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Construction of a Range of Derivatives of the Biological Control Strain *Agrobacterium rhizogenes* K84: a Study of Factors Involved in Biological Control of Crown Gall Disease

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The biological control strain *Agrobacterium rhizogenes* K84 is an effective agent in the control of *Agrobacterium* pathogens, the causative agents of crown gall disease. A number of factors are thought to play a role in the control process, including production of the specific agrocins 84 and 434, which differ in the spectra of pathogenic strains that they inhibit in vitro. A range of derivatives of strain K84 has been developed with every combination of the three resident plasmids, pAgK84, pAgK434, and pAtK84b, including a plasmid-free strain. These derivatives produced either both, one, or neither of the characterized agrocins 84 and 434 and were isolated by plasmid curing, conjugation, and Tn*5* transposon mutagenesis. The ability of the derivative strains to inhibit gall formation on almond roots was compared to that of the wild-type K84 parent. Treatment with the plasmid-free derivative did not result in a significant level of control of an *A. rhizogenes* pathogen based on numbers or dry weight of galls formed on injured almond roots. The presence of plasmid pAgK84, pAgK434, or pAtK84b significantly enhanced the biological control efficacy of K84 derivatives, and the highest level of control was observed with strains harboring two or more plasmids. The results observed with strains deficient in agrocin 434 production suggest that this product may play an important role in the biological control of *A. rhizogenes* pathogens. The involvement of plasmid pAgK84b in biological control has not previously been reported. This study supports the conclusion that multiple factors are involved in the success of strain K84 as a biological control agent.

Crown gall is an economically important disease caused by *Agrobacterium* spp., which infect a wide range of crops, such as stone fruit, roses, and grape vines (5, 8, 21). Biological control of pathogenic strains of *Agrobacterium* has been successful for many years, with the nonpathogenic strain *Agrobacterium rhizogenes* (formerly *Agrobacterium radiobacter*) K84 and, more recently, a genetically engineered derivative of K84 designated K1026 (8, 13, 18, 19, 25, 30, 31). Both strains K84 and K1026 produce an antibiotic-like product, agrocin 84, which specifically inhibits the activity of many *Agrobacterium* pathogens (13, 20, 21). A number of researchers have suggested that other mechanisms of control may also be involved, including competition for nutrients or for attachment sites and production of additional inhibitory agents (8, 9, 22, 39). Early evidence for the involvement of factors other than agrocin K84 was obtained when control was observed with pathogenic strains insensitive to agrocin 84 (8, 22, 25, 38). One of these factors may be the effect of another antibiotic, agrocin 434, which is produced by the *A. rhizogenes* strains K84, K1026, and K434, a K84 derivative strain lacking the agrocin 84 genes (9). Agrocin 434 is less inhibitory to agrocin 434- and 84-sensitive *A. rhizogenes* pathogens than is agrocin 84 in vitro but inhibits a wider range of pathogens. Penalver et al. (28) have also described a third product, ALS84, produced by strains K84, K1026, and a derivative of K84 lacking pAgK84, which inhibited a range of bacterial pathogens in in vitro tests.

Strain K84 carries three plasmids: pAgK84 (48 kb), which encodes production of and immunity to agrocin 84; pAgK434 (300 to 400 kb), involved in agrocin 434 production; and pAtK84b (173 kb), a plasmid which encodes nopaline catabolism. It has been previously suggested that plasmid pAgK84b may be a derivative of a pTiC58-like Ti plasmid which has undergone deletion of the T-DNA and Vir regions (7). The genes involved in biosynthesis of agrocin 84 have been characterized and localized to a 21-kb segment of pAgK84 (39). Donner et al. (9) provided evidence that genes involved in production of the novel agrocin 434 are carried on the large, previously cryptic plasmid pAgK434.

The objectives of this study were to construct a range of derivatives of strain K84 with every combination of the three resident plasmids and to test the efficacy of these strains in the biological control of an *A. rhizogenes* pathogen by using almond seedlings. Evidence is presented that all the resident plasmids in strain K84 may play a role in biological control of susceptible pathogens. In addition, the role of agrocin 434 in the biological control process was examined by using Tn*5-mob* insertion mutants which were defective in agrocin 434 production.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in this study and their relevant characteristics are shown in Table 1. The media used for maintenance of *Agrobacterium* strains and for filter matings were Bergensen's medium (3) and TY medium (tryptone [5 g/liter], yeast extract [3 g/liter], $CaCl_2 \cdot 6H_2O$ [1.3 g/liter], Difco Bacto Agar [15 g/liter]).

Bacterial conjugations. Bacterial conjugations were conducted on 25-mm-diameter, 0.22-μm-pore-size nitrocellulose filters on TY plates, with donor and

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TABLE 1. Bacterial strains and plasmids used in this study

Species and strain	Plasmid(s)	Agrocin(s)	Description and source and/or reference
A. rhizogenes			
K27	pTiK27 pAtK27	None	Pathogenic strain sensitive to agrocins 84 and 434; A. Kerr collection
K84	pAgK84 pAtK84b pAgK434	84, 434	Peach gall, S. Australia (26)
K434	pAtK84b pAgK434	434	Spontaneous mutant of K84 lacking pAgK84 (9)
K1143	pAgK434	434	K434 cured of pAtK84b (9)
K1347		None	K1143 cured of pAgK434; this study
K1352	pAgK84	84	Transconjugant of mating K1347 × K325; this study
K1353	pAgK84 pAtK84b	84	Transconjugant of mating K1347 × K325; this study
K1355	pAgK84::Tn*5* pAgK434	84 434	Transconjugant of mating K1143 × K1295; this study
K1351	pAtK84b	None	Transconjugant of mating K1347 × K815; this study
K1356	pAgK434::Tn*5-mob*	None	Tn*5-mob* insertion mutant in pAgK434; this study
K1357	pAgK434::Tn*5-mob*	None	Tn*5-mob* insertion mutant in pAgK434; this study
A. tumefaciens			
K325	pAgK84 pAtK84b pAtC58	84	Derivative of *A. tumefaciens* C58; Rif^r Str^r Spe^r Ery^r; constitutive for octopine and nopaline catabolism and transfer; A. Kerr collection
K815	pAtK84b	None	Derivative of C58C1RS (10); A. Kerr collection
K1295	pAgK84::Tn*5*	84	Derivative of NT1 (39); Km^r; S. Farrand
E. coli			
S 17-1	pSUP5011		Tn*5-mob* transposon donor strain (32)
C600	pNJ5000		Unstable mobilizing plasmid; Tc^r (14)

recipient strains grown overnight on mating filters before being plated onto selective medium. For selection of transconjugants from bacterial matings with *Agrobacterium tumefaciens* as donor and *A. rhizogenes* as recipient, a medium selective for *A. rhizogenes* was used (6). Multiple patch matings were conducted with a multipronged metal replicator.

Growth tests to differentiate between *A. rhizogenes* and *A. tumefaciens*. (i) 3-Ketolactose production. Confirmation of the identity of *A. rhizogenes* transconjugants was done by using a series of tests which differentiate between *A. rhizogenes* and the *A. tumefaciens* strains used in this study. Although the differential tests are not appropriate for all *A. rhizogenes* and *A. tumefaciens* strains, they were suitable for the strains used in this study. To test 3-ketolactose production, the method of Bernaerts and De Ley (4) was used. The *A. tumefaciens* (formerly biovar 1) strains used in this study give a bright yellow-orange zone around colonies which produce 3-ketolactose.

(ii) Growth on 2% NaCl. Sensitivity to high salt concentrations (*A. rhizogenes* strains are inhibited on media with high levels of salt) was assessed by growing the strains on Oxoid nutrient broth medium with 2% NaCl for 10 days.

(iii) Maximum growth temperature. Nutrient agar plates were inoculated with each strain and incubated at 37°C for up to 10 days. Growth of *A. rhizogenes* strains is inhibited at 37°C. The donor strains used were capable of poor growth at 37°C.

Plasmid isolation and electrophoresis. Plasmids from *Agrobacterium* strains were isolated by an adapted miniprep method essentially as described by Farrand et al. (12). Agarose gel electrophoresis was carried out by standard methods.

Agrocin 434 purification. Crude extracts of agrocin 434 from culture supernatants were obtained by using the method described by Donner et al. (9). Crude extracts were dissolved in deionized water for use in bioassays or characterization by high-voltage paper electrophoresis (HVPE) (35).

HVPE. HVPE of agrocin 434 was carried out with a high-voltage electrophoresis apparatus (35) under conditions, and with detection of agrocin 434 by UV absorbance, as described previously (9).

Agrocin 84 and 434 bioassays. To test agrocin 84 production, the method of Kerr and Htay (20) was used, in which zones of inhibition were detected in lawns of a sensitive strain (*A. rhizogenes* K27) on killed producer colonies with Stonier's agar medium (34). For agrocin 434 bioassays the producer colonies were replaced by filter paper discs with 20 μl of crude agrocin recovered from 1.5 to 3.0 ml of culture supernatant as described above.

Preparation of almond seedlings. Fresh almond seeds, *Prunus amygdalus* cultivar Fritz (a gift from Ali Vezvaei), were soaked in distilled water containing 1 g of the fungicide benomyl/liter and incubated for 3 days in the dark at 4°C to initiate germination. The seeds were then planted, one per pot, in 7-in. pots in University of California soil mix and were maintained moist under field conditions for 5 months. The seedlings were then used for almond seedling assays.

Almond seedling assay for biological control of crown gall (root inoculation). The method of Htay and Kerr (17) was used for root inoculation of almond cv. Fritz. Unsterilized soil (standard University of California soil mix) was placed in 72 pots (27-cm diameter), with eight replicates for each treatment, and inoculated with 3-day-old cultures of K27 at about 10^7 cells/ml to give approximately 10^6 cells per g of soil. The actual distribution of K27 in the soil was not examined. The soil was kept for 2 days prior to planting. The 3-day-old cultures of biological control test strains were suspended in 3 liters of nonchlorinated water. The suspensions were estimated by optical density measurements to contain about 10^7 cells/ml (optical density at 600 nm of approximately 0.02). The 5-month-old almond seedlings were removed from their pots, the soil was shaken gently from their roots with water, and the taproots were trimmed to a length of approximately 10 cm. The plants were dipped in water or a suspension of the biocontrol strains. They were then replanted in the K27-infested soil. The pots were watered regularly when necessary. After 6 months of incubation outdoors, the plants were removed and the roots were washed in running water. Assays with genetically altered bacterial strains (those containing Tn*5* insertions) were conducted in a containment greenhouse with separate control treatments. The experiments were set up with a randomized complete block design.

Statistical analysis. An analysis of variance was performed on the data with the Genstat 5 statistical package and, if the variation permitted ($P < 0.05$), the residual degrees of freedom and residual mean square were used to calculate Tukey's honestly significant difference (HSD) (15). Tukey's statistic was used for multiple comparison of means, with means differing by more than the calculated HSD value considered significantly different at the 5% significance level.

RESULTS

Construction of K84 derivatives. Donner et al. (9) described the isolation of strains K434 and K1143. Strain K434 was a spontaneous cured derivative of strain K84 lacking pAgK84 (agrocin 84$^-$ agrocin 434$^+$), and strain K1143 was obtained by curing strain K434 of the nopaline-catabolic plasmid pAtK84b. New derivatives of K84 carrying various combinations of the three resident plasmids were isolated as follows.

Tn*5* mutagenesis of plasmid pAgK434. Tn*5-mob* insertions in pAgK434 were obtained as follows. Transposon Tn*5-mob* was transferred into strain K1143 by using the suicide donor plasmid pSup5011 as described previously (9, 32). Transconjugants carrying inserts in plasmid pAgK434 were isolated by identifying transconjugant strains capable of transferring the Tn*5*-encoded kanamycin resistance at high frequency in multiple patch matings with *A. tumefaciens* K749 as a recipient. As pAgK434 is not thought to be self-transmissible, it was necessary to transfer the mobilizing plasmid pNJ5000 (14) from *Escherichia coli* C600 into all the transconjugants in a multiple patch mating as the first step of this process. The selection used was Bergensen's medium (3) with 200 μg of kanamycin/ml and 10 μg of tetracycline/ml. Multiple patch matings were then conducted with strain K749 as a recipient, and the strains giving high rates of transfer in patches on TY agar with rifampin (25 μg/ml), streptomycin (250 μg/ml), and kanamycin (200 μg/ml) were putatively identified as carrying plasmid insertions. Several independent filter matings between *E. coli* S17-1(pSup5011) and *A. rhizogenes* K1143 resulted in the isolation of approximately 4,000 transconjugants carrying insertions either in the chromosome or plasmid pAgK434. Of these, 300 were capable of transfer of kanamycin resistance to strain K749 at high frequency. After purification, the K749 transconjugants were screened by HVPE for their ability to produce agrocin 434. Two of the transconjugants did not produce agrocin 434, and the corresponding K1143::Tn*5-mob* donors were also deficient in agrocin 434 production. Loss of ability to produce agrocin 434 was confirmed by bioassay, and plasmid analysis showed that these strains retained plasmids indistinguishable in size from plasmid pAgK434 (data not shown). This was important, as a previous attempt to isolate Tn*5* inserts in pAgK434 had resulted in isolation of a strain in which loss of agrocin 434 production was accompanied by a large deletion in pAgK434 and a coincidental insertion of Tn*5* in a chromosomal location. The two Tn*5* insertion derivatives of K1143 were designated K1156 and K1157. Southern hybridization analysis of the insertions in these two strains, which were isolated in independent transposon mutagenesis experiments, has confirmed the presence of different, single Tn*5-mob* insertions in pAgK434 (data not shown), and further analysis of the sites of insertion is in progress.

Isolation of plasmid-free strain K1347. The isolation of a plasmid-free derivative of strain K1143 was achieved by heat curing of a derivative of K1143 carrying a Tn*5*-encoded kanamycin resistance marker on plasmid pAgK434 isolated as described above. Little or no growth of strain K1143 or its derivatives was observed at temperatures of 35°C or above, and therefore the heat curing was carried out at a temperature of 34.5°C. After 3 days of subculture at this temperature loss of the kanamycin resistance marker was observed at frequencies of 5 to 10%. Kanamycin-sensitive colonies were tested by HVPE and bioassay for the ability to produce agrocin 434, and all of the strains tested had lost the ability (data not shown). One representative strain, K1347, was tested for plasmid content and was shown to have lost plasmid pAgK434 (Fig. 1). Strain K1347 was then used as a recipient to complete the range of derivatives of K84 which carried all possible combinations of the three plasmids pAgK84, pAtK84b, and pAgK434.

Isolation of strain K1351(pAtK84b). Plasmid AtK84b was transferred from K815 [*A. tumefaciens*(pAtK84b)] as the donor to K1347 in a filter mating on TY agar medium with nopaline (125 mg/liter) at 28°C overnight. Transconjugants were selected on *A. rhizogenes*-selective medium with nopaline (125 mg/ml) as the sole nitrogen source. After purification on the same medium, the transconjugants were tested by agarose gel electrophoresis for 3-ketolactose production, growth on 2% NaCl medium, growth at 37°C, and the presence of pAtK84b plasmid. One resulting transconjugant was designated K1351, and the presence of the single plasmid, pAtK84b, is shown in Fig. 1.

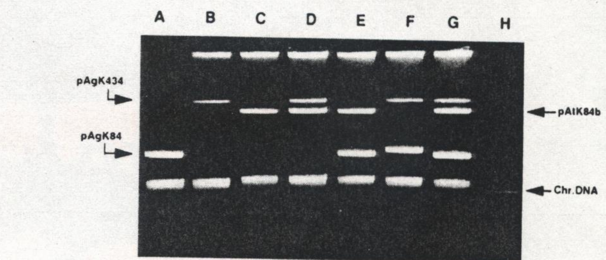

FIG. 1. Plasmid profiles of *A. rhizogenes* K84 and its derivatives. Lanes: A, K1352; B, K1143; C, K1351; D, K434; E, K1353; F, K1355; G, K84; H, K1347. Chr. DNA, chromosomal DNA.

Isolation of K1352(pAgK84). Plasmid pAgK84 was transferred from K325 [*A. tumefaciens*(pAgK84, pAtK84b)] as donor to K1347 in a filter mating on TY agar at 28°C. Transconjugants were selected on *A. rhizogenes*-selective medium (6). As there was no direct selection for transfer of pAgK84, 300 colonies arising on the selective plates (either recipient strains or transconjugants) were screened for production of agrocin 84 by using a bioassay with K27 as the indicator. Transconjugants which inhibited strain K27 were then tested for 3-ketolactose production, growth on 2% NaCl, and growth at 37°C. One resulting transconjugant, which inhibited strain K27 in the bioassay and had growth characteristics of *A. rhizogenes*, was designated strain K1352. Gel electrophoresis of plasmid DNA isolated from strain K1352 confirmed that it contained a single plasmid, pAgK84.

Isolation of K1353(pAgK84, pAtK84b). Plasmids pAtK84b and pAgK84 were transferred from K325 to K1347 in a patch mating on TY agar medium with nopaline (125 mg/liter) at 28°C for 24 h. Transconjugants of this mating were selected on *A. rhizogenes*-selective medium minus NH_4NO_3 with nopaline (125 mg/liter). The transconjugants were then purified on the same medium and were screened for agrocin 84 production, as described above. One resulting transconjugant, designated strain K1353 and having growth characteristics of *A. rhizogenes*, was shown to contain the two plasmids pAgK84 and pAtK84b (Fig. 1).

Isolation of K1355(pAgK434, pAgK84::Tn*5*). Plasmid pAgK84::Tn*5* was transferred from *A. tumefaciens* K1295 to strain K1143, and transconjugants were selected on medium selective for *A. rhizogenes* with 200 μg of kanamycin/ml. The resulting transconjugants were tested for agrocin 84 and 434 production and confirmed as *A. rhizogenes* by growth tests. One representative transconjugant, designated strain K1355, was shown to have plasmids pAgK84::Tn*5* and pAgK434 (Fig. 1).

Assessment of biological control of pathogen *A. rhizogenes* K27 by almond seedling root bioassay. The *A. rhizogenes* strain K27 is sensitive to both agrocin 84 and agrocin 434 in in vitro assays. The ability of the K84 derivatives described above (except strain K1355, which was isolated at a later date) to inhibit gall formation by strain K27 on almond seedling roots was tested. Injured roots of almond cv. Fritz seedlings were inoculated with *Agrobacterium* strains by dipping them prior to planting them in pathogen-infested soil. This experimental design mimics the practical application procedure for the commercial K84-derived biological control products. Two sets of experiments were performed: seedlings treated with derivatives without Tn*5-mob* insertions were incubated in the open air under ambient conditions, whereas the seedlings treated

TABLE 2. Effect of pretreatment of almond roots with strain K84 or its derivatives on gall formation (number of galls and gall dry weight) after incubation of almond trees (cv. Fritz) in soil inoculated with pathogen *A. rhizogenes* K27

Inoculant strain(s) (agrocin(s) produced)	No. of galls[a,b]	Gall dry wt[a,c]
None	0.1^{1}	$<0.1^{1}$
K27 pathogen	11.4^{3}	4.2^{2}
K27 pathogen + K84 (84 + 434)	0.9^{1}	0.1^{1}
K27 pathogen + K434 (434)	1.6^{1}	0.3^{1}
K27 pathogen + K1143 (434)	3.6^{1}	0.6^{1}
K27 pathogen + K1347 (none)	$9.9^{2,3}$	3.9^{2}
K27 pathogen + K1351 (none)	$5.1^{1,2}$	0.9^{1}
K27 pathogen + K1352 (84)	4.0^{1}	0.9^{1}
K27 pathogen + K1353 (84)	2.3^{1}	0.4^{1}

[a] Superscript numbers indicate strains which are significantly different ($P < 0.05$); i.e., a strain with superscript 1 is significantly different from a strain with superscript 2, and a strain with superscript 1,2 is not significantly different ($P < 0.05$) from strains with superscript 1 or 2.
[b] Tukey's HSD, 5.5.
[c] Tukey's HSD, 2.5.

with the Tn*5-mob* insertion mutants, K1356 and K1357, were incubated in a separate experiment in a containment greenhouse with control strains. There were eight replicates for each treatment. After 6 months, the galls were removed from the roots, counted, dried (3 days at 65°C), and weighed individually.

The results for strain K1347, K1143, K1351, K1352, K1353, K434, and the wild type, K84, are shown in Table 2. Although a high level of variation was observed in the bioassay test, there was a significant difference ($P < 0.05$) in biological control ability between strains which contained one or more plasmids, compared to treatment with the pathogen alone. There was no significant difference between treatment with the pathogen alone and treatment with the pathogen plus the plasmid-free strain, K1347, based on either number of galls or gall dry weights. The highest level of control was observed with the wild-type strain, K84, followed by the strains carrying two plasmids, K434 and K1353.

Gall formation was significantly reduced on treatment with K1351 compared with treatment with the pathogen alone, which suggests that plasmid pAtK84b, which does not encode any known agrocins or alternative inhibitory agents, may play a role in the biological control process.

Comparison of pathogen inhibition by strain K1143 and Tn*5* insertion mutants deficient in agrocin 434 production. Strain K1143, which carries only plasmid pAgK434 (involved in agrocin 434 production), had a significant effect on the number and dry weight of galls compared to treatment with the pathogen alone, whereas the plasmid-free strain, K1347, had no significant effect on either the number or dry weight of galls in either experiment, compared to treatment with the pathogen alone. Although the only function which has been ascribed to plasmid pAgK434 is involvement in agrocin 434 production, it is possible that additional factors involved in the biological control process may be encoded by this large (300- to 400-kbp) plasmid. To determine whether production of agrocin 434 itself was the major control factor encoded by pAgK434, the ability of two Tn*5-mob* insertion mutants, strains K1356 and K1357, to control the pathogen K27 was also assessed in almond seedling root bioassays. These experiments were carried out in a separate experiment in a containment greenhouse, with strain K1347 as an additional control. Only strain K1143 exhibited a significant effect ($P < 0.05$) on inhibition of the pathogen K27 compared to treatment by the pathogen alone, on the basis of number of galls and gall dry weights (Table 3). As both K1356 and K1357 are derivatives of strain K1143, with distinct single insertions in pAgK434 and the accompanying loss of ability to produce agrocin 434, it is likely that reduced biocontrol ability is correlated with loss of production of agrocin 434.

DISCUSSION

The successful biological control of crown gall pathogens by *A. rhizogenes* K84 is probably the result of a number of activities exhibited by this strain. Factors which have been identified to date include the potent agrocin 84, effective against pathogens which carry nopaline-agrocinopine A-type Ti plasmids with associated *acc* genes (16). Donner et al. (9) also described a second agrocin produced by strain K84 and its derivatives, which inhibited all *A. rhizogenes* strains tested except producer strains. A third product, ALS84, produced by strain K84, inhibited a range of phytopathogenic bacteria in addition to *Agrobacterium* spp. in vitro (28), although the nature and genetic basis of this product have not yet been determined, to our knowledge. Attributing biological control ability to specific factors or activities is complicated when test strains produce a range of products which may act synergistically. In this study, we isolated a range of derivatives of strain K84 which were deficient in the production of one or both of the known agrocins 84 and 434 and carried the Ti plasmid pAtK84b, with the T-DNA and Vir regions deleted. The derivatives included strains with every combination of the three plasmids present in strain K84, allowing differences in pathogen inhibition to be attributed to the presence or absence of a single plasmid. These strains included a plasmid-free strain, K1347.

Strain K1347 was ineffective as a biological control strain against the pathogen K27, as shown by the almond seedling tests used in two independent long-term greenhouse and pot experiments. This strain does not produce agrocin 84 or 434, and the results suggest that chromosomally determined characteristics alone do not play an important role in biocontrol with this pathogen and test system. A number of workers have suggested previously that blockage of or competition for infection sites may explain the biological control of strains resistant to agrocin 84 (8, 11, 22, 38). Other possible mechanisms which may play a role in control include substrate competition in soil (22) and a superior root colonizing ability of biocontrol strains

TABLE 3. Effect of pretreatment of almond roots with strain K1143 or its derivatives on gall formation (number of galls and gall dry weight) after incubation of almond trees (cv. Fritz) for 9 months in soil inoculated with pathogen *A. rhizogenes* K27

Inoculant strain(s) (agrocin produced)	No. of galls[a,b]	Gall dry wt[a,c]
Pathogen K27	19.5^{1}	4.8^{1}
K27 pathogen + K1143 (434)	4.4^{2}	0.7^{2}
K27 pathogen + K1347	23.5^{1}	4.3^{1}
K27 pathogen + K1356	$15.0^{1,2}$	$1.8^{1,2}$
K27 pathogen + K1357	20.7^{1}	4.9^{1}

[a] Superscript numbers indicate strains which are significantly different ($P < 0.05$); i.e., a strain with superscript 1 is significantly different from a strain with superscript 2, and a strain with superscript 1,2 is not significantly different ($P < 0.05$) from strains with superscript 1 or 2.
[b] Tukey's HSD, 12.4.
[c] Tukey's HSD, 3.9.

(31). However, there is little direct evidence to support these explanations, and the results from this study suggest that effective biological control may require the presence of one or more of the plasmids in strain K84. It is possible that the presence of one or more plasmids may enhance the potential for competition with pathogens at or near injury sites. This could occur, for example, if activities important in initial stages of the pathogenic process are still encoded on plasmid pAtK84b, although there is no direct evidence for this.

The ability of strains producing only agrocin 84 or 434 (strains K1352 and K1143, respectively) to control the pathogen K27 shows that both of these agents may play an important role in the inhibition of *Agrobacterium* pathogens in vivo. For agrocin 434, this conclusion was supported by the reduced efficacy of strain K1356 and K1357, derivatives of K1143 which were deficient in agrocin 434 production only, presumably due to a Tn*5-mob* insertion into genes required for agrocin 434 production. Further characterization of these strains and confirmation of the basis of this deficiency is in progress and will play an important part in the characterization of the genetic basis for agrocin 434 production. There was little difference between the inhibitions observed with strains producing agrocin 84 and those producing agrocin 434, taking into account the large variation observed with the bioassay test used. This is the first evidence for a role for agrocin 434 in biological control in an in vivo test. There was a significant effect on both the number of galls and gall weight ($P < 0.05$) following treatment of roots with the pathogen K27 and strain K1143 compared to treatment with the pathogen alone. This effect was seen in both experiments, and the mean numbers of galls and gall dry weight were consistent following treatment with strain K1143 under both test conditions (in the greenhouse trial and in the open air). The test strain used was sensitive to both agrocin 84 and agrocin 434 in vitro. In the past the inhibition of pathogens resistant to agrocin 84 has been attributed to factors such as competition for nutrients and for attachment sites. An alternative explanation for *A. rhizogenes* or biovar 2 pathogens could be inhibition by agrocin 434, which, although apparently a less potent agent than agrocin 84 in in vitro tests, is effective against all *A. rhizogenes* strains tested to date except producer strains (9).

The involvement of plasmid pAtK84b in the control of strain K27 was a novel result and warrants further investigation. Plasmid pATK84b is thought to be a pTiC58-type Ti plasmid, disarmed in the oncogenic T-DNA and Vir regions but retaining genes involved in catabolism of nopaline (7). A number of explanations are possible for the effect of plasmid pAtK84b. The ability of host strains to catabolize nopaline is unlikely to be important, as this would take effect only after infection and gall formation; however, it may provide a mechanism for competition with pathogens in the soil following initial infection, reducing further infection and gall formation. A more likely reason is that pAtK84b carries genes which are important in early stages of the pathogenic process, e.g., attachment and the potential for competition with or inhibition of pathogens. This possibility is supported by studies which have shown that nopaline-type Ti plasmids enhance attachment (23, 29). Alternatively, pAtK84b may enhance chemotaxis to wound sites, as Ti plasmids have been shown to play a role in chemotaxis to wound exudates (1, 2). Both of these possibilities would enhance the potential for competition or inhibition of pathogenic strains at the site of infection. Thus, competition may play a role in the control effect but may require the presence of pAtK84b-encoded activities for effective pathogen inhibition. As both chromosomal and plasmid-borne genes are thought to play a role in the early stages of the pathogenic process (23, 24, 27, 29, 36), the role of pAtK84b in the biological control process is likely to be complex.

A further possibility which cannot be discounted is that pAtK84b encodes production of an additional, unknown inhibitory agent. The activity of ALS84 (28) has been demonstrated only in in vitro tests, and the product itself and the genetic basis for its production have not been identified. In this study we did not test the ability of derivative strains to produce ALS84 and so cannot rule out the possibility that this or other inhibitory agents are encoded on pAtK84b.

It should also be noted that derivatives which did not retain pAtK84b but which produced agrocin 84 or 434 (strains 1352 and 1143, respectively) were effective inhibitors of gall formation by the pathogen K27. This suggests that pAtK84b is not absolutely necessary for pathogen inhibition when other potent inhibitory compounds are produced by control strains. The highest level of control was exhibited by strains which produced multiple agents; thus, strain K84 itself was the most effective inhibitor, followed by strains which retained two of the three plasmids, although the variability observed in the test assay means that these differences were not statistically significant.

The biological control of *Agrobacterium* pathogens by strain K84 is a complex process with a number of factors playing roles in the inhibition process. The relative contribution of individual factors in any control situation will depend on the pathogens present, the ratio of the pathogens to the biocontrol strain, the host plant, and the method of application of the control strain used. Thus, in the control of agrocin 84-resistant *A. rhizogenes* pathogens, agrocin 434 is likely to be an important component, whereas in the control of *A. tumefaciens* pathogens, agrocin 434 is unlikely to play a major role. This study has confirmed the efficacy of agrocin 84 as a potent inhibitor and has presented evidence that agrocin 434 may also be an important component in the biological control of *A. rhizogenes* strains. As would be expected, the presence of multiple control factors appears to enhance the control effect. The role of pAtK84b in the control process has yet to be elucidated. Studies are being undertaken to examine the attachment and interaction of pathogens and biocontrol derivatives directly on plant surfaces, using strains tagged with fluorescence markers. This may provide an explanation for the role of pAtK84b and lead to an enhanced understanding of this complex but successful biological control system.

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REFERENCES

1. **Ashby, A. M., M. D. Watson, and C. H. Shaw.** 1987. A Ti-plasmid determined function is responsible for chemotaxis of *Agrobacterium tumefaciens* towards the plant wound product acetosyringone. FEMS Microbiol. Lett. **41**:189–192.
2. **Ashby, A. M., M. D. Watson, G. J. Loake, and C. H. Shaw.** 1988. Ti plasmid-specified chemotaxis of *Agrobacterium tumefaciens* C58C[1] toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J. Bacteriol. **170**:4181–4187.
3. **Bergersen, F. J.** 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. **14**:349–360.
4. **Bernaerts, M. J., and J. De Ley.** 1963. A biochemical test for crown gall bacteria. Nature **197**:406–407.
5. **Braun, A. C.** 1982. A history of the crown gall problem, p. 155–210. *In* G. Kahl and J. S. Schell (ed.), Molecular biology of plant tumors. Academic Press, New York, N.Y.
6. **Brisbane, P., and A. Kerr.** 1983. Selective media for three biovars of *Agrobacterium*. J. Appl. Bacteriol. **54**:425–431.
7. **Clare, B. G., A. Kerr, and D. A. Jones.** 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.

8. **Cooksey, D. A., and L. W. Moore.** 1982. Biological control of crown gall with an agrocin mutant of *Agrobacterium radiobacter*. Phytopathology **72:**919–921.
9. **Donner, S. C., D. A. Jones, N. C. McClure, G. M. Rosewarne, M. E. Tate, A. Kerr, N. N. Fajardo, and B. G. Clare.** 1993. Agrocin 434, a new plasmid encoded agrocin from the biocontrol *Agrobacterium* strains K84 and K1026, which inhibits biovar 2 agrobacteria. Physiol. Mol. Plant Pathol. **42:**185–194.
10. **Ellis, J. G., P. J. Murphy, and A. Kerr.** 1982. Isolation and properties of transfer regulatory mutants of the nopaline Ti plasmid, pTiC58. Mol. Gen. Genet. **186:**275–281.
11. **Farrand, S. K., and C. Wang.** 1992. Do we really understand crown gall control by *Agrobacterium radiobacter* strain K84?, p. 287–293. *In* E. C. Tjamos, G. C. Papavizas, and R. J. Cook (ed.), Biological control of plant diseases. Plenum Press, New York, N.Y.
12. **Farrand, S. K., J. E. Slota, J.-S. Shim, and A. Kerr.** 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.
13. **Farrand, S. K.** 1990. *Agrobacterium radiobacter* strain K84: a model biocontrol system, p. 679–691. *In* R. R. Baker and P. E. Dunn (ed.), New directions in biological control: alternatives for suppressing agricultural pests and diseases. Wiley-Liss, New York, N.Y.
14. **Grinter, N. J.** 1983. A broad host range cloning vector transposable to various replicons. Gene **21:**133–143.
15. **Groebner, D. F., and P. W. Shannon.** 1990. Business statistics: a decision-making approach, 3rd ed., p. 520–527. Merrill Publishing Co., Columbus, Ohio.
16. **Hayman, G. T., and S. K. Farrand.** 1988. Characterization and mapping of the agrocinopine-agrocin 84 locus on the nopaline Ti plasmid pTiC58. J. Bacteriol. **170:**1759–1767.
17. **Htay, K., and A. Kerr.** 1974. Biological control of crown gall: seed and root inoculation. J. Appl. Bacteriol. **37:**525–530.
18. **Jones, D. A., and A. Kerr.** 1989. *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative of strain K84, for biological control of crown gall. Plant Dis. **73:**15–18.
19. **Jones, D. A., M. H. Ryder, B. G. Clare, S. K. Farrand, and A. Kerr.** 1988. Construction of a Tra$^-$ deletion mutant of pAgK84 to safeguard the biological control of crown gall. Mol. Gen. Genet. **212:**207–214.
20. **Kerr, A., and K. Htay.** 1974. Biological control of crown gall through bacteriocin production. Physiol. Plant Pathol. **4:**37–44.
21. **Kerr, A., and M. E. Tate.** 1984. Agrocins and the biological control of crown gall. Microbiol. Sci. **1:**1–4.
22. **Lopez, M. M., M. T. Gorris, C. I. Salcedo, A. M. Montojo, and M. Miro.** 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on stone fruit trees. Appl. Environ. Microbiol. **55:**741–746.
23. **Matthysse, A. G., P. M. Wyman, and F. V. Holmes.** 1978. Plasmid-dependent attachment of *Agrobacterium tumefaciens* to plant tissue culture cells. Infect. Immun. **22:**516–522.
24. **Matthysse, A. G.** 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **169:**313–323.
25. **Moore, L. W., and G. Warren.** 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Annu. Rev. Phytopathol. **17:**163–179.
26. **New, P. B., and A. Kerr.** 1972. Biological control of crown gall: field measurements and glasshouse experiments. J. Appl. Bacteriol. **35:**279–287.
27. **Parke, D., N. L. Ornston, and E. W. Nester.** 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. J. Bacteriol. **169:**5336–5338.
28. **Penalver, R., B. Vicedo, C. I. Salcedo, and M. Lopez.** 1994. *Agrobacterium radiobacter* strains K84, K1026 and K84 Ag$^-$ produce an antibiotic-like substance, active *in vitro* against *A. tumefaciens* and phytopathogenic *Erwinia* and *Pseudomonas* spp. Biocontrol Sci. Technol. **4:**259–267.
29. **Pu, X.-A., and R. N. Goodman.** 1993. Attachment of agrobacteria to grape cells. Appl. Environ. Microbiol. **59:**2572–2577.
30. **Ryder, M. H., and D. A. Jones.** 1990. Biological control of crown gall, p. 45–63. *In* D. Hornby (ed.), Biological control of soil-borne plant pathogens. CAB International, Wallingford, United Kingdom.
31. **Shim, J.-S., S. K. Farrand, and A. Kerr.** 1987. Biological control of crown gall: construction and testing of new biocontrol agents. Phytopathology **77:**463–466.
32. **Simon, R.** 1984. High frequency mobilization of Gram-negative bacterial replicons by the *in vitro* constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196:**413–420.
33. **Smith, V. A., and J. Hindley.** 1978. Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells. Nature **276:**498–500.
34. **Stonier, T.** 1960. *Agrobacterium tumefaciens* Conn. II. Production of an antibiotic substance. J. Bacteriol. **79:**889–898.
35. **Tate, M. E.** 1968. Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). Anal. Biochem. **23:**141–149.
36. **Thomashow, M. F., J. E. Karlinsey, J. R. Marks, and R. E. Hurlbert.** 1987. Identification of a new virulence locus in *Agrobacterium tumefaciens* that affects polysaccharide composition and plant cell attachment. J. Bacteriol. **169:**3209–3216.
37. **Van Zyll, F. G. H., B. W. Strijdom, and J. L. Staphorst.** 1986. Susceptibility of *Agrobacterium tumefaciens* strains to two agrocin-producing *Agrobacterium* strains. Appl. Environ. Microbiol. **52:**234–238.
38. **Vicedo, B., R. Peñalver, M. J. Asins, and M. M. López.** 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.
39. **Wang, C.-L., S. K. Farrand, and I. Hwang.** 1994. Organization and expression of the genes on pAgK84 that encode production of agrocin 84. Mol. Plant-Microbe Interact. **7:**472–481.
40. **Watson, B., T. C. Currier, M. P. Gordon, M.-D. Chilton, and E. W. Nester.** 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **123:**255–264.