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Fate of *Agrobacterium radiobacter* K84 in the Environment†

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***Agrobacterium radiobacter* K84 is an effective, commercially applied, biological control agent for the plant disease crown gall, yet little is known about the survival and dissemination of K84. To trace K84 in the environment, spontaneous antibiotic-resistant mutants were used. Growth rates and phenotypes of streptomycin- or rifampin-resistant K84 were similar to those of the parental K84, except the rifampin-resistant mutant produced less agrocin 84 as determined by bioassay. K84 and a strain of *Agrobacterium tumefaciens* established populations averaging 10^5 CFU/g in the rhizosphere of cherry and persisted on roots for 2 years. K84 established rhizosphere populations between 10^4 and 10^6 CFU/g on cherry, ryegrass, and 11 other herbaceous plants. Populations of K84 declined substantially in fallow soil or water over a 16-week period. K84 was detected in the rhizosphere of ryegrass located up to 40 cm from an inoculum source, indicating lateral dissemination of K84 in soil. In gall tissue on cherry, K84 established populations of 10^5 CFU/g, about 10- to 100-fold less than that of the pathogen. These data demonstrate that K84 persists for up to 2 years in a field environment as a rhizosphere inhabitant or in association with crown gall tissue.**

Agrobacterium radiobacter (Beijerinck and van Delden) Conn K84 is used commercially to control crown gall, a tumorigenic plant disease caused by a ubiquitous soil-borne pathogen, *Agrobacterium tumefaciens* (41, 42). Losses due to crown gall can be extensive, particularly in the nursery industry, where each galled plant must be culled. Wounds caused by the routine practice of root pruning may be colonized and infected by *A. tumefaciens* when seedlings are planted in nursery soils infested with the pathogen. During the infection process, *A. tumefaciens* transfers the T-DNA region of the tumor-inducing plasmid (pTi) into the plant cell (58). Incorporation of T-DNA into the plant genome results in the formation of a hyperplastic growth called a gall. The gall provides a nutrient-rich environment for further bacterial growth. Galls can weaken, reduce the aesthetic quality, and eventually kill the host plant. There are no effective chemical controls currently available for crown gall.

Strain K84 has demonstrated remarkable and widespread success as an agent for the biological control of crown gall (40). K84 has been used commercially for more than a decade in many regions of the world, including Australia, Greece, Israel, Italy, Japan, New Zealand, South Africa, Spain, and the United States (50). In the Pacific Northwest region of the United States, K84 is applied routinely to roots of stone fruit seedlings after pruning and before seedlings are planted into nurseries (39). Cells of K84 protect root wounds from infection by *A. tumefaciens*, in part because of the in situ production of agrocin 84, an antibiotic with specific toxicity against sensitive strains of *A. tumefaciens* (18–20, 28). Genes determining the biosynthesis of agrocin 84 and the immunity of the host bacterium to agrocin 84 are present on pAgK84, an indigenous, conjugative plasmid of strain K84 (51). Sensitivity of *A. tumefaciens* to agrocin 84 and to biological control by K84 is determined by a gene required for the uptake of both agrocinopines and agrocin 84, which is present on tumor-inducing plasmids of sensitive strains (17, 19, 20, 49). Cells that harbor both pAgK84 and pTi are pathogenic, because of virulence genes present on pTi, and produce and are immune to agrocin 84, because of genes present on pAgK84. Cells harboring both pAgK84 and pTi may arise through conjugal plasmid transfer between K84 and strains of *A. tumefaciens*, which is known to occur in culture and in crown gall tissue (22, 24, 27, 29, 57). In response to concerns that the predominance of such cells in a field may reduce the efficacy of biological control (45), a derivative strain of K84, lacking a region (*tra*) required for conjugal transfer of pAgK84, has been developed (25, 26). The use of this genetically engineered Tra^- strain could minimize the risk of pAgK84 transfer and the potential breakdown of effective biological control of crown gall.

Despite the widespread use of strain K84 for biological control of crown gall, little is known of the survival or dispersal of this strain in the field. Its capacity to establish populations in crown gall tissue and in the rhizosphere of agricultural plants, to persist in soil or surface water, or to disperse from points of inoculation is virtually unknown. Such information is useful to enhance our understanding of the biology of *A. radiobacter* and to evaluate the potential for plasmid exchange between K84 and other agrobacteria in agricultural fields. Because the behavior of a bacterial strain is often an excellent indicator of that of genetically manipulated derivatives (30, 56), an understanding of the survival and persistence of K84 would be applicable to questions regarding the environmental fate of a Tra^- derivative of K84. In this report, we demonstrate the capacity of K84 to persist in crown gall tissue, in the rhizosphere of agricultural plants, and, for limited periods, in surface water.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study were *A. radiobacter* K84 (39) and *A. tumefaciens* B49c (13). The pathogen, B49c, is resistant to agrocin 84, which is produced by K84. Spontaneous mutants of K84 resistant to either 500

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μg of streptomycin sulfate per ml or 100 μg of rifampin per ml were selected. Growth rates in liquid culture media and reactivity to strain-specific polyclonal antibodies in Ouchterlony double-diffusion assays of mutant isolates were compared to those of the parental strain (6). Agrocin 84 production by K84 and antibiotic-resistant derivative strains was detected by an inhibition zone assay with *A. tumefaciens* strains K27, C58, or NT1(pTiC58Tra^c) in the overlay agar as indicators (28). NT1(pTiC58Tra^c), obtained from Stephen Farrand, University of Illinois, is constitutive for pTi transfer and more sensitive to agrocin 84 than the parental strain, *A. tumefaciens* C58, as indicated by an increase in the diameter of the zone of inhibition on bioassay plates (3).

A spontaneous mutant of *A. tumefaciens* B49c that was resistant to 100 μg of rifampin per ml and similar phenotypically to the parental strain was selected. The stability of antibiotic resistance of mutant strains was evaluated after growth with no selection pressure for over 100 generations. Pathogenicity of B49c and the rifampin-resistant derivative strain was evaluated by artificial inoculations on tomato seedlings maintained in a greenhouse (41).

Further tests of the pathogenicity and biocontrol competence of the derivative strains were conducted in experimental plots in Corvallis and Aurora, Oreg., and in Ephrata and Moses Lake, Wash., by inoculation of Mazzard cherry rootstocks. To evaluate pathogenicity of *A. tumefaciens* B49c and B49c Rf^r, root-pruned cherry seedlings were dipped in a suspension that contained 10^7 CFU/ml. Biocontrol efficacy of strains K84 and K84 Sm^r was tested by first dipping roots in a 10^8-CFU/ml suspension of the biocontrol bacteria and then a 10^7-CFU/ml suspension of pathogenic strains of *A. tumefaciens*. The pathogens used were an agrocin-sensitive *A. tumefaciens* K27 or an agrocin-resistant strain, B49c Rf^r. The inoculated seedlings were planted in a randomized complete block design with four repetitions per treatment and 25 seedlings per repetition. Crown gall incidence was assessed 7 months after inoculation.

Bacterial strains were stored at −80°C in nutrient broth amended with 15% glycerol. Bacteria were grown routinely on mannitol-glutamate-yeast extract agar. Aqueous suspensions from 5-day-old lawns were used as inocula.

Field plots. All field plot experiments on the survival and dissemination of strain K84 were conducted on a Newberg fine sandy loam soil at the Oregon State University Botany and Plant Pathology Farm near Corvallis, Oreg. All cherry seedlings (*Prunus avium* cv. Mazzard rootstock) used in the experiments were 1 year old and generously donated by Oregon and Washington State Nurserymen. Plots were irrigated as needed, receiving about 2 cm of water weekly during the summer.

Survival of agrobacteria on cherry roots and in cherry galls. One hundred fifty cherry seedlings were root pruned and immediately inoculated by dipping the tap root into a 10^8-CFU/ml suspension of K84 Sm^r for 5 min. Seedlings were planted immediately after inoculation in May 1989. Seedlings were sampled over 6 months to monitor populations of K84 Sm^r in the rhizosphere. At each sampling, 10 seedlings were dug by hand, shaken to dislodge loose soil from the roots, and the tap and lateral roots were excised. After weighing, root samples were submerged in sterile 10 mM phosphate buffer containing 1% peptone, pH 7.1, and agitated for 30 min. Root washings were dilution plated onto Kerr 2E medium (7) containing 500 μg of streptomycin sulfate per ml.

Three hundred freshly root-pruned cherry seedlings were treated with a 10^8-CFU/ml suspension of strain K84 Sm^r and were inoculated 1 h later with a 10^7-CFU/ml suspension of *A. tumefaciens* B49c Rf^r. Seedlings were planted within 6 h after treatment in May 1988. Rhizosphere populations of K84 Sm^r and B49c Rf^r were monitored over 18 months by the methods described above. Roots from 10 seedlings were harvested at each sample and plated onto Kerr 2E medium containing 500 μg of streptomycin sulfate per ml or 100 μg of rifampin per ml to enumerate K84 Sm^r and B49c Rf^r, respectively. To monitor populations of B49c Rf^r and K84 Sm^r in gall tissue, at least five galled cherry seedlings were harvested at each sampling from the plot. A single gall from each seedling was excised and rinsed well with distilled water to remove adhering soil. A sample of the gall tissue was weighed, diced finely, and suspended in phosphate-peptone buffer. After agitation for 30 min, the suspension was dilution plated onto Kerr 2E amended with either 500 μg of streptomycin sulfate per ml or 100 μg of rifampin per ml.

TABLE 1. Incidence of crown gall on Mazzard cherry rootstocks in 1988 field trials in Oregon and Washington

Strain or treatment	Disease incidence[a] (%) at following location:			
	Oregon		Washington	
	Corvallis	Aurora	Moses Lake	Ephrata
B49c	52 A	31 A	83 A	94 A
B49c Rf^r	55 A	30 A	76 A	73 B
K84 Sm^r + B49c Rf^r	20 B	18 B	32 B	61 C
K84	1 D	0 C	6 C	4 E
K84 Sm^r	1 D	2 C	0 C	14 DE
Water	10 C	2 C	5 C	17 D

[a] Means within the same column followed by the same letter are not significantly different by using the Waller-Duncan procedure at $P = 0.05$.

Survival of K84 on herbaceous plants and grasses. Herbaceous plants were either collected from the field plot location or grown from commercial seed (Table 1). Plants were planted in 10-cm^2 diameter plastic pots containing field soil that was passed through a 200-mesh screen. Plants were maintained in a greenhouse with a 14-h photoperiod at 25°C. Prior to inoculation with strain K84 Sm^r, five plants of each species were assayed to determine background levels of Sm^r agrobacteria in the rhizosphere. Inoculation was accomplished by drenching the soil to field capacity with a 10^4- to 10^5-CFU/ml suspension of K84 Sm^r. Three, 14, 28, and 56 days after inoculation, plants were harvested, the root systems were excised, and loosely adhering soil was removed by shaking. The roots were weighed, placed in 10 ml of sterile phosphate-peptone buffer, and vortexed for 2 min. The suspensions were dilution plated on Kerr 2E medium amended with 500 μg of streptomycin sulfate per ml to estimate the rhizosphere population size of K84 Sm^r.

To evaluate survival of strain K84 on grass roots grown under field conditions, plots 15 cm wide and 200 cm in length were arranged in a completely randomized block, with nine repetitions per treatment. Plots were seeded with annual ryegrass (*Lolium temulentum*) in early May 1989. After grass seedling emergence, each plot was drenched to field capacity with a suspension of K84 Sm^r or K84 Rf^r. The final concentration of bacteria added was 10^5 CFU/g (dry weight) of soil. Root samples were harvested 1, 2, 4, 8, 12, and 16 weeks after infestation, and rhizosphere population sizes of K84 mutants were determined by dilution plating.

Infestation and recovery of K84 from soil. Fallow soil plots measuring 15 by 200 cm were established in a completely randomized block, with nine repetitions per treatment. Plots were hoed every 4 to 8 days to suppress weed growth. Soil

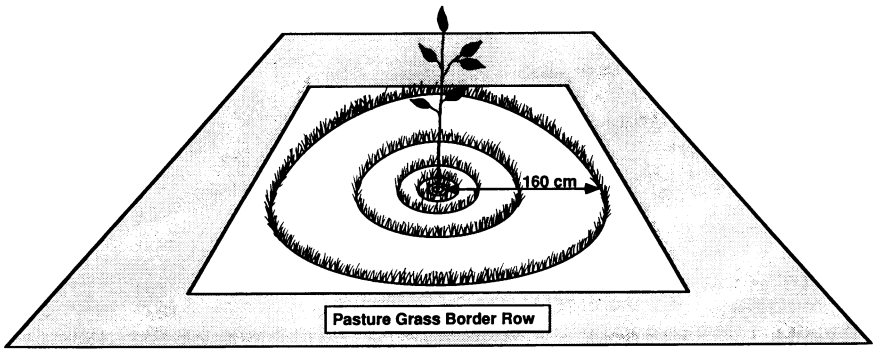

FIG. 1. Schematic representation of the grass ring systems used to monitor lateral dissemination of strain K84. Ryegrass seed was planted in rings with radii of 5, 10, 20, 40, 80, and 160 cm. Fallow soil separated the rings. A K84-inoculated cherry seedling was planted in the center of the ryegrass rings. The entire system was enclosed with a border row of pasture grass.

plots were infested in May 1989 by drenching the plots to field capacity with suspensions of strain K84 Sm^r or K84 Rf^r to achieve a final concentration of 10^5 CFU/g (dry weight) of soil in each plot. Bacterial populations in the soil were assayed 1, 2, 4, 8, 12, and 16 weeks after infestation. Three samples from each plot were collected with an ethanol-flamed soil corer, and the upper 5 cm of soil was mixed thoroughly in a polyethylene bag. Three subsamples were then extracted by agitation for 30 min in phosphate-buffered peptone and dilution plated on Kerr 2E medium amended with the appropriate antibiotics. Three additional subsamples were taken to measure soil moisture content. As bacterial populations decreased below levels detectable by standard dilution plating techniques, soil suspensions were enriched for 24 h at 25°C with agitation in mannitol-glutamate-yeast extract broth prior to dilution plating.

Detection of lateral movement of K84 through fallow soil with soil cores. Soil cores were taken five to 25 cm from cherry seedlings treated with strain K84 Sm^r as described previously. Soil core samples were collected 2 and 6 weeks after planting the seedlings. The upper 10 cm of soil from each core was mixed thoroughly and passed through a 200-mesh screen to remove gravel. Three subsamples were taken from each soil sample, weighed, and suspended in phosphate-peptone buffer. After agitation for 30 min, the suspensions were dilution plated on Kerr 2E medium with 500 μg of streptomycin sulfate per ml or enrichment cultured for 24 h prior to plating.

Detection of lateral movement of K84 through soil with biological trap plants. Annual ryegrass (*L. temulentum*) was grown as trap plants around strain K84-inoculated cherry seedlings to estimate the distance of lateral dissemination of the bacterium through the soil (Fig. 1). Ryegrass seeds were planted in concentric rings with radii of 5, 10, 20, 40, 80, and 160 cm in May 1989. A total of 25 ring systems was planted with a 1-m-wide border row of pasture grass mixture separating each ring system. After emergence of the grass, a cherry seedling inoculated with 10^8 CFU of K84 Sm^r per ml was planted in the center of each ring system. The soil between the rings of annual ryegrass was kept fallow by hoeing every 4 to 8 days. To avoid contamination by soil adhering to the hoe, hoeing progressed from outer to inner circles and hoes were cleaned and sterilized between circles. Ryegrass plants were removed from each ring 1, 2, 4, 8, and 12 weeks after planting the cherry seedlings. The harvested ryegrass roots were assayed for the presence of K84 Sm^r by dilution plating on Kerr 2E amended with 500 μg of streptomycin sulfate per ml. To detect populations of K84 Sm^r below 10^2 CFU per root more consistently, samples were also enriched in mannitol-glutamate-yeast extract broth for 24 h prior to plating. The identity of recovered bacteria was confirmed by an agrocin-production bioassay and Ouchterlony double-diffusion assay with strain-specific polyclonal antibodies.

Survival of K84 in water. Survival of strain K84 in distilled and natural sources of water was tested. The natural water sources chosen were (i) surface water from a field that was flooded with rain water at the OSU Botany and Plant Pathology Experimental Farm, (ii) the Willamette River, and (iii) a pond 100 m from the Willamette River near Corvallis, Oreg. Water was collected in sterile 1-liter nalgene bottles from the sites in February 1989. Aliquots (50 ml) of water from each source were placed in a sterile 250-ml flask. A suspension of K84 Sm^r was added to the water samples to give a mean population size of 2×10^4 CFU/ml. Flasks were incubated at 22°C with and without shaking (200 rpm). Population sizes of K84 in water were determined by dilution plating.

Data analysis. Bacterial populations were subjected to base 10 logarithmic transformation (36) prior to further analysis with Statistical Analysis System software (SAS Institute, Cary, N.C.), using analysis of variance and Waller-Duncan's procedure at $P = 0.05$ for mean comparison. Samples with bacterial populations below the detection level were not included in the mean. Disease incidence data were subjected to arcsine square root transformation prior to statistical analysis.

RESULTS

Derivation of antibiotic-resistant mutants. The B49c Rf^r isolate used in these experiments was phenotypically similar to the parental strain with respect to growth rate, pathogenicity, colony morphology, and Ouchterlony double-diffusion pattern to strain-specific polyclonal antibodies. K84 Sm^r and K84 Rf^r mutants were similar to the parental strain in colony morphology, Ouchterlony double-diffusion reaction to strain-specific antibodies, growth rate in liquid culture, and plasmid profile by agarose gel electrophoresis. Agrocin 84 production by K84 and K84 Sm^r was similar, as detected by bioassay. In contrast, production or export of agrocin 84 by K84 Rf^r mutants was less than that of the parental strain. Of the 20 K84 Rf^r mutants tested for agrocin 84 production, none produced quantities of agrocin 84 that

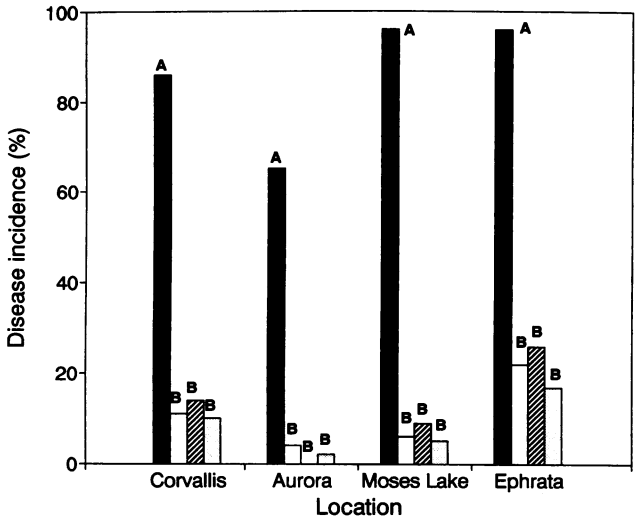

FIG. 2. Biological control efficacy of K84 (□) and its streptomycin-resistant derivative (▨) against crown gall on cherry seedlings grown in field plots in four locations and inoculated with an agrocin-sensitive *A. tumefaciens* K27. Disease incidence of K27-induced crown gall on seedlings not treated with K84 or K84 Sm^r is shown with the solid bar (■). Background incidence of indigenous crown gall on water-treated seedlings is included (▨). Different letters above the bars indicate significant difference in disease incidence by using the Waller-Duncan test at $P = 0.01$.

were detectable in bioassays that used K27 or C58 as the indicator strain. In 12 of the K84 Rf^r mutants, agrocin 84 production was detected by the NT1(pTiC58Tra^c) indicator strain. The K84 Rf^r mutant used in the field experiments had an average inhibition zone diameter of 48 mm, which was 26% smaller than the average inhibition zone produced by the parental strain K84.

Disease incidence of crown gall. Inoculation of Mazzard cherry rootstocks with *A. tumefaciens* B49c or B49c Rf^r resulted in a significant increase ($P = 0.05$) in the incidence of crown gall in all four field sites compared with that in the water-treated control (Table 1). There was no significant difference in disease incidence between the parental strain B49c and its rifampin-resistant derivative in three of four sites.

Both the parental strain K84 and the K84 Sm^r derivative significantly ($P = 0.01$) reduced the incidence of crown gall on cherry seedlings inoculated with the agrocin-sensitive pathogen K27 (Fig. 2). Application of K84 Sm^r also significantly ($P = 0.05$) reduced the disease incidence on seedlings inoculated with the agrocin-resistant *A. tumefaciens* B49c Rf^r in three of four sites (Table 1), but not to the same magnitude as control of the agrocin-sensitive pathogen (Fig. 2).

Survival of agrobacteria on cherry roots. One hour after inoculation of cherry seedlings with a suspension of K84 Sm^r at 10^8 CFU/ml, a population size of 10^7 CFU/g of root was recovered. The rhizosphere population size of K84 Sm^r decreased to 10^6 CFU/g after 1 week in the field (Fig. 3A) and further declined to 10^5 CFU/g of root over 4 weeks. The rhizosphere population size of K84 Sm^r stabilized at 10^5 CFU/g over the following 18 weeks. Statistically similar ($P = 0.05$) population sizes were recovered from tap and lateral roots, which were first harvested 4 weeks after planting. K84 Sm^r was recovered from all of the cherry roots sampled.

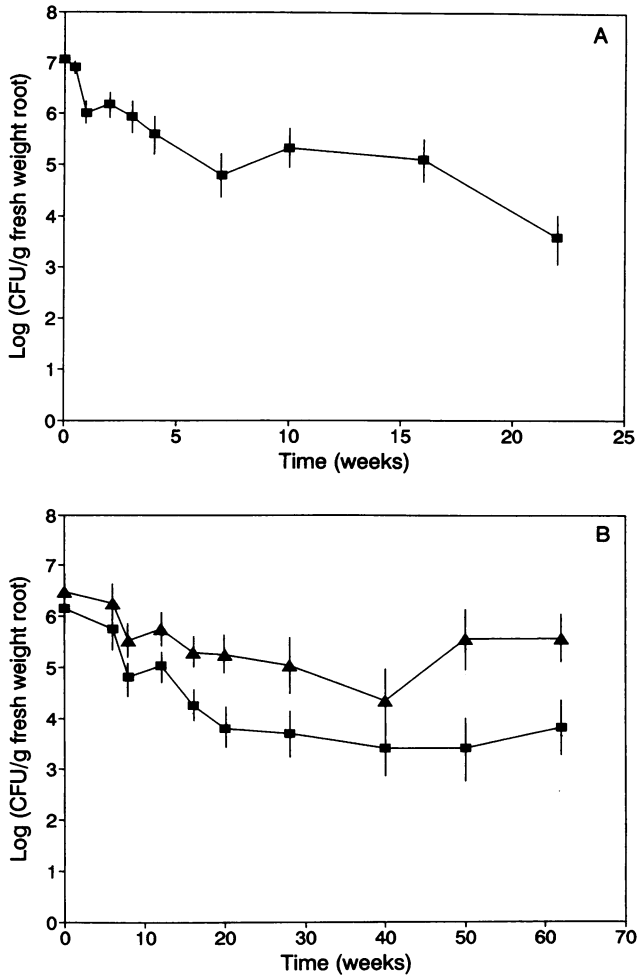

FIG. 3. (A) Average rhizosphere population size of *A. radiobacter* K84 Sm^r on cherry seedlings; (B) average rhizosphere population sizes of K84 Sm^r (■) and *A. tumefaciens* B49c Rf^r (▲) on coinoculated cherry seedlings. Seedlings were maintained in field plots near Corvallis, Oreg. Vertical bars represent ±1 standard deviation.

The initial population size of K84 Sm^r on cherry roots challenge inoculated with the pathogen, B49c Rf^r, was approximately 10^6 CFU/g (Fig. 3B), or about 10-fold less than that recovered from cherry seedlings treated only with K84 Sm^r (Fig. 3A). During the first 12 weeks, the rhizosphere population size of K84 Sm^r on cherry seedlings also inoculated with the pathogen averaged about 10^5 CFU/g, which was similar to the population size maintained on seedlings inoculated with K84 Sm^r only. At about week 18, the K84 Sm^r population size decreased to 10^4 CFU/g. The population size of K84 Sm^r was stable at 10^3 to 10^4 CFU/g from weeks 20 to 62 (the winter of 1988 to the summer of 1989) on cherry seedlings challenge inoculated with the pathogen.

The average initial population size of the pathogen, B49c Rf^r, was 5×10^6 CFU/g on cherry roots treated with K84 Sm^r (Fig. 3B) and declined to 5×10^5 by 8 weeks after inoculation. A stable population size of 10^5 to 10^6 CFU/g was maintained on cherry roots through week 30 (December 1988). Populations of B49c Rf^r declined to 10^4 CFU/g in February 1989 (week 40) but then increased and remained

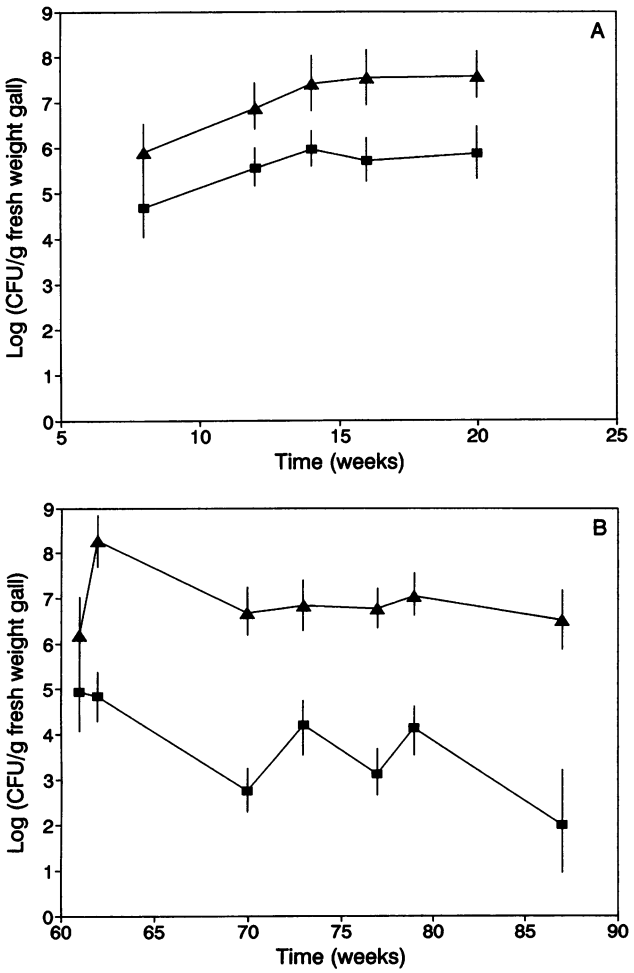

FIG. 4. Average population sizes of K84 Sm^r (■) and *A. tumefaciens* B49c Rf^r (▲) in cherry gall tissue. (A) Populations during first 20 weeks of gall development; (B) populations during second year of gall development. Vertical bars represent ±1 standard deviation.

stable at 10^5 CFU/g in the spring and summer of 1989 (weeks 50 to 62). Both of the agrobacteria, K84 Sm^r and B49c Rf^r, were recovered from all cherry roots harvested for the duration of the experiment.

Survival of agrobacteria in cherry galls. Galls were observed first on seedlings inoculated with B49c Rf^r and K84 Sm^r 8 weeks after inoculation. The population size of B49c Rf^r recovered from gall tissue initially averaged 10^6 CFU/g of gall. The population size of the pathogen increased to 10^7 CFU/g and was stable over 20 weeks (Fig. 4A). The population size of B49c Rf^r in the second year, 60 to 87 weeks after inoculation, averaged 10^6 to 10^7 CFU/g of gall (Fig. 4B). The pathogen was recovered from all galls sampled during both years. The average initial population size of K84 Sm^r in galls was 5×10^4 CFU/g. The population size of K84 Sm^r increased in galls and was maintained at approximately 10^5 CFU/g of gall tissue for the first year (Fig. 4A). During the first 20 weeks of gall development, K84 Sm^r was detected in all galls sampled. In the second year, the incidence of recovery of K84 Sm^r decreased to 70% of the galls sampled. The average population size of K84 Sm^r in gall tissue during the second year also was more variable than during the first year and ranged from 10^2 to 10^4 CFU/g (Fig. 4B). The average population sizes of B49c Rf^r and K84 Sm^r in galls harvested in January 1990, 87 weeks after seedling inoculation, were 5×10^6 and 1×10^2 CFU/g, respectively, although K84 Sm^r was recovered from only two of the six galls assayed.

TABLE 2. Rhizosphere population sizes of K84 on herbaceous plants grown in a greenhouse

Host plant	Source[a]	Population size[b] following weeks after soil infestation:			
		0.5	2	4	8
Agosaris spp.	Field	4.90	4.30	4.08	2.94
Allium vineale	Field	5.20	4.41	3.72	4.04
Cardaria draba	Field	5.81	5.66	4.78	NT[c]
Cerastium arvense	Field	5.88	5.40	5.80	NT
Cirsium spp.	Seed	5.54	4.14	4.68	4.25
Geranium molle	Field	5.66	4.49	4.62	NT
Ipomoea hederacea	Seed	5.11	4.62	4.46	3.25
Lamium amplexicaule	Field	6.07	5.34	5.23	NT
Lolium temulentum	Seed	5.40	4.60	4.72	4.63
Poa annua	Field	5.53	5.18	4.82	NT
Portulacaceae spp.	Field	5.30	5.28	5.08	NT
Trifolium incarnatum	Seed	5.36	3.92	4.23	3.48

[a] Field, plant was transplanted from the field plot; seed, plants were grown from commercial seeds.
[b] Population size is given as log (CFU/grams [fresh weight] of root).
[c] NT, not tested.

Survival of K84 on herbaceous plants and grasses. No strain K84 or streptomycin-resistant agrobacteria were recovered from the roots of herbaceous plants prior to inoculation. K84 Sm^r survived on the roots of the 12 species of plants tested in greenhouse trials (Table 2), suggesting that K84 Sm^r could survive on native weed species. The rhizosphere population size of K84 Sm^r generally did not increase on the plants tested. The smallest observed population size was on false dandelion (*Agosaris* spp.) 8 weeks after inoculation, whereas the population size of K84 Sm^r on the roots of greenhouse-grown annual ryegrass (*L. temulentum*) was fairly consistent at 5×10^4 CFU/g (fresh weight) of root.

In field tests, K84 Sm^r and K84 Rf^r maintained steady population sizes ranging from 10^4 to 10^5 CFU/g (fresh weight) of root in the rhizosphere of annual ryegrass (*L. temulentum*) for 4 months (Fig. 5). The rhizosphere population sizes of these strains on annual ryegrass in field plots were similar to those observed in the greenhouse (Table 2). The population sizes of rifampin- and streptomycin-resistant strains on annual ryegrass roots were similar.

Infestation and recovery of K84 from soil. The population sizes of strains K84 Sm^r and K84 Rf^r in fallow soil were similar over a 16-week growing season (Fig. 6). Both strains maintained large populations in fallow soil (10^5 CFU/g [dry weight] of soil) for only 1 week, after which the population sizes of the K84 mutants in fallow soil decreased from 10^5 to 10^2 CFU/g over the following 8 weeks (Fig. 6). By 16 weeks after infestation of soil plots, K84 Sm^r was detected by dilution plating of fallow soil of only two of nine plots. Enrichment plating of samples increased detection to seven of nine plots. Sixteen weeks after infestation, K84 Rf^r was recovered from one-third of the plots in directly dilution-plated soil samples and in four of nine plots after enrichment culture.

Detection of lateral movement of K84 though soil. K84 Sm^r

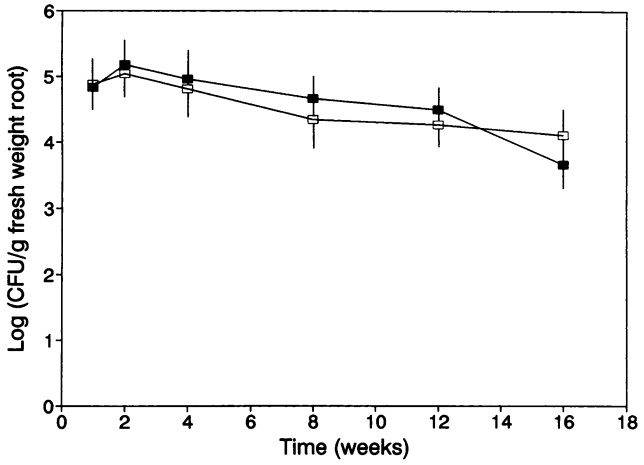

FIG. 5. Rhizosphere population sizes of K84 Sm^r (■) and K84 Rf^r (□) on annual ryegrass grown in field plots. Vertical bars represent ±1 standard deviation.

was detected in one-third of the soil cores taken 5 cm from the treated cherry seedling at 3 and 6 weeks after planting. The population size of K84 Sm^r was small and ranged from 10^1 to 10^3 CFU/g (dry weight) of soil. The bacterium was not detected in soil cores taken at greater distances from cherry seedlings, even after enrichment culture.

One and 2 weeks after planting K84 Sm^r-inoculated cherry seedlings into the centers of the grass ring systems (Fig. 1), K84 Sm^r was readily recovered from the rhizosphere of grass seedlings planted 5 cm away from the cherry seedling. Four weeks after planting, K84 Sm^r was recovered consistently 10 cm away from the seedling and, in one ring system, on grass roots planted 20 cm from a cherry plant. Eight and 12 weeks after planting, K84 Sm^r was detected 40 cm distal to the inoculated seedlings in 32% of the grass ring systems. After 12 weeks, K84 Sm^r was also recovered from grass rings planted 80 cm from cherry seedlings in 12% of the grass ring systems. K84 did not skip grass rings but progressively moved from inner rings to outer rings over the growing season. Additionally, K84 was not recovered from the rhizosphere of grasses planted 160 cm from cherry seedlings or from pasture grass in border rows. Thus, K84 Sm^r moved across at least 40 cm of fallow soil, from grass roots of the 40-cm ring to the roots of the 80-cm ring. These data are in contrast to detection limits for lateral movement of 5 cm or less from the point source when the bacterium was extracted from cores of fallow soil around cherry seedlings.

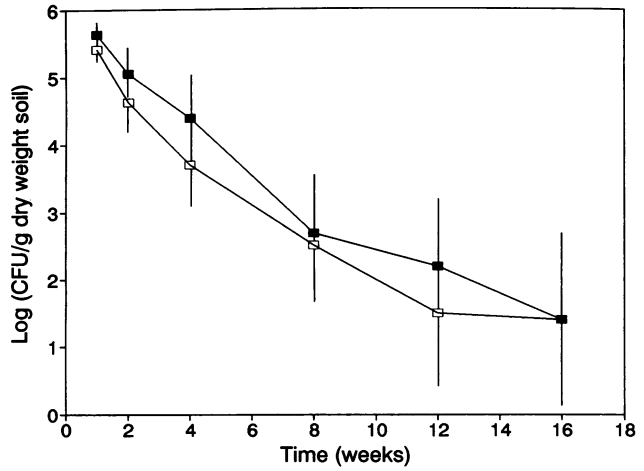

FIG. 6. Average population sizes of K84 Sm^r (■) and K84 Rf^r (□) in fallow soil field plots. Vertical bars represent ±1 standard deviation.

TABLE 3. Survival of K84 in water

Water source	Population size[a] at following weeks after inoculation:					
	0	1	2	3	6	12
Flooded field	4.30 A	3.74 A	3.34 A	3.28 A	2.69 A	2.44 A
Willamette River	4.30 A	2.78 B	1.36 B	1.54 B	1.44 B	1.53 B
Pond	4.32 A	2.67 B	1.63 B	1.72 B	1.41 B	1.58 B
Distilled	4.31 A	0.32 C	<D[b]	<D	NT[c]	NT

[a] Population size is given as log (CFU/milliliters). Means within the same column followed by the same letter are not significantly different by using the Waller-Duncan procedure at $P = 0.05$.
[b] <D, population size was below the detection limit.
[c] NT, not tested.

Survival of K84 in water. Strain K84 survived in water from a flooded field plot, the Willamette River, and a pond for 12 weeks but was only detected in distilled water for 1 week (Table 3). The population sizes of K84 in river and pond water were similar. The populations decreased 100-fold during the first 2 weeks and then stabilized. K84 maintained larger population sizes in water obtained from a flooded field plot compared with those obtained with the other sources. Agitation during incubation of the water samples did not affect the population sizes of K84.

DISCUSSION

The use of antibiotic resistance markers made it possible to follow the movement and survival of K84 in the environment. Streptomycin sulfate and rifampin were chosen for their specificity in recovery of K84 mutants when coupled with a semiselective medium for biovar II agrobacteria. Streptomycin resistance has been an effective marker with other rhizosphere inhabitants (8), and this marker had no apparent effect on the phenotype or the biological control efficacy of K84. In contrast, resistance to rifampin resulted consistently in a decrease in the size of the inhibition zone in agrocin production bioassays. It was not determined if K84 Rf^r synthesizes or exports less agrocin 84 compared with that by the parental strain. Alternatively, inhibition of NT1 (pTiC58Tra^c) by K84 Rf^r could be due to production of another agrocin or inhibitory substance, if it is assumed that no agrocin 84 is exported by K84 Rf^r and that strains K27 and C58 are insensitive to these other inhibitory compounds. Pleiotropic effects of antibiotic resistance have been reported for other plant-associated microbes (9, 16, 47). Although K84 Rf^r produced smaller inhibition zones in the in vitro bioassay, its survival in soil and on ryegrass roots was similar to that of K84 Sm^r. Rifampin resistance did not appear to have a measurable detrimental effect on the phenotype of the pathogen B49c, as the incidence of crown gall on cherry rootstocks caused by the rifampin-resistant derivative and the parental strain was similar in field trials. For these studies on survival and dissemination of the agrobacteria, it appeared that resistance to either streptomycin or rifampin was a useful marker.

Both *A. tumefaciens* and *A. radiobacter* established rhizo-

sphere populations of 10^5 CFU/g on field-grown cherry seedlings that were comparable to those reported earlier (4, 11, 38, 39). The population sizes of K84 on cherry roots were also similar to those reported for other rhizosphere bacteria (10, 23, 34, 46). The population size of K84 on cherry seedlings immediately after application was less following challenge inoculation with the pathogen compared with that recovered from seedlings not inoculated with the pathogen. This difference may only reflect mechanical displacement of the bacterium due to dipping the roots into an aqueous suspension of the pathogen. One week after treatment and thereafter, K84 maintained similar population sizes on cherry roots regardless of treatment with *A. tumefaciens*. The rhizosphere population size of the pathogenic agrobacterium was consistently 10- to 100-fold greater than that of the biocontrol agent, K84. Because *A. tumefaciens* B49c is immune to agrocin 84, in situ production of the bactericide by K84 was not expected to affect adversely the population dynamics of B49c.

In commercial applications, K84 is applied as a biocontrol agent of crown gall primarily on nursery rootstocks of woody plants. In greenhouse and field experiments, K84 also survived on the roots of several species of herbaceous plants and grasses. Like other rhizosphere inhabitants (2, 44), K84 did not express specificity for root systems of woody plants. A number of pathogenic agrobacteria have large host ranges and are able to survive on several host plants (1, 32, 35, 54). Additionally, several strains of agrobacteria have been recovered from native grasses in an undisturbed prairie (5), even though grasses are not a host to crown gall disease. This experiment indicated that herbaceous plants or grasses in orchards could serve as reservoirs for the agrobacteria.

Although K84 did not express host plant specificity, root systems were important for survival of the bacterium in the environment. In fallow soil plots, the population size of the bacterium decreased dramatically. The rhizosphere/soil ratio of populations of K84 around cherry or ryegrass roots averaged from 2.5 to 3.5 at 4 months after treatment, underscoring the preference of the bacterium for the rhizosphere. The population size of K84 also decreased in water from a river or surface water from a field. The lack of detection of K84 in distilled water after 1 week could be related to the unfavorable osmotic pressure of distilled water. Alternatively, K84 may have remained viable but could no longer be cultured (12). Although the survival of K84 in soil and water was poor compared with the survival on root systems, the bacterium was detected in water or soil for several months after infestation. Water from rain or irrigation could be an important inoculum source for the bacteria and could be important in their dissemination. It was suggested that crown gall epidemics in peach rootstock nurseries were caused by using irrigation water infested with *A. tumefaciens*; however, there were no attempts to isolate the pathogen directly from the water source (52, 53). In contrast, irrigation water has been shown to be an important inoculum source for other phytopathogenic bacteria (14, 21).

The dissemination of K84 within a field was studied by assay of cores of fallow soil and ryegrass roots around K84-treated cherry seedlings. K84 was recovered from soil cores taken at a maximum distance of only 5 cm from an inoculated cherry seedling. In contrast, K84 was recovered from an inoculum source at greater distances when ryegrass roots were present to provide a substrate that supported the growth of the bacterium. Lateral dissemination of K84 through fallow soil was commonly detected between grass rings 20 cm apart and also was detected at a distance of 40 cm from an inoculum source. The progressive outward colonization of ryegrass roots by K84 from the innermost ring to the outer rings indicated that dispersion was not a one-time event. The colonized roots of the grasses in each ring may have become a new inoculum source of the bacterium for more distal rings. We suspect that dissemination was passive, aided by irrigation (33) and perhaps soil-inhabiting insects (31). Dissemination of the bacterium by root-to-root contact probably occurred within the grass rings, but not between the grass rings, because of hoeing on a weekly basis. The hoe was sanitized before use in the soil between each ring system to minimize spread of the bacterium with the implement. While we felt that it was important to remove weeds without the use of herbicides, obviously the soil structure was disturbed by hoeing and this may have increased the distance of lateral dissemination of the bacterium. Nevertheless, similar distances of lateral dissemination of rhizosphere bacteria through soil have been reported (2, 30). Lateral migration of *Pseudomonas aureofaciens* was limited to 18 cm through soil, although occasionally the bacterium was detected on wheat roots grown 36 cm from the inoculum source (30). *Azospirillum brasilense* was detected on wheat roots growing 30 cm from the inoculum source (2). In field trials, horizontal movement of *A. brasilense* was detected on wheat roots 160 cm distal to the inoculum source if native flora that supported growth of the bacterium were growing in the plots; otherwise, movement was restricted to 5 cm.

Galls developed on cherry seedlings by 8 weeks after inoculation and *A. tumefaciens* B49c was recovered from each gall. The biocontrol agent, K84 also was found consistently in cherry galls. The pathogen maintained a 100-fold-greater population size within galled tissue compared with K84. The high incidence of strain K84 in gall tissue was not surprising, as it has been shown that galls do not have an epidermal layer of cells to act as a barrier to ingress; nonpathogenic agrobacteria and pseudomonads are isolated commonly from galls (13, 43, 55).

Gall tissues maintained large population sizes of pathogenic agrobacteria and the biocontrol agent K84 for several months, indicating that the potential for plasmid transfer between these bacteria exists under field conditions. The population data were obtained by dilution plating; thus, the spacial distribution of the *Agrobacterium* species within galls was not determined. Although large populations of both bacteria may be recovered from tissues, they may grow as discrete microcolonies several plant cells apart and thus may not be in contact for conjugative plasmid transfer between the bacterial species. Immunomicroscopy or immunoblots of galls may provide information regarding the proximity of the bacterial species within galls.

Transfer of pAgK84 into pathogenic agrobacteria could compromise effective biocontrol of crown gall. Although antibiosis is an important factor of biocontrol in this system, it is not the sole mechanism, as non-agrocin-producing mutants of K84 still exhibit some residual biocontrol activity (15). Furthermore, K84 has been shown to be partially effective in protecting plants from infection by agrocin-resistant strains of *A. tumefaciens* in this study (Table 1) and others (37, 57). Nevertheless, a derivative Tra^- strain of K84 lacking the transfer genes for pAgK84 has been constructed (26). It has been suggested that use of this engineered strain could stabilize biocontrol of crown gall by minimizing the risk of acquisition of resistance to agrocin 84 via plasmid transfer. The Tra^- mutant of K84 has shown considerable promise as an effective biological control agent for crown

gall (25, 48, 57). Information of the survival and persistence of K84 should provide indicators of how genetically engineered derivatives will behave in an agroecosystem.

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