AN ABSTRACT OF THE THESIS OF

Donald Arthur Cooksey for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on August 31, 1981

Title: Biological Control of Crown Gall with Bacterial and Fungal Antagonists

Abstract approved: Redacted for privacy
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Thirty five different fungi and bacteria isolated from nursery soils in Oregon and Washington inhibited the growth of pathogenic Agrobacterium strains in vitro. Seven of the 35 antagonists also prevented infection of tomato seedlings by biotype 1 and 2 A. tumefaciens strains in greenhouse tests. In field trials, isolates of Penicillium, Aspergillus, Bacillus, Pseudomonas, and A. radiobacter reduced the incidence of crown gall on mazzard cherry seedlings by some of the pathogens tested. With some antagonists, a greater reduction in crown gall was observed when cherry seedlings were inoculated with antagonists. One Penicillium antagonist reduced the incidence of infection to a level below that of the wounded controls when used against some pathogenic strains.

To study the mechanisms of biological control by the antagonist A. radiobacter K84, an agrocin$^-$ mutant of K84 was obtained by mitomycin C curing of the 30 megadalton bacteriocinogenic plasmid in K84. This mutant (designated K84Agr$^-$) no longer prevented crown gall induction on tomato stems when coinoculated with the agrocin-sensitive pathogen A. tumefaciens K24. However, it was effective in reducing

infection when inoculated 24 hr before the pathogen. In addition, either K84 or K84Agr$^-$ reduced infection by agrocin-resistant A. tumefaciens B6 when the antagonists were applied 24 hr before the pathogen. Tumor weights were reduced when K84Agr$^-$ was coinoculated with K24 or B6, or when it was applied 24 hr before the pathogens. The data suggest that other mechanisms, such as a physical blockage of infection sites, are involved in biological control by K84 in addition to the production of agrocin 84.

The rate of mutation of Agrobacterium tumefaciens to agrocin 84 resistance in vitro was 4.2×10^{-4}, 2.6×10^{-4}, and 2.0×10^{-4} for strains C58, K24, and B234, respectively, and 2.5×10^{-3} for A. rhizogenes A4. The mutations were shown to be spontaneous for C58 by a replica-plating technique. The percentages of resistant mutants that were avirulent were 68%, 80%, 28%, and 100% for strains C58, K24, B234, and A4, respectively. Most of the avirulent mutants had lost the tumor-inducing (Ti) plasmid carrying genes for agrocin sensitivity, but a few had large deletions from the Ti-plasmid. When selecting resistant mutants from plates containing agrocin 84, the largest colonies retained the Ti-plasmid and the smallest colonies had lost the plasmid. The high rate of mutation to resistance accompanied by plasmid loss suggests that the Ti-plasmid is not very stable in these strains. The number of virulent resistant mutants was also significant and emphasizes the need for alternatives to A. radiobacter K84 for biological control.

Growth of Rhizobium leguminosarum 128C56 was inhibited by agrocin 84. Agrocin-resistant mutants of R. leguminosarum developed quickly in the presence of agrocin. Of 18 agrocin-resistant mutants examined,

all but one had lost a 93 megadalton plasmid. RP4-mediated transfer of this plasmid to avirulent A. tumefaciens strain NT1 produced a transconjugant that was sensitive to agrocin 84. It was concluded that genes controlling agrocin sensitivity in R. leguminosarum 128C56 are located on the 93 megadalton plasmid.

Biological Control of Crown Gall with Bacterial and Fungal Antagonists

by

Donald Arthur Cooksey

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Completed August 31, 1981

Commencement June 1982

APPROVED:

Redacted for privacy

Associate Professor of Botany and Plant Pathology
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Date thesis is presented August 31, 1981

Typed by Donald Arthur Cooksey

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INTRODUCTION

Crown gall disease, caused by Agrobacterium tumefaciens, can occur on plants from 93 families (8). The major economic loss due to crown gall occurs on nursery crops where galled plants are destroyed by growers because they are unsalable. Present control measures are therefore directed toward the prevention of crown gall at the nursery level. In addition to proper sanitation and cultural practices, the use of the biological control agent, A. radiobacter K84, has now become a widely used preventive measure for the disease (30).

Biological control of crown gall was first reported by New and Kerr (33). It had been observed that a high ratio of virulent to avirulent Agrobacterium strains existed in soil near infected plants while soil around healthy plants contained a high proportion of avirulent strains. Several avirulent strains were used to artificially increase the proportion of avirulent bacteria on wounded tomato stems in an attempt to prevent infection. One strain, A. radiobacter K84, was very effective in reducing infection. It was later shown also to control crown gall of peach when inoculated to the seed before planting to field soil (17). Subsequent tests in many parts of the world have confirmed the effectiveness of K84 as a biological control agent, although instances of ineffective control with K84 do exist (1, 30). The instances of ineffective control have encouraged workers to look for other antagonists for biological control, but none has

been as effective as K84 (5, 13, 20, 27).

Control of crown gall by K84 involves the production of a bacteriocin (agrocin 84) that inhibits the growth of certain A. tumefaciens strains. Sensitivity to agrocin 84 appears to involve active transport of the agrocin molecule into sensitive strains by means of a periplasmic binding protein (32) coded for by genes on the tumor-inducing (Ti) plasmid (12). Agrocin 84 has been reported to inhibit nucleic acid and protein synthesis (7, 24). It has also been reported to disrupt the cell envelope of A. tumefaciens, thus reducing its ability to bind to plant cells (37), an essential event in the infection process (22).

There is a correlation between sensitivity of a pathogen to agrocin 84 in vitro and biological control on plants; pathogenic strains sensitive to agrocin 84 are generally prevented by K84 from infecting tomato seedlings, while insensitive strains generally are not controlled (19). However, biological control in the field is not always correlated with the production of agrocin 84, since some agrocin-resistant pathogenic strains have been controlled by K84 on field-grown Prunus seedlings (5, 27, 35). This suggests that additional mechanisms other than bacteriocin production are involved in biological control by K84.

This study was initiated to investigate interactions of Agrobacterium with other soil bacteria and fungi that might lead to alternative methods for biological control. Studies were also undertaken to further our understanding of the mechanisms of biological control by A. radiobacter K84 and the development of resistance to

agrocin 84. This information may help us in selecting new antagonists for biological control. Finally, as part of the study on interactions of Agrobacterium with other soil microbes, the genetic basis for sensitivity of Rhizobium leguminosarum to agrocin 84 was investigated, since several Rhizobium strains are sensitive to agrocin 84 (Anderson and Moore, unpublished data). This interaction is of concern because fields planted with trees treated with K84 are often followed by legume cover-crops.

I. BIOLOGICAL CONTROL OF CROWN GALL WITH FUNGAL AND BACTERIAL ANTAGONISTS

INTRODUCTION

Successful biological control of crown gall using strain K84 of Agrobacterium radiobacter has been reported in several countries, and has been reviewed recently (18, 28, 30). However, some strains of A. tumefaciens were insensitive to the bacteriocin (agrocin 84) produced by strain K84 in vitro (19, 20, 28, 35), and in some instances K84 did not prevent tumor production by these pathogens on susceptible hosts (20, 28). The success of K84 has encouraged workers to look for new antagonists for the agrocin 84-resistant pathogens, but other A. radiobacter strains that inhibit pathogenic Agrobacterium species in vitro have been ineffective as control agents on plants (13, 20, 27).

Soil fungi are a potential source of antagonists that have been largely ignored in crown gall research. Deep and Young (9) showed that preplanting fungicide treatments increased the incidence of crown gall of cherry seedlings, suggesting the presence of natural fungal competitors. They later used a suspension of unidentified fungi as a preplanting treatment and reduced galling by about 25%. The purpose of the present study was to isolate fungal and bacterial antagonists for biological control of crown gall, particularly for control of A. tumefaciens strains not subject to control by K84.

MATERIALS AND METHODS

Isolation of Antagonists

Soil samples from nursery fields in Oregon and Washington were collected as composite samples consisting of six 15 cm core samples (2 cm in diameter) from each field. The soil samples were mixed thoroughly and screened for microorganisms antagonistic toward pathogenic Agrobacterium strains by a plate-spraying technique similar to that of Stessel et al. (38). Soil dilutions were plated on Difco potato-dextrose agar (PDA), allowed to incubate at room temperature for 1-3 days, and were then sprayed with standardized cell suspensions of either strain B6 of A. tumefaciens (a biotype 1 strain insensitive to agrocin 84) or strain B234 of A. tumefaciens (biotype 2, sensitive to agrocin 84). Inoculum for these two test strains was prepared from late log-phase cultures grown in a yeast-dextrose-peptone broth (27) on a rotary shaker. Test strain cultures were diluted to about 1×10^8 cells/ml with sterile distilled water, and sprayed onto plates until evenly wetted, but not to runoff. Microbial colonies exhibiting zones of inhibition against the test strains were picked off the agar and obtained in pure culture.

Antibiotic Production In Vitro

Each antagonist selected in the initial screening was retested in vitro against strains B6 and B234, and was tested against an additional biotype 1 pathogen that was agrocin 84-resistant (EU-8) and three additional biotype 2 pathogens that were agrocin 84-sensitive (Q51,

K27, K29). A modification of the method of Pridham et al. (34) was used for antagonists other than the A. radiobacter strains. Seven mm plugs cut from agar plate cultures of the antagonists were placed on PDA plates that had just been sprayed with the test strain, thus avoiding spread of non-chloroformed antagonists over the agar surface. This method provided clear zones of inhibition against the pathogens by all antagonists except agrobacteria. A method similar to Stonier's (39) was used for A. radiobacter strains which were spotted at the center of mannitol-glutamate (MG) agar (16) plates, and then grown for 3 days at room temperature before being sprayed with the test strains. The antagonists were not killed with chloroform after the initial incubation period as described by Stonier, since little or no spread of the colonies resulted from spraying the test strains. Zones of inhibition appeared after 1 to 2 days in lawns of sensitive strains of A. tumefaciens. Antagonists that inhibited all six pathogenic test strains, or strains that were resistant to strain K84, were screened for control of galling of tomato seedlings in the greenhouse.

Greenhouse Experiments

Assays for the prevention of galling were made by wounding 4-week old tomato seedlings (Lycopersicon esculentum cv. Bonny Best) in the first internode with dissecting needle, forming a slit 2-3 mm long and 1-1.5 mm deep in the stem, and placing 0.01 ml of inoculum on the wound. Inoculum was prepared by washing cells from PDA or MG agar slants with sterile distilled water, and diluting to the desired concentration. Inocula of the pathogen and antagonist were mixed together

in a 1:10 ratio and applied to the wounds. Seedlings were then grown for 4 weeks before making final readings. On the basis of these tomato assays, seven antagonists were selected for field trials, including species of Penicillium, Aspergillus, Bacillus, Pseudomonas, and Agrobacterium.

Further tomato assays were conducted with the seven antagonists against mixtures of pathogenic strains which were also used in field trials. Mixtures of pathogenic strains were used because natural populations of A. tumefaciens can contain more than a single strain. Evidence for a heterogeneous pathogen population comes from the observation that agrobacteria of different biotypes, and with different host specificities, can be isolated from a single tumor (2). In the present study, two groups of pathogens were used. Group I consited of four pathogen strains (A49, A432, A20, A329) that were insensitive to strain K84 in vitro. Group II consisted of two agrocin-resistant strains (B6, EU-8) and two agrocin-sensitive strains (B234, U-3). Pathogen-antagonist mixtures were inoculated directly to stem wounds, as described for the tomato assays, or the pathogens were applied 24 hr after the antagonists.

Field Tests for Biological Control

Before planting, mazzard cherry seedlings were root-pruned and dipped in inoculum suspensions of individual antagonists, then immediately dipped in suspensions of the pathogens. This sequence of inoculation gave effective control of crown gall with A. radiobacter K84 in previous work (27). Alternatively, seedlings were inoculated with

antagonists, held for 24 hr in a cool, moist condition, and inoculated with pathogens just before planting. The 24 hr delay was used because root-pruned, inoculated plants are sometimes held for extended periods before planting if excessive rain occurs, and because delay between inoculation with A. radiobacter K84 and pathogens enhances control (27). Mixtures of A. tumefaciens were prepared in nonchlorinated tap water in the field from strains grown separately on MG medium. Viability counts of the inocula were made within 24 hr of use by plating serial dilutions on PDA.

RESULTS

Antibiotic Production In Vitro

In the initial screening from soil, 35 different fungi and bacteria inhibited the test strains B6 or B234 of A. tumefaciens. Twelve of these antagonists inhibited B6, B234, and an additional four pathogenic test strains (Q51, K27, K29, EU-8). Four antagonists were effective against only the biotype 1 pathogens, B6, and EU-8 (Table 1). The remaining 19 antagonists were effective only against one or two biotype 2 strains, and were not included in greenhouse tests.

Greenhouse Experiments

Six of the 16 antagonists tested on tomato seedlings reduced or prevented galling by the biotype 2 strain B234 below that of the control (Table 2). Five of these antagonists also reduced galling by strain EU-8, a biotype 1 strain not controlled by A. radiobacter K84.

For some antagonists, there was a low correlation between inhibition of agrobacteria in vitro (Table 1) and biological control of crown gall in the initial tomato assay (Table 2). One Pseudomonas antagonist (78-18) prevented galling by strains B234 and EU-8, but neither of these strains was inhibited by the Pseudomonas strain in vitro. More commonly, antagonists, such as A. radiobacter W1, failed to prevent galling by pathogenic strains that were sensitive in vitro.

Seven of the 16 antagonists were also tested against two mixtures of pathogenic strains to be used in field trials (Table 3). Both groups of pathogens contained agrocin 84-insensitive strains, and as expected strain K84 failed to prevent galling when coinoculated with these pathogens. However, strain K84 gave complete protection against all pathogens when inoculated 24 hr before the pathogens (Table 3). Of the new antagonists tested in this study, only the Penicillium and the Aspergillus strains gave complete control of galling on tomato by both pathogen groups. Complete control by the Penicillium isolate occurred only when inoculation with the pathogen was delayed 24 hr; in contrast, Aspergillus controlled crown gall only when mixed directly with the pathogens (Table 3).

Field Tests for Biological Control

Most of the new antagonists were effective in reducing galling by both groups of pathogens when the pathogens were applied 24 hr after the antagonists (Table 4). Biological control was enhanced by the 24 hr delay for both fungal antagonists and for some of the bacterial antagonists. In addition, the Penicillium antagonist reduced

galling by group II pathogens to a level below that of the wounded uninoculated controls, whether or not pathogen inoculation was delayed 24 hr. However, none of the antagonists isolated in this study were as effective as strain K84 for overall reduction of galling by both pathogen groups. There was no observable phytotoxicity from any of the antagonists; treated plants were as healthy as untreated controls.

DISCUSSION

Biological control of crown gall was achieved using fungal and bacterial antagonists other than Agrobacterium radiobacter strain K84. Strain K84 was previously the only antagonist reported to give successful control of crown gall, although many Agrobacterium strains have been tested (13, 20, 27). The level of control achieved with some of our new antagonists was comparable to that reported initially by Kerr (17) using strain K84. In his first report, Kerr found that the incidence of galling was reduced from 79% to 31% when peach seeds were treated with strain K84. Results from the present study showed that the incidence of crown gall of cherry seedlings could by reduced from 61.9% to 4.1% using a Penicillium antagonist against group II pathogens. The same antagonist reduced galling by group I pathogens from 81.4% to 32.8%. In addition, the mean level of naturally occurring infection (wounded uninoculated control) was reduced from 9.1% to 0.0% by both the Penicillium antagonist and Bacillus 77-135. The reduction in naturally occurring infection is one of the most important considerations, because the number of cells in A. tumefaciens culture inoculum far exceeds the natural level. Some biological con-

trol was achieved by the remaining antagonists, but not always with both groups of pathogens.

Biological control was enhanced when some of the antagonists were inoculated into wounded plant tissues 24 hr before the pathogens. The 24 hr delay may have helped the antagonists become established at the wound site, resulting in greater antibiotic production or better physical competition against the pathogen for infection sites. It is also possible that the antagonists stimulated production of substances by the host that inhibited the pathogens. In field tests, the 24 hr delay may have simply allowed more propagules of the antagonists to adsorb onto the cherry seedling roots, so they were not washed off in the subsequent pathogen dip. If true, the method of coinoculating antagonists and pathogens in the field may have favored the pathogens. Wound healing following a 24 hr delay between application of the antagonists and pathogens probably did not enhance control, because Moore (26) showed that 85% of mazzard cherry seedlings remain susceptible to infection for 3 days after wounding; Braun (3) also showed that Kalanchoe tissues were more susceptible to infection 24 hr after wounding. There were also instances in greenhouse and field tests where the 24 hr delay adversely affected control by some antagonists. Inoculum survival and colonization may have been poor during the 24 hr period for the Aspergillus antagonist on the aerial tomato stem, and for the bacterial strain 78-11 on cherry seedlings.

There was a low correlation between in vitro activity and biological control with some of the antagonists. A. radiobacter W1

inhibited A. tumefaciens EU-8 in vitro, but was ineffective in biological control on tomato. Kerr and Panagopolous (20) have also isolated several bacteriocin-producing A. radiobacter strains that failed to control sensitive pathogenic strains on tomato. The failure to control sensitive pathogenic strains may result from (i) a lack of bacteriocin production by the antagonists in vivo, (ii) mechanisms involved in biological control other than bacteriocin production, or (iii) poor growth of the antagonists at the wound site (10). On some hosts, A. radiobacter K84 has been shown to control pathogenic strains that were insensitive to agrocin 84 in vitro (27, 35). This may suggest that other mechanisms, such as competition for infection sites (22), are utilized by strain K84 to prevent infection of some host species. In our work, K84 also prevented infection by insensitive strains on cherry seedlings, and on tomato when inoculated 24 hr before the pathogens. However, we have recently found that these "insensitive" pathogens are inhibited by strain K84 in vitro when grown on PDA, or on a minimal medium (mannitol-glutamate) to which glucose was added (D. Cooksey and L. Moore, unpublished data). Since sensitivity of the "insensitive" pathogens to strain K84 can be demonstrated in vitro using a specific growth medium, bacteriocin production is still a probable mechanism by which strain K84 controls these pathogens. The effect of different media on sensitivity of pathogens to strain K84 may also explain why the group I pathogens were controlled on cherry and pear, but not on apple seedlings (28) or tomato seedlings. The sensitivity of A. tumefaciens to agrocin 84 may be influenced by root exudates or other factors that differ between hosts.

The results of this investigation show that fungi and bacteria other than A. radiobacter K84 can be used as biological control agents for crown gall. The data also support the conclusion of Deep and Young (9) that fungal competitors play an important role in the natural incidence of crown gall. The Penicillium antagonist provided protection from naturally occurring A. tumefaciens strains throughout the growing season, and the initial inoculum level was comparable to that found naturally in soil from one Washington nursery. The possibility of recolonizing fumigated nursery soils with selected antagonists is also being investigated.

Table 1. In vitro sensitivity of Agrobacterium tumefaciens to bacterial and fungal antagonists

Antagonist	Diameter of inhibition zone (cm) against various A. tumefaciens strains[a]					
	B6	EU-8	B234	Q51	K27	K29
Aspergillus 77-148	3.5	2.2	2.0	0.0	0.0	1.9
Aspergillus 77-149	1.5	1.1	0.0	0.0	0.0	0.0
Penicillium 77-15	1.5	1.1	0.0	0.0	0.0	0.0
Penicillium 77-104	3.1	2.6	5.5	3.7	4.6	4.0
Penicillium 77-166	1.0	3.0	0.0	0.0	0.0	0.0
Penicillium 77-170	1.2	3.0	0.0	0.0	0.0	0.0
Penicillium 77-174	1.6	1.2	0.0	0.0	0.0	0.0
Trichoderma 77-119	3.0	1.2	2.7	1.5	1.7	2.5
Trichoderma 77-171	3.6	1.6	2.0	1.5	1.7	1.7
Bacterial sp.78-10	1.3	1.2	1.7	1.1	1.5	1.1
Bacterial sp.78-11	2.6	1.6	2.8	0.0	0.0	0.0
Bacillus 77-102	4.2	5.0	2.0	5.0	2.0	4.0
Bacillus 77-135	1.7	1.8	2.6	2.0	4.4	1.2
Bacillus 77-144	4.1	2.8	2.2	2.3	2.0	2.1
Pseudomonas 78-18	3.5	0.0	0.0	0.0	0.0	0.0
A. radiobacter W1	2.5	2.8	0.0	0.0	0.0	0.0
A. radiobacter K84	0.0	0.0	5.0	4.8	5.0	5.0

[a]Strains W1 and K84 were spotted at the center of mannitol-glutamate agar plates and grown for 3 days before being sprayed with suspensions of the test strains. All other antagonists were placed on the PDA plates in 7-mm diameter plugs from agar plate cultures immediately after the PDA plates were sprayed with the test strains.

Table 2. Effect of fungal and bacterial antagonists on formation of tumors on tomato seedlings by biotype 1 and biotype 2 strains of *Agrobacterium tumefaciens*

Antagonists	Viable propagules per ml of inoculum	Galled seedlings (%)[a]	
		EU-8	B234
Bacterial sp. 78-11	4.0×10^7	100	0
Pseudomonas 78-18	1.6×10^8	40	40
Bacillus 77-102	3.0×10^8	67	0
Bacillus 77-135	1.2×10^9	27	0
A. radiobacter W1	1.7×10^9	100	100
A. radiobacter K84	1.8×10^9	100	0
Penicillium 77-104	9.4×10^6	13	0
Aspergillus 77-148	4.9×10^6	0	13
Inoculated control		100	100

[a]Mean of five replications of three tomato seedlings per pot. The concentration of strain EU-8 was 1.5×10^7 viable cells/ml, and B234 was 7.0×10^6 viable cells/ml. Control seedlings wounded and inoculated with antagonists alone or with distilled water were not galled.

Table 3. Effect of bacterial and fungal antagonists on galling of tomato seedlings by two mixtures of *Agrobacterium* pathogens

Antagonists	Viable propagules per ml of inoculum	% Galling[a]			
		Group I pathogens		Group II pathogens	
		Coinoculated	24 hours[b]	Coinoculated	24 hours
Bacterial sp. 78-11	3.6×10^7	100	100	100	100
Pseudomonas 78-18	7.2×10^7	100	56	100	71
Bacillus 77-102	6.3×10^8	100	100	67	100
Bacillus 77-135	3.6×10^8	11	89	89	100
A. radiobacter W1	2.6×10^8	100	56	100	78
A. radiobacter K84	1.8×10^8	100	0	100	0
Penicillium 77-104	9.0×10^6	11	0	56	0
Aspergillus 77-148	8.5×10^7	0	100	0	100
Inoculated control		100	100	100	100

[a]Mean of three replications of three tomato seedlings per pot. Group I pathogens included strains A49, A432, A20, and A329 of *A. tumefaciens* at 2.9×10^7, 4.4×10^7, 1.1×10^8, and 3.3×10^7 viable cells/ml of inoculum. Group II included strains U-3, B6, B234, and EU-8 at 5.0×10^7, 1.9×10^6, 2.5×10^8, and 1.0×10^8 viable cells/ml of inoculum. Control seedlings wounded and inoculated with antagonists alone or with distilled water were not galled.

[b]Inoculations with the pathogens were 24 hr after inoculations with the antagonists.

Table 4. Effectiveness of bacterial and fungal antagonists on crown gall of mazzard cherry seedlings in the field

Treatment	Viable propagules per ml of inoculum	% Galling[a]				
		Antagonist alone	Group I pathogens		Group II pathogens	
			Immediate[b]	24 hours[c]	Immediate	24 hours
Bacterial sp. 78-11	3.0×10^6	7.6x	63.2	71.9	24.6	30.8
Pseudomonas 78-18	3.5×10^7	3.7x	69.0x	23.7	44.2x	10.2
Bacillus 77-102	1.3×10^6	2.7	65.9x	38.1	34.0	19.4
Bacillus 77-135	1.2×10^6	0.0	57.1	28.3	36.7	70.6y
A. radiobacter W1	3.3×10^6	12.0x	55.5	53.0	35.6	11.2
A. radiobacter K84	9.0×10^5	1.1	7.6	14.9	4.4	0.0
Penicillium 77-104	5.3×10^6	0.0	62.5	32.8	4.1	8.7
Aspergillus 77-148	4.0×10^6	4.1x	80.8x	52.2	17.6	21.1
Inoculated control			81.4x	...d	61.9x,y	...d
Wounded control		9.1x				

[a]Mean of 100 cherry seedlings per treatment. Group I pathogens included strains A49, A432, A20, and A329 of *A. tumefaciens*. Group II pathogens included strains U-3, B6, B234, and EU-8. The concentration of the group I inoculum mixture was 5×10^4 viable cells/ml, and the group II mixture was 4.7×10^5 viable cells/ml. Means in a column followed by the same letter do not differ significantly (p=0.01), using the Z test.

[b]Seedlings were dipped in suspensions of individual antagonists 10 min before dipping in pathogen mixtures.

[c]Seedlings were dipped in suspensions of antagonists 24 hr before pathogen mixtures.

[d]Not tested (see discussion).

II. BIOLOGICAL CONTROL OF CROWN GALL WITH AN AGROCIN$^-$ MUTANT OF *AGROBACTERIUM RADIOBACTER* K84

INTRODUCTION

Biological control of crown gall with *Agrobacterium radiobacter* K84 involves the production of a bacteriocin (agrocin 84) that inhibits growth of certain strains of *A. tumefaciens* in vitro (11, 19). There is a good correlation between agrocin 84 sensitivity in *A. tumefaciens* and biological control by K84 when the pathogen and antagonist are coinoculated to wounded tomato stems (19). However, when K84 is applied to wounds in tomato stems 24 hr before the pathogen, infection by agrocin-resistant strains can be prevented (5). In addition, agrocin-resistant strains have been controlled by K84 in field tests with *Prunus* seedlings (5, 27, 35). These data suggest that in addition to the production of agrocin 84, other mechanisms are involved in biological control by K84. A better understanding of these mechanisms may help us in selecting new antagonists for situations where K84 is not effective (1, 30).

As a prerequisite to the study of other mechanisms of biological control, a mutant of K84 that no longer produced agrocin 84 was needed. Since the genes coding for agrocin production reside on a 30 megadalton plasmid in K84 (11), curing of this plasmid should result in an agrocin$^-$ mutant. This paper describes the selection of an agrocin$^-$ mutant of K84 using mitomycin C and tests for biological control with this mutant.

MATERIALS AND METHODS

Mitomycin C Treatment

Agrobacterium radiobacter K84 cells grown on a Difco potato-dextrose agar slant were suspended in sterile distilled water, washed once, and resuspended to about 10^6 cells/ml in 100 ml volumes of mannitol-glutamate (MG) broth (16). Mitomycin C was then added to 0.1, 1.0, or 2.0 µg/ml. The cultures were incubated at 28 C on a rotary shaker for 18 hr and then diluted and spread on MG agar plates. Individual colonies appearing on MG agar were spotted with sterile toothpicks to fresh MG plates (12 colonies/plate), incubated at 28 C for 24 hr, and sprayed with a suspension (about 10^6 cells/ml) of A. tumefaciens K24. Colonies were observed for production of agrocin 84 against K24 after 48 hr incubation at 28 C.

Examination of Plasmid DNA

The protease cell lysis and plasmid isolation procedure of Currier and Nester (6) was used except that the DNA shearing step was omitted. Cells were harvested after growth in MG broth (supplemented with 0.025% yeast extract) to a density of about 10^7-10^8 cells/ml. All steps were performed in 50 ml centrifuge tubes using gentle inversion to mix reactants.

A horizontal slab gel apparatus with gel dimensions of 13 cm X 42 cm X 4 mm was used for electrophoresis of plasmid DNA. Seakem (ME) agarose at 0.7% was dissolved in tris-borate buffer (25). Ten µl of DNA was mixed with 5 µl of tracking dye (20% Ficoll, 0.2% SDS, 0.025%

bromophenol blue) before loading to 30 µl wells. Electrophoresis was for 5 hr at 250 V. Gels were stained for 60 min in a 0.5 µg/ml ethidium bromide solution in tris-borate buffer and photographed on a short-wave Ultra-violet Products transilluminator with Polaroid type 47 film.

Greenhouse Tests for Biological Control

Tomato seedlings were wounded and inoculated as described previously (5). Ten seedlings were used per treatment with five seedlings per pot. Pathogens and antagonists were either coinoculated in about a 1:10 ratio immediately following wounding, or the pathogen was applied 24 hr after the stem was wounded and inoculated with the antagonist. Controls consisted of noninoculated, wounded stems and pathogen inoculations immediately after wounding or 24 hr after wounding. Wounds were examined for the presence or absence of tumors after 4 wk. Fresh weights of tumor tissues were then determined by cutting 2.5 cm stem segments, including the wounded area, and weighing them individually. The average weight of ten noninoculated, wounded stems was subtracted from the values for inoculated stems.

RESULTS

Mitomycin C Plasmid Curing

Mutants that no longer inhibited the growth of A. tumefaciens K24 were recovered from the 2.0 µg/ml mitomycin C treatment at a frequency of about 10^{-2} mutants per viable cell. At this concentration

of mitomycin C, survival was about 0.1% after 18 hr of incubation. One of the mutants was purified and retested for agrocin production (Fig. 1) and designated K84Agr$^-$. Examination of plasmid DNA from K84Agr$^-$ showed that it had lost the 30 megadalton bacteriocinogenic plasmid (Fig. 2).

Biological Control on Tomato Seedlings

When coinoculated with agrocin 84-sensitive A. tumefaciens K24 to wounded tomato stems, the wild-type K84 completely prevented infection, but K84Agr$^-$ allowed 90% infection (Table 5). However, when K84Agr$^-$ was inoculated 24 hr before K24, infection was reduced to 30%. In addition, infection by agrocin-resistant A. tumefaciens B6 was reduced to 40% by K84 and 50% by K84Agr$^-$, when either antagonist was inoculated 24 hr before B6.

Even when infection occurred, tumor weight was reduced by K84 and K84Agr$^-$ in all treatments (Table 6). When the two antagonists were inoculated 24 hr before B6, the resulting tumors were so small that they were not detected by tumor weight measurements.

DISCUSSION

When K84 was cured of the 30 megadalton plasmid coding for agrocin 84 production, it no longer prevented infection if coinoculated with an agrocin-sensitive pathogenic strain. However, when K84Agr$^-$ was inoculated 24 hr before the pathogen, infection was reduced. Tumor weight was also reduced when K84Agr$^-$ was either coinoculated with the pathogen or inoculated 24 hr before the pathogen. These results show

that some biological control activity by K84 is independent of agrocin 84 production.

Another probable mechanism for biological control by K84 is a physical blockage of infection sites. Infection by A. tumefaciens requires an attachment of the bacterial lipopolysaccharide to the host cell wall (22, 23, 42). In the pinto bean leaf assay, K84 cells or lipopolysaccharide from K84 were shown to reduced infection when inoculated together with A. tumefaciens (42). This suggested that K84 was able to compete with the pathogen for infection site binding. Our data on the reduction of tumor weights by K84 also suggest a blockage of infection sites. Since tumor size is generally proportional to the size of the wound (45), competition for attachment sites should result in smaller tumors.

Although the production of agrocin 84 is clearly an important mechanism of biological control by K84, selecting new antagonists on the basis of agrocin production alone has not been very effective (13, 20, 27). Thus, selection of antagonists on the basis of other mechanisms, such as the ability to bind to infection sites, should be employed in addition to the selection for agrocin production.

Table 5. Biological control of crown gall on tomato seedlings by *Agrobacterium radiobacter* K84 and K84Agr$^-$

Sequence of inoculation following wounding[a]	Percent infection by *A. tumefaciens*	
	K24	B6
K84 + pathogen	0	90
K84Agr$^-$ + pathogen	90	100
Pathogen control	100	100
K84, wait 24 hr, pathogen	0	50
K84Agr$^-$, wait 24 hr, pathogen	30	40
Wait 24 hr, pathogen control	70	90

[a]0.01 ml of pathogen-antagonist mixtures was applied to wounds in tomato stems. The number of viable cells applied to wounds was 9.6×10^5 for K84, 1.2×10^6 for K84Agr$^-$, 2.7×10^5 for K24, and 1.5×10^5 for B6.

Table 6. Effect of inoculations with Agrobacterium radiobacter K84 and K84Agr$^-$ on tomato seedling tumor weights

Sequence of inoculation following wounding	A. tumefaciens K24		A. tumefaciens B6	
	Tumor weight (g)	Percent reduction from control	Tumor weight (g)	Percent reduction from control
K84 + pathogen	0.00 ± 0.00[a]	100	0.47 ± 0.55	64
K84Agr$^-$ + pathogen	0.28 ± 0.22	60	0.47 ± 0.44	64
Pathogen control	0.70 ± 0.17	0	1.32 ± 0.55	0
K84, wait 24 hr, pathogen	0.00 ± 0.03	100	0.00 ± 0.02	100
K84Agr$^-$, wait 24 hr, pathogen	0.06 ± 0.11	84	0.00 ± 0.00	100
Wait 24 hr, pathogen control	0.38 ± 0.29	0	1.50 ± 0.31	0

[a]Standard error of the mean.

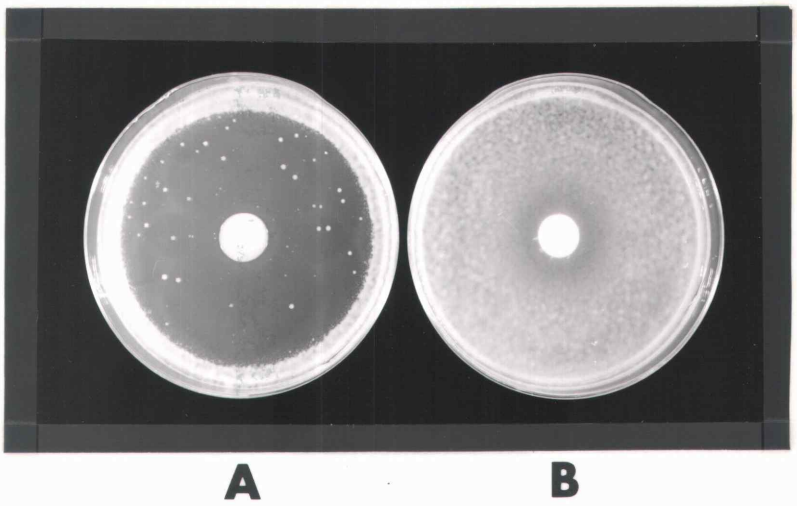

Fig. 1. Lack of agrocin production by a mutant of Agrobacterium radiobacter K84. A, Wild-type K84 inhibiting growth of A. tumefaciens K24 by production of agrocin 84. B, Mutant of K84 that no longer produces agrocin 84. A. radiobacter strains were spotted in the center of mannitol-glutamate (16) agar plates, grown for 3 days, and sprayed with a suspension of A. tumefaciens K24.

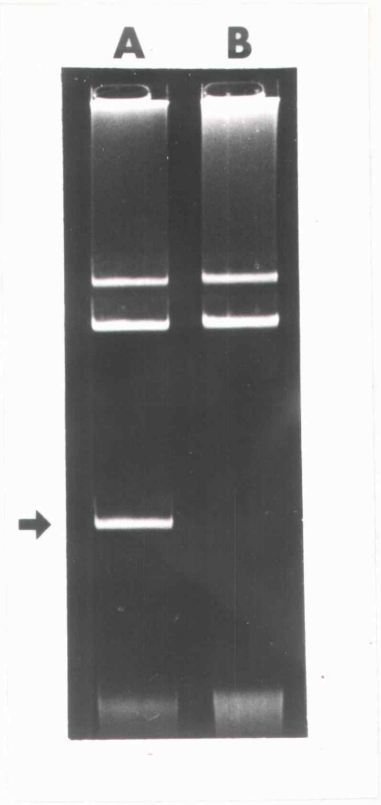

Fig. 2. Agarose gel electrophoresis of plasmid DNA from Agrobacterium radiobacter K84. A, Wild-type A. radiobacter K84. The arrow indicates the 30 megadalton bacteriocinogenic plasmid band. B, K84Agr$^-$, a mutant of K84 cured of the 30 megadalton plasmid that no longer produces agrocin 84.

III. HIGH FREQUENCY SPONTANEOUS MUTATIONS TO AGROCIN 84 RESISTANCE IN *AGROBACTERIUM TUMEFACIENS* AND *A. RHIZOGENES*

INTRODUCTION

Agrocin-resistant mutants of normally sensitive strains of *A. tumefaciens* develop readily when grown in the presence of agrocin 84 (19). Initially it was reported that most of the resistant mutants had lost virulence (19) and no longer carried the Ti-plasmid (12). Thus, the development of resistance to agrocin 84 did not appear to be an immediate threat to the use of K84 as a biological control agent. However, in a more recent study, the agrocin-resistant mutants examined were predominantly virulent and retained the Ti-plasmid (40).

In the present study, the rate of mutation to agrocin 84 resistance was measured in vitro as a preliminary assessment of the potential for a breakdown in effectiveness of K84 as a biological control agent. In addition, the pathogenicity and plasmid content of agrocin-resistant mutants were examined.

MATERIALS AND METHODS

Bacterial Strains and Media

The bacterial strains are listed in Table 7.

Cultures were maintained on Difco potato-dextrose agar with 1% calcium carbonate. Mannitol-glutamate (MG) medium (16) was used in all agrocin sensitivity tests.

Preparation of Agrocin 84 Extract

A crude extract of agrocin 84 was used in all experiments. K84 was grown on dialysis membrane placed over 50 ml of MG agar in 150 X 15 mm plastic petri dishes. After 3 days at 28 C the membrane was lifted from the agar, thus removing the bacteria and most of the bacterial capsule. Each plate was then soaked twice with 50 ml sterile distilled water for 12 hr at 4 C. The washes were combined and lyophilized, and the residue was suspended in distilled water to 1/20 of the initial volume. The suspension was centrifuged at 12,000 X g for 10 min, and the supernatant was filter-sterilized and frozen at -20 C.

Rate of Mutation to Agrocin Resistance

Cells grown in MG broth to log phase were subcultured to 100 ml fresh MG broth and grown to a final concentration of about 10^4 cells/ml on a shaker at 200 rev/min at 28 C. Samples of the culture were removed at 2 hr intervals, starting three to four cell generations after subculturing, and plated on MG agar and on MG agar plus agrocin. MG agar with agrocin was prepared by spread-plating 0.1 ml crude agrocin onto 15 ml MG agar in 100 X 15 mm plastic petri dishes and drying under a sterile transfer hood for 30 min. Colony counts were made after 36-48 hr incubation at 28 C.

The rate of mutation to agrocin resistance during log phase growth was calculated using the formula $M = \log_e 2\ (M_2-M_1)/(N_2-N_1)$, where M_1 and M_2 are the number of resistant colonies from plating on the agrocin

MG medium at times 1 and 2, and N_1 and N_2 are the corresponding total cell counts from MG plates (14). Values for M_1, M_2, N_1, and N_2 were taken from the lines obtained by linear regression of the log of colony forming units against time (Fig. 3).

Spontaneous Mutations to Agrocin Resistance

Culture samples removed during the mutation rate determinations were serially diluted and replica-plated to MG agar plus agrocin; three replicas on agrocin MG were made for each dilution.

Plasmid Isolation and Electrophoresis

The protease cell lysis and alkaline denaturation procedure of Currier and Nester (6) was used for plasmid isolations, but the shearing step was omitted. Cells were harvested after growth in 100 ml of MG (supplemented with 0.025% yeast extract) to 10^7-10^8 cells/ml. All steps were performed in 50 ml centrifuge tubes using gentle inversion to mix reagents.

A horizontal slab gel apparatus with gel dimensions of 130 X 420 X 4 mm was used for electrophoresis of the plasmid preparations. Seakem (ME) agarose at 0.7% was dissolved in tris-borate buffer to form the gel (25). Ten µl of the DNA sample was mixed with 5 µl tracking dye (20% Ficoll, 0.2% SDS, 0.05% bromophenol blue) before loading to 30 µl wells formed in the agarose by a lucite comb with 12 teeth; electrophoresis was for 4 hr at 270 V. Gels were stained for 90 min in a 0.5 µg/ml ethidium bromide solution and photographed on a

short-wave Ultra-violet Products transilluminator with Polaroid type 47 film.

RESULTS

Rate of Mutation to Agrocin Resistance

Mutations to agrocin resistance occurred at unusually high rates in all strains tested (Table 8). The three A. tumefaciens strains did not differ significantly in their mutation rates, since the variation was high between trials for a given strain. However, the rate of mutation of A. rhizogenes A4 was consistently about ten times higher than A. tumefaciens.

Spontaneous Mutations

When a bacterial lawn of C58 was replica-plated to three agrocin MG plates the resistant colonies developed in identical patterns on all three plates (Fig. 4), indicating that the agrocin-resistant colonies were present in the bacterial lawn before any exposure to agrocin (21). In addition, the number of agrocin-resistant colonies on the replica plates agreed with the plate counts on agrocin MG obtained during the determination of mutation rates. Thus, the high rate of mutation to agrocin resistance can be explained solely on the basis of spontaneous events.

Examination of Plasmids in Agrocin-resistant Derivatives

Plasmid analysis of 25-50 agrocin-resistant colonies of each A.

tumefaciens strain showed that 28-68% of the agrocin resistant mutants had lost the entire Ti-plasmid (Table 9). Many other resistant mutants had Ti-plasmids banding the same as the wild-types, and a few had large deletions (Fig. 5). Strains with intact Ti-plasmids were still virulent; those with large deletions were avirulent (Table 9).

In A. rhizogenes A4, agrocin resistance was always associated with the loss of the smallest and the largest of the three wild-type plasmids. In contrast to A. tumefaciens, none of the agrocin resistant derivatives of A4 was virulent.

Correlation of Colony Size to Plasmid Content

The agrocin-resistant colonies appearing on agrocin MG agar varied greatly in size. These colonies were rated on scale from one to five, with five being the largest. Six colonies from C58 in each size class were streaked for purity three times on agrocin MG and examined for the presence or absence of the Ti-plasmid. All six colonies of the largest class retained the Ti-plasmid, while all colonies of the smallest class had lost the Ti-plasmid (Table 10).

DISCUSSION

Our results show that mutations to agrocin resistance occur spontaneously at a surprisingly high rate. The majority of the agrocin-resistant mutants from C58 and K24 had lost the entire Ti-plasmid, suggesting that the Ti-plasmid is lost spontaneously from these strains at a high rate. However, a significant number of resistant mutants from all three A. tumefaciens strains retained the Ti-

plasmid and were virulent; in B234, this class of resistant mutants was more common than those with complete plasmid loss. The high rates of mutation to agrocin resistance therefore are not due entirely to instability of the Ti-plasmid. The appearance of mutants with various large deletions from the Ti-plasmid suggest mutational events from elements such as insertion sequences that would be more consistent with the high mutation rates than classical point mutations. Sule and Kado (40) have suggested the involvement of insertion sequences in mutation to agrocin resistance because of the appearance of sensitive revertants with a diversity of sensitivity levels.

In previous studies using strain C58, there were conflicting reports that agrocin-resistant mutants either lost the entire Ti-plasmid and were avirulent (12), or that they had retained the Ti-plasmid and were still virulent (40). In our work, the largest colonies of C58 selected from MG agar containing agrocin all retained the Ti-plasmid, while the smallest colonies had lost the entire plasmid. It is possible that the discrepancies in the literature occurred by a preferential selection for a certain colony size. Based on our data, random selection should yield a majority of plasmidless Colonies of C58, but selection of large colonies would yield mutants retaining the Ti-plasmid. The use of higher levels of agrocin could favor the selection of large colonies, since the smaller colonies are slow to develop when exposed to high agrocin concentrations (Cooksey and Moore, unpublished data).

The resistant mutants of A. rhizogenes A4 showed complete loss of both the largest and the smallest plasmid bands. It has been suggested

that the largest plasmid in A4 is a cointegrate of the two smaller plasmids (43). Therefore, instability of the smallest plasmid could account for our findings. Since all of the agrocin-resistant mutants in our study were avirulent, it would appear that virulence genes are carried on the smallest plasmid along with the genes for agrocin sensitivity. Similar results were obtained when A4 was treated with ethidium bromide (31), yielding mutants that were avirulent, agrocin resistant, and had lost the smallest and largest plasmid bands (Cooksey and Moore, unpublished data). In contrast, strain 15834 of A. rhizogenes was found to carry virulence genes on the second largest plasmid and agrocin sensitivity on the smallest plasmid (43). Although the plasmids from 15834 and A4 showed almost identical sequence homology (44), it is possible that the dissociation of the largest plasmid into the two smaller plasmids differs somewhat between these two strains causing the virulence and agrocin sensitivity genes to be segregated into the two smallest plasmids in 15834.

Mutation to agrocin resistance with retention of virulence has long been recognized as a potential threat to the continued use of K84 as a biological control agent. The high rate of spontaneous mutation to agrocin resistance in vitro described here emphasizes the need to study mutations to resistance in vivo, and to search for alternate methods of controlling the disease. A few antagonists have already been found that provided some control of crown gall incited by agrocin-resistant strains (5). The use of mixtures of several antagonists is also being investigated and should reduce the threat from mutations to resistance.

Table 7. Bacterial strains of Agrobacterium and their source

Strain	Biotype	Source
A. tumefaciens		
C58	1	E. Nester, University of Washington, Seattle
K24	1	A. Kerr, University of Adelaide, Australia
B234	2	J. DeVay, University of California, Davis
A. rhizogenes		
A4	2	R. Durbin, University of Wisconsin, Madison
A. radiobacter		
K84	2	A. Kerr

Table 8. Mutation rates of selected Agrobacterium strains to agrocin resistance

	A. tumefaciens			A. rhizogenes
Trial	C58	K24	B234	A4
1	7.0×10^{-4}	4.8×10^{-4}	2.0×10^{-4}	1.5×10^{-3}
2	2.7×10^{-4}	1.0×10^{-4}		2.6×10^{-3}
3	2.9×10^{-4}	2.0×10^{-4}		3.4×10^{-3}
Mean	4.2×10^{-4} $\pm 1.4 \times 10^{-4}$[a]	2.6×10^{-4} $\pm 0.86 \times 10^{-4}$		2.5×10^{-3} $\pm 0.55 \times 10^{-3}$

[a]Standard error of the mean.

Table 9. Agrocin-resistant mutants of Agrobacterium examined for Ti-plasmids and virulence

	Number of mutants examined	Percent of mutants with Ti-plasmids			Virulence[a]
		Intact	Deleted	Cured	
A. tumefaciens					
C58	50	32			+
			4		-
				64	-
K24	25	20			+
			12		-
				68	-
B234	25	72			+
			0		
				28	-
A. rhizogenes					
A4	25	0			
			0		
				100[b]	-

[a]Virulence tests were made on wounded Bonny Best tomato stems for A. tumefaciens (5) and on carrot slices for A. rhizogenes (31).

[b]Agrocin-resistant mutants all lacked the smallest and largest of the three wild-type plasmid bands (Fig. 3).

Table 10. Colony size and plasmid content of agrocin-resistant mutants of Agrobacterium tumefaciens C58

Colony size class[a]	Number of mutants with Ti-plasmid	
	Present	Absent
1	0	6
2	1	5
3	1	5
4	5	1
5	6	0

[a]Colony sizes were rated on a scale of one to five with five being the largest.

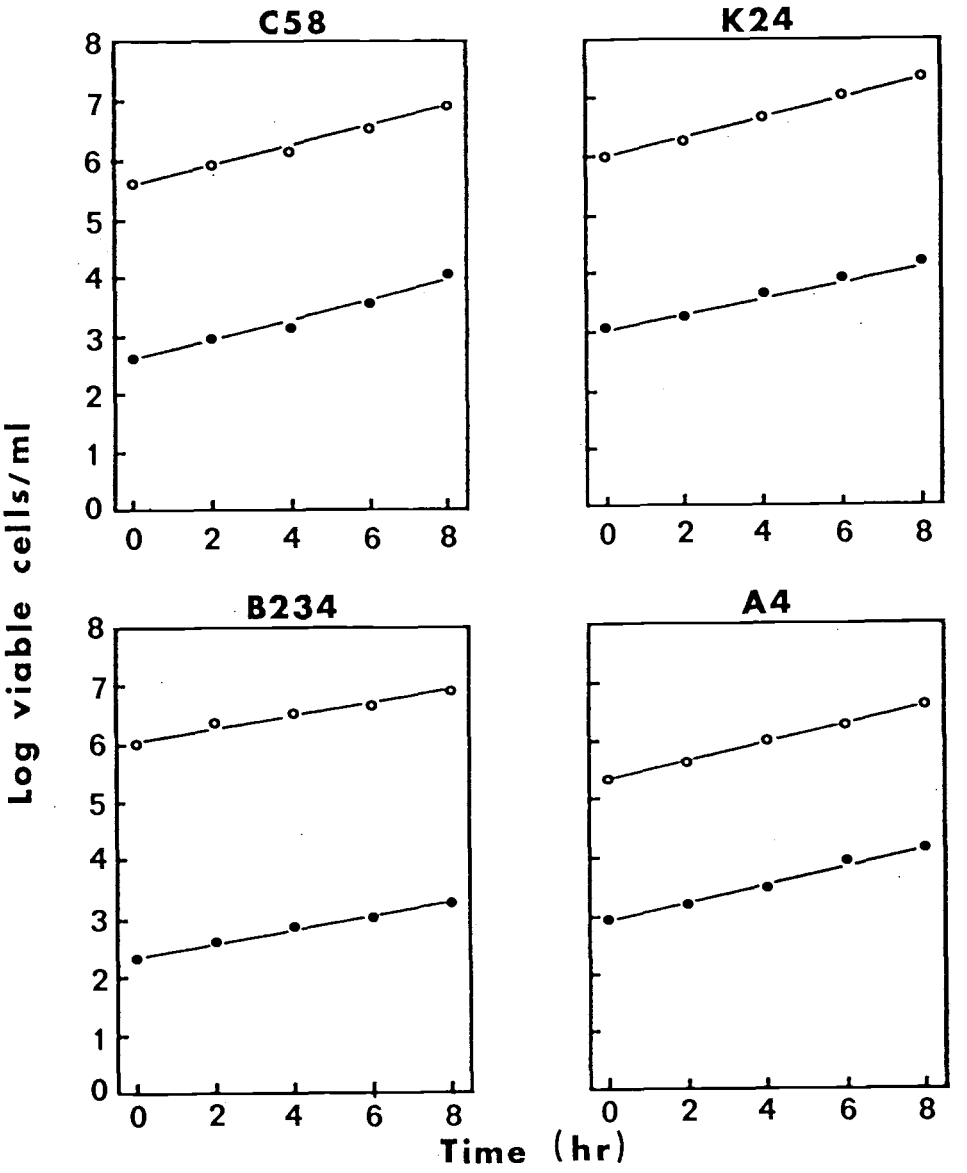

Fig. 3. Mutation to agrocin 84 resistance in liquid culture.
●, Agrocin-resistant mutants; O, total total viable cells.
Samples were taken from mannitol-glutamate (16) broth cultures during log phase growth at 28 C.

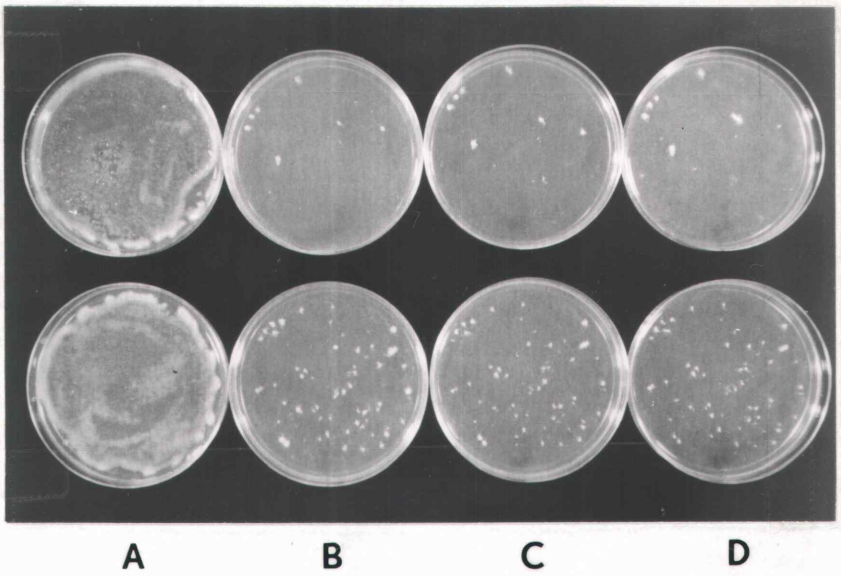

Fig. 4. Spontaneous mutations to agrocin 84 resistance. Bacterial lawn (A) of Agrobacterium tumefaciens C58 was replica-plated to three plates (B, C, and D) containing agrocin 84. Mutant colonies appear in identical patterns on all three replicas, indicating the spontaneous nature of mutations to agrocin resistance.

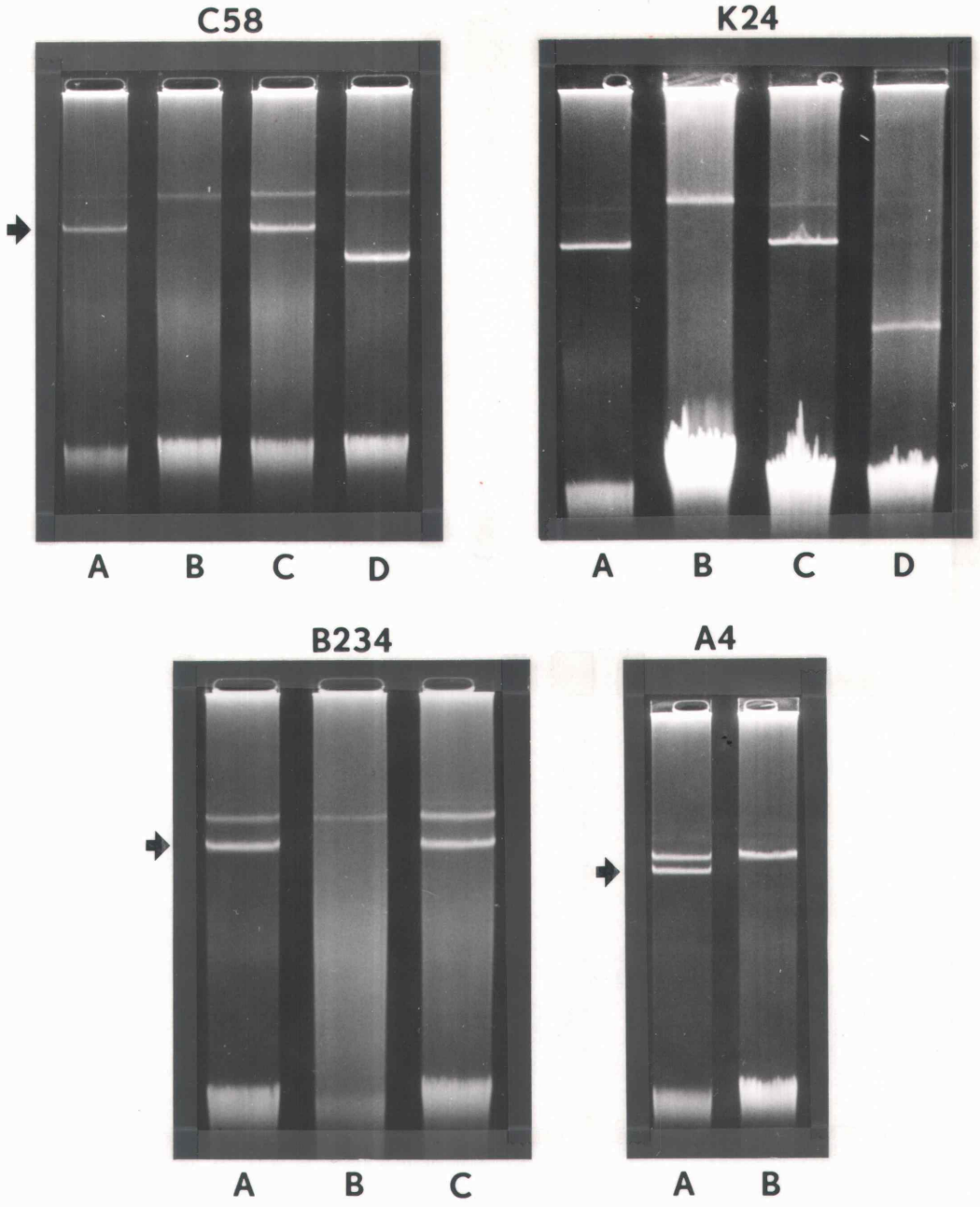

Fig. 5. Agarose gel electrophoresis of plasmid DNA from Agrobacterium tumefaciens strains C58, K24, and B234 and A. rhizogenes A4. Lanes labeled A are wild-types. Lanes labeled B are agrocin-resistant mutants that have lost the entire Ti-plasmid. Lanes labeled C are agrocin-resistant mutants that have retained the Ti-plasmid. Lanes labeled D are agrocin-resistant mutants with large deletions from the virulence plasmid.

IV. AGROCIN 84 SENSITIVITY IN *RHIZOBIUM LEGUMINOSARUM* ASSOCIATED WITH PLASMID DNA

INTRODUCTION

Agrobacterium radiobacter strain K84 is used as a biological control agent for the prevention of crown gall disease of plants (30). The mechanism of control is attributed to the production of a bacteriocin (agrocin 84) by K84 that inhibits sensitive strains of the crown gall pathogen, *A. tumefaciens* (19). Sensitivity to agrocin 84 in *A. tumefaciens* is associated with large plasmids (called Ti-plasmids) that are involved in tumor induction of plant tissues (12).

Agrocin 84 sensitivity has also been reported in *Rhizobium trifolii* (36), *R. leguminosarum*, and *R. meliloti* (Anderson and Moore, unpublished data). Because of taxonomic similarities between *Rhizobium* and *Agrobacterium*, it occurred to us that sensitivity to agrocin 84 in *Rhizobium* might be a plasmid-borne trait. In this paper we report the association of agrocin 84 sensitivity with a large plasmid in one strain of *R. leguminosarum*.

MATERIALS AND METHODS

Bacterial strains

R. leguminosarum 128C56 was obtained from H. J. Evans. *A. radiobacter* K84 was obtained from A. Kerr, and avirulent *A. tumefaciens* strain NT1 was from E. Nester.

Media

Cultures were maintained on Difco potato-dextrose agar (PDA) with 0.5% calcium carbonate. Mannitol-glutamate (MG) agar (16) at pH 7.0 was used in agrocin sensitivity tests.

Isolation of Agrocin-resistant Rhizobia

A. radiobacter K84 was spotted at the center of MG agar plates and incubated for 3 days at 28 C. Suspensions of log phase Rhizobium were then sprayed over the plates, and zones of inhibition appeared after 24-36 hr of incubation at 28 C (Fig. 6). Eighteen resistant colonies from the inhibition zones were purified and retested for agrocin resistance by spraying cell suspensions on MG plates with strain K84 grown in the center.

RP4-mediated Transfer of Rhizobium Plasmid DNA to Avirulent A. tumefaciens NT1

The RP4 plasmid was transferred from Escherichia coli J53 to R. leguminosarum 128C56 by a filter-mating technique similar to that of Van Larebeke et al. (41). Cell suspensions (about 10^7 cells/ml) of donor and recipient strains were mixed in a 1:1 ratio, and 3 ml were forced through a 0.4 µm Nucleopore filter (25 mm diameter). The filter was placed on a PDA plate and incubated at 28 C for 48 hr. Then the filter was suspended in 10 ml of sterile distilled water and vortexed for 30 sec. Dilutions of this suspension were plated on MG agar with 200 µg/ml kanamycin. The auxotrophic donor J53 did not

grow on MG agar, and only those recipients receiving kanamycin resistance from RP4 formed colonies. Transconjugants were apparent after 5 days at 28 C. 128C56 carrying RP4 was crossed with A. tumefaciens NT1 (a Ti-plasmid-cured derivative of strain C58 with resistance to rifampicin and chloramphenicol) by the technique just described. The selective medium used in this cross was PDA containing 200 µg/ml kanamycin, 100 µg/ml rifampicin, and 100 µg/ml chloramphenicol. Colonies were visible after 4 days at 28 C.

Isolation of Plasmid DNA

The protease cell lysis and plasmid isolation procedure of Currier and Nester (6) was used except that the DNA shearing step was omitted. Cells were harvested after growth in 100 ml of MG broth (supplemented with 0.025% yeast extract) to about 10^7-10^8 cells/ml. All steps were performed in 50 ml centrifuge tubes using gentle inversion to mix reactants.

Agarose Gel Electrophoresis of DNA

A horizontal slab gel apparatus was used with gel dimensions of 13 cm X 42 cm X 4 mm. Seakem agarose at 0.7% was dissolved in tris-borate buffer (25). Fifteen µl of DNA was mixed with 5 µl of tracking dye (20% Ficoll, 0.2% SDS, 0.05% bromophenol blue) before loading to 30 µl wells formed by a lucite comb with 12 teeth. Electrophoresis was for 5 hr at 250 V. Gels were stained for 60 min in a 0.5 µg/ml ethidium bromide solution and photographed on a short-wave Ultraviolet Products transilluminator with Polaroid type 55 film.

RESULTS

Correlation of Agrocin 84 Resistance with the Loss of a Large Plasmid

Eighteen agrocin-resistant derivatives of R. leguminosarum 128C56 were independently isolated from inhibition zones formed by agrocin 84 (Fig. 6). All but one of these isolates had lost a large plasmid of about 93 megadaltons (Md) (Fig. 7b). The wild-type 128C56 strain (Fig. 7a) carried two plasmids of 123 Md and 144 Md (Table 11) in addition to the 93 Md plasmid absent in the resistant derivatives. Agrocin-resistant derivative no. 2 (Fig. 7c) carried three plasmids that appeared to be identical to the wild-type by electrophoretic measurements.

RP4-mediated Transfer of Agrocin Sensitivity Genes from R. leguminosarum to A. tumefaciens NT1

Transconjugants from the E. coli J53 X 128C56 cross were recovered at a frequency of about 10^{-5} per recipient. These transconjugants were shown to harbor a 36 Md plasmid (RP4) in addition to the three wild-type plasmids in 128C56 (Fig. 7d). When 128C56(RP4) was subsequently used as a donor in a cross with avirulent A. tumefaciens NT1 (containing only a large cryptic plasmid), transconjugants carrying kanamycin resistance and the recipient phenotypes were recovered in a frequency of about 10^{-3} per recipient. Of the 30 transconjugants tested for agrocin sensitivity, one showed full wild-type sensitivity (Fig. 6). Plasmid isolation from these strains

showed that the sensitive transconjugant had received a 108 Md plasmid (Fig. 7e), assumed to be a cointegrate of RP4 (36 Md) and the 93 Md pRle-128C56a plasmid carrying genes for agrocin sensitivity; stable cointegrates between RP4 and the Ti-plasmids of A. tumefaciens have been reported (15). The cointegrate in the present study was smaller by about 21 Md than one would expect for a pRle-128C56a::RP4 cointegrate, indicating that some DNA was lost from one of the plasmids.

DISCUSSION

Agrocin sensitivity in R. leguminosarum 128C56 was found to be associated with a plasmid of about 93 Md. This conclusion is based on 1) the correlation between agrocin resistance and the loss of the 93 Md plasmid, and 2) the transfer of this plasmid and agrocin sensitivity to A. tumefaciens NT1 by RP4 mobilization.

Agrocin 84 is taken up by means of a plasmid-coded binded protein in sensitive A. tumefaciens strains (32). If Rhizobium sensitivity involves a similar permease system, this would have interesting implications as to the origin of Rhizobium and Agrobacterium plasmids. Skotnickni and Rolfe (36) have reported interesting similarities between R. trifolii T1K and A. tumefaciens that include agrocin 84 sensitivity, nopaline utilization, and the ability to induce tumors. However, it was not demonstrated that these characteristics were determined by a plasmid.

Plasmid-borne bacteriocin sensitivity in Rhizobium may provide

a valuable marker for genetic studies. Plasmids carrying genes for agrocin sensitivity in A. tumefaciens apparently are unstable in the presence of agrocin, since agrocin 84-resistant colonies occur at a high frequency (Cooksey and Moore, unpublished data). In resistant cells, the plasmid coding for agrocin sensitivity is often missing (12). Thus, plasmids with agrocin 84 sensitivity genes can be selectively cured, and the resulting strain can be screened for the loss of other suspected plasmid-borne traits.

Sensitivity to agrocin 84 in Rhizobium is also of interest because nursery plantings treated with strain K84 as a biological control agent are often followed by legume cover-crops. However, repeated tests in our lab (29) have shown that coinoculation of alfalfa seedling roots with sensitive R. meliloti strains and strain K84 did not prevent nodulation, and the mean growth of these seedlings was equivalent to seedlings inoculated only with R. meliloti.

Table 11. Plasmid molecular weights determined by electrophoretic mobility

Strain	Plasmid designation	Plasmid molecular weight (Md)
Rhizobium leguminosarum 128C56	pRle-128C56a	93.58 ± 3.94
	pRle-128C56b	128.08 ± 8.43
	pRle-128C56c	142.66 ± 6.07
128C56(RP4) X *Agrobacterium tumefaciens* NT1	pRle-128C56::RP4	108.19 ± 5.36

[a]Plasmid molecular weights were determined by electrophoretic mobility as described by Meyers et al. (25). Plasmids used as standards were from strains K27 and C58 of *A. tumefaciens*, K84 of *A. radiobacter*, 102F51 of *R. meliloti*, and *Escherichia coli* J53.

[b]Plasmid designations followed those of Casse et al. (4) for *R. leguminosarum*.

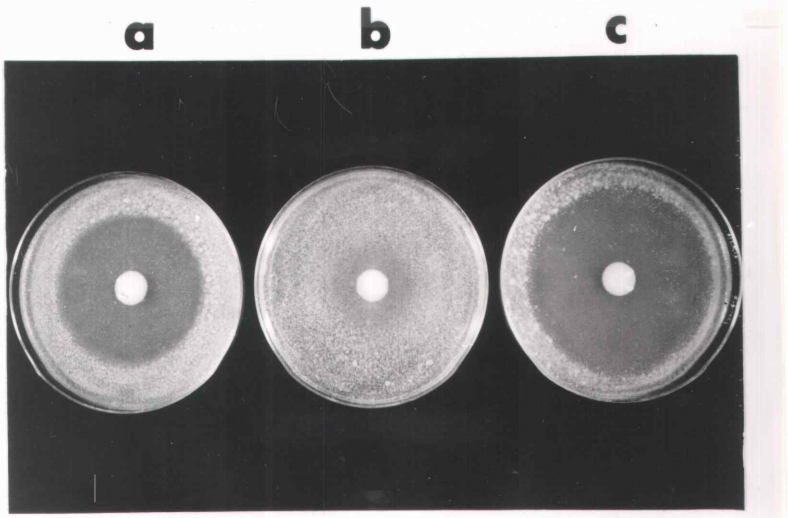

Fig. 6. Inhibition of Rhizobium leguminosarum 128C56 by Agrobacterium radiobacter K84. A) Wild-type 128C56 lawn inhibited by K84 (center colony); B) Agrocin-resistant derivative 128C56R1; C) Transconjugant between 128C56(RP4) and agrocin-resistant A. tumefaciens NT1. Neither the recipient strain NT1 or NT1 carrying the RP4 plasmid were sensitive to agrocin 84.

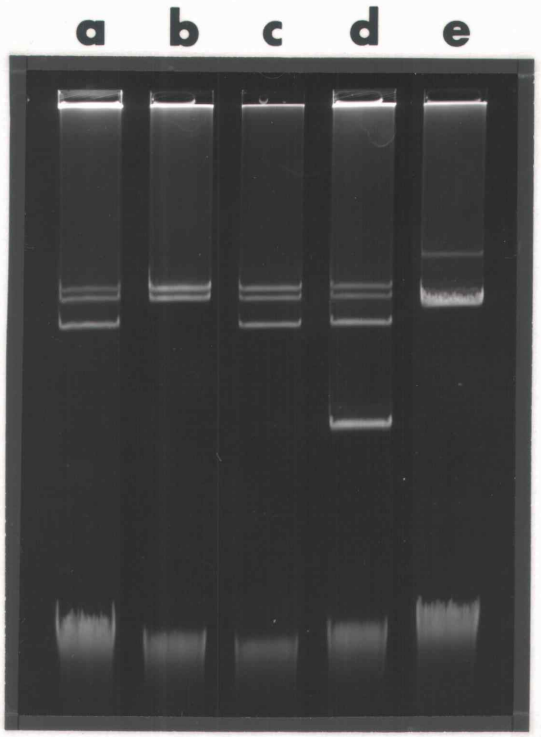

Fig. 7. Agarose gel electrophoresis of plasmid DNA. A) Plasmid pattern of the wild-type Rhizobium leguminosarum 128C56; B) Agrocin-resistant derivative 128C56R1 lacking the smallest of the three wild-type plasmids; C) Agrocin-resistant derivative 128C56R2 retaining all three plasmids; D) 128C56(RP4); E) Transconjugant between 128C56(RP4) and Agrobacterium tumefaciens NT1. A plasmid assumed to be a cointegrate of RP4 and the smallest plasmid is present below the very large NT1 cryptic plasmid.

SUMMARY AND CONCLUSIONS

Through the study of antagonistic interactions of soil bacteria and fungi with Agrobacterium, several new antagonists were found that reduced crown gall infections in both greenhouse and field tests. Although some antagonists provided protection from certain pathogenic strains, none was as effective as A. radiobacter K84 for overall prevention of crown gall. This led to the study of the mechanisms that make K84 a successful antagonist. The development of an agrocin$^-$ mutant of K84 made it clear that production of agrocin 84 was not the only mechanism of biological control by K84. The substantial reduction of tumor weight by this mutant suggested that K84 reduces the available area for infection by physically blocking infection sites, because tumor size is generally proportional to the size of wounded tissue (45). Further work is needed to confirm that binding to infection sites is an important mechanism for control by K84. The development of an in vitro cell binding assay would be useful for this purpose and also for the selection of new antagonists with the ability to bind to plant cells.

The surprisingly high rate of mutation to agrocin resistance in A. tumefaciens and A. rhizogenes emphasizes the need for new antagonists. These experiments should be extended to the measurement of agrocin-resistant mutations in the field. Agrocin-resistant A. tumefaciens strains have been found in Washington (28) and for the first time in Oregon in 1981 (L. Moore, personal communication). The use of several antagonists should reduce the threat from mutations

to agrocin resistance.

The inhibition of Rhizobium species by A. radiobacter K84 is of interest because nursery plantings where K84 has been used are often followed by legume cover-crops. This study demonstrates that, as in A. tumefaciens, agrocin sensitivity genes in Rhizobium leguminosarum 128C56 are coded for by a large plasmid. This suggests that in addition to the taxonomic similarities between Rhizobium and Agrobacterium, there may be similarities between plasmids of these two genera.

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