Attachment of *Agrobacterium tumefaciens* B6 and *A. radiobacter* K84 to Tomato Root Tips

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***Agrobacterium tumefaciens* B6 and the avirulent *Agrobacterium radiobacter* strain K84 attached to in vitro-cultured tomato root tips, but the binding of strain B6 to root tips was greater than the binding of strain K84. Strain K84 was not able to block the attachment of *A. tumefaciens* B6 to in vitro-cultured tomato root tips.**

Agrobacterium tumefaciens causes crown gall disease in several plant species (5). Tumor formation is based on the ability of virulent *Agrobacterium* strains to transfer a particular DNA segment, the T-DNA, from the Ti plasmid of the bacterium to the plant genome during the bacterium-plant cell interaction. *Agrobacterium radiobacter* K84 is used commercially to control crown gall and has been successfully employed for biocontrol for more than 20 years on different plant hosts (3, 12). The main mechanism involved in this biocontrol is the biosynthesis and secretion of a highly specific antibiotic, called agrocin 84, by strain K84 (13). Agrocin 84 inhibits the growth of certain tumorigenic strains. *A. tumefaciens* strains susceptible in vitro to agrocin 84 have been generally controlled in vivo by K84 (13, 15, 17). However, there are some cases in which strains resistant to agrocin 84 have also been controlled by K84 (1, 4, 7, 15, 24). Furthermore, in several experiments, the biocontrol of strains resistant to agrocin 84 was similar using strain K84 or a mutant of K84 that did not produce agrocin 84, called strain K84 Agr⁻ (4, 15, 20). Taken together these data suggest that, in addition to agrocin 84 production, other factors must be involved in crown gall biocontrol using strain K84. Production of other antibiotic activities by strain K84 and attachment to specific wound sites on roots have also been proposed as mechanisms involved in this biocontrol (9, 14, 21).

An early step in the process of tumor induction is the attachment of *A. tumefaciens* to plant roots. Hawes and Pueppke (10) reported that a high binding reaction was strongly correlated with susceptibility to tumorigenesis in a survey of the binding of *A. tumefaciens* to plant cells from 48 species. A number of *Agrobacterium* mutants were reported to show differences in attachment to plant cells (6, 16). These mutants were avirulent on many plant species (11). Specific binding of *A. tumefaciens* to a host wound site on roots is an essential early event in the crown gall tumor initiation process (14). Inhibition of tumor formation was obtained in pinto bean leaves with mixed inocula of virulent and avirulent bacteria and when the addition of the avirulent strain preceded the virulent strain but not when the virulent preceded the avirulent strain.

It was proposed that the inhibition of tumor initiation obtained with avirulent *A. radiobacter* was due to its ability to exclude pathogenic *A. tumefaciens* from specific sites at the wound tissues (14). Incubation of tomato plant cells with *A. tumefaciens* reduced subsequent binding of radiolabeled *A. tumefaciens* by 60 to 75% (18). Competition between strain K84 and *A. tumefaciens* in attaching to plant roots and the blockage of root wound sites by *A. radiobacter* K84 have been suggested as possible mechanisms involved in the biocontrol of *A. tumefaciens* strains resistant to agrocin 84 (4, 9, 15), but they have never been demonstrated. Nothing is known about the attachment of strain K84 to roots in host plants in which biocontrol of resistant strains has been observed. *A. tumefaciens* B6 is innately resistant to agrocin 84, but it has been successfully controlled by strain K84 in tomato greenhouse bioassays (4) and in other hosts under field conditions (15, 20). The objective of this study was to compare the attachment of *A. tumefaciens* B6 and *A. radiobacter* K84 to tomato root tips and to assess the effect of root tip preincubation with strain K84 on the subsequent attachment of strain B6.

Hybrid 741505-45 of *Lycopersicon esculentum* Mill × *Lycopersicon peruvianum* was used as the source of tissue to obtain in vitro-cultured root tips. Roots were obtained by culturing leaf disk explants on agar solidified medium (8) containing 1 mg of indole-3-acetic acid per liter. Leaflets were surface sterilized with 2% sodium hypochlorite and rinsed five times in sterile distilled water. Leaf disk explants were obtained by using a no. 5 cork borer. Leaf disks were placed with the lower leaf surface and cut perimeter in contact with the medium. Four leaf explants were cultured in each petri dish and were maintained at 28 to 30°C in the dark. Root tips measuring 1 cm were excised and transferred into 25-mm-diameter glass tubes containing 2 ml of the same medium without indole-3-acetic acid and kept in a Bellco roller drum at 100 rpm. After 2 to 4 days, root tips measuring 1 cm were used.

A. tumefaciens B6 (2437 NCPPB) and *A. radiobacter* K84 were used. *Escherichia coli* V517 (678 CECT; Colección Española de Cultivos Tipo, Burjassot, Valencia, Spain) was used as a negative control to evaluate background attachment. *Agrobacteria* were maintained on PYGA medium (24). Bacteria were grown on liquid minimal medium (18) with agitation at 24°C for *Agrobacterium* strains or 37°C for *E. coli*. Bacterial suspensions in exponential phase (16 to 24 h) were filtered (8-μm pore size; Millipore) to remove bacterial aggregates. Bacterial suspensions were concentrated by centrifugation to 10⁸ to 10⁹ cells per ml. Radioisotope labeling of bacteria was

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FIG. 1. Comparative attachment of *A. tumefaciens* B6 and *A. radiobacter* K84 to in vitro-cultured tomato root tips, at similar initial concentrations in the attachment mixture. (a) Bacterial concentrations in the attachment mixture were 1.4×10^6 cells per ml (SR = 0.077 dpm per cell) and 1.1×10^6 cells per ml (SR = 0.123 dpm per cell) for strains B6 and K84, respectively. (b) Bacterial concentrations in the attachment mixture were 3.4×10^6 cells per ml (SR = 0.024 dpm per cell) and 3.9×10^6 cells per ml (SR = 0.017 dpm per cell) for strains B6 and K84, respectively. Datum points are means from seven root tips per incubation time and strain from one representative experiment. Standard deviations of the data are shown by bars.

performed with agitation at 200 to 300 rpm for 2 to 3 h with 20 μ Ci of [35 S]methionine (>600 Ci/mmol) per ml of bacterial suspension. Vials free of radioisotope were prepared in parallel to estimate bacterial concentration (cold vials). Radiolabeled bacteria were washed three times with 10 mM K-phosphate (pH 6.0) to remove the unincorporated radioisotope. A rough estimation of bacterial cell number in the radiolabeled bacterial suspensions was accomplished by measuring the optical density of the cold vials at 660 nm. Proper amounts of the radiolabeled bacteria were resuspended in the same buffer to obtain the desired bacterial concentration for attachment assays. Accurate counts of viable bacteria (cells per milliliter) and radioactivity of the labeled bacterial suspension (disintegrations per minute per milliliter) were calculated to determine the specific radioactivity (SR) of the radiolabeled bacterium (disintegrations per minute per cell) in each attachment experiment. The standard attachment assays were performed in 4-cm-diameter wells of six-well tissue culture plates (Nunc) by dispensing 4 ml of 35 S-labeled bacterial suspension and 4 to 10 root tips in each well per incubation time. Attachment assays were carried out at 25°C on a rotary shaker at 50 rpm. Root tips were periodically removed from the labeled bacterial suspension and washed twice in 4 ml of the same buffer for 3 min at 50 rpm to eliminate the bacterial cells not tightly bound to root tips. Root tips were transferred to a glass microfiber filter (Whatman GF/C), and the root tip radioactivity (disintegrations per minute) was measured. Root tips incubated with nonlabeled bacteria were also used in each assay to estimate background disintegrations per minute, which values were subtracted from the values obtained from root tips containing radiolabeled bound bacteria. The number of bound bacterial

cells per root tip was calculated by using the SR of the radiolabeled bacterial suspension for each assay (disintegrations per minute per cell) and the root tip radioactivity (disintegrations per minute). The quenching of GF/C filter and bacterial suspensions was taken into account to report radioactivity values as disintegrations per minute. Root tips were also incubated for 2 h with nonlabeled bacterial suspensions of *A. radiobacter* K84 prepared by the procedure described previously. Root tips were washed twice in K-phosphate buffer as described above before attachment assays using radiolabeled *A. tumefaciens* B6 were performed. Root tips with and without preincubation in nonlabeled K84 suspensions were used to study subsequent attachment of radiolabeled strain B6 as previously described. Because of unavoidable variation in the absolute number of bacteria in different experiments, datum points presented are averages from one representative experiment. Two to four replicates were done per experiment reported and yielded the same result as the reported experiment. Data were examined by a series of one-way analysis of variance in which each treatment or strain was compared with the control ($P = 0.05$). This statistical analysis was performed using the StatGraphics computer program (Statistical Graphics Corp., Inc.). Data were expressed as means $\pm$ standard deviations of the number of bound bacterial cells per root tip at each incubation time studied.

Bacterial attachment was confirmed by light and scanning electron microscopy (SEM). Tomato root tips were incubated with two bacterial suspensions (10^8 and 10^7 cells per ml) of strains B6 and K84 and processed following the described protocol. Bacterial suspensions and untreated root tips were also observed as controls. All the root tips were observed at 5,

FIG. 2. Micrographs of tomato root tips incubated with *A. tumefaciens* B6 and *A. radiobacter* K84 after 2 h of coinoculation. (a) Bacterial suspension (10^7 cells per ml) of strain B6. (b) Untreated root hair used in these assays. (c) Root hair incubated with strain B6. Clusters of bacteria were often observed on the root hair surface. (d) Root hair incubated with strain K84. Clusters of bacteria were often observed on the root hair. Panels a, b, c, and d are light micrographs. (e) SEM micrograph of tomato root tips incubated with strain B6. Higher magnification shows individual cells and clusters of bacteria attached to the root and root hair surface (arrowheads). (f) SEM micrograph of root tips incubated with strain K84. Bacteria were observed attached to the root surface and root hair. Bars equal 10 μ m.

FIG. 3. Attachment of radiolabeled *A. tumefaciens* B6 to in vitro-cultured tomato root tips preincubated with nonlabeled *A. radiobacter* K84 for 2 h. The bacterial concentration in the attachment mixture of radiolabeled *A. tumefaciens* B6 was 5.9×10^6 cells per ml (SR = 0.021 dpm per cell). Datum points are the mean from seven root tips per incubation time and treatment from one representative experiment. Standard deviations of the data are shown by bars. Buffer, number of radiolabeled cells of *A. tumefaciens* B6 bound to root tips preincubated with buffer for 2 h; K84 E6, number of radiolabeled cells of *A. tumefaciens* B6 bound to root tips preincubated with nonlabeled *A. radiobacter* K84 at concentration of 3.75×10^6 cells per ml for 2 h; K84 E7, number of radiolabeled cells of *A. tumefaciens* B6 bound to root tips preincubated with nonlabeled *A. radiobacter* K84 at 1.9×10^7 cells per ml for 2 h; K84 E8, number of radiolabeled cells of *A. tumefaciens* B6 bound to root tips preincubated with nonlabeled *A. radiobacter* K84 at 1.6×10^8 cells per ml for 2 h.

60, and 120 min after incubation. For SEM, root tips from attachment assays were fixed in Karnovsky's fixative (1.25% [wt/vol] glutaraldehyde and 1% paraformaldehyde in 0.12 M phosphate buffer [pH 7.2 to 7.4]) for 4 to 12 h, washed twice for 15 min in phosphate-buffered saline (PBS), fixed in 1% (wt/vol) osmium tetroxide for 2 h, and washed twice in PBS for 15 min. The root tips were dehydrated through an ethanol gradient and an amyl-acetate series prior to critical-point drying under amyl-acetate. The dried material was then mounted on SEM stubs, which were sputter coated with gold-palladium prior to viewing.

When *A. tumefaciens* B6 cells were incubated with tomato root tips, a quick attachment kinetic was observed (Fig. 1). No significant differences were observed in the extent of B6 binding between 0.5 and 2 h of incubation. The number of cells of strain B6 attached to root tips was significantly higher than that of *E. coli* used as a negative control to determine the background binding (data not shown). The number of K84 cells bound to tomato root tips after 5 min of coincubation was similar to that observed for *E. coli*. However, after 15 min of coincubation, the number of cells of strain K84 attached to root tips was significantly higher than that of *E. coli* (data not shown). Binding of strain K84 to tomato root tips increased with an increasing number of bacteria added. Comparative

attachment of *A. tumefaciens* B6 and *A. radiobacter* K84 to tomato root tips is shown in Fig. 1. When the attachment of strains B6 and K84 to root tips was compared at 1.4×10^6 versus 1.1×10^6 cells per ml, the extent of binding of B6 was significantly greater than that of K84 after 30 min of coincubation (Fig. 1a). The extent of binding of B6 was also greater than that of K84 in comparisons of 3.4×10^6 versus 3.9×10^6 cells per ml after 2 h of coincubation (Fig. 1b), showing that *A. tumefaciens* B6 attached to root tips with greater efficiency than K84.

Light microscopy and SEM observations confirmed in vitro binding of strains B6 and K84 to tomato root tips. Bacteria were observed attached to the root surface and also to the root hairs (Fig. 2). Frequently, large clusters of bacteria were associated with the root surface and root hairs (Fig. 2c, d, and e), whereas bacteria incubated in fresh buffer did not aggregate (Fig. 2a). Both bacteria were not bound uniformly through the root surface, and free areas seemed to be still available for further binding (Fig. 2e and f).

The attachment kinetics of radiolabeled *A. tumefaciens* B6 to tomato root tips preincubated with nonlabeled *A. radiobacter* K84 or buffer for 2 h is shown in Fig. 3. Attachment of strain B6 to tomato root tips at a concentration of 5.9×10^6 cells per ml was not affected by the preincubation of the root tips with

nonlabeled K84 suspension of 10^6 , 10^7 , or 10^8 cells per ml for 2 h.

Agrobacterium binding to plant tissues is exceedingly complicated, and it is influenced by experimental conditions, plant hosts, and tissues and not so simple as first envisioned (23). Binding of *A. tumefaciens* to plant cells has generally been quantified by viable-cell counts for low bacterial concentrations (fewer than 10^4 cells per ml) and by detecting radioisotope-labeled attached bacteria for experiments involving high concentrations of bacteria (23). In this study, tomato root tips were preferred to cultured cells in an attempt to simulate natural infection conditions. The attachment of both strains B6 and K84 to tomato root tips increased with increasing numbers of bacteria added, as reported earlier for *A. tumefaciens* (2, 18, 19). At similar bacterial concentrations, the levels of B6 cells attached to root tips after 2 h of coinocubation were higher than those of K84. In a different study, using grape callus cells, strain K84 attached to plant cells with lower efficiency than the pathogenic strain, and in that case, K84 scarcely attached to grape cells after 2 h of incubation (22). We show that the preincubation of tomato root tips with nonlabeled *A. radiobacter* K84 had no effect on the subsequent attachment of radiolabeled *A. tumefaciens* B6.

Our *in vitro* attachment results show that strain K84 is not capable of blocking the binding of *A. tumefaciens* B6 to tomato root tips. Competition between strain K84 and *A. tumefaciens* for root surface attachment seems not to be a mechanism that can explain the effectiveness achieved by strain K84 in controlling crown gall on tomato incited by strain B6. The bacterial concentrations used in this work were similar to those observed in biocontrol experiments. Strains B6 and K84 established populations averaging 10^4 to 10^6 and 10^6 CFU/g of root, respectively, 8 months after inoculations in biocontrol assays (20). Furthermore, the presence of strain K84 in the roots did not affect the ability of strain B6 to colonize the rhizosphere (20a). This suggests that the efficiency in the biocontrol of agrocin 84-resistant strains by K84 is not related to the competition for binding to the plant root surface. The fact that strain K84 does work at the commercial level makes it a particularly attractive system in which to identify the relevant components (9).

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