Colonization of Tomato Plants by Two Agrocin-Producing Strains of *Agrobacterium tumefaciens*

SHARMANE MACRAE,^{1,2} JENNIFER A. THOMSON,^{3*} AND JOHANNES VAN STADEN²

Council for Scientific and Industrial Research, Division of Processing and Chemical Manufacturing Technology, P.O. Box 395, Pretoria 0001,¹ University of Natal Council for Scientific and Industrial Research Unit for Plant Growth and Development, Department of Botany, University of Natal, Pietermaritzburg 3200,² and Department of Microbiology, University of Cape Town, Rondebosch 7700,³ South Africa

Received 18 May 1988/Accepted 28 September 1988

For a bacterium to be a successful biocontrol agent against crown gall disease, it must produce an effective agrocin specific for *Agrobacterium tumefaciens* and be able to colonize host plants efficiently. The colonization abilities of K84 and J73, successful and potential biocontrolling strains, respectively, were compared both in vivo and in vitro. Both strains produced fibrils attaching them to tomato root surfaces and had similar colonization efficiencies up to 14 days after inoculation. However, the ability of J73 to colonize plants for longer periods was significantly less than that of K84. Thus, the presence of fibrils is not sufficient to ensure colonization. No correlation was found between hydrophobicity and colonization.

Successful biological control of crown gall disease of plants by means of nonpathogenic *Agrobacterium tumefaciens* strain K84 has been reported for a variety of susceptible plants (9). Biocontrol by strain K84 is due to its production of an agrocin which is toxic to *A. tumefaciens* pathogens carrying an agrocinopine type Ti plasmid. Its effectiveness is also enhanced because of the fact that it colonizes plants efficiently. This was shown by Ellis et al. (5), who transferred the agrocin plasmid, pAgK84, to *A. tumefaciens* C58C1 and found that one of the transconjugants failed to control crown gall induction. This failure was correlated with poor growth of the organism in the inoculation sites, and it was concluded that a biological control agent must have two properties; it must be able to produce sufficient amounts of an effective antibiotic substance, and it must be able to colonize the plant efficiently. It was pointed out that it might be difficult to find a new biological control agent that combines both these requirements.

In view of the fairly narrow spectrum of *A. tumefaciens* sensitivity to K84, which is limited to pathogenic *A. tumefaciens* biotype 1 and 2 strains harboring an agrocinopine Ti plasmid, it was decided to search for agrocin-producing strains with wider host ranges. One such strain is J73, which synthesizes an agrocin active against strains from all three biotypes of *A. tumefaciens* regardless of whether they carry a Ti plasmid (16). Although J73 was originally pathogenic, it has been cured of its Ti plasmid (J. Webster and J. A. Thomson, Mol. Gen. Genet., in press) and greenhouse trials have been performed to determine its ability to protect plants from crown gall disease. Preliminary experiments indicated that in those cases where strains of *A. tumefaciens* are sensitive to both K84 and J73, K84 was the more effective biocontrol agent. As both strains produce comparable levels of agrocin in vitro (16), it was postulated that differences in biological control may be accounted for by different plant colonization efficiencies. This paper reports on the results of colonization studies carried out on tomato seedlings.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. *A. tumefaciens* K84 and J73 are both biotype 2, agrocin-producing strains (9, 16). Spontaneous antibiotic-resistant mutants were isolated to facilitate selection; K84 Nal and J73 Rif were resistant to nalidixic acid and rifampin, respectively, at levels of 100 µg/ml. All strains were grown in GT medium (3) at 25°C.

Colonization in vivo. Tomato seeds or seedlings were inoculated with the antibiotic-resistant strains by incubation for 20 min at room temperature with late-logarithmic-phase bacterial suspensions adjusted turbidometrically to densities of approximately 10⁸ CFU/ml. The seeds and seedlings were removed from the bacterial suspensions without rinsing and planted in nonsterile soil. At various time intervals, the seedlings were dug up and loosely adhering soil was removed. The roots were cut off at the point of seed attachment, and the mass of the roots and closely adhering soil was determined. The colonization efficiencies of the stem, rhizosphere (zone of soil closely associated with the root which is influenced by root exudates), and rhizoplane (root surface) were determined. The roots and closely adhering soil were washed, placed in 10 ml of 0.25× Ringer solution (1× Ringer solution is 2.25 g of NaCl, 0.105 g of KCl, 0.06 g of CaCl₂, 0.05 g of NaHCO₃ per liter) and shaken vigorously for 5 s. The roots were removed, dried between filter paper, and weighed. This gave the fresh mass of the roots. The mass of the rhizosphere soil was obtained by determining the difference. The rhizosphere bacteria were enumerated by plating on the appropriate antibiotic-containing GT medium (100 µg of nalidixic acid per ml for K84 and 100 µg of rifampin per ml for J73). Data were expressed as CFU per gram of rhizosphere soil. The roots were then placed in 10 ml of 0.25× Ringer solution in vessels containing four glass beads and shaken vigorously for 15 min on a wrist-action shaker, and the released rhizoplane bacteria were enumerated as described above. Data were expressed as CFU per gram of fresh mass of root. Bacterial colonization of the stem was determined as for the rhizoplane by using 40-mm stem segments and taken from the point of seed attachment. The fresh mass was determined, and data were expressed as CFU per gram. In all cases, six replicates for each treatment were run.

* Corresponding author.

FIG. 1. In vivo colonization of tomato seedlings by J73 (□) and K84 (▨). The bars above the histograms indicate standard deviations, and the letters indicate statistically significant differences; bars bearing the same letter indicate that there is no statistical difference between those values. RS, Rhizosphere; RP, rhizoplane; S, stem.

Colonization in vitro. Two methods to assay in vitro colonization were used. In the first, surface-sterilized seeds (sterilized by suspension in 3.5% [wt/vol] sodium hypochlorite for 10 min, followed by three washes in sterile distilled water) or sterile seedlings (grown aseptically in petri dishes from sterile seeds on sterile filter paper premoistened with distilled water) were aseptically inoculated with the test strains described earlier. These were planted in sterile test tubes containing sterile soil moistened with 0.5× Hoagland solution (7) and incubated in a growth room with a 16-h light/8-h dark cycle at 22°C. At various time intervals, rhizosphere, rhizoplane, and stem colonization efficiencies were determined as described. In the second method, the in vitro colonization of tomato roots in liquid culture (6) was determined by aseptically excising 15-mm root tip segments from tomato root stocks, inoculating with the test strains by dipping, and incubating at 25°C in 7 ml of Miller medium (12) in the light with agitation at 80 rpm. At intervals, the root segments were aseptically removed and the rhizoplane colonization efficiency was determined. Six replicates were run for each treatment.

Statistical analysis of data. Analysis of variance of data collected from the various tests was carried out by using the Stat Graphics computer program. Together with this, the Duncan multiple range test (4) was used to determine whether there were statistically significant differences in the data obtained.

Scanning electron microscopy. Root material was fixed overnight in 3% (wt/vol) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2), washed twice for 30-min durations in 0.05 M cacodylate buffer, fixed in 2% (wt/vol) osmium tetroxide for 2 h, and washed in 0.05 M cacodylate buffer for 30 min. The root material was dehydrated through an ethanol gradient series prior to critical-point drying under amyl acetate. The dried material was then mounted on scanning electron micrograph stubs, which were sputter coated with gold-palladium prior to viewing.

Hydrophobicity assay. The hexadecane assay based on the assay of Rosenberg et al. (13) was used. The bacteria were grown in GT medium at 25°C to late log phase (10⁷ to 10⁸ CFU/ml), harvested, washed twice in PUM buffer (pH 7.2)

(16.95 g of K₂HPO₄ per liter, 7.26 g of KH₂PO₄ per liter, 1.8 g of urea per liter, 0.2 g of MgSO₄ per liter), and suspended in buffer to an A₄₃₆ of 0.6. Samples (3 ml) of the bacterial suspension were dispensed into test tubes, and 100-μl aliquots of hexadecane were added. The tubes were vigorously agitated for 1 min on a vortex mixer and allowed to stand for 15 min. The aqueous phase was removed, and the A₄₃₆ was determined. Six replicate samples and controls containing no hexadecane were prepared for each bacterial sample, and the test was repeated three times. Hydrophobicity was expressed as the percentage of cells bound to hexadecane.

RESULTS

In vivo colonization. In vivo colonization of tomato seedlings was tested on a daily basis for 14 days after inoculation. Both strains colonized with similar efficiencies during this period (results not shown). However, by 28 and 65 days after inoculation, the numbers of K84 had either remained constant or increased slightly, while those of J73 had decreased by up to 2 orders of magnitude (Fig. 1). There was no statistically significant difference in the K84 population levels between 14 and 28 days. However, there was a significant decrease in all of the J73 populations between 14 and 28 days. Colonization of seedlings grown from inoculated seeds (Fig. 2) confirmed the relative inefficiency of J73 at 25 days after inoculation.

In vitro colonization. In vitro assays in sterile soil again showed that J73 could colonize efficiently in the early stages but that by 28 days numbers had declined by 3 to 4 orders of magnitude (Fig. 3). Colonization of seedlings grown from inoculated seeds confirmed the relative inefficiency of J73 at 25 days after inoculation (Fig. 4).

In vitro colonization of roots in liquid culture. To determine whether the poor colonizing ability of J73 was due to its inability to adhere to the tomato seedlings, in vitro colonization in liquid culture was studied. It was only possible to maintain the cultures for 10 days, but during this time there was no significant difference between the two strains (results not shown). Scanning electron micrographs showed the presence of attachment strands from both J73 and K84 onto

FIG. 2. In vivo colonization of tomato seedlings grown from inoculated seeds by J73 (□) and K84 (▨). The bars above the histograms and abbreviations are as for Fig. 1.

the surface of the tomato roots (Fig. 5). These were first observed at 48 h after inoculation. The bacteria attached mainly in and around the root hair region, covering approximately 10% of the surface.

FIG. 4. In vitro colonization of tomato seedlings grown in sterile soil under sterile conditions from inoculated seeds by J73 (□) and K84 (▨). The bars above the histograms and abbreviations are as for Fig. 1.

FIG. 3. In vitro colonization of tomato seedlings grown in sterile soil under sterile conditions by J73 (□) and K84 (▨). The bars above the histograms and abbreviations are as for Fig. 1.

FIG. 5. Scanning electron micrographs showing attachment strands between bacteria and tomato roots.

Hydrophobicity assays. A number of studies have suggested a relationship between bacterial hydrophobicity and adherence (17). However, the hexadecane binding assay indicated that there was no significant difference between K84 and J73 (results not shown).

DISCUSSION

The first step in the colonization process is site-specific attachment of bacteria to the plant. Cellulose fibrils produced by *A. tumefaciens* and *Rhizobium leguminosarum* have been implicated in aspects of this process (10, 15). The lack of these fibrils does not render bacteria noninfective, and the extracellular polysaccharide products of chromosomal genes *chv* and *exoC* (1, 2), as well as some unidentified surface polypeptides (11), are required for these processes. However, cellulose fibrils may be important in colonization by biocontrolling bacteria.

The results reported in this paper from both in vivo and in vitro colonization of tomato seedlings, whether by seed or seedling inoculation, showed that in all cases J73 was significantly less efficient than K84. Up to 14 days after inoculation, however, there was no difference between the two strains. Scanning electron micrographs showed the presence of attachment strands from the bacteria onto the tomato root surfaces during a 10-day period. This suggests that J73, while attaching efficiently with the aid of fibrils, is unable to maintain this initial colonization. Thus, while fibrils may be important in the initial attachment of bacteria to plants, their presence is not sufficient to ensure longer-term colonization. The fact that both in vivo and in vitro assays gave similar results indicates that the poor colonization by J73 in nonsterile soil was not due simply to competition with indigenous bacteria but was due to some property inherent in this strain.

The numbers of bacteria colonizing the rhizoplane in liquid culture rose from approximately 2×10^3 to 4×10^3 CFU/cm of root after 1 day to approximately 2×10^4 after 10 days, an increase of 5- to 10-fold. This is not surprising, as James et al. (8) found that a number of rhizobacteria, including *A. tumefaciens*, adhered very rapidly to roots. The rhizobacteria reached half-maximum adherence by 5 min, and after 25 days James et al. observed an increase in colonization of 67-fold.

Contradictory results exist for the role played by hydrophobic properties of cell surfaces in bacterial adhesion (8, 14). Hydrophobic interactions may stabilize the adhesive reaction, and in some instances, loss in hydrophobicity has been associated with loss of the ability to adhere. Results from this study support the findings of James et al. (8), as both K84 and J73 had similar hydrophobic properties. This rules out any major role of hydrophobic interactions in the colonization process.

ACKNOWLEDGMENTS

We acknowledge the staff of the Electron Microscope Unit, University of Natal, Pietermaritzburg, for their technical assistance, Richard Beckett for help with the statistical analysis of data, and Jocelyn Webster for helpful discussions.

LITERATURE CITED

1. Cangelosi, G. A., L. Hung, V. Puvanesarajah, G. Stacey, D. A. Ozga, J. A. Leigh, and E. W. Nester. 1987. Common loci for *Agrobacterium tumefaciens* and *Rhizobium meliloti* exopolysaccharide synthesis and their roles in plant interactions. *J. Bacteriol.* 169:2086-2091.
2. Douglas, C. J., R. J. Staneloni, R. A. Rubin, and E. W. Nester. 1985. Identification and genetic analysis of *Agrobacterium tu-*

- mefaciens* chromosomal virulence region. J. Bacteriol. **161**:850–860.
3. Douglas, J., A. Qanber-Agha, and V. Phillips. 1974. Medium for the propagation and assay of lactic and other phages. Lab. Pract. **23**:2–4.
 4. Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics **11**:1–42.
 5. Ellis, J. G., A. Kerr, M. van Montagu, and J. Schell. 1979. *Agrobacterium*: genetic studies on agrocin 84 production and biological control of crown gall. Physiol. Plant Pathol. **15**:311–319.
 6. Finnie, J. F., and J. van Staden. 1985. Effect of seaweed concentrate and applied hormones on *in vitro* cultured tomato roots. J. Plant Physiol. **120**:215–222.
 7. Hoagland, D. R., and D. I. Arnon. 1939. The water-culture method for growing plants without soil. California Agricultural Experiment Station circular no. 347. California Agricultural Experiment Station.
 8. James, D. W., T. V. Suslow, and K. E. Steinback. 1985. Relationship between rapid, firm adhesion and long-term colonization of roots by bacteria. Appl. Environ. Microbiol. **50**:392–397.
 9. Kerr, A. 1980. Biological control of crown gall through production of agrocin 84. Plant. Dis. **64**:25–30.
 10. Matthysse, A. G. 1983. Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. J. Bacteriol. **154**:906–915.
 11. Matthysse, A. G. 1987. Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **169**:313–323.
 12. Miller, C. O. 1965. Evidence for the natural occurrence of zeatin and derivatives: compounds from maize which promote cell division. Proc. Natl. Acad. Sci. USA **54**:1052–1058.
 13. Rosenberg, M., D. Gutnick, and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9**:29–33.
 14. Rutter, P. R., and B. Vincent. 1980. The adhesion of microorganisms to surfaces: physico-chemical aspects, p. 79–92. In R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (ed.), Microbial adhesion to surfaces. Ellis Horwood, Ltd., Chichester, United Kingdom.
 15. Smit, G., J. W. Kijne, and B. J. J. Lugtenberg. 1987. Involvement of both cellulose fibrils and a Ca^{2+} -dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. J. Bacteriol. **169**:4294–4301.
 16. Webster, J., and J. A. Thomson. 1986. Agrocin-producing *Agrobacterium tumefaciens* strain active against grapevine isolates. Appl. Environ. Microbiol. **53**:217–219.
 17. Weis, E., M. Rosenberg, H. Judes, and E. Rosenberg. 1982. Cell surface hydrophobicity of adherent oral bacteria. Curr. Microbiol. **7**:125–128.