Transfer of pAgK84 from the Biocontrol Agent *Agrobacterium radiobacter* K84 to *A. tumefaciens* Under Field Conditions

V. O. Stockwell, M. D. Kawalek, L. W. Moore, and J. E. Loper

First and fourth authors: Horticultural Crops Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Corvallis, OR 97330; second and third authors: Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331.
Current address of M. D. Kawalek: Department of Environmental Toxicology, University of California, Riverside 92521.
This research was supported in part by grant CR-81434 from the U.S. Environmental Protection Agency Biotechnology Risk Assessment Program and by the Oregon and Washington State Nurseryman's associations.
We thank M. Canfield, D. Pitkin, G. DeGardea, and S.-F. Lu for assisting in experiments; Willow Drive Nursery, Ephrata, WA, and Van Wells Nursery, Moses Lake, WA, for providing plant materials and field space; and S. K. Farrand, J. Kraus, J. Costa, and A. Muehlchen for insightful comments on the manuscript.
Accepted for publication 2 October 1995.

ABSTRACT

Stockwell, V. O., Kawalek, M. D., Moore, L. W., and Loper, J. E. 1996. Transfer of pAgK84 from biocontrol agent *Agrobacterium radiobacter* K84 to *A. tumefaciens* under field conditions. Phytopathology 86:31-37.

Agrobacterium radiobacter strain K84 effects biological control of crown gall disease caused by *A. tumefaciens* in part by the plasmid-conferred production of agrocin 84, an antibiotic with specific activity against certain strains of the pathogen. A transmissible plasmid (pAgK84) resident in the biocontrol bacterium contains genes for agrocin 84 biosynthesis and immunity. The frequency of pAgK84 transfer from K84 to *A. tumefaciens* strain B49c, a naturally occurring strain that is insensitive to agrocin 84, in gall tissue of cherry seedlings was evaluated under field conditions. Putative transconjugants were detected by colony hybridization in 4 of 13 galls evaluated. The identity of transconjugants recovered from one of those galls was confirmed by serology, agrocin production, pathogenicity, and Southern blot analysis using agrocin 84 biosynthesis, T-DNA, and nopaline synthase gene probes. In the four galls in which transconjugants were detected, the estimated frequency of plasmid transfer from K84 to B49c was approximately 10^{-4} transconjugants per recipient. A transconjugant strain and B49c did not differ in their capacities to colonize the rhizosphere of cherry or to cause crown gall under field conditions. The transconjugant retained pAgK84 for up to 7 months in the rhizosphere of field-grown plants. These results confirm that *Agrobacterium* strains harboring both a tumor-inducing plasmid and pAgK84 can develop from plasmid transfer in the field and that such newly derived strains can persist under field conditions.

Crown gall is a tumorigenic disease of many dicotyledonous plants that is caused by the ubiquitous soilborne bacterium *Agrobacterium tumefaciens*. Crown gall results in significant economic losses to the stone fruit industry because galls can weaken or kill the host plant. Losses to the nursery industry can be particularly severe, because symptomatic plants must be culled. Root pruning of stone fruit seedlings prior to transplanting into nursery fields is a routine practice, resulting in wounds that facilitate entry of the pathogen, which commonly inhabits nursery soils. During the infection process, the bacterium attaches to plant cells and transfers the T-DNA region of its endogenous tumor-inducing (Ti) plasmid into the plant cell (42). Incorporation of T-DNA into the plant genome results in overproduction of plant growth regulators and subsequent formation of the hyperplastic growth that is characteristic of crown gall (18). In addition, the T-DNA encodes the biosynthesis of opines, members of an uncommon class of metabolites found in crown gall tissue. Opines support growth of *Agrobacterium* spp. and induce conjugal plasmid transfer among agrobacteria (10). Nutrients, phenolic compounds, and other metabolites produced by the host plant also are present in crown gall tissue and are available to the pathogen and secondary invaders, such as other agrobacteria and pseudomonads that are common inhabitants of plant tumors (3,28).

A. radiobacter strain K84 (K84) is an effective biological control agent for crown gall disease on stone fruit rootstocks (21,24, 31,34,36,38,39). In commercial settings, K84 commonly is applied to roots of nursery plants immediately after pruning to protect wounds from infection by *A. tumefaciens* (26). K84 produces an antibiotic called agrocin 84, a disubstituted adenine analogue that inhibits DNA replication (20). Agrocin 84 is transported into cells of *A. tumefaciens* via agrocinopine permease, a periplasmic protein required for uptake of the opine agrocinopine (27). Genes encoding the agrocinopine permease are carried on the Ti plasmid of sensitive strains of *A. tumefaciens* (15).

Two observations indicate that agrocin 84 production is an important determinant in biological control of crown gall by K84: (i) mutants of K84 that do not produce agrocin 84 are only partially effective in biological control of crown gall caused by agrocin 84-sensitive strains of *A. tumefaciens* (5), and (ii) K84 is more effective in protecting plants from agrocin 84-sensitive than from agrocin 84-insensitive strains of *A. tumefaciens* (21,36). Nevertheless, K84 provides some protection against strains of *A. tumefaciens* that are insensitive to agrocin 84 (21,36,38), possibly through site or nutrient competition. Because production of agrocin 84 contributes to the biocontrol activity of K84, development and proliferation of agrocin 84-insensitive strains of the pathogen could diminish the capacity of strain K84 to suppress crown gall in the future.

Corresponding author: V. O. Stockwell; E-mail address: stockwell@bcc.orst.edu

Publication no. P-1996-1120-03R

Agrocin 84-sensitive strains of *A. tumefaciens* can become insensitive to the antibiotic by one of several mechanisms. After exposure to agrocin 84, variants with resistance to the antibiotic can be selected readily from a population of *A. tumefaciens* that is sensitive to agrocin 84 (6,19,37). Certain variants are nonpathogenic due to loss of the Ti plasmid (including genes encoding agrocinopine permease) (6,19) or deletions extending into Ti plasmid-borne genes required for virulence (6); these variants pose no direct threat as K84-insensitive pathogens. Other agrocin 84-insensitive variants are virulent (6,37) and, if ecologically fit, could persist as pathogens with reduced sensitivity to biocontrol by strain K84.

Horizontal gene transfer from *A. radiobacter* K84 to *A. tumefaciens* also is a mechanism by which agrocin 84-insensitive pathogens may develop (10). The agrocin 84 biosynthesis genes and the genes encoding immunity to agrocin 84 are present on pAgK84, a conjugative plasmid of K84 (32,41). Conjugal transfer of pAgK84 to strains of *A. tumefaciens* occurs in culture and in crown gall tissue of infected plants (11,39). Cells of *A. tumefaciens* that harbor both pAgK84 and a Ti plasmid are pathogenic, due to virulence genes present on the Ti plasmid. They produce and are immune to agrocin 84, due to genes present on pAgK84. *A. tumefaciens* strains that produce agrocins and have plasmids similar to pAgK84 have been isolated from various parts of the world (23, 35,38). Although the origin of these strains is unclear, their existence suggests that conjugal transfer of plasmids occurred in the environment and that strains containing pAgK84 are ecologically fit.

Interstrain gene transfer undoubtedly occurs between bacteria in the environment (9). Nevertheless, the frequency at which gene transfer occurs, and the persistence and impact of resultant strains in the environment are poorly understood. Although there is evidence that pAgK84 can transfer to *A. tumefaciens* in agricultural fields (39), the frequency of transfer and the environmental fate of transconjugant strains that have received pAgK84 are unknown. Knowledge of the transfer frequency of pAgK84 under conditions found in the field and the relative fitness and pathogenicity of transconjugants must be understood to assess the risk for loss of effective disease control associated with horizontal gene transfer.

In this report, we examined the transfer of pAgK84 from the biological control bacterium *A. radiobacter* K84 to an agrocin 84-insensitive strain of *A. tumefaciens* in gall tissue of field-grown cherry seedlings. The pathogenicity, survival, and stability of transconjugants on cherry seedlings in field plots also were evaluated. We used an agrocin 84-insensitive pathogen for three reasons: (i) to maximize the probability that populations of both K84 and the pathogen would be present in crown gall tissue; (ii) to reduce biocontrol efficacy of K84 so an adequate supply of coinhabited galls would be available for analysis; and (iii) to minimize selective pressure for transconjugants, which may have a competitive advantage over an agrocin 84-sensitive parental strain in galls also supporting populations of K84. Differential fitness of the recipient and transconjugant strains could skew estimates of plasmid transfer frequency.

MATERIALS AND METHODS

Bacterial strains. *A. radiobacter* K84 contains pAgK84, a plasmid that carries genes for immunity to and synthesis of agrocin 84 (32,41), and pAtK84b, which carries genes for nopaline (4) catabolism. *A. tumefaciens* B49c is a naturally-occurring biovar II strain isolated from a crown gall tumor on apple in Washington (3). Strain B49c is pathogenic on cherry and insensitive to agrocin 84, presumably due to lack of uptake of the antibiotic (36). Strain K84Sm, a streptomycin sulfate (Sm)-resistant mutant of *A. radiobacter* strain K84, and strain B49cRf, a rifampicin (Rf)-resistant mutant of *A. tumefaciens* strain B49c, were described previously (36). K84Sm is similar to the wild-type strain in growth rate, agrocin 84 production, serology, and biocontrol efficacy in field trials. B49cRf is similar to its parental strain with respect to growth rate, serological reaction, insensitivity to agrocin 84, and pathogenicity under field conditions. The resistances of K84 and B49c resistance to Sm and Rf, respectively, are stable phenotypes in laboratory and field experiments (36).

K84Sm and B49cRf were cultured on mannitol-yeast agar (19) amended with 2 g of glutamate (MGY) per liter and Sm (500 µg/ml) or Rf (100 µg/ml), respectively. Plasmid pAgK84 was introduced into B49cRf by transformation, and a transformant, B49cRf(pAgK84), was detected by colony hybridization with an agrocin 84 biosynthesis gene probe (*agn*). *A. tumefaciens* strain K439, a derivative of *A. tumefaciens* C58 with enhanced sensitivity to agrocin 84 (8), was provided by S. K. Farrand, University of Illinois, Urbana. *Agrobacterium* strains used for evaluating the specificity of the agrocin 84 biosynthesis gene probe were obtained from the culture collection of L. W. Moore. One hundred eighty-three strains of *Agrobacterium*, 105 of which were pathogenic on tomato ('Bonnie Best'), were originally isolated from the United States, Africa, Australia, Europe, and Asia from host plants that included almond, apple, birch, blueberry, boysenberry, carrot, cedar, cherry, dahlia, euonymous, grape, peach, photinia, rhododendron, rose, tomentosa, walnut, and willow. Three of the *Agrobacterium* strains (K84, B49c(pAgK84), and *A. tumefaciens* NT-1(pAgK84)) harbored pAgK84. Twenty-five additional soil-inhabiting bacteria representing genera other than *Agrobacterium* also were tested for homology to the probe. These strains, including species of *Alcaligenes, Bradyrhizobium, Erwinia, Enterobacter, Pseudomonas,* and *Streptomyces*, were from the culture collection of J. E. Loper. All bacterial strains were stored at –80°C in nutrient broth amended with 15% glycerol.

Detection of *Agrobacterium* spp. containing agrocin 84 biosynthesis genes by colony hybridization. The *agn* probe consisted of a 3.7-kb *Sma*I fragment of pAgK84, designated fragment G, which contains portions of the *agnA*, *agnB*, and *immA* loci (41) cloned into pUC8 (40). The 3.7-kb *Sma*I fragment cloned into pSa152 (35) was provided by S. K. Farrand.

The sensitivity of colony hybridization in detecting single transconjugants in a lawn of the recipient strain was evaluated. A 10-fold dilution series of a B49cRf cell suspension was spiked with a constant volume of a B49cRf(pAgK84) cell suspension to obtain final cell ratios of 1 CFU of B49cRf(pAgK84) per 100 CFU of B49cRf and decreasing on a logarithmic scale up to 1 CFU of B49cRf(pAgK84) per 10^6 CFU of B49cRf. The resulting suspensions (100 µl each) were spread on nylon membranes (Nytran, Schleicher and Schuell, Keene, NH) placed on the surfaces of MGY agar. Plates were incubated for 2 to 3 days at 27°C until colonies were 2 to 3 mm in diameter. The cells were lysed, and the DNA was fixed to the nylon membrane by standard methods (33). The DNA probe was labeled with [α-^{32}P]dCTP (New England Nuclear, Boston) using a random primer labeling kit (GIBCO-BRL Life Technologies, Gaithersburg, MD). Membranes were placed in hybridization solution for 12 to 18 h at 42°C (33) prior to incubation with ^{32}P-labeled *agn* probe for 24 h at 42°C. Membranes were washed for at least 10 min three times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate at 25°C, then twice for 20 min in 1× SSC at 55°C. Autoradiographs were prepared by exposing X-ray film to the membranes with a single intensifying screen (regular X-Omatic screen, Eastman Kodak Co., Rochester, NY) for 6 to 7 days at –80°C.

Field plot locations. Experiments to measure the frequency of pAgK84 transfer from *A. radiobacter* K84Sm to *A. tumefaciens* B49cRf in crown gall tissue of 1-year-old cherry seedlings (*Prunus avium* L. 'Mazzard') were conducted at the Oregon State University Botany and Plant Pathology Farm near Corvallis and in commercial nurseries in Ephrata and Moses Lake, WA. K84 had not been introduced to any of these field plots previously. Survival and

pathogenicity of a recovered transconjugant, B49cRf(pAgK84), were evaluated in cherry seedlings at the Oregon State University Botany and Plant Pathology Farm.

Inoculation of cherry seedlings. K84Sm and B49cRf were cultured for 5 days on MGY agar amended with Sm or Rf, respectively. Bacterial cells were suspended in water to approximately 10^8 CFU of K84Sm per ml or approximately 10^7 CFU of B49cRf per ml. Freshly root-pruned cherry seedlings were dipped in the suspension of K84Sm for 5 min. The seedlings were air-dried for 1 h and dipped in the cell suspension of B49cRf for 5 min. Seedlings were planted within 6 h after treatment in 12 blocks, with 25 seedlings per block, at each site for each of two successive years.

Analysis of agrobacteria in gall tissue. Seedlings were harvested from plots 6 months after planting in Washington or 18 months after planting in Oregon. Crown gall tissue from each of 79 seedlings was placed in resealable plastic bags and transported to the laboratory in an ice chest. Individual galls were rinsed with distilled water, weighed, diced finely with a sterile razor blade, and suspended in sterile 10 mM potassium phosphate buffer (pH 7.2) containing 1% peptone. After agitation for 30 min, gall extracts were diluted, and samples of dilutions were spread onto Kerr 2E medium (2) amended with Sm at 500 μg/ml or Rf at 100 μg/ml to enumerate K84Sm or B49cRf, respectively. Gall extracts were stored at 4°C for 4 to 30 days before analysis by colony hybridization.

Extracts from 13 galls that contained at least 10^2 CFU/g fresh weight of K84Sm gall tissue and at least 5×10^5 CFU/g fresh weight of B49cRf gall tissue were selected for colony hybridization to detect and recover transconjugants, i.e., B49cRf harboring pAgK84. Gall extracts were diluted to approximately 10^4 CFU of B49cRf per ml, and 100-μl samples from each gall were spread on 10 replicate plates of Kerr 2E medium amended with Rf at 100 μg/ml and cycloheximide at 200 μg/ml. A dilution series of gall extracts also was spread on the medium to quantify culturable cells of B49cRf in the extract at the time of colony hybridization. Plates were incubated at 27°C for 3 days until colonies were approximately 1 mm in diameter. Colonies were transferred with a sterile velvet fabric replicator from the 10 master plates to nylon membranes placed on the surface of Kerr 2E medium containing Rf and cycloheximide. Plates were incubated for 1 to 2 days at 27°C until colonies on the nylon membranes were 2 to 3 mm in diameter before colony hybridization. As a control, extracts of two galls from plants inoculated only with B49cRf also were processed. Membranes were processed for colony hybridization as described above and incubated with ^{32}P-labeled *agn* probe. Bacterial colonies were recovered from master plates that corresponded to locations of hybridization with the *agn* probe on autoradiographs, streaked for purity, and evaluated again by colony hybridization. Colonies that hybridized to the *agn* probe in the second colony hybridization experiment were evaluated as putative transconjugants.

Characterization of putative transconjugant strains from galls. The identity of the recovered transconjugants as B49cRf was confirmed by testing for resistance to Rf, pathogenicity on tomato (25), and the presence of a unique precipitin band with B49c-specific antiserum in Ouchterlony double-diffusion assays (1,36). The presence of pAgK84 in putative transconjugants was assessed by the agrocin 84 production assay, using K439 as the sensitive indicator strain, as described previously (36,41).

Selected transconjugants were characterized by plasmid profiles and Southern blot analysis with *agn*, T-DNA, or nopaline synthase gene probes. The T-DNA probe was a 7.5-kb *Eco*RI fragment containing tumor morphology genes, *tms, tmr*, and *tml*, of pTiB$_6$806 (14). The nopaline synthase (*nos*) gene probe was a 3.2-kb *Hin*dIII fragment, designated fragment 23 of pTi37 (7). The plasmids containing these fragments, pEco7 and pHindIII-23, were donated by A. L. Montoya (deceased), Ciba-Geigy, Research Triangle Park, NC. Plasmids of putative transconjugants were isolated by an alkaline lysis method (12) and electrophoresed in 0.5% agarose in 0.5× TBE (Tris-borate-EDTA) buffer (33) for 16 h at 4 to 5 V/cm. Linearized DNA was separated by gel electrophoresis for 4 h at 1 to 2 V/cm in 0.7% agarose with 0.5× TBE buffer. DNA in gels was stained with ethidium bromide and visualized with UV light. DNA fragments to be probed were transferred to nylon membranes by standard methods (33) and incubated with ^{32}P-labeled probes, and hybridizing fragments were visualized by autoradiography.

Survival of B49cRf and B49cRf(pAgK84) on cherry rootstocks. The survival and pathogenicity of B49cRf and transconjugants recovered from a cherry gall were compared in a field plot at the Oregon State University Botany and Plant Pathology Farm. The tap roots of freshly root-pruned cherry seedlings were placed in 10^7 CFU/ml suspensions of B49cRf or B49cRf(pAgK84) for 5 min before planting. To monitor survival of bacteria in the rhizosphere of cherry, inoculated seedlings were planted in a randomized complete block design, with 20 seedlings per block and 10 blocks per treatment. A 1.5-m zone of fallow soil was maintained around each block. Ten seedlings per treatment were harvested monthly. The tap and lateral roots were excised, weighed, placed in sterile 10 mM potassium phosphate buffer (pH 7.2) containing 1% peptone, and agitated for 30 min. Dilutions of the root washings were spread on Kerr 2E medium containing Rf at 100 μg/ml. At each sampling time, 48 randomly selected colonies recovered from each treatment were tested for the precipitin banding pattern with B49c-specific antiserum and for agrocin 84 production and export, using K439 as the indicator strain. The presence of pAgK84 was determined by gel electrophoresis of plasmids from 48 isolates per treatment recovered during the final harvest and from 10 isolates recovered from each earlier harvest.

To compare pathogenicity of B49cRf and an agrocin plasmid-containing derivative strain under field conditions, 100 cherry seedlings each were inoculated with B49cRf and B49cRf(pAgK84). Inoculated seedlings were planted in a randomized complete block design, with five blocks per treatment and 20 seedlings per block and a 1.5-m-wide fallow soil buffer between blocks. All plants were harvested at 7 months after planting, and the incidence of crown gall was recorded. Isolates were recovered from gall tissues and characterized by the methods described above.

Data analysis. Bacterial population data were subjected to logarithmic transformation to achieve normality before analysis with Statistical Analysis System software (SAS Institute, Cary, NC) using analysis of variance and Fisher's LSD procedure at $P = 0.05$ for mean comparison. Disease incidence data were subjected to square root-arcsine transformation prior to statistical analysis.

RESULTS

Specificity of the *agn* probe and sensitivity of colony hybridization for detection of pAgK84. Of the 183 strains of *Agrobacterium* evaluated, only the 3 strains harboring pAgK84 (K84, B49c(pAgK84), and *A. tumefaciens* NT-1(pAgK84)) hybridized to the *agn* probe. The *agn* probe did not hybridize to B49c. Thus, the *agn* probe was useful for specific detection of pAgK84 in strains of *Agrobacterium*. The specificity of the probe was further tested by hybridization to other genera of soil-inhabiting bacteria. Of the 25 different strains of soil bacteria evaluated, only strains of *Enterobacter* spp. hybridized to the *agn* probe, presumably because indigenous plasmids or chromosomal DNA hybridized to pUC8.

The sensitivity of the colony hybridization method was sufficient to detect single colonies of B49c(pAgK84) in a background of 10^5 B49c colonies (Fig. 1). About 20 single colonies of B49cRf (pAgK84) were detected by colony hybridization in the presence of 1×10^3 and 1×10^4 CFU per plate of B49cRf (plate and autoradiogram A and B in Fig. 1, respectively). Eight colonies of B49cRf(pAgK84) were detected on membranes containing $1 \times$

10^5 CFU of B49cRf (plate and autoradiogram C in Fig. 1). No colonies of B49cRf(pAgK84) were detected by colony hybridization with higher cell numbers of B49cRf (plate and autoradiogram D and E in Fig. 1). Therefore, it appeared that colony hybridization would be useful for detection of pAgK84-containing cells among a population of less than 10^5 *Agrobacterium* colonies isolated from plants and soil.

Detection of putative transconjugants. The pathogen B49cRf was recovered from all of the 79 galls assayed and had an average population size of 6.84 log(CFU/g fresh weight of gall). K84Sm was recovered from 68 of the 79 galls. The average population size of K84Sm in galls was 3.83 log(CFU/g fresh weight of gall). The population size of K84Sm in gall tissues was consistently less than that of B49cRf; in 29% of the sampled galls, however, the difference in population sizes of the two bacteria was less than 2 log units. Extracts prepared from a subsample of galls that contained detectable populations of both bacteria were analyzed for the presence of transconjugants. Putative transconjugants of B49cRf containing pAgK84 were detected by colony hybridization in 4 of the 13 galls evaluated (3 galls from Corvallis, OR, and 1 gall from Ephrata, WA). The population sizes of the recipient and donor strains in each of the galls containing putative transconjugants were (i) 1.1×10^7 and 5.3×10^5; (ii) 3.8×10^6 and 2.5×10^5; (iii) 1×10^7 and 1.2×10^5; and (iv) 4×10^6 and 5×10^2 CFU/g fresh weight of B49cRf and K84Sm galls, respectively. The frequency of detection of putative transconjugants ranged from 1.7 to 3.8×10^{-4} transconjugants per recipient among the four galls. From one gall, seven regions of hybridization to the *agn* probe were detected; the colonies at corresponding locations on master plates were streaked to purity. Of the 200 purified colonies that were evaluated again by colony hybridization, 47 hybridized to the *agn* probe; these were considered putative transconjugants. No colonies from gall extracts of seedlings inoculated with B49cRf alone hybridized with the *agn* probe.

Characterization of transconjugants of *A. tumefaciens* B49cRf. The 47 putative transconjugants had a precipitin banding pattern with B49c-specific antiserum that was characteristic of strain B49cRf in Ouchterlony double-diffusion assays. Furthermore, all of the 47 putative transconjugants induced galls on tomato seedlings, were resistant to Rf at 100 μg/ml, and produced detectable amounts of agrocin 84 in bioassays.

Plasmid profiles revealed that the 47 transconjugants contained pAgK84 and a large cryptic plasmid (pAtB49c1) of B49c, but the sizes of pTiB49c and a second cryptic plasmid (pAtB49c2) of B49cRf varied among the transconjugants (Fig. 2). Transconjugants fell into two groups based on plasmid profiles: (i) in 13% of the transconjugants, pTiB49c and pAtB49c2 were the same size as in B49cRf, and (ii) in the remainder of the recovered transconjugants, both pTiB49c and pAtB49c2 were altered. Two distinct plasmid bands of consistent size were observed in the second group of transconjugants.

Plasmids isolated from six of the recovered B49cRf(pAgK84) strains, representing the two types of plasmid profiles, were evaluated by Southern hybridization analysis using *agn*, T-DNA, and *nos* gene probes. *Bam*HI fragments of plasmid DNA from K84 and the six B49cRf(pAgK84) transconjugants that hybridized to the *agn* probe were indistinguishable in size (Fig. 3), confirming the presence of pAgK84. The *Sma*I fragments of plasmid DNA from B49cRf and from B49cRf(pAgK84) transconjugants that hybridized to the T-DNA probe were indistinguishable in size, indicating that the *tms*, *tmr*, and *tml* genes present in the T-DNA of B49c also were present in the transconjugants (Fig. 4). Plasmid DNA from K84 did not hybridize to the T-DNA probe. The *nos* probe hybridized to pTiB49c and pAtK84b, the nopaline catabolism plasmid of K84, but the size of hybridizing restriction fragments differed between the two plasmids, so they could be distinguished on Southern blots. The sizes of *Bam*HI, *Sma*I, and *Eco*RI fragments of plasmids isolated from transconjugants that hybridized

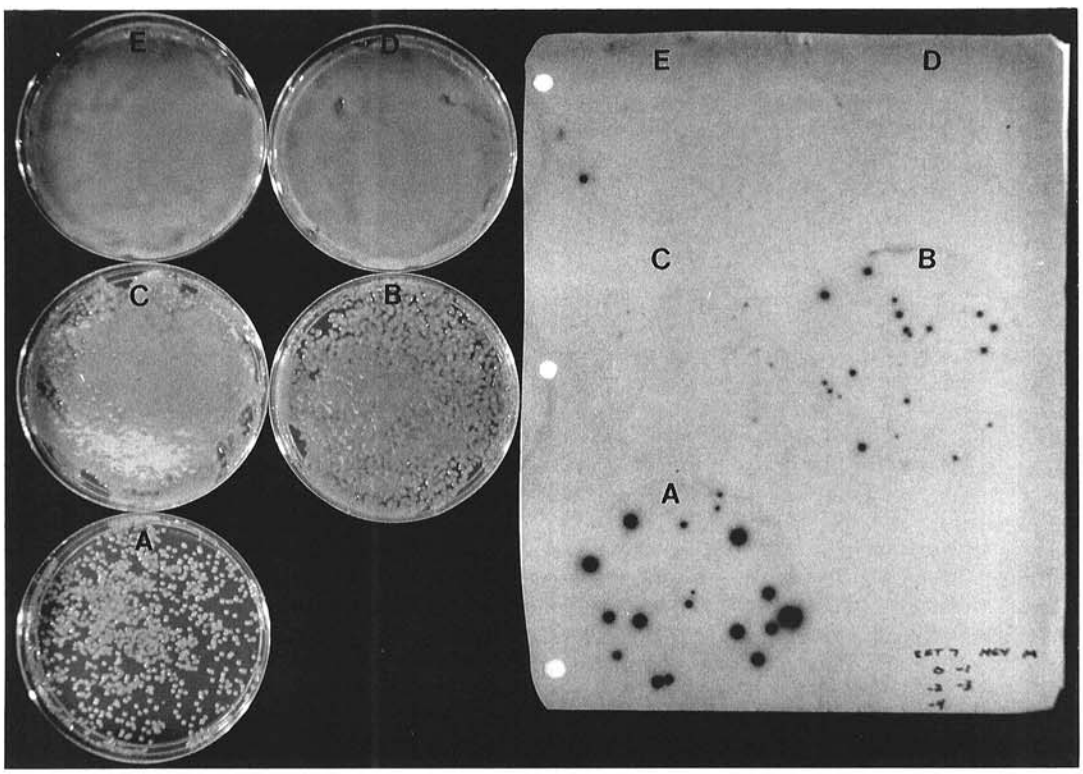

Fig. 1. Detection of *Agrobacterium tumefaciens* transformant B49cRf(pAgK84) present in a population of *A. tumefaciens* B49cRf by colony hybridization using the agrocin 84 biosynthesis gene probe. Each plate was inoculated with approximately 20 CFU of B49cRf(pAgK84) and **A–E,** 10^3, 10^4, 10^5, 10^6, or 10^7 CFU of B49cRf, respectively. Colonies of B49cRf(pAgK84) were detected on autoradiographs corresponding to master plates inoculated with $\leq 10^5$ CFU of B49cRf (**A, B, and C**).

to the *nos* probe were indistinguishable from hybridizing fragments of pTiB49c and differed from hybridizing fragments of pAtK84b (data not shown).

Survival and pathogenicity of B49cRf and B49cRf(pAgK84) on cherry. One transconjugant (Fig. 2, lane 8, a strain that had an unaltered plasmid profile) was further tested for pathogenicity and rhizosphere colonization of cherry in a field experiment. The incidence of crown gall on field-grown cherry seedlings inoculated with B49cRf or B49cRf(pAgK84) did not differ significantly and averaged 27 and 28%, respectively. The severity of crown gall also was similar; both strains induced the formation of one or two galls on each of the infected seedlings. B49cRf and B49cRf(pAgK84) established similar rhizosphere population sizes ranging from 10^5 to 10^7 CFU/g fresh weight of root (Fig. 5). The population sizes of the bacteria were statistically similar at all sampling times, except at 26 weeks after planting, when the population size of B49cRf(pAgK84) was less than that of B49cRf.

Rf-resistant bacteria recovered throughout the growing season from the rhizosphere or galls of trees treated with B49cRf or B49cRf(pAgK84) reacted with a precipitin banding pattern characteristic of B49c with B49c-specific antiserum in double-diffusion assays. Bacteria recovered from seedlings treated with B49cRf (pAgK84) inhibited growth of K439, whereas none of the bacteria recovered from B49cRf-treated seedlings produced inhibition zones in the bioassay. Plasmids comigrating with pAgK84, pTiB49c, pAtB49c1, and pAtB49c2 in agarose gels were detected consistently in Rf-resistant bacteria reisolated from the rhizosphere or galls of B49cRf(pAgK84)-treated cherry seedlings. Rf-resistant bacteria recovered from B49cRf-treated seedlings contained plasmids comigrating with pTiB49c, pAtB49c1, and pAtB49c2 in agarose gels but did not contain a plasmid comigrating with pAgK84.

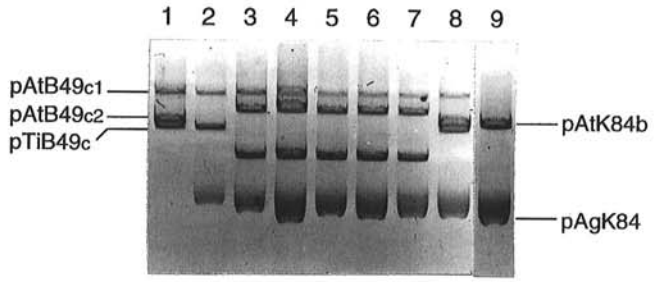

Fig. 2. Plasmid profiles of lane 1, *Agrobacterium tumefaciens* B49cRf; lane 2, *A. tumefaciens* transformant B49cRf(pAgK84); lanes 3–8, field transconjugants (B49cRf(pAgK84)) recovered from a cherry gall; and lane 9, *A. radiobacter* K84Sm. Lane 1, bands corresponding to pAtB49c1 and pAtB49c2, two cryptic plasmids of strain B49c, and pTiB49c are indicated. Lane 9, bands corresponding to pAtK84b and pAgK84, two plasmids of strain K84, are indicated.

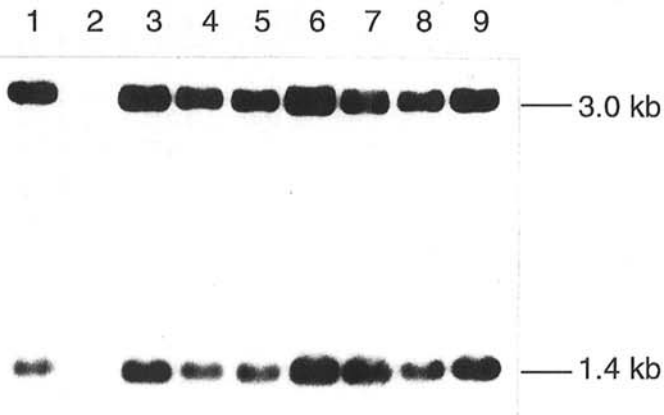

Fig. 3. Southern hybridization with the agrocin 84 biosynthesis gene probe of *Bam*HI-digested plasmid DNA of lane 1, *Agrobacterium radiobacter* K84Sm; lane 2, *A.* B49cRf; lane 3, *A. tumefaciens* transformant B49cRf(pAgK84); and lanes 4–9, recovered field transconjugants (B49cRf(pAgK84)).

DISCUSSION

We have demonstrated conclusively that pAgK84, which carries the agrocin 84 synthesis and immunity genes from *A. radiobacter* K84, transfers to *A. tumefaciens* in galls of cherry seedlings under field conditions in the Pacific Northwest of the United States. Transfer of pAgK84 was detected at moderate frequencies (approximately 10^{-4}) and low incidence (4 of 13 galls) in this study. It is noteworthy that other researchers detected pAgK84 transfer from K84 to *A. tumefaciens* in galls from which fewer than 500 colonies of *Agrobacterium* spp. were screened (29,39). There are several possible explanations for the success that others have had in recovery of transconjugants when so few colonies were screened: (i) transfer frequency may have been higher than observed here due to greater receptivity of the recipient to conjugation; (ii) different host plants may support greater population sizes of the pathogen and K84, and thus, transfer frequency could increase due to increased probability of interaction between strains

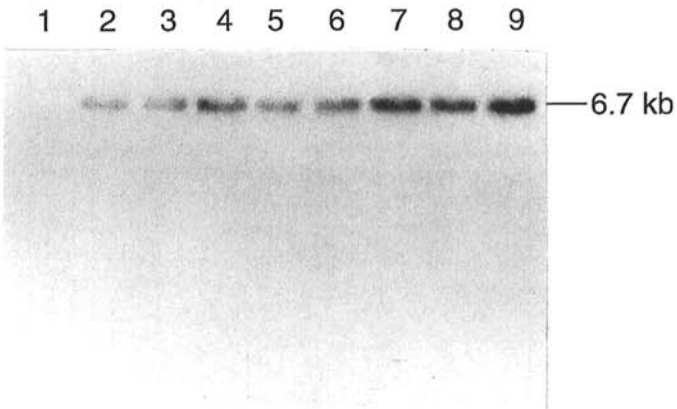

Fig. 4. Southern hybridization with the T-DNA probe of *Sma*I-digested plasmid DNA of lane 1, *Agrobacterium* K84Sm; lane 2, *A. tumefaciens* B49cRf; lane 3, *A. tumefaciens* transformant B49cRf(pAgK84); and lanes 4–9, recovered field transconjugants (B49cRf(pAgK84)).

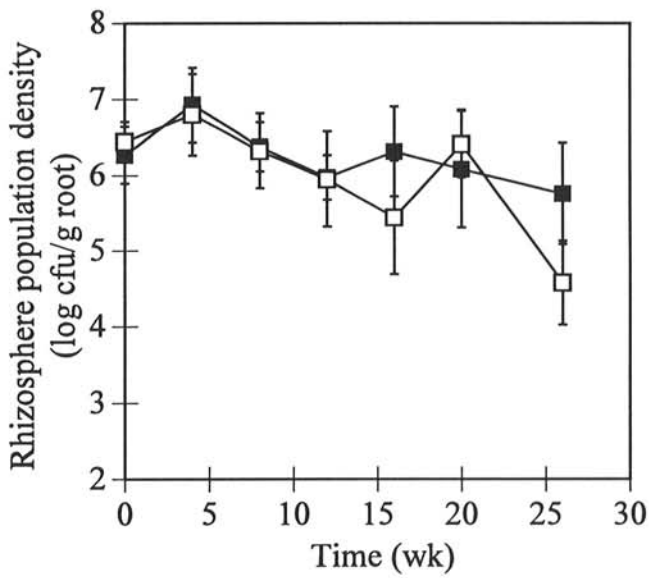

Fig. 5. Average rhizosphere population size of *Agrobacterium tumefaciens* B49cRf (■) and *A. tumefaciens* field transconjugant B49cRf(pAgK84) (□) on cherry seedlings grown in field plots near Corvallis, OR. Vertical bars represent ±1 SD.

or due to cell density effects on the conjugation process (30); and (iii) transconjugants that acquire agrocin 84 immunity may have a selective advantage over agrocin 84-sensitive parental strains in galls colonized by strain K84; therefore, the transconjugants could constitute a greater proportion of the pathogenic population and be recovered at a greater incidence.

Agrobacteria present in crown gall tissue are both diverse and abundant, constituting a significant background that obscures the detection of transconjugants in field studies. For example, strains of *A. tumefaciens* that produce an antibiotic and induce tumors have been described (23,35,38), and their occurrence discredits the coincident expression of those phenotypes as a sufficient criterion for conclusive identification of a transconjugant. In this study, an antibiotic resistance marker was used to select an introduced strain of *A. tumefaciens* from among other bacteria present in gall tissue. Serological tests with strain-specific antibodies provided additional evidence that the Rf-resistant isolates had characteristics of the introduced strain. The virulence phenotype conferred by pTiB49c and the restriction pattern of the T-DNA region were indistinguishable among strain B49c and the transconjugants harboring pAgK84. Nevertheless, the majority of transconjugants recovered from cherry gall tissue had variations in their plasmid profiles. In contrast, migration of pTiB49c isolated from laboratory-constructed transconjugants and transformants that contained pAgK84 was as expected. Rearrangements in the Ti plasmid of pathogenic *Agrobacterium* strains recovered from field plots have been reported by others (39). Fortin et al. (13) established that exposure to acetosyringone, a phenolic in plants, results in mutations in the Ti plasmid and particularly in the *vir* gene cluster. We did not detect variations in pTiB49c restriction fragment length polymorphism analysis with a limited number of DNA probes. Further efforts to identify the source and the potential ecological effects of plasmid profile variation among the field transconjugants were beyond the scope of this study.

Hybridization to the *agn* probe was an effective method for specific detection of agrobacteria harboring pAgK84 in environmental samples. In addition to K84, other strains of *Agrobacterium* harbor plasmids related to pAgK84 (35). Nevertheless, such strains did not interfere with detection of transconjugants in this study. No colonies isolated from galls obtained from field plots inoculated with B49cRf alone hybridized to the *agn* probe. Furthermore, colonies from 9 of the 13 galls evaluated (approximately 90,000 Rf-resistant colonies screened) from cherry coinoculated with K84Sm and B49cRf did not hybridize to the *agn* probe. Plasmids isolated from putative transconjugants comigrated with pAgK84 and had restriction patterns indistinguishable from that of pAgK84, providing strong evidence that these transconjugants harbored pAgK84 rather than a similar but distinct plasmid.

The use of colony hybridization, coupled with selective media containing antibiotics to recover and detect transconjugants in gall extracts, allowed us to screen a large number of colonies and to estimate the proportion of transconjugants in a population of virulent *A. tumefaciens*. In the four galls in which B49cRf(pAgK84) transconjugants were detected, they were present at a proportion of 10^{-4} transconjugants per recipient. We consider this proportion to be a relatively good estimate of pAgK84 transfer frequency, because the presence of K84 in gall tissue did not select for growth of transconjugants relative to the parental strain of the pathogen, which is insensitive to agrocin 84 (36). While it was difficult to recover colonies from the master plates that corresponded to those hybridizing to the *agn* probe on X-ray film, it was done successfully with one gall sample. The recovered colonies were identified as B49cRf(pAgK84) transconjugants.

In addition to pAgK84, K84 contains another self-conjugal plasmid, pAtK84b, that carries genes for nopaline catabolism (4,12). We found no evidence of transfer of pAtK84b into B49c. pAtK84b and pTiB49c are of similar size, comigrate in agarose gel electrophoresis (Fig. 2), and, thus, are difficult to distinguish. Nevertheless, through Southern hybridization analysis of pTiB49c, pAtK84b, and transconjugant plasmids with the *nos* gene probe, it was apparent that transconjugants harbored pTiB49c and not pAtK84b. The absence of pAtK84b in transconjugants harboring pTiB49c is probably due to known incompatibility between pAtK84b and Ti plasmids (4,12).

The biological control bacterium K84 has been used successfully to control crown gall for several decades in agricultural fields throughout the world. The reported failures have been few and have been attributed to the presence of indigenous populations of agrocin 84-insensitive strains of pathogenic agrobacteria. Improper application of the biological control agent could facilitate the development of agrocin 84-resistant strains of *A. tumefaciens*. For example, if K84 is applied at populations too low to control disease, then agrocin 84-sensitive *Agrobacterium* isolates can infect the plant and develop galls. K84 could subsequently colonize the developing galls and plasmid transfer could occur in the gall tissue. Recently, Lu (22) reported the recovery of several pathogenic *Agrobacterium* isolates containing pAgK84 from galls on cherry and raspberry that developed on K84-treated plants grown in commercial nurseries in Oregon and California, respectively.

Establishment of large populations of pathogenic agrobacteria harboring pAgK84 could threaten the sustained efficacy of K84 as a biocontrol agent. To minimize the risk of acquisition of pAgK84 by *A. tumefaciens*, a derivative of K84 (*A. radiobacter* K1026) that produces agrocin 84 but lacks the *tra* region required for conjugal transfer of pAgK84 has been developed (17,34). The efficacy of *A. radiobacter* K1026 is similar to that of K84, and transfer of pAgK84Δ*tra* into pathogenic agrobacteria has not been detected in experimental plots (16,31,39). The use of *A. radiobacter* K1026 is expected to minimize the risk of pAgK84 transfer into pathogenic *Agrobacterium* strains and to help preserve the efficacy of *A. radiobacter* for biological control of crown gall. Although the incidence of pAgK84 plasmid transfer observed in the field studies described here was low, transconjugants were as virulent as the parental strain, persisted in galls and the rhizosphere, and retained both pAgK84 and the Ti plasmid in these environments. Thus, efforts to minimize pAgK84 transfer through judicious use of K84 or K1026 should be encouraged as a means of maintaining the efficacy of biocontrol.

LITERATURE CITED

1. Bouzar, H., Moore, L. W., and Schaad, N. W. 1986. Serological relationship between 50 S ribosomal units from strains of *Agrobacterium* and *Rhizobium*. Phytopathology 76:1265-1269.
2. Brisbane, P. G., and Kerr, A. 1983. Selective media for three biovars of *Agrobacterium*. J. Appl. Bacteriol. 32:348-351.
3. Canfield, M. L., and Moore, L. W. 1991. Isolation and characterization of opine utilizing *Agrobacterium tumefaciens* and fluorescent strains of *Pseudomonas* spp. from rootstocks of *Malus*. Phytopathology 81:440-443.
4. Clare, B. G., Kerr, A., and Jones, D. A. 1990. Characteristics of the nopaline catabolic plasmid in *Agrobacterium* strains K84 and K1026 used for biological control of crown gall disease. Plasmid 23:126-137.
5. Cooksey, D. A., and Moore, L. W. 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. Phytopathology 72:919-921.
6. Cooksey, D. A., and Moore, L. W. 1982. High frequency spontaneous mutations to agrocin 84 resistance in *Agrobacterium tumefaciens* and *A. rhizogenes*. Physiol. Plant Pathol. 20:129-135.
7. Depicker, A., Stachel, S., Dhaese, P., Zambryski, P., and Goodman, H. M. 1982. Nopaline synthase: Transcript mapping and DNA sequence. J. Mol. Appl. Genet. 1:561-573.
8. Ellis, J. G., Murphy, P. J., and Kerr, A. 1982. Isolation and properties of transfer regulatory mutants of the nopaline Ti plasmid of pTiC58. Mol. Gen. Genet. 186:275-281.
9. Farrand, S. K. 1992. Conjugal gene transfer on plants. Pages 345-362 in: Microbial Ecology: Principles, Methods, and Applications. M. A. Levin, R. J. Seidler, and M. Rogul, eds. McGraw-Hill, New York.
10. Farrand, S. K. 1993. Conjugal transfer of *Agrobacterium* plasmids. Pages 255-291 in: Bacterial Conjugation. D. B. Clewell, ed. Plenum Press, New York.

11. Farrand, S. K., Slota, J. E., Shim, J.-S., and Kerr, A. 1985. Tn*5* insertions in the agrocin 84 plasmid: The conjugal nature of pAgK84 and the locations of determinants for transfer and agrocin 84 production. Plasmid 13: 106-117.
12. Farrand, S. K., Wang, C.-L., Hong, S.-B., O'Morchoe, S. B., and Slota, J. E. 1992. Deletion derivatives of pAgK84 and their use in the analysis of *Agrobacterium* plasmid functions. Plasmid 28:201-212.
13. Fortin, C., Nester, E. W., and Dion, P. 1992. Growth inhibition and loss of virulence in cultures of *Agrobacterium tumefaciens* treated with acetosyringone. J. Bacteriol. 174:5676-5685.
14. Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P., and Nester, E. W. 1981. Genetic analysis of crown gall: Fine structure map of T-DNA by site-directed mutagenesis. Cell 27:143-153.
15. Hayman, G. T., and Farrand, S. K. 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. J. Bacteriol. 170:1759-1767.
16. Jones, D. A., and Kerr, A. 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. Plant Dis. 73:15-18.
17. Jones, D. A., Ryder, M. H., Clare, B. G., Farrand, S. K., and Kerr, A. 1988. Construction of a Tra$^-$ deletion mutant of pAgK84 to safeguard the biological control of crown gall. Mol. Gen. Genet. 212:207-214.
18. Kado, C. I. 1991. Molecular mechanisms of crown gall tumorigenesis. Crit. Rev. Plant Sci. 10:1-32.
19. Kerr, A., and Htay, K. 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. 4:37-44.
20. Kerr, A., and Tate, M. E. 1984. Agrocins and the biological control of crown gall. Microbiol. Sci. 1:1-4.
21. López, M. M., Garris, M. T., Salcedo, C. I., Montojo, A. M., and Miró, M. 1989. Evidence of biological control of *Agrobacterium tumefaciens* sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. Appl. Environ. Microbiol. 55:741-746.
22. Lu, S.-F. 1994. Isolation of putative pAgK84 transconjugants from commercial cherry and raspberry plants treated with *Agrobacterium radiobacter* strain K84. M.S. thesis. Oregon State University, Corvallis.
23. Macrae, S., Thomson, J. A., and Van Staden, J. 1988. Colonization of tomato plants by two agrocin-producing strains of *Agrobacterium tumefaciens*. Appl. Environ. Microbiol. 54:3133-3137.
24. Moore, L. W. 1977. Prevention of crown gall on *Prunus* roots by bacterial antagonists. Phytopathology 67:139-144.
25. Moore, L. W., Kado, C., and Bouzar, H. 1988. *Agrobacterium*. Pages 16-36 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria. 2nd ed. N. W. Schaad, ed. The American Phytopathological Society, St. Paul, MN.
26. Moore, L. W., and Warren, G. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Annu. Rev. Phytopathol. 17:163-179.
27. Murphy, P. J., and Roberts, W. P. 1979. A basis for agrocin 84 sensitivity in *Agrobacterium radiobacter*. J. Gen. Microbiol. 114:207-213.
28. Nautiyal, C. S., and Dion, P. 1990. Characterization of the opine-utilizing microflora associated with samples of soil and plants. Appl. Environ. Microbiol. 56:2576-2579.
29. Panagopoulos, C. G., Psallidas, P. G., and Alivizatos, A. S. 1979. Evidence of a breakdown in the effectiveness of biological control of crown gall. Pages 569-678 in: Soil-borne Pathogens. B. Schippers and W. Gams, eds. Academic Press, London.
30. Piper, K. P., Beck von Bodman, S., and Farrand, S. K. 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature (Lond.) 362:448-450.
31. Ryder, M. H., and Jones, D. A. 1991. Biological control of crown gall using *Agrobacterium* strains K84 and K1026. Aust. J. Plant Physiol. 18: 571-579.
32. Ryder, M. H., Slota, J. E., Scarim, A., and Farrand, S. K. 1987. Genetic analysis of agrocin 84 production and immunity in *Agrobacterium* spp. J. Bacteriol. 169:4184-4189.
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. Shim, J.-S., Farrand, S. K., and Kerr, A. 1987. Biological control of crown gall: Construction and testing of new biocontrol agents. Phytopathology 77:463-466.
35. Slota, J. E., and Farrand, S. K. 1982. Genetic isolation and physical characterization of pAgK84, the plasmid responsible for agrocin 84 production. Plasmid 8:175-186.
36. Stockwell, V. O., Moore, L. W., and Loper, J. E. 1993. Fate of *Agrobacterium radiobacter* K84 in the environment. Appl. Environ. Microbiol. 59:2112-2120.
37. Süle, S., and Kado, C. I. 1980. Agrocin resistance in virulent derivatives of *Agrobacterium tumefaciens* harboring the pTi plasmid. Physiol. Plant Pathol. 17:347-356.
38. Van Zyl, F. G. H., Strijdom, B. W., and Staphorst, J. L. 1986. Susceptibility of *Agrobacterium tumefaciens* strains to two agrocin-producing *Agrobacterium* strains. Appl. Environ. Microbiol. 52:234-238.
39. Vicedo, B., Penalver, R., Asins, M. J., and López, M. M. 1993. Biological control of *Agrobacterium tumefaciens*, colonization, and pAgK84 transfer with *Agrobacterium radiobacter* K84 and the Tra$^-$ mutant strain K1026. Appl. Environ. Microbiol. 59:309-315.
40. Vieira, J., and Messing, J. 1982. The pUC plasmids and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.
41. Wang, C.-L., Farrand, S. K., and Hwang, I. 1994. Organization and expression of the genes on pAgK84 that encode production of agrocin 84. Mol. Plant-Microbe Interact. 7:472-481.
42. Zambryski, P. C. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Mol. Biol. 43:465-490.