Biological Control of Crown Gall with an Agrocin Mutant of *Agrobacterium radiobacter*

D. A. Cooksey and L. W. Moore

Graduate research assistant and associate professor, respectively, Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331. Present address of senior author: Department of Plant Pathology, University of California, Riverside 92521.
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ABSTRACT

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A mutant of *Agrobacterium radiobacter* K84 that no longer produced agrocin 84 was obtained by mitomycin C curing of the 30 Mdalton bacteriocinogenic plasmid in K84. This mutant (designated K84Agr$^-$) no longer prevented crown gall of tomato stems when coinoculated with an agrocin-sensitive pathogen *A. tumefaciens* K24. However, it effectively reduced infection when it was placed on the host plant 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 physical blockage of infection sites, are involved in biological control by K84 in addition to production of agrocin 84.

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 (4,7). There is a good correlation between agrocin 84 sensitivity in *A. tumefaciens* and biological control by K84 when wounded tomato stems are coinoculated with the pathogen and antagonist (7). However, when K84 is applied to tomato stems 24 hr before the pathogen, infection by agrocin-resistant strains can be prevented (2). In addition, agrocin-resistant strains have been controlled by K84 in field tests with *Prunus* seedlings (2,12,14). These data suggest that in addition to the production of agrocin 84, other mechanisms are involved in biological control by K84. A full understanding of these mechanisms may be an aid in selecting new antagonists for situations in which K84 is not effective (1,13).

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 Mdalton plasmid in K84 (4), 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 $\sim 10^6$ cells per milliliter in 100-ml volumes of mannitol-glutamate (MG) broth (6). Mitomycin C was then added to 0.0, 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 transferred with sterile toothpicks to fresh MG plates (12 colonies per plate), incubated at 28 C for 24 hr, and sprayed with a suspension (about 10^6 cells per milliliter) of *A. tumefaciens* K24. Colonies were observed for production of agrocin 84 against K24 after 48 hr of incubation at 28 C.

Examination of plasmid DNA. The protease cell lysis and plasmid isolation procedure of Currier and Nester (3) was used except that the DNA-shearing step and the purification on cesium chloride-ethidium bromide gradients were 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 per milliliter. All steps were performed in 50-ml centrifuge tubes using gentle inversion to mix reagents.

A horizontal slab gel apparatus with gel dimensions of 13 cm × 42 cm × 4 mm was used for electrophoresis of plasmid DNA. Seakem (ME) agarose at 0.7% was dissolved in tris-borate buffer (11). Ten microliters 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. Electrophoresis was for 5 hr at 6.4 V/cm. Gels were stained for 60 min in a 0.5 μg/ml ethidium bromide solution in tris-borate buffer and photographed with Polaroid type 47 film on a Model C-61 shortwave transilluminator (Ultra-Violet Products Inc., San Gabriel, CA 91778).

Greenhouse tests for biological control. Tomato seedlings were wounded and inoculated as described previously (2). Ten seedlings were used per treatment (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 uninoculated 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 10 uninoculated, wounded stems was subtracted from the values for inoculated stems.

RESULTS

Mitomycin C plasmid curing. Mutants of K84 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. No agrocin$^-$ mutants were found in 200 colonies examined from each of the 0.0, 0.1, or 1.0 μg/ml mitomycin C treatments. One of the agrocin$^-$ 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 Mdalton bacteriocinogenic plasmid (Fig. 2).

Biological control on tomato seedlings. When coinoculated with

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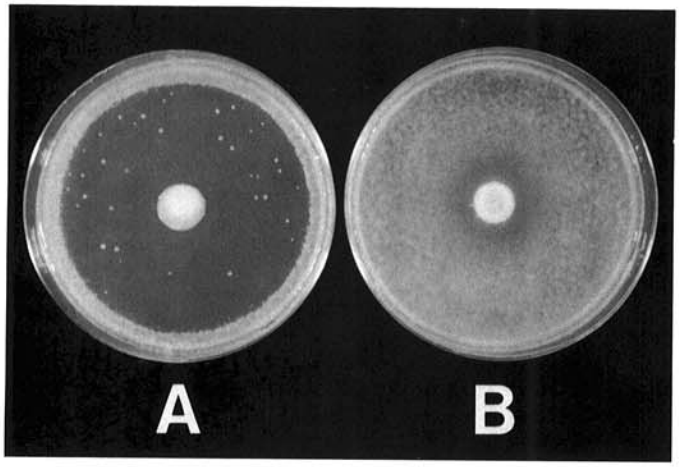

Fig. 1. Lack of agrocin production by a mutant of *Agrobacterium radiobacter* K84. **A,** Wild-type K84 inhibiting growth of *A. tumefaciens* K24 by production of agrocin 84. **B,** Mutant of K84 that no longer produces agrocin 84. *A. radiobacter* strains were spotted in the center of mannitol-glutamate (6) agar plates, grown for 3 days, and sprayed with a suspension of *A. tumefaciens* K24.

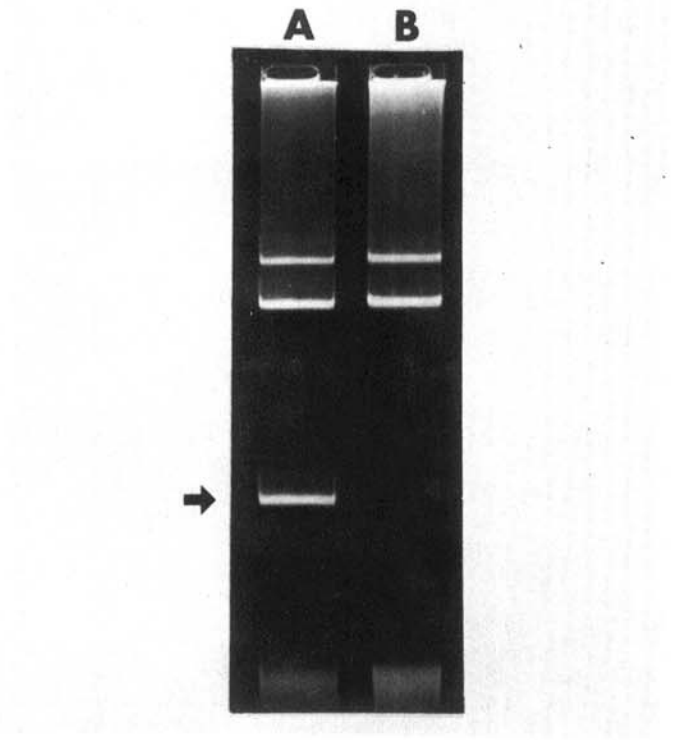

Fig. 2. Agarose gel electrophoresis of plasmid DNA from *Agrobacterium radiobacter* K84, **A,** Wild-type *A, radiobacter* K84. The arrow indicates the 30-Mdalton bacteriocinogenic plasmid band. A 124 Mdalton nopaline plasmid (15) and a large cryptic plasmid (4) are visible above the 30-Mdalton plasmid. The broad band at the bottom of the gel is chromosomal DNA. **B,** K84 Agr$^-$, a mutant of K84 cured of the 30 M dalton plasmid that no longer produces agrocin 84.

A. tumefaciens K24 on wounded tomato stems, the wild-type K84 completely prevented infection, but K84Agr$^-$ allowed 90% infection (Table 1). However, when the seedlings were preinoculated with K84Agr$^-$ 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 the seedlings were preinoculated with either antagonist 24 hr before being inoculated with B6.

Even when infection occurred, tumor weight was reduced by K84 and K84Agr$^-$ in all treatments (Table 2). When seedlings were inoculated with the two antagonists 24 hr before B6, the resulting tumors were so small that they were not detectable by tumor weight measurements.

TABLE 1. Biological control of crown gall on tomato seedlings by *Agrobacterium radiobacter* K84 and K84Agr$^-$

Sequence of inoculation[a] following wounding	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	40
K84Agr$^-$, wait 24 hr, pathogen	30	50
Wait 24 hr, pathogen control	70	90

[a] Inoculum concentrations were 9.6×10^7, 1.2×10^8, 2.5×10^7, and 1.5×10^7 viable cells per milliliter for K84, K84Agr$^-$, K24, and B6, respectively.

TABLE 2. 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.

DISCUSSION

When K84 was cured of the 30 Mdalton plasmid coding for agrocin 84 production, it no longer prevented infection if coinoculated with an agrocin-sensitive pathogenic strain. However, infection was reduced in seedlings inoculated with K84Agr$^-$ 24 hr before the pathogen. 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 (9,10,16). In the pinto bean leaf assay, K84 cells or lipopolysaccharide from K84 reduced infection when inoculated together with *A. tumefaciens* (16). 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 (17), 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 (5,8,12). 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.

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