Interactions of Wild-Type and a Cellulose-Minus Mutant of *Agrobacterium tumefaciens* with Tobacco Mesophyll and Tobacco Tissue Culture Cells

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ABSTRACT

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The interactions of *Agrobacterium tumefaciens* wild-type strain A6 and *A. tumefaciens* strain Ce-12, a cellulose-minus transposon mutant of A6, with tobacco mesophyll cells, tobacco suspension culture cells, and tobacco callus cells were examined by using scanning electron microscopy. Bacteria of both strains were seen in association with tobacco cell walls of all three cell types within 90 min of inoculation. Cells of *A. tumefaciens* strain A6 synthesized cellulose fibrils during their attachment to tobacco suspension culture cells. Such fibrils served to entrap nonattached agrobacteria and resulted in the formation of large clumps of bacteria attached to the tobacco cell surface. A lesser, but still substantial, number of fibrils was formed by *A. tumefaciens* which were associated with tobacco callus cells. A more modest number of fibrils was formed by bacteria associated with tobacco mesophyll cells. Thus, exposure to increasingly wet environments resulted in increased fibril formation by *A. tumefaciens* wild-type strain A6. No fibrils were formed by, or in response to, cells of *A. tumefaciens* strain Ce-12 even 17.5 hr after inoculation. This indicates that all fibrils formed were probably of bacterial origin.

Additional key words: *Nicotiana tabacum*.

Crown gall disease results from the infection of dicotyledonous plants with *Agrobacterium tumefaciens* (Smith & Townsend) Conn. Crown gall is worldwide in distribution and can infect many species of woody and herbaceous plants. During the early stages of infection, bacteria transfer tumor-inducing (pTi) plasmid DNA to the host plant cells. Attachment of bacteria to specific sites in the wounded plant may be required for infection (3). Work in our laboratory has demonstrated that during attachment of *A. tumefaciens* strain A6 to carrot (*Daucus carota* L.) suspension culture cells (5) and carrot protoplasts (6), bacteria synthesize cellulose fibrils which entrap other bacteria. The aggregates of entrapped and attached bacteria eventually become so extensive that they bind the tissue culture cells into large clusters (5).

Cellulose production by *A. tumefaciens* strain A6 is not required for virulence (4). Mutants with altered ability to synthesize cellulose were constructed by introducing the transposon Tn5 into the genome of *A. tumefaciens* strain A6 (4) by the method of Beringer et al (1). Such bacterial mutants were able to produce tumors on *Bryophyllum daigremontianum* (Hamet & Perr.) A. Berger and tobacco (*Nicotiana tabacum* L.) but did not make cellulose. The ability of the cellulose-minus mutants to induce tumors in *B. daigremontianum* was reduced by washing the inoculation site with water. Washing with water did not affect the ability of the parent strain to induce tumors (4).

The interesting phenomenon of cellulose production associated with attachment of *A. tumefaciens* to carrot suspension culture cells and protoplasts stimulated our interest in examining other cell types. Cultivated tobacco was chosen as it is susceptible to crown gall and because it is possible to compare cells from intact plants with tissue culture cells. We included the cellulose-minus mutant *A. tumefaciens* strain Ce-12 in our study so that we could distinguish fibrils formed by the bacteria from any that might be formed by the host plant cells.

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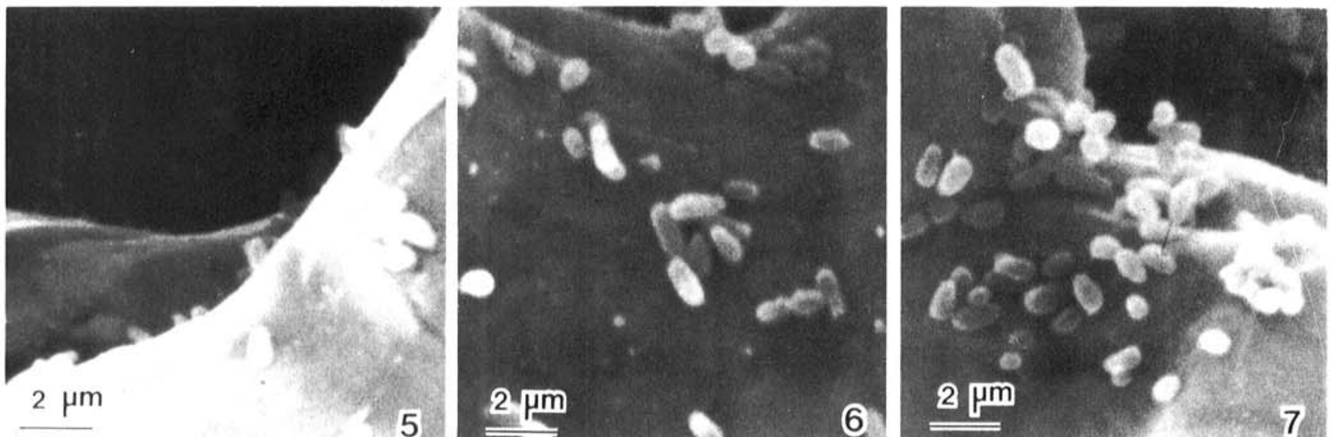
We report here a direct microscopic comparison of the attachment of *A. tumefaciens* strains A6 and Ce-12 to tobacco leaf mesophyll cells, agar-grown tobacco callus cells, and tobacco suspension culture cells.

MATERIALS AND METHODS

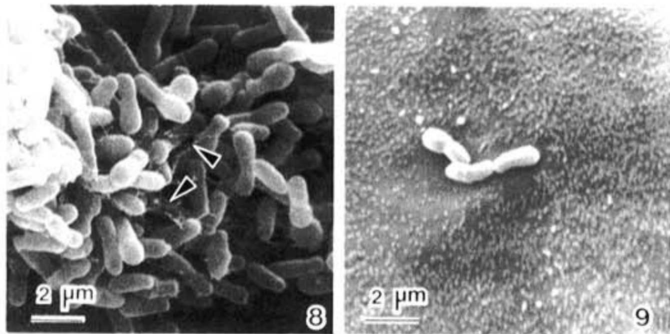
Cultures. *A. tumefaciens* A6 was obtained from A. Braun, Rockefeller University, New York, NY. *A. tumefaciens* Ce-12 is a cellulose-minus mutant of *A. tumefaciens* A6 obtained in our laboratory (4). *A. tumefaciens* strains were maintained as previously described (4,5). Bacteria used for inoculation showed few, if any, fibrils and were not aggregated (5). The transfer of the bacteria from Luria broth to plant tissue culture medium represents a nutritional "shift down." The bacteria enter a lag phase and there is no increase in bacterial number for the first 2–3 hr after inoculation with the bacteria (7). After this lag phase, the bacteria grow with a doubling time of between 90 and 150 min, depending on the medium and temperature (A. G. Matthysse, *unpublished*). Suspension cultures of *N. tabacum* 'Wisconsin 38' were a gift from S. Flashman, North Carolina State University. Suspension culture cells were grown on a rotary shaker in Murashige and Skoog (8) medium containing the following amounts of plant hormones (per liter): 3 mg of indoleacetic acid, 0.3 mg of dimethylallylaminopurine, 0.3 mg of *p*-chlorophenoxyacetic acid, and 0.1 mg of 2,4-dichlorophenoxyacetic acid (S. Flashman, *personal communication*). Tobacco callus cells were grown from *N. tabacum* Coker 319, on Murashige and Skoog agar medium, containing 2 mg of kinetin and 5 mg of naphthaleneacetic acid per liter. Tobacco plants (*N. tabacum* 'Coker 319') were grown in individual pots under ordinary greenhouse conditions.

Studies with tobacco mesophyll. Cultures of *A. tumefaciens* strains A6 and Ce-12 were grown overnight in Luria broth. Bacteria were harvested by centrifugation at 10,000 *g* for 10 min. Cells were resuspended in distilled water, transferred to 0.5-ml tuberculin syringes and injected into the underside of leaves of a young tobacco plant. Leaf tissue was sampled before injection and at the following times after injection: 30 min, 90 min, and 17.5 hr. Leaf disks were collected with a cork borer and fixed overnight in 2% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2). The disks were rinsed twice in 0.1 M sodium cacodylate and then

Figs. 1–4. Scanning electron micrographs of mesophyll from uninfected tobacco leaf and mesophyll from tobacco leaves injected with wild-type, cellulose-positive *Agrobacterium tumefaciens* strain A6. **1,** Tobacco leaf mesophyll (×2,400). **1a (inset),** Higher magnification of mesophyll cell surface at the junction of two cells (×5,200). **2,** Strain A6 bacteria associated with mesophyll cell surface, 30 min after injection (×5,200). **3,** Chain of bacteria bridging an open space 90 min after injection (×5,200). **4,** Cluster of bacteria held together by cellulose fibrils (arrows), 90 min after injection (×5,200). The surface of uninoculated mesophyll cells was smooth. No fibrillar material was visible. Very few fibrils were seen 30 min after injection with A6. After 90 min, fibrils were readily visible.



Figs. 5–7. Scanning electron micrographs of mesophyll from tobacco leaves injected with the cellulose-minus transposon mutant, *Agrobacterium tumefaciens* strain Ce-12. Bacteria associated with tobacco mesophyll: **5,** at 30 min after injection (×5,200); **6,** at 90 min after injection (×5,200); and **7,** at 17.5 hr after injection (×5,200). No cellulose fibrils were visible at any time after the injection of strain Ce-12, even after prolonged incubation (17.5 hr).



Figs. 8–9. Scanning electron micrographs of tobacco callus cells inoculated with **8,** cellulose-positive *Agrobacterium tumefaciens* strain A6 or **9,** its cellulose-minus transposon mutant *A. tumefaciens* Ce-12, both at 8 hr after inoculation (×3,900). Fibrils were visible surrounding cells of strain A6 (arrows), but no fibrils were seen surrounding those of strain Ce-12.

postfixed in 1% osmium tetroxide (OsO_4) in the same buffer for at least 4 hr. After two buffer rinses, the leaf disks were placed in Flo-Thru specimen capsules (Martin Instrument Co., Greenville, SC), dehydrated in an acetone series and critical-point dried. Dried leaf disks were placed on a stub covered with double-stick tape. Another stub covered with the same tape was pressed onto the leaf disk and then removed. In this way, the epidermal surfaces of the leaf became attached to the tape and the mesophyll was revealed. This technique was modified from one described by Sigee and Al-Issa (9). Leaf disks were coated for 30 sec with gold-palladium and were examined with an ETEC Autoscan scanning electron microscope.

Studies with tobacco callus cells. Callus cultures growing on agar medium were inoculated by pipetting 0.5 ml of a stationary phase culture of bacteria directly onto the callus surface. Callus samples were fixed before inoculation and at the following times after inoculation with bacteria: 30 min, 90 min, 4 hr, and 8 hr. Callus cells were processed for observation by scanning electron microscopy as

Figs. 10–15. Scanning electron micrographs of uninfected tobacco suspension culture cells (Fig. 10) and cells inoculated with wild-type, cellulose-positive *Agrobacterium tumefaciens* strain A6 (Figs. 11–13), or its cellulose-minus transposon mutant *A. tumefaciens* strain Ce-12 (Figs. 14 and 15). **10,** Surface of a suspension culture cell (×5,200). **11,** A6, suspension culture cell with associated bacteria, 90 min after inoculation (×5,200). **12,** A6, cluster of bacteria 90 min after inoculation; arrows denote fibrils (×5,200). **13,** A6, 8 hr after inoculation; arrows denote fibrils (×5,200). **14,** Ce-12, 4 hr after inoculation (×5,200). **15,** Ce-12, 8 hr after inoculation (×5,200). Very few fibrils were visible immediately after inoculation with strain A6. After 90 min of incubation, fibrils were visible in some areas. At 8 hr, large bacterial clusters with associated fibrils were visible. No fibrils were seen in cultures incubated with strain Ce-12, although some microcolonies were visible after 4 hr.

previously described (4).

Preparation of tobacco suspension culture cells. About 10^7 bacteria per milliliter were added to a suspension containing 80 mg of tobacco culture cells per milliliter. Suspension culture cells were sampled before inoculation and at the following times after inoculation: 30 min, 90 min, 4 hr, and 8 hr. Cells were processed for observation by scanning electron microscopy as previously described (4).

RESULTS

Tobacco mesophyll. Preparation of tobacco leaf tissue by the method we describe is very useful in that the surfaces of many mesophyll cells can be examined (Fig. 1).

At 30 min after injection, bacteria were seen in association with the mesophyll surface (Figs. 2 and 5). By 90 min after injection, strain A6 had synthesized a small number of fibrils that tended to bind bacteria into small clusters and allow bacteria to bridge open spaces between tobacco cells (Figs. 3 and 4). Bacterial cellulose fibrils were formed less frequently than with callus or suspension culture cells at 5 and 17.5 hr after injection. *A. tumefaciens* Ce-12 made no fibrils during the experiment (Figs. 5–7) and neither did the tobacco mesophyll cells. The *A. tumefaciens* Ce-12 cells may have undergone cell division while inside the leaf. Single bacteria were present at 30 min and 90 min after injection, but microcolonies appeared at the later times. Neither strain of *A. tumefaciens* showed any preference for specific locations on the mesophyll cells. A number of bacteria tended to accumulate in crevices such as those between adjacent tobacco cells (*unpublished*). Such accumulation may be due to the drying down of the liquid in which the bacteria were suspended during injection (2). Tumors developed in injected leaves after 2–3 wk indicating that at least some of the injected bacteria were attached to the plant cells in a manner which allowed the transfer of Ti plasmid DNA.

Tobacco callus cells. By 30 min after inoculation, *A. tumefaciens* strains A6 and Ce-12 were both associated with the tobacco callus cell surface (*unpublished*). Some of the cells of *A. tumefaciens* A6 had synthesized a small number of fibrils by 90 min. However, fibrils were not prevalent at this time. Fibrils were more evident by 4 and 8 hr after inoculation (Fig. 8). *A. tumefaciens* Ce-12 was found associated with the tobacco callus cells infrequently and did not form or induce any fibrils during the experiment (Fig. 9).

Tobacco suspension culture cells. Attachment of *A. tumefaciens* strains A6 and Ce-12 occurred within 90 min after exposure to tobacco suspension culture cells (Figs. 10 and 11). By this time, some of the strain A6 bacteria had synthesized a small number of fibrils (Fig. 12), which served to trap bacteria in small clusters and formed bridges between cells. By 8 hr after inoculation, large clusters of bacteria were visible and large aggregates of attached and enmeshed bacteria had almost covered the cells (Fig. 13).

Attachment of *A. tumefaciens* strain Ce-12 was infrequent at 30 min after inoculation. By 90 min after inoculation, several bacteria were attached to the same tobacco cells (*unpublished*). By 4 hr after inoculation, small clusters of bacteria were present on the tobacco cells (Fig. 14). The clusters of bacteria seemed larger by 8 hr after inoculation (Fig. 15). This phenomenon may be indicative of bacterial growth. No fibrils were seen at any time with strain Ce-12.

DISCUSSION

Cells of *A. tumefaciens* A6 formed fibrils during interactions with all three tobacco cell types. However, no fibrils were formed by *A. tumefaciens* Ce-12 under the same circumstances. As these two strains are isogenic except for the inability of Ce-12 to form fibrils, it is probable that all fibrils seen in association with the cells of *A. tumefaciens* A6 were produced by the bacteria and not by the plant cells.

Fibril formation by *A. tumefaciens* strain A6 varied with the situation in which bacteria were tested. The largest amount of fibrils was formed by bacteria incubated with suspension culture cells. An intermediate amount was formed by bacteria incubated with callus cells. The least amount of fibrils was formed by bacteria injected into leaves. Thus, the availability of free water and dissolved nutrients may have an effect on cellulose production by wild-type *A. tumefaciens*. Under conditions in which water and nutrients are diminished such as inside tobacco leaves, the wild type and the cellulose-minus mutant appear to have similar opportunities to become associated with the mesophyll cell walls. However, under conditions of extreme moisture, the wild type has the advantage of being able to anchor itself to the plant cell surface.

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