Biological Control of Crown Gall: Construction and Testing of New Biocontrol Agents

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ABSTRACT

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Transfer deficient (Tra$^-$) Tn5 insertion mutants of the agrocin 84 plasmid pAgK84 were transformed into two chromosomal backgrounds and the transformants tested as agents for biological control of crown gall on almonds and for colonization of almond seedling roots. Efficiency of root colonization and of biological control was influenced by the chromosomal background. Two Tra strains were as effective as the commercial Tra$^+$ strain K84 in controlling crown gall. Strains of Agrobacterium radiobacter which contained a high copy number mutant of pAgK84 and overproduced agrocin 84 were no more effective, and in one case, significantly less effective in controlling crown gall than strains containing the normal copy number of pAgK84.

Additional key word: Agrobacterium tumefaciens.

The biological control of crown gall on stone fruit and rose by strain K84 of *Agrobacterium radiobacter* (Beijerinck & van Delden 1902) Conn 1942 is used commercially in many countries, including Australia, Greece, Israel, Italy, Japan, New Zealand, South Africa, Spain, and the United States. Strain K84 produces agrocin 84, a novel nucleotide antibiotic (26) that inhibits the causal organism, *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942 if the latter contains a nopaline-type tumor-inducing (Ti) plasmid (7,14). By this means, infection of susceptible plants by such a strain is prevented. The synthesis of agrocin 84 is encoded by a 47.7 kb (kilobase) plasmid, pAgK84 (5,23).

Although this method of control has generally proved very efficient, there have been some reports that control is not always effective (1,9,17,19). Not all strains of *A. tumefaciens* are sensitive to agrocin 84, especially strains isolated from grapevine, apple, chrysanthemum, and blackberry, but biological control of crown gall by strain K84 has never been recommended for these crops. Even on stone fruit where biological control is usually very effective, some isolates are resistant to agrocin 84 (13). These innately resistant strains probably account for many reports of unsatisfactory control. Some pathogenic strains become resistant to agrocin 84 after field use of strain 84 (19; M. Lopez, *personal communication*). This is due to transfer of pAgK84 from strain K84 to *A. tumefaciens* (4) and poses a serious threat to the continued success of biological control of crown gall. This threat could be markedly reduced if pAgK84 were made transfer deficient (Tra$^-$).

The transfer region of pAgK84 has been located (8), and this paper reports that a Tra$^-$ insertion mutant in a suitable chromosomal background is an efficient agent of biological control. In addition, mutants that overproduce agrocin 84 are tested as biological control agents.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Media and buffers. The following media were used: Citrate-glutamate-glucose (CGG) medium (18); selective media 1A and 2E (13) for biovars 1 and 2, respectively; Bergerson's medium (2); Stonier's medium (24), L-broth (15); nutrient agar (NA) (Difco, Detroit, MI); yeast extract broth (YEB) (27), and yeast mannitol agar (YM) (5).

The following buffers were used: buffered saline (BS) (0.02 M sodium phosphate 0.85% NaCl, pH 7.3), LTE (8), and TEB (89 mM Tris, 2.5 mM EDTA, 89 mM boric acid).

Standard bioassay for agrocin production. The method of Stonier (24) as modified by Kerr and Htay (12) was used. Strain K198 was used as the indicator.

Quantitative bioassay for agrocin production. Fresh YM agar cultures were suspended in sterile distilled water (SDW) and the A_{680nm} values adjusted to 0.45; 100-μl aliquots were inoculated into 20 ml of CGG medium in 100-ml flasks and incubated at 26 C on a rotary shaker. At appropriate times A_{680nm} was measured, 1-ml samples removed and centrifuged, and 150 μl of supernatant added to 9-mm-diameter wells in plates of Stonier's agar. Three replicates of each culture were used. The plates were treated with chloroform to kill bacteria and left for 3 hr to allow agrocin 84 to diffuse into the agar before being overlaid with the indicator strain K198.

Isolation of an agrocin 84 overproducing mutant. A purified preparation of the Tra$^-$ insertion derivative, pAgK84A1 was prepared from strain A1 and mutagenized in vitro with hydroxylamine essentially as described (15). The treated DNA was precipitated with ethanol, the pellet washed four times with 70% ethanol, and redissolved in 50 μl of LTE. A 20-μl aliquot was used to transform strain NT-1. Kanamycin-resistant transformants were picked to master NA+Km plates and also to Stonier's plates, which were incubated for 3 hr at 26 C. This is the minimum time necessary to detect agrocin 84 production by strain A1. After this incubation, the Stonier's plates were exposed to chloroform and overlayed with the sensitive indicator strain, C58. Colonies giving zones of inhibition larger than that of the parent strain, A1, were recovered from the master plates and reassayed for increased levels of agrocin 84 production.

TABLE 1. Bacterial strains

Strain	Biovar	Plasmid[a]	Agrocin 84 production	Source and description
K27	2	pTiK27	...	Peach gall, S. Australia. Tumorigenic nopaline strain. Sensitive to agrocin 84
K84	2	pAgK84; pAtK84b	Normal	Peach gall, S. Australia (16)
K198	1	pTiK27	...	pTiK27 transconjugant (22)
K434	2	pAtK84b	...	Spontaneous mutant of strain K84 lacking pAgK84
NT1	1	...	...	pTi-cured C58 (28)
A1	1	pAgA1	Normal	NT1 transformed with pAgK84::Tn5 A1 (Tra⁻) (8)
K657	1	pAgK657	Excess	NT1 containing a hydroxylamine induced, high copy number mutant of pAgA1; this study
K823	1	pAgA1; pAtK84b	Normal	pAtK84b transconjugant of A1; this study
K907	2	pAgA1; pAtK84b	Normal	K434 transformed with pAgA1; this study
K911	2	pAgK657; pAtK84b	Excess	K434 transformed with pAgK657; this study
K969	1	pAgK657; pAtK84b	Excess	pAtK84b transconjugant of K657; this study

[a] All strains contain a large cryptic plasmic, in addition to those listed.
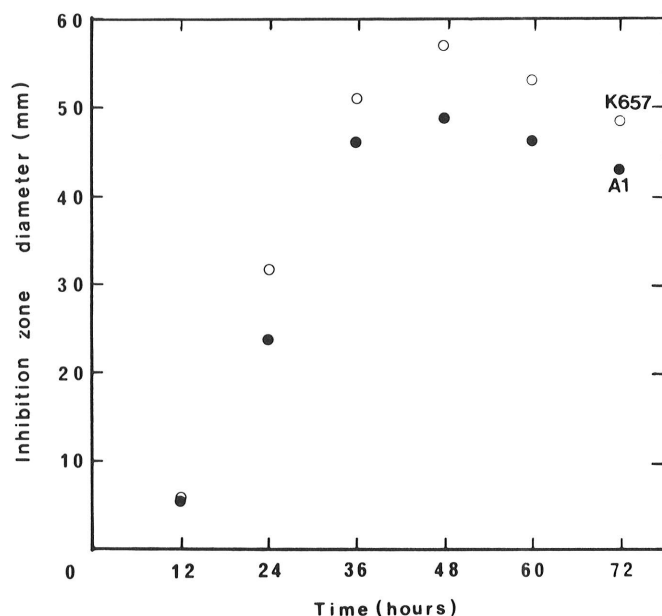

Fig. 1. Quantitative bioassay for agrocin 84 production. A comparison of strains A1 and K657. Each value is the mean of three reps of each culture.

Plasmid isolation. Plasmid DNA was extracted and partially purified by a modification of the alkaline miniprep procedure (15) described by Farrand et al (8).

Quantitative measurement of plasmid DNA. Bacteria were inoculated into 3 ml of L-broth containing 25 μg ml⁻¹ of kanamycin and incubated at 26 C overnight on a rotary shaker. The cultures were adjusted to $A_{680nm} = 0.4$ and 1 ml used for extraction of plasmid DNA. The partially purified DNA was redissolved in 100 μl of LTE and subjected to electrophoresis (20 μl per well) for 3.5 hr. Gels were stained and photographed and the negative films used to prepare microdensitograms (25). Relative amounts of plasmid DNA were estimated by measuring the areas under the peaks.

Gel electrophoresis. Gel electrophoresis was performed in a horizontal submerged gel apparatus (Bethesda Res. Lab.) using 0.7% Seakem ME agarose in TEB buffer, pH 8.0, at 100 mA constant current. DNA was stained with 0.5 μg ml⁻¹ ethidium bromide in TEB buffer.

Transformation. *Agrobacterium* strains were transformed by the freeze-thaw method as described by Holsters et al (10). After freezing and thawing, the mixtures were diluted fivefold in YEB, incubated at 28 C for 24 hr and 100-μl aliquots spread on NA containing 200 μg ml of kanamycin. (Biovar 2 strains are relatively resistant to kanamycin and high levels are necessary to select for transformants.) Transformants appeared after 3 to 4 days.

Transfer of pAtK84b by conjugation. The nopaline catabolic plasmid pAtK84b (16) was induced to transfer by growing donor strains on nopaline (6). Recipients that contained PAgK84::Tn5

were suspended in SDW at 10⁹ cells per milliliter and 100 μl spread over a 9-cm plate of Bergerson's medium containing 2 mg ml⁻¹ nopaline as sole source of carbon and nitrogen, and 50 μg ml⁻¹ of kanamycin. After 24 hr, the donor, which had been grown for 48 hr on the same medium lacking kanamycin, was suspended in BS and 5-μl drops spotted onto the recipient lawn. Transconjugants that combined the kanamycin resistance of the recipient and the nopaline catabolism of the donor appeared within the droplet zones after 3 days.

Colonization of roots. Uninoculated, unsterilized soil was used. Almond seedlings were inoculated with test strains (10⁷ cells ml⁻¹) and planted in soil in pots in the open. At regular intervals, plants were dug up, loosely adhering soil removed, and the taproot cut 3 cm below the point of seed attachment. A 2.5-cm length of taproot was cut off and placed in 10 ml of BS in McCartney bottles. Samples were vigorously shaken for 5 sec and placed at 4 C for 2 hr. After vortexing for 30 sec, the sample washings were diluted 10⁻¹, 10⁻² and 10⁻³. Three 5-μl aliquots of each dilution were placed on 1A and 2E selective media. After 4 days' incubation at 27 C, colonies were counted. The approximate surface area of root was calculated and results expressed as cells per unit area.

Almond seedling assay for biological control of crown gall. The method of Htay and Kerr (11) was used. Unsterilized soil (15 kg) was placed in pots in the open and inoculated with a pathogen to give about 10⁶ cells per gram of soil. Two days later, almond seedlings (cv. Challeston) were dipped in a suspension of the biocontrol strain (about 10⁷ cells per milliliter) and planted in the inoculated soil. After 6 mo, plants were removed and washed in running water. Number of galls per plant was counted and fresh weight of gall tissue measured.

RESULTS

Agrocin 84 overproducing mutant. After hydroxylamine treatment of pAgK84A1 DNA, the frequency of transformation of strain NT-1 to kanamycin resistance was reduced some 90-fold when compared with untreated DNA. Transformants (582) were recovered and tested for increased levels of agrocin 84 production. One transformant, K657, was identified that consistently produced a very wide zone of inhibition in the standard agrocin 84 bioassay.

Strains K657 and A1 were quantitatively assayed for agrocin 84 production in liquid medium. There was no significant difference in growth of the two strains, as measured by A_{680nm}. Samples were taken every 12 hr for 72 hr. Strain K657 produced significantly more agrocin 84 than did strain A1 (Fig. 1). Maximum production was at 48 hr for both strains. As inhibition zone diameter is directly related to log concentration of agrocin 84 (26), a twofold dilution series of the supernatant from a 48-hr culture of strain K657 was used to obtain the straight line relationship represented by

$$Y = 50.55 - 3.7x$$

where Y is diameter of zone of inhibition and x is the dilution expressed as 2^{-x}. Using this equation and data from an assessment of agrocin 84 production by the two strains at 48 hr, it was

calculated that strain K657 produced 4.9 times more agrocin 84 than did strain A1.

Plasmid DNA was extracted from both strains and analyzed by electrophoresis. A much stronger band of DNA with the same mobility as pAgK84::Tn5 was present in the extract from strain K657 than from strain A1. The procedure was repeated using seven replicates and the quantity of plasmid DNA estimated (Table 2). Samples from strain K657 contained 4.7 times more plasmid than did those from strain A1. When the plasmid extract of strain K657 was diluted fivefold, there was no significant difference between band intensities from extracts of A1 and K657 (Table 2). We conclude that strain K657 contains a copy number mutant of pAgK84::Tn5 A1.

Transformation of pAgA1 and pAgK657 into strain K434. Strains A1 and K657 would appear to have considerable potential as agents of biological control. Both contain Tra⁻ mutants of pAgK84 and therefore the danger of transfer of the agrocin plasmid to a pathogen is avoided. Both produce agrocin 84 but strain K657 produces about five times more than strain A1. However, as the ultimate aim is to find a replacement for strain K84, it was decided to transfer the agrocin 84 plasmids into the chromosomal background of strain K84.

The recipient strain was K434, a spontaneous mutant of K84 lacking pAgK84. Transformants were selected for resistance to Km and assayed for production of agrocin 84. Those containing pAgK657 produced wider zones of inhibition than did those containing pAgA1; the difference was similar to that shown by the parent strains A1 and K657 (data not shown). The presence of pAgK657 and pAgA1 in these strains was confirmed by gel electrophoresis (data not shown). Two strains were selected for further study, strain K907 containing pAgA1 and strain K911 containing pAgK657.

Conjugative transfer of pAtK84b to strains A1 and K657. As strains K84, K907, and K911 all contain the nopaline catabolic plasmid pAtK84b (16) as well as an agrocin 84 plasmid, it was decided to introduce pAtK84b to strains A1 and K657 before testing them as biocontrol agents. Otherwise, it could be argued that any difference between strains might be due to the presence or absence of pAtK84b.

Both recipients were mated with strain K434 and putative transconjugants appeared after 4 days. After purification, they were subjected to various tests; all belonged to biovar 1 (13) and combined agrocin 84 production of the recipient with nopaline catabolism of the donor. Their plasmid complement was determined by agarose gel electrophoresis and confirmed that they were indeed transconjugants (data not shown). The transconjugant strains K823 and K969 were tested as biocontrol agents.

Colonization of roots. An effective biocontrol strain must be ecologically competent and able to colonize the roots of susceptible plants. Colonization of almond roots by the newly constructed strains K823, K907, K911, or K969 was compared with that by strain K84.

Young almond seedlings were inoculated with the potential biocontrol strains before planting in soil. Number of bacterial cells on roots was determined after 39, 89, 112, and 140 days. There were 10 replicates of each treatment at every sampling time and a randomized block design was used. Data were statistically analyzed (two-way analysis of variance) after a cube root transformation which gave a normal distribution of data and homogeneous variances. Results show that there was a significant difference between treatments at all sampling times (Fig. 2). At

sampling times 2, 3, and 4, strains K84, K907, and K911 were significantly more numerous than strains K823 and K969 ($P <$ 0.01).

Biological control. The newly constructed strains K823, K969, K907, and K911 were tested as agents of biological control and compared with the commercial strain K84.

Soil was inoculated with the pathogenic strain K27 and young almond seedlings inoculated with the biocontrol strains before planting in soil. Untreated seedlings were used as controls. There were 10 replicates of each treatment in randomized blocks and results were assessed after 6 mo (Table 3). For statistical analysis (two-way analysis of variance), data were transformed by $\ln(x + 1)$.

Strain K84 and its derivatives gave significantly more efficient control than did the strains with the C58 chromosomal background. Strain K969, which overproduces agrocin 84 in culture, did not significantly control crown gall whereas the same strain (K823), which produces the normal amount of agrocin 84, did. There was no significant difference in efficiency of control between strain K84 and its derivatives, including K907, which overproduces agrocin 84.

DISCUSSION

We believe that the main threat to the continued success of the biological control of crown gall is the ability of the agrocin 84 plasmid, pAgK84, to transfer from the controlling agent, strain K84, to pathogenic strains, rendering them resistant to agrocin 84 and not subject to biological control. The main aim of this project was to determine if transfer deficient (Tra⁻¹) strains are efficient biocontrol agents and to determine if the chromosomal background for the plasmid is important.

Strain K907, a K84 derivative harboring a Tra⁻ Tn5 insertion of pAgK84 efficiently controlled crown gall, whereas strain K823, a C58 derivative harboring the same plasmid was much less efficient.

TABLE 3. Biological control of strain K27 on almond seedlings

Treatment[a]	Biovar	Mean no. galls/plant		Mean fresh wt. (g) galls/plant	
		Actual (x)	ln(x + 1)	Actual (x)	ln(x + 1)
None	⋯	11.6	2.26	7.36	1.98
K969	1	10.6	2.28	4.40	1.59
K823	1	3.8	1.32	3.89	1.02
K911	2	0.9	0.43	1.15	0.40
K907	2	0.0	0.0	0.0	0.0
K84	2	0.0	0.0	0.0	0.0
Least significant difference					
(P = 0.05)			0.75		0.55
(P = 0.01)			1.01		0.74

[a] Treatment consisted of dipping almond seedlings in a bacterial suspension of the strains indicated before planting in unsterilized soil inoculated with the pathogenic strain K27.
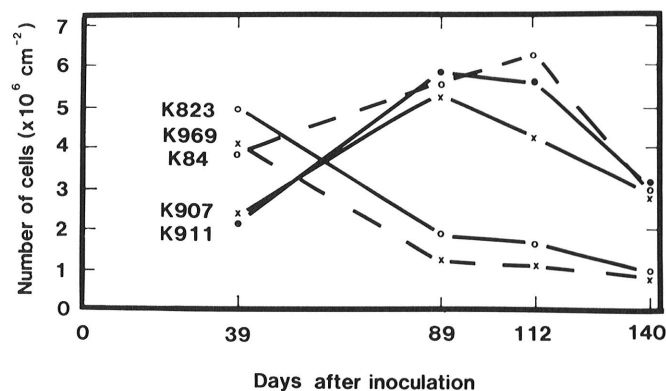
TABLE 2. The relative amounts of agrocin 84 plasmid DNA in strains A1 and K657

Ratio A1:K657	Strain	
	A1	K657
1:1	1.61 ± 0.50[a]	7.60 ± 0.69
1:0.2	1.29 ± 0.43	1.23 ± 0.57

[a] Means of seven replicates ± 95% confidence limits.

Fig. 2. Colonization of almond roots by various potential biocontrol strains. K823 and K969 are strains with C58 background; K907 and K911 are strains with a K84 background.

The latter strain appears to be ecologically inefficient. Thirty-nine days after being inoculated onto the roots of young almond seedlings, there were about 5×10^6 cells per square centimeter of root but numbers fell with time, until, after 140 days there were only about 10^6 cells per square centimeter of root (Fig. 2). Presumably, it is this relatively poor root colonization that explains the inefficient control of crown gall by this strain (Table 3) although it should be noted that the maximum difference in numbers between the two strains was less than threefold. If numbers are important, it would imply that most infection occurs after 39 days, but we have no direct evidence to support this. Strain K823 belongs to biovar 1, but it must not be assumed that all biovar 1 strains will give the same result. Panagopoulos et al (20) have reported that an agrocin 84-producing biovar 1 strain efficiently controls crown gall. Presumably their strain is ecologically competent. Our results reemphasize previous findings (5) that production of agrocin 84 by a strain does not automatically make it an efficient biocontrol agent. One surprising result was obtained. If biological control operates through agrocin 84 production, one might expect a strain that produces more agrocin 84 to give better control. Our data do not confirm this expectation. In culture, strains carrying a high copy number mutant of pAgK84::Tn5 produce about five times more agrocin 84 than do strains with a normal plasmid number. Yet they are no more efficient as biocontrol agents and indeed are significantly less efficient when in the chromosomal background of C58. The reason for this is not known.

Should Tra⁻ strains replace the commercial strain K84? Eventually, yes, but our present constructs contain the transposon Tn5 and have two main disadvantages. First, they can revert to Tra⁺ through loss of the transposon. Secondly, they carry resistance to the antibiotics kanamycin, neomycin, and streptomycin (21). Although these antibiotics are not extensively used in medicine, it is probably unwise to encourage widespread release of such strains in horticulture because of the risk of transferring antibiotic resistance to human pathogenic bacteria. Tra⁻ deletion mutants would have neither of these disadvantages and we are constructing such strains.

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