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Elaboration of Cellulose Fibrils by *Agrobacterium tumefaciens* During Attachment to Carrot Cells

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The attachment of virulent strains of *Agrobacterium tumefaciens* to plant cells is the first step in the bacterial induction of tumors. Binding of *A. tumefaciens* to carrot tissue culture cells occurred as a two-step process. The initial step was the attachment of the bacteria to the plant cell wall. Living plant cells were not required. Bacterial attachment to heat-killed or glutaraldehyde-fixed carrot cells proceeded with only slightly altered kinetics and unaltered bacterial strain specificity. After the bacteria bound to the carrot cell surface, scanning electron microscopy showed that fibrils developed, surrounded the bacteria, and anchored them to the plant cell surface. These fibrils were synthesized by the bacteria and not by the plant cell since they were also made after the attachment of *A. tumefaciens* to dead carrot cells and since under some conditions the bacteria synthesized fibrils in the absence of plant cells. Calcofluor staining, acid hydrolysis, enzymatic digestion studies, and infrared spectroscopy showed that the fibrils were composed of cellulose. The formation of these cellulose fibrils occurred during the attachment of virulent strains of *A. tumefaciens* to plant cells in vitro. The fibrils anchored the bacteria to the plant cell surface and entrapped additional bacteria. The multiplication of entrapped and attached bacteria resulted in the formation of large clusters of bacteria held close to the plant cell wall and plasma membrane by cellulose fibrils. This high concentration of bacteria may facilitate transfer of Ti plasmid deoxyribonucleic acid to the plant cell resulting in the formation of tumors.

Crown gall disease of dicotyledenous plants is the only known example of a bacterially induced tumor (15). The tumors result from the extracellular infection of a wound site by *Agrobacterium tumefaciens* (15). Virulent strains of *A. tumefaciens* harbor a large tumor-inducing (Ti) plasmid (34, 35, 39). During the course of infection, the Ti plasmid is transferred from the bacterium to the plant host cell in which a portion of the Ti DNA is maintained and transcribed (4, 10, 17, 33). The mechanism of this transfer is unknown.

The first step in tumor formation in vivo is the site-specific attachment of *A. tumefaciens* to the plant (13). We have demonstrated specific attachment of *A. tumefaciens* to tissue culture cells. The attachment is dependent on the presence of the bacterial Ti plasmid (18). This manuscript reports further studies of bacterial attachment to tissue culture cells and describes the development of fibrils attaching the bacteria to the host cell wall. Evidence is presented that these fibrils are composed of cellulose elaborated by the bacterium.

MATERIALS AND METHODS

Bacteria were grown, and viable cell counts were determined as previously described (18). Sources of virulent strains of *A. tumefaciens* A6, C58 and B6, avirulent strains NT1 and IIBNV6, and avirulent *Agrobacterium radiobacter* strains 6467, S1005, and TR1 were the same as previously listed (18). *Escherichia coli* 23739 was obtained from the American Type Culture Collection. Suspension cultures of *Daucus carota* were obtained from Wendy Boss, North Carolina State University. They were maintained in Murashige and Skoog medium (20) with weekly transfers.

Attachment studies were carried out by adding a suspension of bacteria to the carrot cell cultures. The bacteria had been well dispersed by Vortex mixing. The percentage of the bacterial inoculum attached was determined as previously described (18). A known concentration of bacteria was introduced into the carrot cell suspension. Samples were removed at various times and filtered through Miracloth (Calbiochem), which allowed the passage of bacteria but not whole plant cells. The concentration of free bacteria was determined by a viable cell count of the filtrate. For some experiments, the concentration of attached bacteria was determined by a viable cell count of a ho-

mogenate of the carrot cells. For all experiments 1×10^5 to 2×10^5 carrot cells per ml were used. For kinetic experiments, bacteria were added to the carrot cell suspension to a final concentration of 1×10^3 to 3×10^3 bacteria per ml. For microscopic observations, bacteria were added to a final concentration of 2×10^6 to 10×10^6 bacteria per ml.

For scanning electron microscopy, plant cells incubated with or without bacteria inoculated were fixed after 0, 0.5, 1.5, 4, 8, and 19 h. Cells were fixed in 1% glutaraldehyde for 1 to 5 days and postfixed in 1% osmium tetroxide for 4 to 16 h. Cells in Flow-Thru specimen capsules (Martin Instrument Co.) were sequentially dehydrated in aqueous solutions of 30, 50, 70, 90, 95, and 100% acetone and dried in a Polaron critical-point drying apparatus with liquid CO_2. The cells were attached to aluminum specimen holders with Microstick (Ted Pella Co.), coated in a Ladd rotary coater with about 10 nm of 60% gold–40% palladium, and examined with a JEOL JSM 35U scanning electron microscope with a tungsten filament at 25 kV.

For some attachment studies, carrot cells were killed by heating at 65°C for 20 min. They were collected on a Miracloth (Calbiochem) filter and washed twice with 10 ml of Murashige and Skoog medium. They were then suspended in fresh Murashige and Skoog Medium and used for studies of bacterial attachment. For other experiments, before the addition of bacteria, about 10^8 carrot cells were killed by fixation with 1% glutaraldehyde for 30 min at room temperature. To remove the glutaraldehyde from the cells, 10 ml of fresh medium was added, and the cells were pelleted in a tabletop centrifuge for 5 min at 1,000 rpm. The cells were washed by centrifugation six times, suspended in fresh medium, and used for studies of bacterial attachment.

For scanning electron microscopy of free bacteria, living bacteria were allowed to adhere to glass cover slips coated with 1 mg of poly-L-lysine per ml for 15 min. The cover slips were rinsed in water, followed by 1% bovine serum albumin, and fixed in 1% glutaraldehyde. They were then processed as described above.

For light microscopy, specimens were stained with 0.025% Calcofluor white ST (American Cyanamide), pH 8, which yields fluorescent staining of cellulose, chitin, and other β-linked polysaccharides (12). Stained preparations were examined with a Zeiss Photoscope by using fluorescence microscopy with exciter filter no. 4, which transmits wavelengths of 250 to 400 nm, and barrier filter no. 41, which transmits wavelengths above 410 nm.

Cellulase purified from *Trichoderma viride* (29) was obtained from Ross D. Brown, Jr., Virginia Polytechnic Institute and State University. Samples of bacterial cultures were incubated with 10 μg of cellulase per ml in 0.05 M citrate buffer, pH 4.9, at 45°C for 24 to 72 h until digestion was complete. The enzyme is active and stable under these conditions.

To characterize further the chemical composition of the fibrils, the trifluoroacetic acid extraction procedure of Romanovicz and Brown (26) was used. Samples were extracted with 0.5 N trifluoroacetic acid at 37°C for 3 h and centrifuged at 12,800 × *g* in an Eppendorf microcentrifuge. The pellet was extracted with 2 N trifluoroacetic acid at 121°C for 3 h, collected by centrifugation, washed once with water, placed on Formvar-covered, carbon-coated copper grids, negatively stained with 2% phosphotungstic acid, pH 7.2, and examined in a Philips 400 transmission electron microscope. The washed pellet was also subjected to digestion with 6 N HCl at 100°C for 24 h or to enzymatic digestion with 10 μg of cellulase per ml. The sugars released were identified by comparison with authentic standards by using thin-layer chromatography on Baker cellulose sheets (no. 4468) and visualized with a spay of 1.23% *p*-anisidine and 0.166% phthalic acid in ethanol. The developing solvent was *n*-butanol–pyridine–water (6:4:3) (26). In addition, the pellet was washed once with 1 N NaOH, rinsed with water, and characterized by infrared spectroscopy with a KBr pellet and a Beckman model 4250 spectrophotometer. The spectrum was compared with that of α-cellulose purchased from Sigma Chemical Co.

RESULTS

Formation of fibrils during attachment of *A. tumefaciens* to carrot cells. Virulent strains of *A. tumefaciens* attach to the surface of normal carrot tissue culture cells in increasing numbers during the first 2 h of incubation of the bacteria with the plant cells (Fig. 1). The attachment is specific for virulent strains containing the tumor-inducing (Ti) plasmid (18). Virulent strains A6 and C58 attached to carrot cells, whereas the avirulent strain NT1 obtained by curing C58 of its Ti plasmid (35) did not show significant attachment at the low multiplicity of infection (one bacterium per 100 carrot cells) used here (Fig. 1).

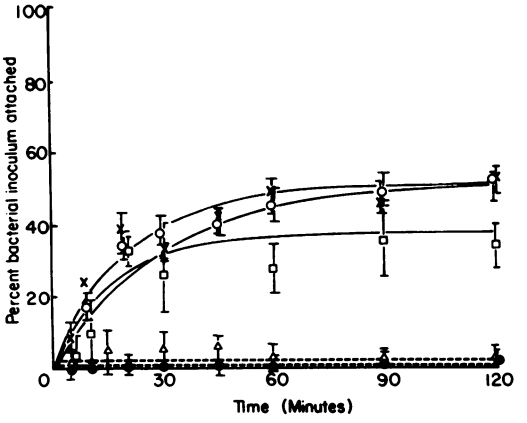

FIG. 1. *Time course of attachment of A. tumefaciens to living and glutaraldehyde-fixed carrot cells. Symbols:* ○, *strain A6 and live carrot cells;* ×, *strain A6 and glutaraldehyde-fixed carrot cells;* □, *strain C58 and glutaraldehyde-fixed carrot cells;* ●, *strain NT1 and live carrot cells;* △, *strain NT1 and glutaraldehyde-fixed carrot cells. Bars indicate standard deviations of a minimum of five experiments.*

Scanning electron microscopy showed that carrot cells grown in liquid suspension culture on a shaker were spherical and grew in clumps of 2 to 20 cells (Fig. 2a). Dividing cells were frequently seen. The surface of the cell wall consisted of a flat mesh of fibrillar material (Fig. 2b). The rough texture of this cell surface may be due to surface wounding caused by the constant agitation of these cells in culture flasks.

A. tumefaciens strain A6 was added to washed carrot cells, and the resulting cellular interactions were observed with scanning electron microscopy at intervals during the subsequent 19 h. By 30 min after their addition, bacteria were attached to the surface of the carrot cells both singly and in small clumps (Fig. 2c and d). By 90 min more bacteria were observed on the surface of the carrot cells, and many of these bacteria were in well-isolated clumps (Fig. 3c). Figure 3a also shows that bacteria could attach to a recently divided carrot cell. It is interesting to note that in both control and inoculated cultures

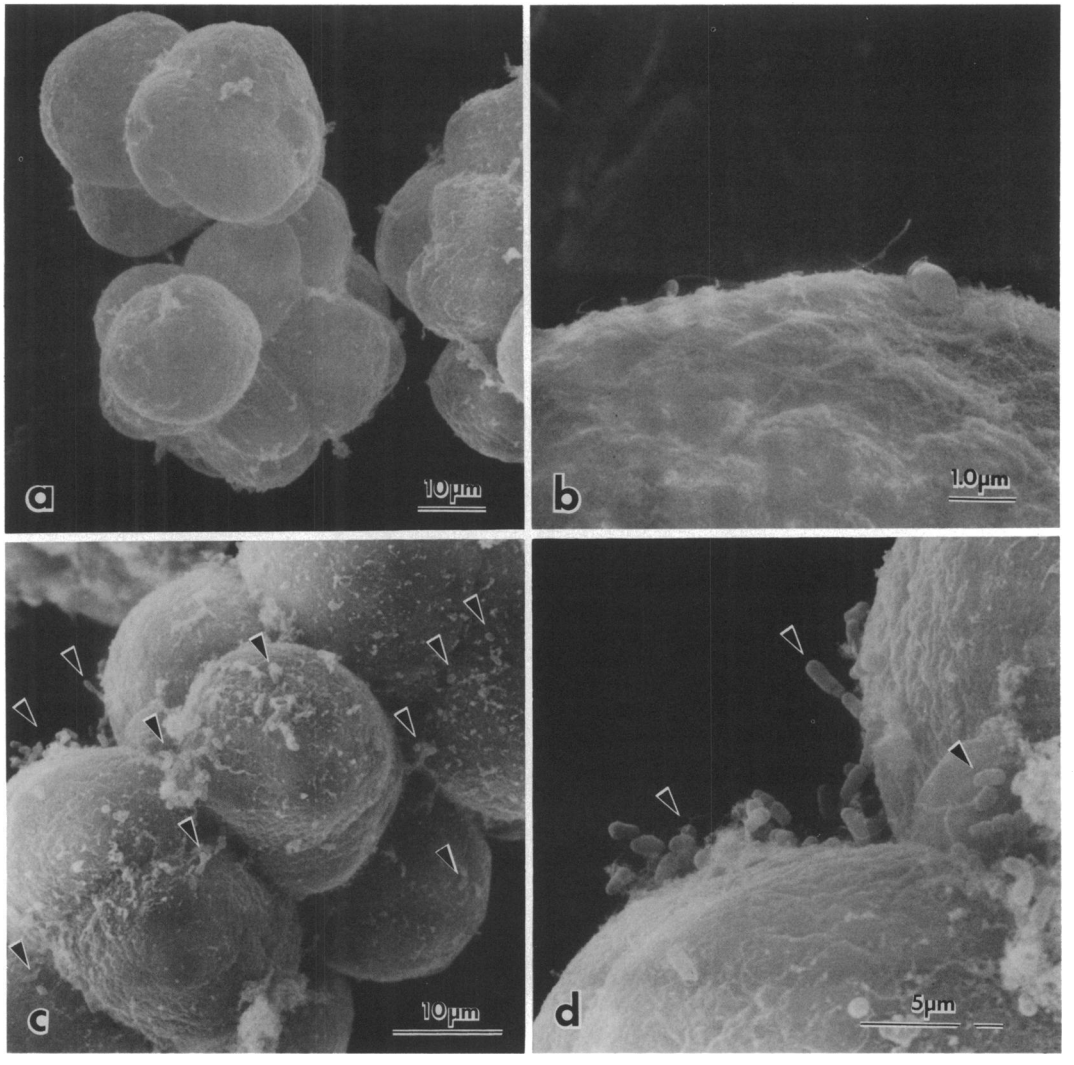

FIG. 2. *Scanning electron micrographs of suspension cultures of D. carota cells before and after binding of the virulent strain A6 of A. tumefaciens. (a) Normal carrot cells appeared as small clusters of spherical cells. (b) The surface of a normal carrot cell showed a finely fibrillar mesh and occasional small blebs. (c) Within 30 min after the addition of A6 bacteria to the plant cell culture, bacteria were observed attached to the surface of the carrot cells (arrowheads). (d) Higher magnification shows that bacteria may be attached to the plant cells by their ends or lateral sides and that bacteria may also attach to other bacteria (arrowheads).*

dividing plant cells typically showed circumferential constriction as shown in Fig. 3. At this time, fibrils were seen at the site of attachment of the bacteria to the plant cell wall. These long, curving fibrils were distinct from the underlying matted fibrils of the plant cell wall; they projected well above the plant cell surface.

From 1.5 to 4 h and from 4 to 8 h, the number of bacteria attached to the carrot cells increased greatly. By 8 h most of the attached bacteria were present as clumps. The number of long fibrils also increased with time; these were seen only at the sites of bacterial attachment. By 19 h the surface of some carrot cells was covered by bacteria aggregated in an extensive network of long fibrils (Fig. 3b and c). These aggregates of bacteria sometimes covered whole groups of carrot cells and attached several clumps of plant cell together. The number of attached bacteria varied greatly from cell to cell, but most cells in the culture had numerous bacteria on their surfaces. In these cultures divisions of both plant

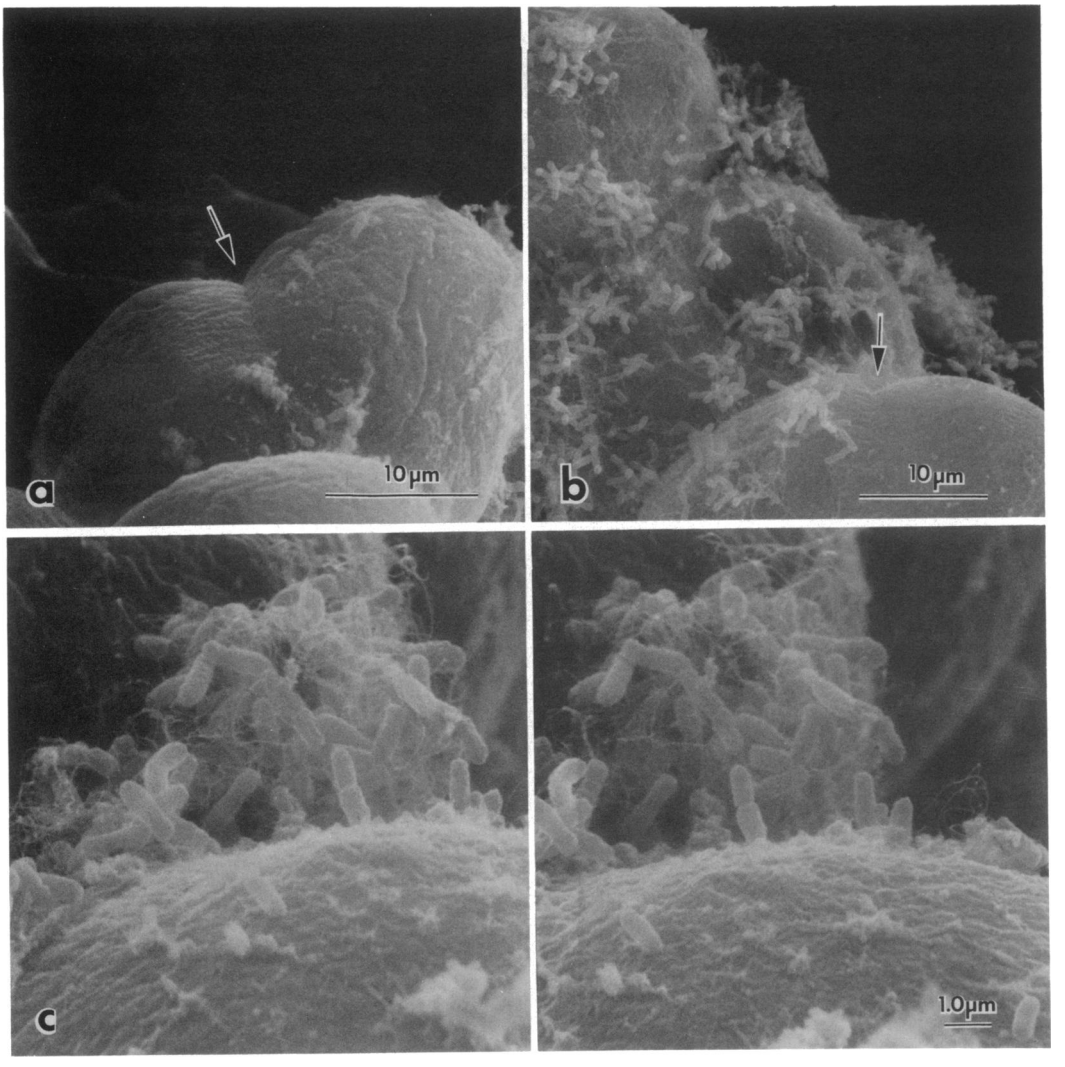

FIG. 3. *Events in the attachment of A. tumefaciens A6 to carrot cells. (a) By 90 min after inoculation, bacteria could be seen singly or in clusters on the surface of many carrot cells including this cell which appears to be dividing (arrows). (b) By 19 h after inoculation, the surface of the plant cell was covered with a large number of bacteria enclosed in a fine mesh of fibrils. The arrow indicates another plant cell which appears to be dividing. (c) A stereo pair showing the long fibrils enmeshing the bacteria on the plant cell surface in three dimensions at 19 h after infection. With stereo glasses it is apparent that the bacteria are surrounded by these fine fibrils.*

cells and bacteria were observed (Fig. 3a and b). Figure 3 shows that the bacteria were attached to the plant cells both singly and in large clusters. There appeared to be no preferential orientation of the bacteria for attachment since bacteria that were attached at their ends or on their sides were observed. A similar observation was made earlier by using light microscopy of living cells (18). In the large clusters, many bacteria were not directly attached to the plant cell but rather were entrapped in a three-dimensional network of long fibrils attached to other bacteria which were directly bound to the surface of the plant cell.

Synthesis of fibrils by *A. tumefaciens*. To determine whether the plant cell or the bacterium synthesized the fibrils, the interaction of bacteria with killed carrot cells was examined. Virulent strains of *A. tumefaciens* attached to carrot cells which had been killed by treatment with 1% glutaraldehyde or by high temperature. The kinetics of attachment to killed plant cells were only slightly different from the kinetics of attachment to living carrot cells (Fig. 1 and 4). The bacterial strain specificity of attachment was the same as that with live plant cells. Virulent *A. tumefaciens* strains A6 and C58 showed attachment to killed cells, whereas the avirulent strain NT1 did not (Fig. 1 and 4). Scanning electron microscopy showed large clumps of bacteria attached to glutaraldehyde- or heat-killed carrot cells after 19 h of incubation (Fig. 5a to d). In some cases, mats of bacteria covered groups of carrot cells (Fig. 5b). Numerous long fibrils were attached to the bacteria (Fig. 5a and d). The fibrils often seemed to arise from the side of the bacterium and frequently appeared twisted together into wide filaments. Although the glutaraldehyde-killed cells were not morphologically distinguishable from cells fixed with glutaraldehyde after bacterial attachment, the surface of heat-killed cells was irregular. Since the long fibrils were made during the attachment of live bacteria to dead carrot cells, it is probable that the bacteria rather than the plant cells synthesize these fibrils.

Characteristics of bacterial growth, aggregation, and fibril formation. Although *A. tumefaciens* was usually grown in LB medium (19), the bacteria also grew on other media. In fresh cultures of *Agrobacterium* growing exponentially in LB medium with vigorous aeration, very little fibrillar material was made, and the bacteria appeared to be well dispersed (Fig. 6b). When the cultures became stationary, some bacterial aggregation was visible to the unaided eye, and small amounts of fibrillar material could be seen in the light microscope after Calcofluor staining.

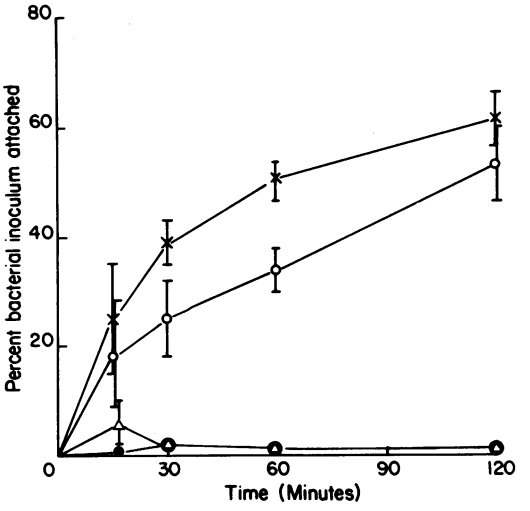

FIG. 4. *Time course of attachment of A. tumefaciens to living or heat-killed carrot cells. Symbols: ○, strain A6 and live carrot cells; ×, strain A6 and heat-killed carrot cells; ●, strain NT1 and live carrot cells; △, strain NT1 and heat-killed carrot cells. Bars indicate standard deviations of a minimum of five experiments.*

Carrot cells grew as small clusters of 4 to 20 cells in Murashige and Skoog medium. They showed no tendency to form larger aggregates (Fig. 6a). The addition of virulent strains of *A. tumefaciens* to carrot cells resulted in the formation of massive aggregates of bacteria and plant cells after 19 h of incubation (Fig. 6c). *Agrobacterium* grew in Murashige and Skoog medium in the presence of carrot cells, but the bacteria did not grow in the absence of the plant cells. However, the addition of 0.02% Soytone (soluble soybean extract [Difco Laboratories]) to this medium allowed good bacterial growth. The bacteria were highly aggregated, and large amounts of fibrillar material were made by both virulent and avirulent agrobacteria. Strains of *Agrobacterium* in which we observed fibril formation and clumping include the virulent *A. tumefaciens* strains A6, B6, and C58, the avirulent *A. tumefaciens* strains NT1 and IIBNV6, and the avirulent *A. radiobacter* strains 6467, S1005, and TR1. Since the avirulent NT1 and the *A. radiobacter* strains lack the Ti plasmid (7, 35, 39) found in the virulent strains and have no known plasmid in common, the genes involved in the synthesis of fibrils are presumably located on the bacterial chromosome.

A. tumefaciens strains A6 and NT1 grown in Murashige and Skoog medium synthesized filaments, aggregated, and attached to Whatman no. 1 filter paper (Fig. 7a and b). By scanning electron microscopy, strain A6 was seen at-

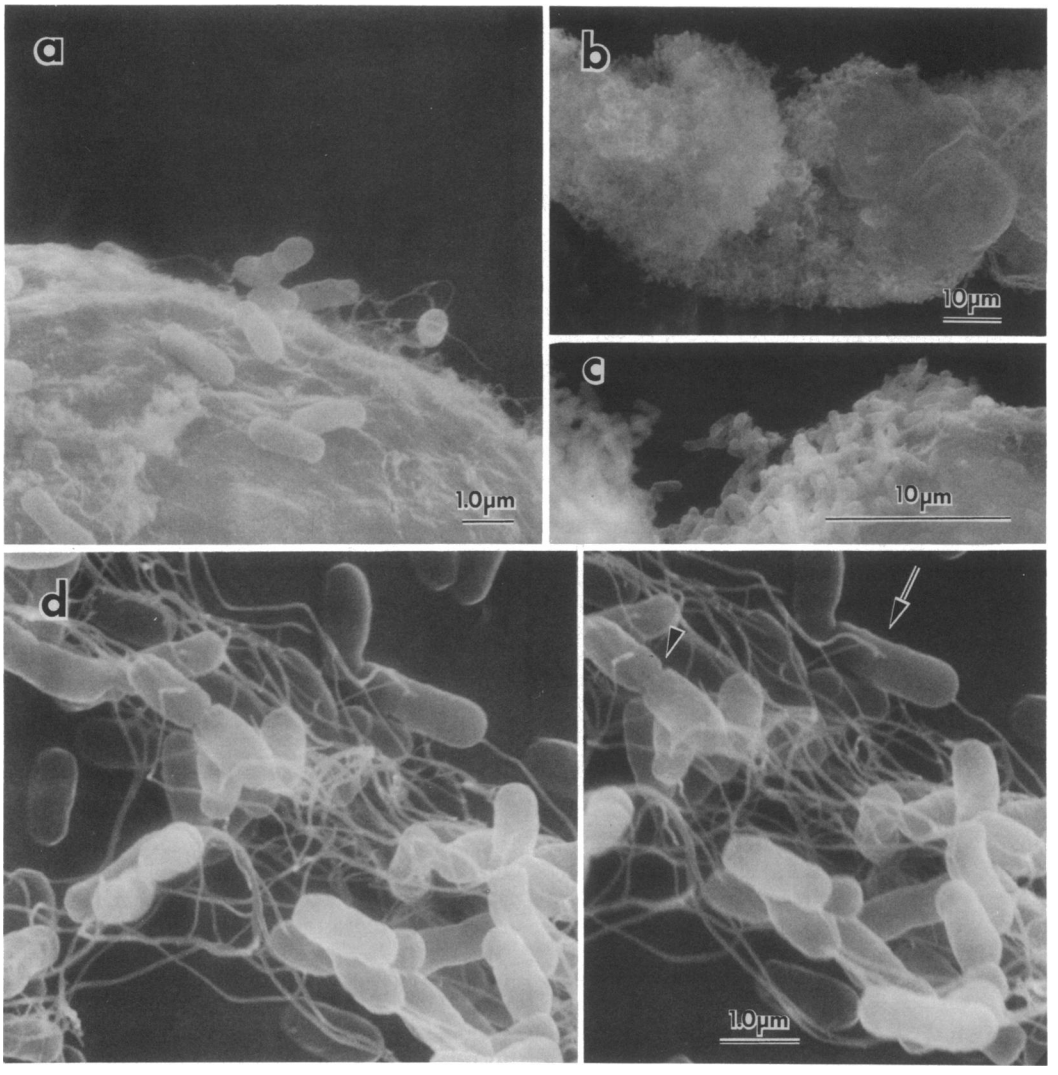

FIG. 5. *Attachment of A. tumefaciens to killed plant cells. (a) Bacteria attached to glutaraldehyde-fixed plant cells, and numerous fine fibrils were elaborated during this process. (b) The surface of this cluster of heat-killed plant cells was nearly covered by a mass of adherent bacteria. (c) At a higher magnification, the individual bacterial cells that were attached to the heat-killed plant cells can be distinguished. (d) At high magnification, a three-dimensional view of bacteria attached to glutaraldehyde-fixed plant cells shows that the numerous long, slender fibrils originated on the sides of bacterial cells (arrow). The fibrils frequently became entwined together to form large fibrils.*

tached to the filter paper apparently by anchoring fibrils which connected to the sides of the bacteria (Fig. 7b). Fibrils were observed to anastomose and to wind around each other. Attachment to filter paper required much longer times, about 24 to 48 h, than attachment to carrot cells.

Aggregation of the A6 strain of *A. tumefaciens* grown in Murashige and Skoog medium with 0.02% Soytone was also observed with bacteria attached to poly-L-lysine-coated glass cover slips. Bacterial aggregation on the cover slips was visible to the unaided eye. In the scanning electron microscope, these aggregates of bacteria could be seen to be held together by extensive networks of fibrils (Fig. 7c). Thus, under certain conditions, *A. tumefaciens* can form fibrils in the absence of the host plant.

The bacteria also possess at least a limited capacity to digest pectin, a component of the

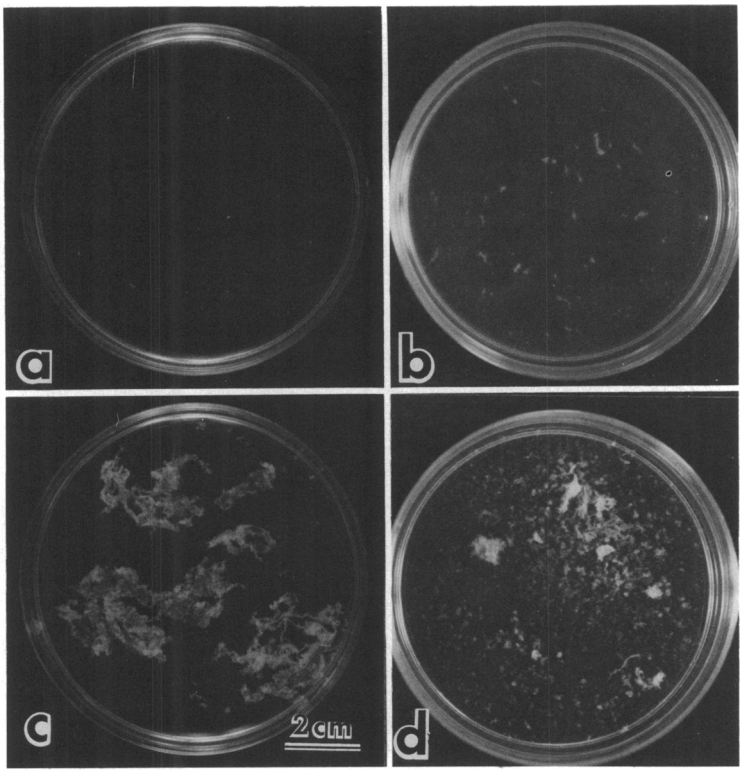

FIG. 6. *Cultures of A. tumefaciens and D. carota. (a) Cultures of D. carota were well dispersed and did not show aggregation. (b) Cultures of A. tumefaciens were also well dispersed and showed minimal clumping when grown in LB medium. (c) A mixture of D. carota cells with A. tumefaciens A6 showed enormous aggregates of plant and bacterial cells after 19 h. (d) A. tumefaciens grown in the presence of Soytone (soluble soybean extract) exhibited massive clumping.*

host cell wall. *A. tumefaciens* A6, C58, and NT1 grew slowly on minimal medium (16) with pectin rather than glucose as the sole carbon source. The pectin was not contaminated with a sufficient amount of glucose or other carbon source to support the growth of *E. coli* 23739, which did not grow on medium with pectin as the sole carbon source, but did grow on medium with glucose as the carbon source.

Composition of bacterial fibrils. When *A. tumefaciens* was grown under conditions which promoted fibril synthesis in the absence of plant cells, long, thin strands of material which fluoresced after Calcofluor staining were observed in the light microscope (Fig. 8a). Cellulose, chitin, and some β-1,3-glucans show fluorescent staining with Calcofluor (12). When these bacterial cultures were treated with purified *Trichoderma* cellulase, no Calcofluor staining was observed. The bacteria themselves showed an intrinsic fluorescence which was not Calcofluor-dependent and which was not affected by cellulase. Thin-layer chromatography of the products of enzymatic digestion showed only cellobiose and glucose at 24 h and only glucose at 72 h. The sole product of hydrolysis with 6 N HCl at 100°C as determined by thin-layer chromatography was glucose. Treatment of the bacterial cultures with proteinase K (100 μg/ml, pH 7.4, at 37°C for 1 to 4 h) did not affect the Calcofluor fluorescent strands and left fibrils which were visible with negative staining in the transmission electron microscope. To determine whether these strands were indeed cellulose, the trifluoroacetic acid extraction procedure of Romanovicz and Brown was used (26). Treatment with 2 N trifluoracetic acid at 121°C for 3 h left visible fibrils which showed fluorescent staining with Calcofluor. These fibrils were also visible after negative staining in the transmission electron microscope (Fig. 8b). An infrared spectrum of the purified fibrillar material was compared with the spectrum of cellulose (Sigma) prepared in a similar manner (Fig. 9). The two spectra

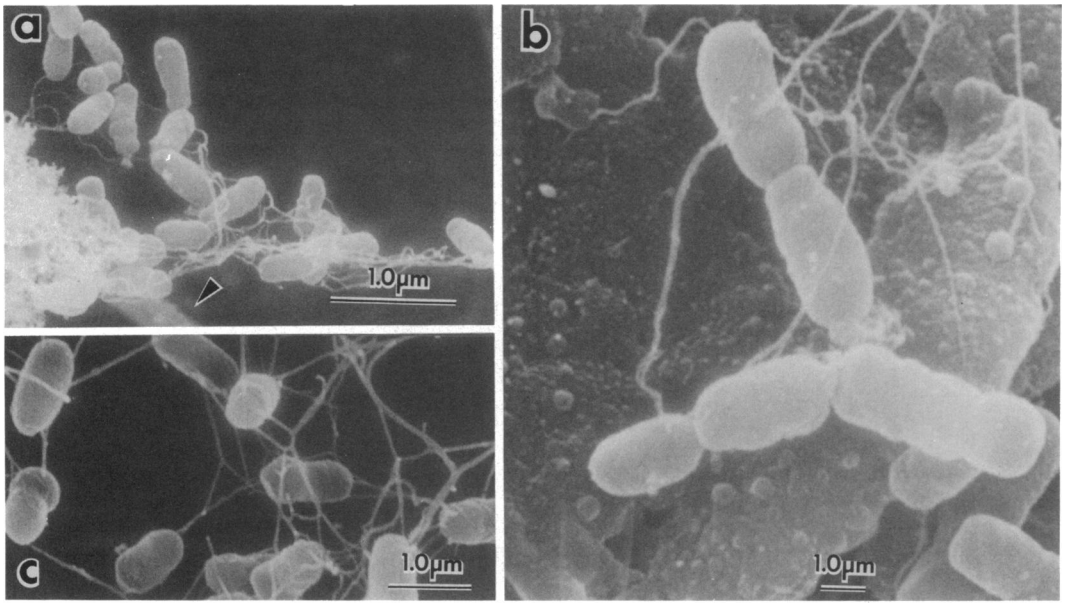

FIG. 7. *Elaboration of fibrils by A. tumefaciens in the absence of plant cells. (a) Bacteria grown in the presence of filter paper developed numerous long fibrils and became aggregated. The arrowhead indicates a portion of the filter paper. (b) At higher magnification, several bacteria growing on the surface of the filter paper can be seen. Long fibrils originate from the sides of the bacteria. (c) Bacteria grown in the presence of soluble plant extract also developed long fibrils. These fibrils formed bridges between adjacent bacteria and lead to massive clumping of the bacteria in the culture.*

were almost identical. Thus, it appears that the fibrils synthesized by *Agrobacterium* are composed of cellulose.

DISCUSSION

Attachment of *A. tumefaciens* to wounded plants and tissue culture cells. The initial step in tumor formation by *A. tumefaciens* is a site-specific attachment of the bacterium to the host plant (13). A similar site-specific attachment has been observed between the bacterium and plant tissue culture cells (18). In the whole plant, the bacterial infection and resulting tumor formation generally occurs in a wound site. In suspension cultures of carrots, the surface of the cells appeared slightly damaged, and occasional cells were broken by the mechanical agitation. This may provide equivalent stimuli and surfaces to those generated by a wound. Both in the wounded plant (36) and in tissue culture (18) bacterial lipopolysaccharide inhibited the interaction between the bacterium and the plant cell suggesting that the bacterial receptor on the plant cell surface may recognize the lipopolysaccharide in the bacterial outer envelope.

Avirulent *A. tumefaciens* strain NT1 inhibited tumor formation in plants apparently by blocking attachment sites for virulent *A. tumefaciens* (14). NT1 did not show significant attachment to tissue culture cells at the low multiplicity of infection used in kinetic studies (Fig. 1). However, data not shown here indicate that at the higher multiplicity of infection used for microscopic studies, NT1 showed some attachment to the carrot cells. NT1 was also capable of fibril formation although at a lower rate that the virulent strain A6.

Specific attachment of *A. tumefaciens* to plant cells has been observed in several in vitro systems including carrot (18), tobacco (18, 32), and *Datura* (22) tissue culture cells and carrot (38) and potato disks (8). Scanning electron microscopy of *A. tumefaciens* attached to habituated tobacco cells suggested that the bacteria generally attached end on to the host cell surface (32), whereas our observations suggest that the bacteria could attach in both polar and lateral orientations to the carrot cell surface.

The attachment of *A. tumefaciens* to *Datura* tissue culture cells has been examined with transmission electron microscopy of sectioned material (22). The bacteria were tightly bound to the surface of the plant cell wall and were surrounded by a network of fine fibrils. Some of the fibrils connected loosely bound bacteria to the host cell wall (22). These fibrils, previously observed in the transmission electron micro-

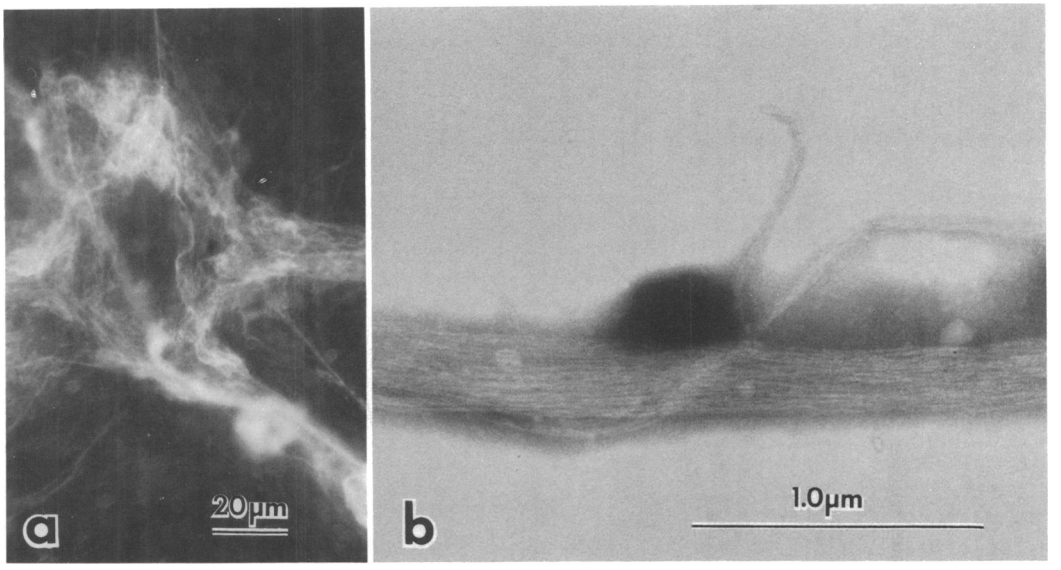

FIG. 8. *Cellulose fibrils elaborated by A. tumefaciens. (a) Calcofluor staining of a bacterial culture grown in the presence of soytone. Calcofluor stains cellulose and other β-linked polysaccharides (12). In other experiments (not shown) in which the bacteria were treated with cellulase before Calcofluor staining no fluorescent stain was observed. (b) Bacterial cultures were extracted with trifluoroacetic acid at 121°C for 3 h. The fibrils which remained were cellulose since they were still positive for fluorescent staining with Calcofluor and appeared as fine filaments in negatively stained preparations.*

scope, are probably identical to the fibrils described here since their size, location, and configuration appear similar.

The presence of large aggregates of bacteria attached to the carrot cell surface noted previously (18) has also been observed with tobacco (32) and *Datura* (22) tissue culture cells. The division of attached bacteria which has been observed with time-lapse microcinematography (R. H. G. Gurlitz and A. G. Matthysse, manuscript in preparation) may be responsible, in part, for the growth of clusters of attached bacteria. However, bacterial growth has a lag time of 2 to 3 h after the addition of the bacteria to carrot cell cultures, and thereafter the bacterial doubling time is about 2 h. Thus, one bacterium which attached to a carrot cell within 1 h of incubation would have been unlikely to have divided at 90 min, but could have given rise to 128 bacteria after 19 h. By 30 min bacterial clumps were visible by scanning electron microscopy (Fig. 2c); these clumps increased in size with longer incubation times. Therefore, the clusters of bacteria seen on the plant cell surface probably arose both from the division of attached bacteria and from the tendency of incoming bacteria to attach near previously attached bacteria (A. G. Matthysse, S. Matthysse, and R. H. G. Gurlitz, manuscript in preparation).

Numerous dividing plant cells were seen by scanning electron microscopy. These cells typically showed a circumferential constriction (Fig. 3a) and retained their spherical shape after division. This method of cytokinesis appeared slightly different from the usual higher plant cell plate formation in which the original cell wall is unaltered in shape.

Role of fibrils in bacterial infections of plants. In contrast to many animal pathogens in which the ability to attach to the host is associated with bacterial virulence, infection of plants by many species of bacterial pathogens is followed by the attachment of avirulent strains to the host cell wall; in general, virulent strains do not attach. Pathogens for which this has been observed include *Pseudomonas putida* (31), *Pseudomonas phaseolicola* (25, 30), *Pseudomonas pisi* (9, 24), *Pseudomonas solanacearum* (28), and *Xanthomonas malvacearum* (2, 3). During attachment of avirulent strains to the host cell wall, the bacteria were covered with fibrils and granular material. The composition of these fibrils is unknown. In some cases a pellicle also covered the attached bacteria. Attachment was usually followed by a hypersensitive response, including changes in membrane permeability and localized necrosis. Saprophytic bacteria such as *E. coli* and *Bacillus subtilis* also attached to the host cell wall but their attachment was not followed by a hypersensitive

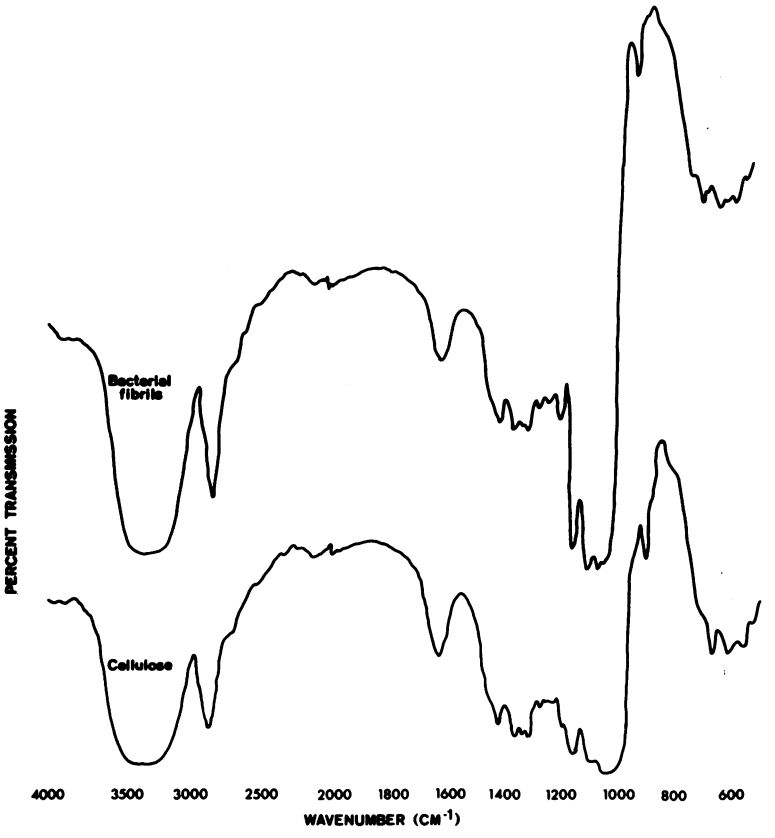

FIG. 9. *Infrared spectra of a purified preparation of bacterial fibrils and cellulose.*

response (28). In the only one of these cases in which the source of the attachment fibrils has been examined, it appeared that the plant, and not the bacterium, was responsible for fibril synthesis. Heat-killed *P. solanacearum* were attached by fibrils to living tobacco mesophyll cells (28). As was the case for virulent strains of *A. tumefaciens,* bacterial lipopolysaccharide appears to be involved in the attachment of avirulent strains of *P. solanacearum* to potato cells (37).

The various species of rhizobia, unlike agrobacteria to which they are closely related (11), have a limited host range (5). Both specific and nonspecific interactions were involved in the initial attachment of *Rhizobium* cells to root hairs (5). Some species of *Rhizobium* have been shown to synthesize cellulose fibrils (5, 21) which could have been involved in the subsequent tighter binding of the bacteria to the root surface. Bacterial lipopolysaccharide appeared to be involved in the initial attachment of *Rhizobium* cells to the plant root hair (5, 27).

Thus, although the interaction of *A. tumefaciens* and other plant pathogens with the host cell surface was similar in some respects, interesting differences were observed. Avirulent strains of most phytopathogens attached to the plant cell, and virulent strains remained free. In contrast, virulent strains of *A. tumefaciens* attached to the plant cell, and avirulent strains remained free. *P. solanacearum* attached to the plant cell by fibrils synthesized by the host, whereas *A. tumefaciens* was attached to the plant cell by fibrils synthesized by the bacterium. These differences may, in part, reflect the difference between an invasive pathogen which destroys host tissue and a noninvasive pathogen whose food supply depends on materials (presumably the opines) excreted by living plant tumor cells (23).

Bacterial cellulose synthesis. The composition of the fibrils involved in the attachment of other phytopathogens to the host is unknown. However, we have shown that in the case of *Agrobacterium* cells, the attachment fibrils are composed of cellulose elaborated by the bacterium. Cellulose synthesis accompanied by bacterial aggregation has previously been observed in several genera of gram-negative bacteria iso-

lated from sewage (6). On the basis of biochemical tests, some of these bacteria were identified as agrobacteria. *Rhizobium trifolii,* which has a 50% chromosomal DNA homology with *A. tumefaciens* (11), has also been shown to synthesize cellulose (21). As with *Agrobacterium* spp. the ability to synthesize cellulose was not correlated with infectivity. However, the cellulose fibrils were apparently involved in the adherence of the bacteria to root hair surfaces (21).

All of the strains of *A. tumefaciens* and *A. radiobacter* that we examined synthesized cellulose, although they differed in the quantity produced. Since these strains do not contain any known plasmids in common, the genes involved may be presumed to be chromosomal.

The ultrastructure of bacterial cellulose synthesis has been examined in *Acetobacter xylinum.* Cellulose microfibrils are synthesized at sites on the side of the bacterium; the microfibrils that formed aggregate into a ribbon of cellulose (1). Cellulose fibrils also appeared to arise from the side of *Agrobacterium* cells; however, the fibrils did not aggregate to form a ribbon.

A model for the attachment of *A. tumefaciens* to the plant host cell. The first step in attachment of *A. tumefaciens* to the plant host cell (Fig. 10.1) appears to be the attachment of a bacterium (probably a motile, flagellated bacterium) to a receptor on the surface of the plant cell. Bacterial lipopolysaccharide appears to be a recognition factor in this step both in the wounded plant (36) and in tissue culture (18). After attachment, the bacterium synthesizes cellulose fibrils (Fig. 10.2). Substances released from wounded plant tissues or from damaged cells in tissue culture may act as Soytone does in synthetic medium to stimulate the synthesis of these cellulose fibrils. The newly developed fibrils anchor the bacteria to the surface of the plant cell (Fig. 10.3). Because the fibrils are made of cellulose, which is also a component of the plant cell wall, the plant host cell does not readily digest the fibrils to detach the bacteria. As the fibrils become longer they may entrap other bacteria and attach them indirectly to the surface of the plant cell (Fig. 10.4). Fibrils may also hold both daughter cells of a dividing bacterium to the surface of the plant cell. Once the bacteria are firmly attached, both directly by interaction with receptor sites and indirectly by cellulose fibrils to the outer surface of the plant cell wall, they probably need to make contact with the host cell plasma membrane to transfer bacterial Ti plasmid DNA to the host cell. How this occurs is unknown. However, we have shown that *Agrobacterium* cells can digest pectin. Possibly other plant cell wall components can be digested as well. This may aid the bacterium in approaching the host plasma membrane (Fig. 10.5). Bacteria which are entrapped in fibrils begin to synthesize fibrils themselves (Fig. 10.6). Steps 2 through 6 may then be repeated to cause the formation of a large cluster of bacteria attached to the plant cell surface by fibrils. At the same time, attached and entrapped bacteria may divide, also increasing the size of the bacterial clump. Unoccupied receptor sites may still be present on the plant cell surface, and additional individual bacteria may attach to them, beginning the sequence again. Thus, at late times, some cells will have large clumps of bacteria representing early initial attachment followed by amplification. Other cells or other areas on the same cell may have individual bacteria or small bacterial clusters resulting from relatively late initial attachment events, with little time available for bacterial entrapment or multiplication.

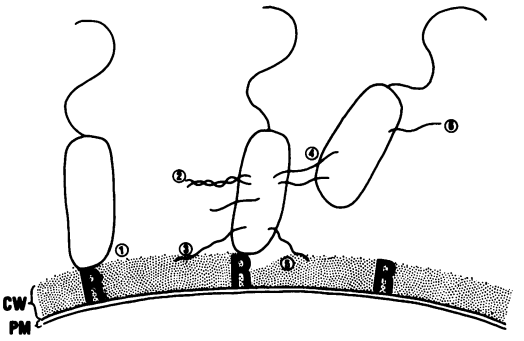

FIG. 10. *A model for the attachment of A. tumefaciens to carrot cells. A bacterium attaches to a specific receptor site (R) on the surface of the plant cell (step 1) and is induced to elaborate cellulose fibrils (step 2). The fibrils anchor the bacterium to the surface of the plant cell wall (CW) (step 3) and entrap additional bacteria (step 4). These entrapped bacteria may themselves begin to synthesize fibrils (step 6). This facilitated attachment and multiplication of attached bacteria result in the development of a bacterial colony attached to the plant cell wall via receptors and cellulose fibrils. The attached bacteria secrete enzymes which digest the plant cell wall (step 5) and may aid in establishing contact of the bacteria with the plant cell plasma membrane (PM) to facilitate transfer of the Ti plasmid from the bacteria to the plant cell.*

The initial step in the pathogenic interaction of *A. tumefaciens* and the plant cell is the attachment of the bacterium to the host cell surface. The studies reported here indicate that this initial interaction is not a simple one-step process. Instead, there exists a complicated series of

events, including bacterial attachment to host receptors, bacterial synthesis of cellulose fibrils, the digestion of a portion of the host cell wall by bacterial enzymes, and amplification of the number of attached bacteria by entrapment and multiplication.

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