrocin 84, and strains 678-2 and 436-3 were resistant to this agent. Strain 678-2 produces a substance that gave a small zone of inhibition (1 cm) of strain K84.

(iii) ***Escherichia coli***. Strains V517 and 1231 were kindly supplied by R. Aznar (Facultad de Ciencias Biológicas, University of Valencia, Valencia, Spain) and S. K. Farrand (University of Illinois, Urbana), respectively.

Root colonization. Montclar and Nemaguard peach (*Prunus persica*) seedlings were inoculated with strain K84 or K1026 by immersion of the crown and roots for 15 min in suspensions of late-exponential-phase cells adjusted turbidometrically to densities of approximately 5×10^8 CFU/ml. The seedlings were planted, without being rinsed, in sterile sand. They were periodically irrigated with sterile Hoagland solution (15). On the day of planting and 5, 13, and 21 days after planting, the seedlings were gently dug up, and the roots were cut off at the point of seed attachment and agitated to remove the adhering sand. The roots were placed in 0.25× Ringer solution (36) containing 0.05% Tween 20 and shaken for 45 min at 200 rpm. The rhizoplane (root surface) bacteria so obtained and the K84 and K1026 cells were counted after dilution in phosphate-buffered saline, pH 7.2 to 7.4. Dilutions were plated on nonselective PGYA medium (20) for counting rhizoplane bacteria and on semiselective New and Kerr medium (31) for counting K84 or K1026 colonies. Production of agrocin 84 by these colonies was used as an identifying trait for K84 or K1026. Data were expressed as log (CFU per gram [fresh weight] of root). In all cases, at least four replicates for each treatment were run after a previous experiment that showed high homogeneity of the number of cells per root among different plants receiving the same treatment at different times.

Biological control. (i) **Sensitive strains.** One experiment under controlled conditions was performed in six concrete open-air containers (5 by 2 by 0.5 m) that were filled with soil typical of the area: loamy, calcareous, sandy clay (pH 7.6). Before planting, the soil in three containers was inoculated with *A. tumefaciens* 804-42, and the soil in the other three was inoculated with *A. tumefaciens* 805-3. Forty liters of bacterial suspension in water (ca. 10^9 CFU/ml) was added to each container. Flood irrigation was done after soil inoculation. The final concentration of bacteria was about 10^6 CFU/g of soil. One hundred ten rooted 1-year-old plants of the peach × almond (*P. persica* × *P. dulcis*) hybrids GF677 and Adafuel were used per treatment.

(ii) **Resistant strains.** The experiment was performed under controlled conditions in open-air pots with sterile substrate

containing 50% peat and 50% sand. Before planting, each pot was inoculated with *A. tumefaciens* 678-2 or 436-3, each of which is resistant to agrocin 84. One-half liter of water containing 1.3×10^7 CFU/ml was added to each pot. The final concentration of bacteria was about 10^6 CFU/g of substrate. Fifty dormant 1-year-old GF677 plants were used per treatment.

(iii) Treatments. The plants were superficially wounded just before planting. One control and two treatments (K84 or K1026) were used in experiments with sensitive or resistant strains. Plants treated with a biocontrol agent were dipped in a suspension of peat preparation of strain K84 or K1026 prepared by mixing 5 kg of commercial inoculated peat with 5 liters of water. The peat inoculum was prepared as previously described (23), and the concentration of the bacteria was about 10^9 CFU/ml. As a control, plants were dipped in a sterile peat suspension. In both experiments, plants were grown for 10 months and then dug up and scored for galls. The number of tumors per plant, the weights of tumors, and the number of plants with galls were recorded for each treatment.

Plasmid transfer. (i) Isolation and characterization of *Agrobacterium* isolates from tumors. Agrobacteria were isolated from tumors appearing in plants receiving the three treatments of the biological control experiments described above. Macerates of tumor slices were directly plated on semiselective media for biovar 1 (37) and biovar 2 (31) strains. After purification of *Agrobacterium*-like colonies, the obtained isolates were assessed for *Agrobacterium* characteristics and classified in biovars according to the classification of Moore et al. (28). Agrocin 84 sensitivity or resistance and bacteriocin production were tested by the Stonier procedure (42) with Stonier medium (41), Dhanvantari's modification of Stonier medium (5), and mannitol glutamate medium (28). Strain C58 was used as the indicator strain for agrocin 84 production; strains producing a zone of inhibition similar to those produced by strains K84 and K1026 were tested against two other strains sensitive to agrocin 84 and two resistant strains. Pathogenicity was determined by inoculation of the *Agrobacterium* isolates onto greenhouse-grown tomato plants (*Lycopersicum esculentum*). Plasmid profiles of the agrocin 84-producing strains were determined by the Eckhart method (8) as modified by Albiach and Lopez (1).

(ii) Preparation of pAgK84 radioactive probe. Whole plasmid pBR322::*Bam*HI C (Table 1) after *Bam*HI digestion or just the insert recovered from the agarose gel was used to prepare probes radiolabeled to high specific activity by the random primer technique (14).

(iii) Southern blotting, hybridization, and washing. Southern blotting was performed with Immobilon-N membranes (0.45-μm pore size; hydrophobic polyvinylidene difluoride). The prehybridization, hybridization, and washing were done at 65°C according to the protocol described in the Millipore manual (26a).

Statistical analysis of data. Analysis of variance of data collected from the root colonization and biological control experiments was performed with the StatGraphics computer program (Statistical Graphics Corporation, Inc.).

RESULTS

Root colonization. The levels of root colonization of Montclar and Nemaguard peach seedlings by strains K84 and K1026 are shown in Fig. 1. The roots were colonized with similar efficiencies during this period by the two strains. No statistically significant differences were observed between

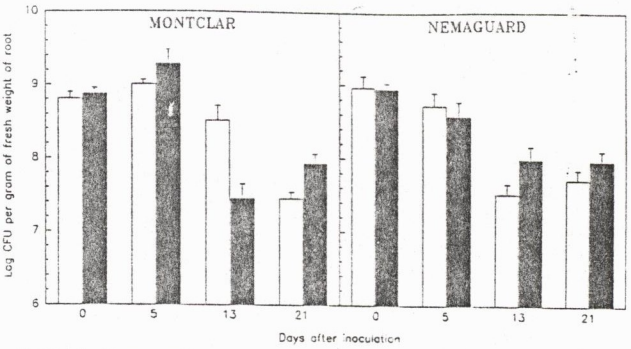

FIG. 1. Root colonization of Montclar and Nemaguard peach seedlings by *A. radiobacter* K84 (□) and K1026 (■). The thin bars indicate standard deviations.

the amounts of K84 and K1026 ($5.33 \times 10^8 \pm 1.33 \times 10^8$ CFU/g [fresh weight] of root versus $8.03 \times 10^8 \pm 1.28 \times 10^8$ CFU/g [fresh weight] of root) in Montclar or Nemaguard roots ($3.84 \times 10^8 \pm 5.97 \times 10^7$ versus $3.82 \times 10^8 \pm 6.17 \times 10^7$ CFU/g [fresh weight] of root, respectively). No significant differences between Montclar and Nemaguard seedlings were found ($6.73 \times 10^8 \pm 1.13 \times 10^8$ versus $3.83 \times 10^8 \pm 1.09 \times 10^8$ CFU/g [fresh weight] of root). Data are expressed as mean ± standard error. About 8×10^8 CFU of K84 and K1026 per g were able to attach to the roots at time zero (Fig. 1). There was a decrease in colonization of about one order of magnitude between 0 and 21 days because the number of CFU per gram (fresh weight) of root remained at about 10^7 in both rootstocks with both strains. The number of rhizoplane bacteria in nonselective medium was between one and two orders of magnitude higher than the number of K84 and K1026 colonies in the semiselective medium. The numbers of rhizoplane bacteria in nonselective medium were similar in the same seedling in plants treated with K84 and plants treated with K1026.

Biological control. (i) Sensitive strains. Biocontrol by strains K84 and K1026 on GF677 and Adafuel rootstocks is shown in Table 2. The incidence of plants with galls was higher in Adafuel than in GF677 rootstocks in this experiment. The results show the effectiveness of strains K84 and K1026 against *A. tumefaciens* 804-42 and 805-3, which are sensitive to agrocin 84. K1026 was as efficient as K84 in controlling crown gall, and among plants treated with the biocontrol agents practically no diseased plants were observed. The incidence of plants with only one gall among the few diseased plants after treatments with K84 or K1026 strains was 100%. The percentage of control plants with one gall was lower; i.e., untreated plants generally had more than one gall per plant.

(ii) Resistant strains. Biocontrol by strains K84 and K1026 on GF677 rootstocks is shown in Table 3. Crown gall was totally controlled in pots inoculated with strain 436-3. Total control was also obtained by using K1026 in pots inoculated with strain 678-2, but in plants treated with K84 some galls appeared. Nevertheless, a clear difference between treated and untreated plants was found.

Isolation and characterization of *Agrobacterium* isolates from tumors. One hundred thirty-nine colonies from tumors that appeared on the control and on the K84- or K1026-treated plants in the experiments were characterized (Table 4). The biovar, pathogenicity, and agrocin production of the isolates are shown in Table 4. Biovar 1 isolates were mostly pathogenic and did not produce agrocin 84, like the inocu-

TABLE 2. Effectiveness of crown gall biocontrol on two peach × almond hybrid rootstocks planted in soil inoculated with *A. tumefaciens* strains sensitive to agrocin 84

Rootstock	Strain	Treatment[a]	% of plants with galls	Mean fresh weight (g) of galls/plant	% of diseased plants with only one gall[b]
GF677	804-42	Control	3.06 ± 0.02	0.14	66.67 ± 0.04
		K84	0.00	0.00	0.00
		K1026	0.00	0.00	0.00
	805-3	Control	12.5 ± 0.03	0.27	75.00 ± 0.04
		K84	0.00	0.00	0.00
		K1026	1.25 ± 0.01	<0.01	100.00
Adafuel	804-42	Control	21.17 ± 0.04	0.54	66.67 ± 0.05
		K84	1.31 ± 0.01	<0.01	100.00
		K1026	0.00	0.00	0.00
	805-3	Control	70.58 ± 0.05	6.41	27.08 ± 0.05
		K84	0.00	0.00	0.00
		K1026	1.17 ± 0.01	<0.01	100.00

[a] Control plants were dipped in a suspension of 5 kg of peat in 5 liters of water. K84- or K1026-treated plants were dipped in a suspension of 5 kg of a peat preparation containing the respective strain in 5 liters of water. One hundred ten rooted plants were planted per treatment.
[b] Mean ± standard deviation based on the total number of plants per treatment.

lated strains. Biovar 2 isolates were all nonpathogenic and produced agrocin 84; they probably were K84 or K1026.

Nine pathogenic isolates of biovar 1 produced an agrocin with a pattern of activity similar to that of agrocin 84. These isolates came from K84-treated plants growing in pots inoculated with the resistant strain 678-2. They produced a zone of growth inhibition of K84 similar to that produced by the inoculated strain 678-2. Plasmid profiles of representative isolates are shown in Fig. 2. The agrocinogenic isolates examined had at least three plasmids. Two of them exhibited electrophoretic mobilities indistinguishable from the plasmids of 124 and 154 kb in strain 678-2. The third one migrated with a mobility similar to that of pAgK84. In six of nine isolates, two bands (Fig. 2A, bands a and b) appeared in the ethidium bromide-stained gel. Band a seemed to have a mobility similar to that of the cryptic plasmid of strain K84. A third band (band c) appeared in one of the isolates from tumors but only in four of eight assays.

Initial screening of the pathogenic isolates from tumors by colony hybridization showed that the pathogenic and agrocin-producing isolates contained sequences that hybridized with the pAgK84 probe. Southern blot analysis confirmed the results of colony blot hybridization. As shown in Fig. 2, the pAgK84 probe hybridized only with the 47.7-kb band.

The specificity of the *Bam*HI C probe of pAgK84 was demonstrated with different *A. tumefaciens* and *Rhizobium* isolates from the collection of the Instituto Valenciano de Investigaciones Agrarias. The probe hybridizes only with plasmids pAgK84 (47.7 kb) and pAgK1026 (41.8 kb).

TABLE 3. Effectiveness of crown gall biocontrol on GF677 peach × almond rootstocks in pots inoculated with *A. tumefaciens* strains resistant to agrocin 84

Strain	Treatment[a]	% of plants with galls[b]	Mean fresh weight (g) of galls/plant	% of diseased plants with only one gall[b]
678-2	Control	50.00 ± 0.07	4.21	65.21 ± 0.07
	K84	16.66 ± 0.06	0.58	100.00
	K1026	0.00	0.00	0.00
436-3	Control	19.44 ± 0.06	0.85	100.00
	K84	0.00	0.00	0.00
	K1026	0.00	0.00	0.00

[a] Control plants were dipped in a suspension of 5 kg of peat in 5 liters of water. K84- or K1026-treated plants were dipped in a suspension of 5 kg of a peat preparation containing the respective strain in 5 liters of water. Fifty pots with GF677 rootstock were planted per treatment.
[b] Mean ± standard deviation.

DISCUSSION

The experiments examining biological control of crown gall by *A. radiobacter* K84 and K1026 demonstrated that strain K1026 was as efficient as strain K84 in controlling various *A. tumefaciens* strains. The effectiveness of K84 was observed on two different hosts growing in soils inoculated with *A. tumefaciens* bv. 1 strains sensitive or resistant to agrocin 84. In all cases, a reduction was observed in the number of plants with galls and in the weight and number of galls per plant, confirming previous results of studies done by López et al. (21). The resistant strains used in these experiments were not inhibited by agrocin 84 in any of the media used, and one of them produced a substance that inhibited strain K84 slightly in vitro. However, biocontrol by strains K84 and K1026 was highly efficient against this strain. Our results confirm the results of previous experiments in which control by K84 of *A. tumefaciens* strains resistant to agrocin 84 was observed (3, 4, 7, 21, 43). Our experiments yielded evidence that strain K1026 is also able to use mechanisms not related to agrocin 84 sensitivity to

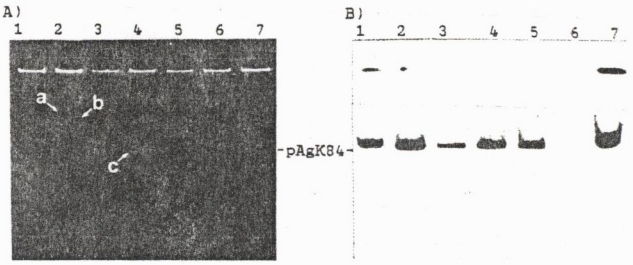

FIG. 2. Electrophoretic and Southern analysis of different *Agrobacterium* isolates. (A) Ethidium bromide-stained gel showing several plasmid profiles. Arrows point out bands a, b, and c, which were not present in strain 678-2 or K84. (B) Autoradiography after hybridization of the corresponding Southern blot with the pAgK84 probe. Lanes 1 and 7, *A. radiobacter* K84; lanes 2 to 5, different isolates of *A. tumefaciens* from tumors; lane 6, inoculated *A. tumefaciens* 678-2.

TABLE 4. Characteristics of *Agrobacterium* isolates from tumors in biocontrol experiments

Inoculated strain	Treatment	No. of isolates[a]							
		Biovar 1				Biovar 2			
		Agrocin 84 producers		Agrocin 84 nonproducers		Agrocin 84 producers		Agrocin 84 nonproducers	
		Pathogenic	Nonpathogenic	Pathogenic	Nonpathogenic	Pathogenic	Nonpathogenic	Pathogenic	Nonpathogenic
804-42	Control	0	0	21	2	0	0	0	0
	K84	0	0	8	0	0	9	0	0
	K1026	0	0	0	0	0	0	0	0
805-3	Control	0	0	11	0	0	0	0	0
	K84	0	0	0	0	0	0	0	0
	K1026	0	0	5	0	0	9	0	0
678-2	Control	0	0	11	0	0	0	0	0
	K84	9	0	31	3	0	10	0	0
	K1026	0	0	0	0	0	0	0	0
436-3	Control	0	0	9	0	0	0	0	0
	K84	0	0	0	0	0	0	0	0
	K1026	0	0	0	0	0	0	0	0

[a] Isolations from tumors that grew during the different treatments were performed on the media described by Schroth et al. (37) and New and Kerr (31). Pathogenicity was determined with tomato plants.

control agrocin 84-resistant strains. Jones and Kerr (16), in the sole previously published article about the practical efficiency of K1026, also described similar efficiencies of K84 and K1026, but only against one *A. tumefaciens* strain sensitive to agrocin 84.

The influence of agrocinopines (10) in the resistant strains and the production of these substances in crown gall tissue were not studied in the experiments.

A role for agrocins other than agrocin 84 in biological control has been recently suggested by Farrand and Wang (13). Production of other agrocins by K84 has been recently described. López et al. (24) observed that strains K84 and K1026 and strain K84 cured of pAgK84 (K84 Agr$^-$) were able to inhibit some phytopathogenic *Pseudomonas*, *Erwinia*, and *Xanthomonas* strains in mannitol glutamate medium. Also, the production of other agrocins in Stonier medium by strain K434, a spontaneous mutant of K84 that has lost pAgK84, has been described (6). Strains K84 Agr$^-$ and K434 do not produce agrocin 84. In our experiments these new agrocins can play a role in the biocontrol of sensitive strains because our agrocin 84-sensitive strains of *A. tumefaciens* were also sensitive to the agrocin produced by strains K84 Agr$^-$ and K434. However, our agrocin 84-resistant strains of *A. tumefaciens* were resistant to both agrocins in mannitol glutamate medium (Table 1). These new agrocins could play a major role in biocontrol against isolates that are resistant to agrocin 84 but sensitive to agrocins produced by strain K84 Agr$^-$ or K434.

Among the mechanisms that contribute to the efficacy of biological control of agrocin 84-resistant strains, efficient root colonization is probably one of the most important. In general, a large population of K84 and K1026 around the roots is necessary to achieve efficient control. K84 has proven to be a good colonizer of the root system in different hosts. Previous results obtained by different authors in colonization experiments showed that the K84 population was stable on tomato and almond roots at 10^6 CFU/g and 10^8 CFU/cm^2 of root, respectively (25, 38). Montclar and Nemaguard seedlings instead of rooted plants were used for our colonization experiment because it was possible to germinate them under sterile conditions and to have clean roots before treatment with K84 or K1026. They were not used for biological control experiments because of their low sensitivity to crown gall. Since GF677 and Adafuel are more sensitive to crown gall than Montclar and Nemaguard rooted plants, the first two rootstocks were used for the biological control experiments. Large populations of strains K84 and K1026 were recovered from the seedlings' roots (10^7 to 10^8 CFU/g [fresh weight] of root), and K1026 was as good a colonizer as K84 during the 21-day study period. This was considered the period of maximum sensitivity of the plants to crown gall.

Mixed populations of K84 and *A. tumefaciens* or K1026 and *A. tumefaciens* were observed in the tumors obtained in the biocontrol experiments. This suggests that the controlling agent and the pathogenic bacteria were colonizing the root system. Furthermore, strain K84 can utilize nopaline, which is produced in the tumors by the strains used in these experiments, and it faces competition for this niche from the tumorigenic agrobacteria that incite the tumors. According to Farrand (11), the role of agrocin 84 is to eliminate this competition, but only if the pathogen is sensitive to agrocin 84. Jones and Kerr (16) observed cocolonization by K84 or K1026 and the *A. tumefaciens* strain used in their experiments 10 months after inoculation. They discuss the apparent anomaly of inhibition of tumorigenesis despite the root colonization by pathogenic and nonpathogenic *Agrobacterium* strains. Strains K84 and K1026 could saturate the sites of infection and prevent a subsequent attack by *A. tumefaciens*. This could explain the efficient biocontrol of agrocin 84-resistant strains.

Transfer of pAgK84 from K84 to *A. tumefaciens* was detected in some galls in our experiments. The soil inoculation and plant treatments simulated closely the conditions found when biological control is used in nurseries. In addition, the percentages of diseased plants obtained were similar to those in cases of natural infection. Therefore, this is the first time that pAgK84 transfer has been demonstrated under conditions very similar to those of natural infection in K84-treated plants. Pathogenic recombinants were first suggested in coinoculation experiments (40). Plasmid transfer had not been observed previously in Spain in plants treated with K84 in our studies (21). Farrand et al. (12) showed that pAgK84 was transferable to the agrocin 84-sensitive tumorigenic strain C58 ex planta. In our experiments, transfer was observed only in tumors caused by the resistant strain 678-2.

However, in experiments using sensitive strains, practically no tumors were observed. Only the pathogenic isolates from tumors were studied; nonpathogenic recombinants were not studied because they did not directly affect the efficiency of the biocontrol. Efficiency of plasmid transfer in the soil was not investigated.

To elucidate the origin of the bands a, b, and c, the pTi plasmid of strain C58 was recovered from the agarose gel and radiolabeled by the random primer technique. This probe hybridizes only to the band corresponding to the 154-kb pTi plasmid of all *A. tumefaciens* strains (data not shown). In spite of this, bands a, b, and c could still be related to other, as yet untested, plasmids of strain K84 or 678-2. It is also possible that they were present in an undetectable amount in 678-2. A third possibility is that they have come by conjugal transfer from other soil bacteria. Further research efforts are aimed at testing these three hypotheses.

Transfer of pAgK84 to the tumorigenic population can result in the appearance of strains no longer controllable by strain K84, because acquisition of the plasmid confers immunity to agrocin 84 (35). In the experiment in which the plasmid transfer to a resistant strain was observed, biocontrol by K84 was efficient, but is possible that, after some time, this transfer can be the cause of a failure in biocontrol, especially if the recombinant strain has a better competitive ability in the rhizosphere than the parent strain. Transfer of pAgK84 to a sensitive strain can affect the efficiency of biological control in a more direct way. In our experiments, no plasmid transfer was observed in the few tumors of the K1026-treated plants. No transfer of pTi from the *A. tumefaciens*-inoculated strains to K84 or K1026 was detected in the different experiments. Incompatibility between pAtK84b (a nopaline-catabolic plasmid from K84) and pTi was described by Clare et al. (2). Nevertheless, Stockwell et al. (40) found transfer of pTi to K84 in 1 of 25 tomato galls.

Inoculation trials have shown that the new Tra$^-$ strain K1026 is as efficient as the progenitor strain K84 in controlling crown gall disease. Strain K1026 is currently in use only in Australia. The results obtained with K1026 by Jones and Kerr (16) in biocontrol of agrocin 84-sensitive strains and the efficient biocontrol of sensitive and resistant strains observed in our experiments suggest the use of strain K1026 as a safer organism wherever strain K84 is used to control crown gall.

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