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Plasmid-Dependent Attachment of *Agrobacterium tumefaciens* to Plant Tissue Culture Cells

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Kinetic, microscopic, and biochemical studies show that virulent Ti (tumor inducing)-plasmid-containing strains of *Agrobacterium* attach to normal tobacco and carrot tissue culture cells. Kinetic studies showed that virulent strains of *A. tumefaciens* attach to the plant tissue culture cells in increasing numbers during the first 1 to 2 h of incubation of the bacteria with the plant cells. Five Ti-plasmid-containing virulent *Agrobacterium* strains showed greater attachment to tobacco cells than did five avirulent strains. Light and scanning electron microscopic observations confirmed that virulent strains attached to the surface of carrot cells while avirulent strains showed little attachment. Bacterial attachment was blocked by prior incubation of the plant cells with lipopolysaccharide extracted from *A. tumefaciens,* but not from *A. radiobacter,* suggesting that bacterial lipopolysaccharide is one of the components involved in the attachment process. At least one other bacterial product may be required for attachment in tissue culture because the avirulent *A. tumefaciens* NT1, which lacks the Ti plasmid, does not itself attach to tobacco cells, but its lipopolysaccharide does inhibit the attachment of virulent strains.

The infection of a wound on a dicotyledenous plant by *Agrobacterium tumefaciens* results in the formation of a tumor at the wound site. The first step in the process of tumor formation has been shown to be the attachment of the bacterium to a wound site (8). The reproducible transformation by *Agrobacterium* of normal plant cells in tissue culture to tumor cells has not yet been achieved. As a first step in obtaining this transformation in tissue culture, we have examined the attachment of *Agrobacterium* to normal tobacco and carrot cells growing in liquid suspension cultures.

MATERIALS AND METHODS

Bacteria were grown to stationary phase in 0.8% nutrient broth, 0.5% sucrose, and 0.1% yeast extract (8). Viable cell counts were determined by dilution in phosphate-buffered saline (Na_2HPO_4, 7 g/liter; KH_2PO_4, 3 g/liter; NaCl, 4 g/liter; and $MgSO_4$; 7 H_2O, 0.2 g/liter) followed by plating on 23 g of nutrient agar per liter with 0.1% yeast extract. The sources of the *A. tumefaciens* strains used were as follows: A6, A. Braun; B6, C. O. Miller; IIBNV6, J. A. and B. B. Lippincott; 15955, the American Type Culture Collection; and strains C58, NT1, and A178, M. D. Chilton. *A. radiobacter* S1005 and TR1 were obtained from J. A. and B. B. Lippincott; strain 6467 was obtained from the American Type Culture Collection; strain K14 was obtained from J. Schell. Suspension cultures of *Daucus carota* were obtained from W. Boss and maintained in Murashige and Skoog medium (11) with weekly transfers.

Callus cultures of *Nicotiana tabacum* var. Coker 319 pith tissue were initiated and maintained on either Murashige and Skoog medium (11) or on Fox medium (5) with 1% agar at 25°C for 3 to 18 months. Suspension cultures of the tobacco cells were prepared by pressing 2 to 3 g of tissue through a wire screen (12 mesh/inch) and transferring the resulting cell clumps to 100 ml of liquid Fox medium. After 1 to 2 days of growth on a shaker at 25°C, a known concentration of bacteria (final concentration, between 5×10^3 and 2×10^4 bacteria per ml) was introduced into either the carrot or tobacco suspension cultures. Samples were removed at various times and filtered through Miracloth (Calbiochem), which allows the passage of bacteria but not whole plant cells. The filtrate was diluted 10-fold and mixed in a Vortex mixer, and 0.1-ml samples from each test were plated on three to six petri plates. For tests of attachment of various strains, time courses were run from 0 to 300 min. The plant cells were resuspended in phosphate-buffered saline and homogenized for 30 s at top speed in a sterile Waring blender, and the number of bacteria present was determined by viable cell counts. (Homogenization in a Waring blender does not affect the viable cell count of *Agrobacterium* cultures.) Light microscope examination of the mixture of bacteria and plant cells showed many free bacteria and, with virulent bacterial strains, some bacteria on the cell walls of the tobacco or carrot cells. Few bacterial aggregates were seen. Photomicrographs of living cells were taken with a Zeiss photoscope. The number of plant cells per ml was determined in a

Sedgewick-Rafter counting chamber and was 5×10^3 cells per ml for tobacco and 5×10^4 cells per ml for carrots.

For scanning electron microscope studies, cells were fixed in 1% glutaraldehyde for 3 days, and postfixed in 1% osmium tetraoxide for 4 h. Cells were then collected on Whatman GF/C glass fiber filters and sequentially dehydrated in aqeuous solutions of 30, 50, 70, 90, 95, 100, and 100% acetone and dried under vacuum at room temperature. Cells on the filters were coated in a Ladd rotary coater in a Denton vacuum evaporator with about 10.0 nm of 60% gold-40% palladium and examined with a JEOL JSM35U scaning electron microscope with a tungsten filament at 25 kV.

Bacterial lipopolysaccharide (LPS) was prepared by extracting stationary phase cells washed three times with 0.12 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) with 0.01 M EDTA following the method of Leive et al. (7) or by hot phenol extraction followed by dialysis, lyophilization, and centrifugation using a modification of the method of Westphal and Jann (15) described by Whatley et al. (16). The concentration of LPS in the extracts was determined by a colorimetric assay for the 2-keto-3-deoxyoctulosonic acid released after acid hydrolysis of the LPS (1, 2, 4, 12). LPS extracts containing 100 μg of KDO per ml

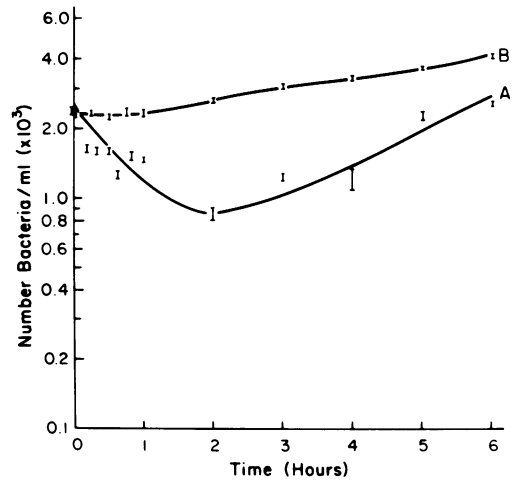


FIG. 1. *Time course of the number of viable A. tumefaciens C58 recovered from the filtrate after the addition of the bacteria to Fox medium. (A) △, Fox medium containing tobacco cells. (B) ○, Fox medium alone. Bars indicate the standard deviations of the points.*

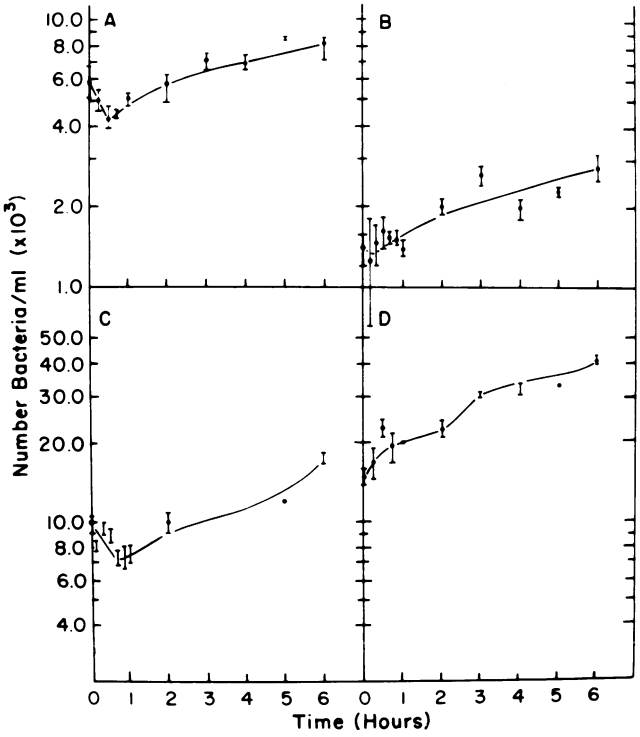
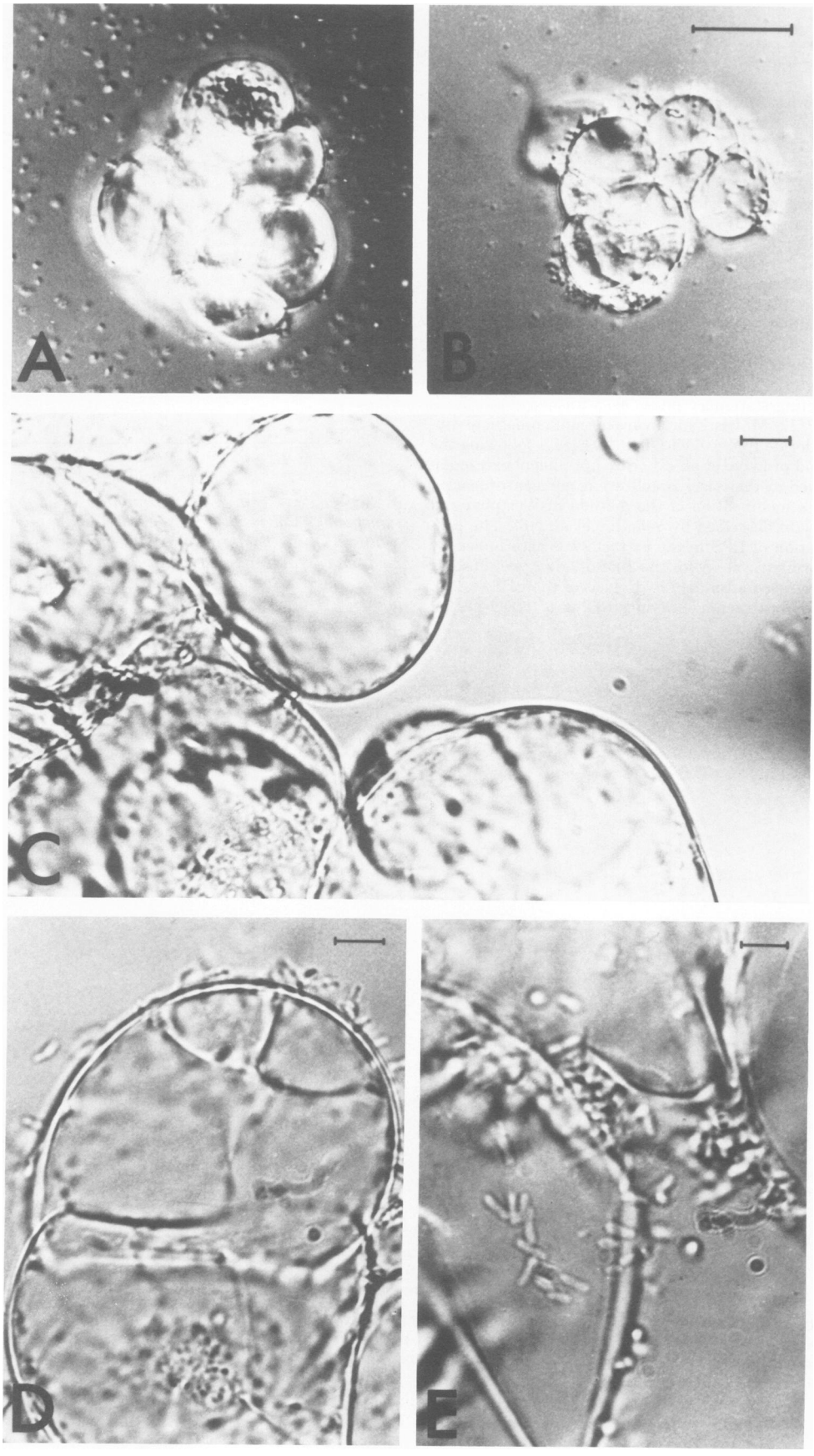


FIG. 2. *Time course of the number of viable agrobacteria recovered from the filtrate after the addition of the bacteria to Fox medium containing tobacco cells. (A) A. tumefaciens A6 (virulent). (B) A. radiobacter 6467 (avirulent). (C) A. tumefaciens C58 (virulent). (D) A. tumefaciens NT1 (avirulent). Bars indicate the standard deviations of the points.*


A
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C
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TABLE 1. *Recovery of bacteria added to plant tissue culture suspensions from the plant-cell free filtrate and from the plant cell homogenate*

Strain	Concn of inoculum[a]	No. recovered in filtrate[a]	No. recovered from plant cell homogenate[a]
B6	97 ± 5[b]	50 ± 8	47 ± 15
15955	81 ± 3	69 ± 5	20 ± 4
A6	70 ± 10	40 ± 5	30 ± 10

[a] Bacterial per ml × 10^{-2}.

[b] Standard error of the mean of a minimum of three separate experiments. Measurements were made at 120 min.

TABLE 2. *Attachment of various strains of Agrobacterium to tobacco tissue culture cells*

Strain	Plasmid present in strain[a]	Virulence[a]	% Bacterial inoculum attached[b]
A. tumefaciens A6	Ti	+	45 ± 9[c]
A. tumefaciens B6	Ti	+	36 ± 13
A. tumefaciens 15955	Ti	+	27 ± 6
A. tumefaciens C58	Ti	+	29 ± 5
A. tumefaciens A178	Ti	+	24 ± 5
A. tumefaciens NT1	None	−	8 ± 9
A. tumefaciens IIBNV6	Ti deletion	−	4 ± 6
A. radiobacter 6467	Cryptic	−	2 ± 3
A. radiobacter S1005	None	−	5 ± 8
A. radiobacter TR1	None	−	7 ± 8

[a] References for virulence and plasmids of the various strains are given in the text.

[b] Initial bacterial concentration varied with the experiment, but was between 5 × 10^3 and 1.5 × 10^4 per ml.

[c] Standard error of the mean of a minimum of four separate experiments. Measurements were made at 0, 15, 30, 60, 90, 120, 180, 240, and 300 min.

had optical densities at 260 and 280 nm of less than 0.05, indicating that they contain little protein or nucleic acid. LPS extracts were added to the plant cell suspensions 15 min before the addition of the bacteria.

RESULTS

Characteristics of the interaction between tobacco cells and *A. tumefaciens*. When a virulent strain of *A. tumefaciens* was added to a suspension of tobacco cells, the number of bacteria in the plant cell-free filtrate was gradually reduced due to attachment of the bacteria to the plant cells (Fig. 1 and 2; Table 1). After a lag time of 2 to 4 h the bacteria began to grow. This growth masked any further reduction in the number of free bacteria. Both the disappearance of the free bacteria and the rate of bacterial growth were dependent on the presence of the tobacco cells in the culture medium (Fig. 1). The bacteria which were not recovered from the plant cell-free filtrate of the medium could be recovered from a homogenate of the plant cells retained by the filter (Table 1), suggesting that these bacteria were attached to the tobacco cells.

TABLE 3. *Attachment of various strains of Agrobacterium to carrot tissue culture cells*

Strain	Plasmid present in strain[a]	Virulence[a]	% Bacterial inoculum attached[b]
A. tumefaciens K14	Ti	+	55 ± 5[c]
A. tumefaciens A6	Ti	+	50 ± 7
A. tumefaciens C58	Ti	+	24 ± 6
A. tumefaciens A178	Ti	+	23 ± 5
A. tumefaciens NT1	None	−	8 ± 5
A. radiobacter TR1	None	−	3 ± 3

[a] References for virulence and plasmids of the various strains are given in the text.

[b] Initial bacterial concentration varied with the experiment, but was between 5 × 10^3 and 2 × 10^4 per ml.

[c] Standard error of the mean of a minimum of four separate experiments. Measurements were made at 0, 15, 30, 60, 120, and 180 min.

Bacterial strain specificity of attachment. Virulent strains of *A. tumefaciens* contain a large (ca. 120 × 10^6 daltons) plasmid (17). The presence of this tumor-inducing (Ti) plasmid is required for virulence of agrobacteria (12, 14, 17), and DNA sequences complementary to Ti plasmid DNA have been found in bacteria-free tumor cells (3, 10). The ability of *Agrobacterium* strains to attach to tobacco tissue culture cells appears to correlate with the presence of the Ti plasmid in the strains surveyed (Fig. 2; Table 2). *A. tumefaciens* A6, B6, 15955, K14, and C58 contain the Ti plasmid (14, 17) and attach to tobacco cells (our B6 strain, unlike that of Genetello et al. [6], contains only one plasmid as judged by gel electrophoresis of *Eco*RI restric-

FIG. 3. *Photomicrographs of various Agrobacterium strains (10^7 cells per ml) and normal carrot cells (10^5 cells per ml) after incubation together in culture for 20 h. (A) A. tumefaciens NT1 (avirulent). Nomarski optics. Bar, 50 μm. (B) A. tumefaciens C58 (virulent). Nomarski optics. Bar, 50 μm. (C) A. radiobacter TR1 (avirulent). Bar, 50 μm. (D) A. tumefaciens A6 (virulent). Bar, 50 μm. (E) A. tumefaciens A6 (virulent). Bar, 10 μm.*

tion endonuclease digests of plasmid preparations). *A. radiobacter* S1005, TR1, and 6467 do not contain the Ti plasmid (17), are avirulent (8), and do not attach to tobacco tissue culture cells (strain 6467 contains a small cryptic plasmid unrelated to the Ti plasmid; A. G. Matthysse, unpublished data). Strain IIBNV6, which also does not attach to tobacco tissue culture cells, is an avirulent derivative of the virulent strain IIB (13). It contains a plasmid smaller than the Ti plasmid (9), which has some sequence homology with the Ti plasmid from C58 (as judged by hybridization with the C58 Ti plasmid, Matthysse, unpublished data). Avirulent strain NT1 was derived by curing the virulent strain C58 of its Ti plasmid (14). The virulent strain, A178, was obtained by reintroducing the Ti plasmid from strain K27 into an antibiotic-resistant derivative of NT1 (14). Both strains C58 and A178 attach to tobacco tissue culture cells, but no significant attachment by NT1 was observed. Thus, the ability of various strains of *Agrobacterium* to show significant attachment to tobacco tissue culture cells correlates with the presence of the Ti plasmid in these strains, suggesting that the products of some gene or genes on the plasmid are required for this attachment.

The attachment of *A. tumefaciens* A6, K14, TR1, C58, NT1, and A178 to carrot suspension cultures was also measured (Table 3). The results were similar to those obtained with tobacco cells. Virulent strains A6, C58, K14, and A178 all showed a greater degree of attachment than did the avirulent strains NT1 and TR1.

Light and electron microscopic observations

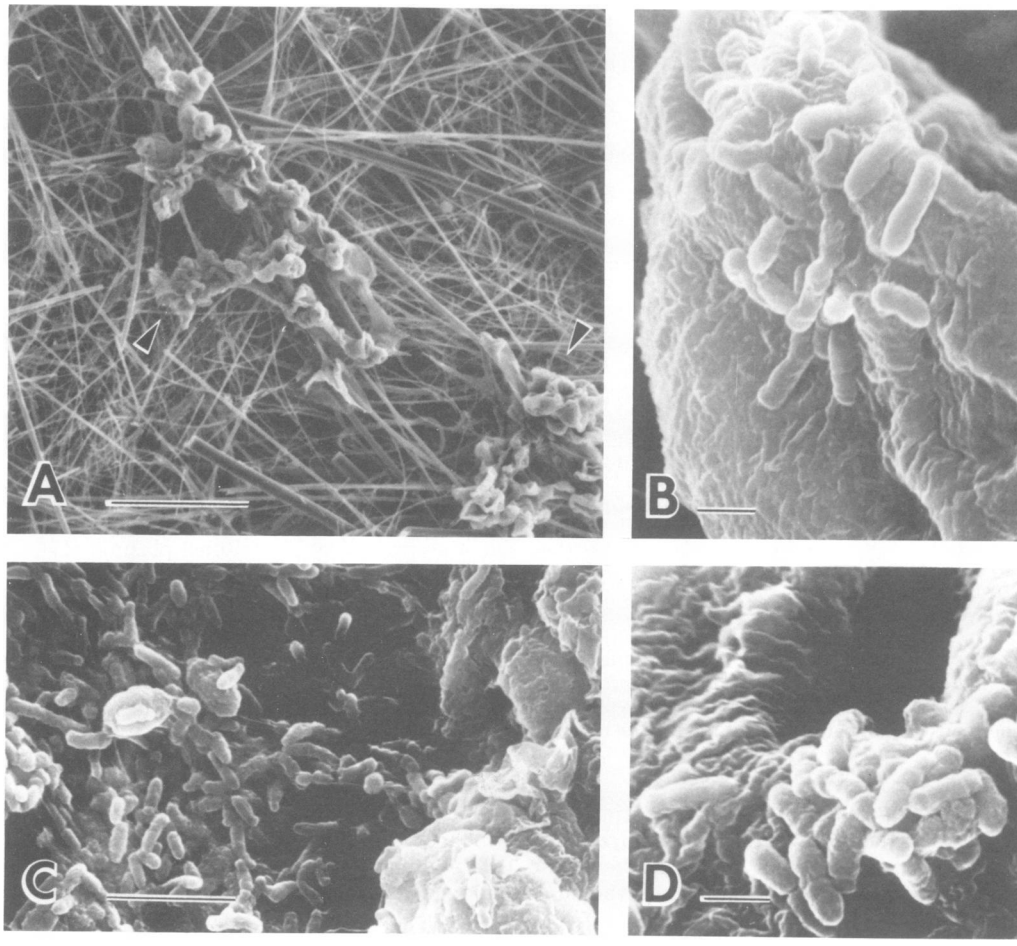

FIG. 4. *Scanning electron micrographs of virulent A. tumefaciens K14 (10^7 cells per ml) adhering to the surface of carrot cells (10^5 cells per ml) after incubation together in culture for 19 h. (A) Clusters of carrot cells (arrowheads) enmeshed in the fibers of the glass GF/C filter are shown at low magnification. Bar, 100 μm (B), (C), and (D) At higher magnification, clusters of various numbers of bacteria are shown adhering to the convoluted surface of the carrot cells. (B) and (D) Bar, 1 μm; (C) bar, 5 μm.*

confirmed that virulent bacteria attached to carrot cells, whereas avirulent bacteria showed very little attachment. Figure 3 shows photomicrographs of living carrot cells incubated with virulent or avirulent bacteria for 20 h. After incubation with avirulent bacterial strains NT1 and TR1, carrot cells had few adherent bacteria (Fig. 3A and C). In contrast, Fig. 3B, D, and E show that virulent strains A6 and C58 attached to the surface of the carrot cells singly or often in large clusters.

At higher magnification, scanning electron microscopy of carrot cells fixed and dehydrated after incubation with the virulent bacterial strain K14 also showed large clusters of adherent bacteria on the cell surface. Although the carrot cells were partially collapsed (Fig. 4A) and the cell surface appeared somewhat convoluted (Fig. 4B), probably due to dehydration, the bacteria appeared well-preserved. Large clusters of smooth-walled bacterial cells of uniform diameter but irregular length were found on the surface of most of the cells in the culture (Fig. 4A to D). Occasionally, long, curved, single, polar flagellae were observed projecting from the end of the bacterial cells (Fig. 5A and B).

Role of bacterial LPS. Whatley et al. (16) have shown that LPS extracted from virulent strains of agrobacteria inhibits tumor formation by the bacteria if applied to wounded bean leaves 15 min before the application of the bacteria. Therefore, the effect of LPS on the attachment of *A. tumefaciens* A6 to tobacco tissue culture cells was examined (Table 4). Similar to the results of Whatley et al. (16) for whole plants, LPS extracted from the virulent strains A6 and C58 inhibited the attachment of the bacteria to tobacco cells, whereas LPS from the avirulent strain 6467 had no effect on this attachment. LPS from the avirulent strain NT1 inhibited tumor formation in whole plants (16) and attachment to tissue culture cells (Table 4).

DISCUSSION

Kinetic, ultrastructural, and biochemical studies showed that virulent Ti plasmid-containing strains of *A. tumefaciens* attach to tobacco and carrot tissue culture cells. In kinetic studies, five virulent Ti-plasmid-containing strains of *Agrobacterium* showed greater attachment than did five avirulent strains. Light and scanning electron microscopic studies confirmed that virulent strains of *A. tumefaciens* attach to the surface of carrot cells. Whether this attachment is identical to the attachment of the bacteria to the plant cells during tumor induction in wounded whole plants is not yet known. However, the interactions between the bacteria and plant cells in tissue culture and in wounded plants do show certain similarities. Both interactions are inhibited by the addition of bacterial LPS of the virulent strains A6 and C58 and the avirulent strain NT1 and are not affected by the addition of LPS of *A. radiobacter* 6467 (16). Both interactions occur with virulent strains C58, A6, 15955, and B6 and do not occur with avirulent strains 6467, TR1, and S1005 (16). Because the whole plant system relies on the measurement of the inhibition by an avirulent strain of tumor formation by a virulent strain, it is not, perhaps, surprising that the two systems differ with regard to results for NT1 and IIBNV6. Both NT1 and IIBNV6 are capable of inhibiting tumor

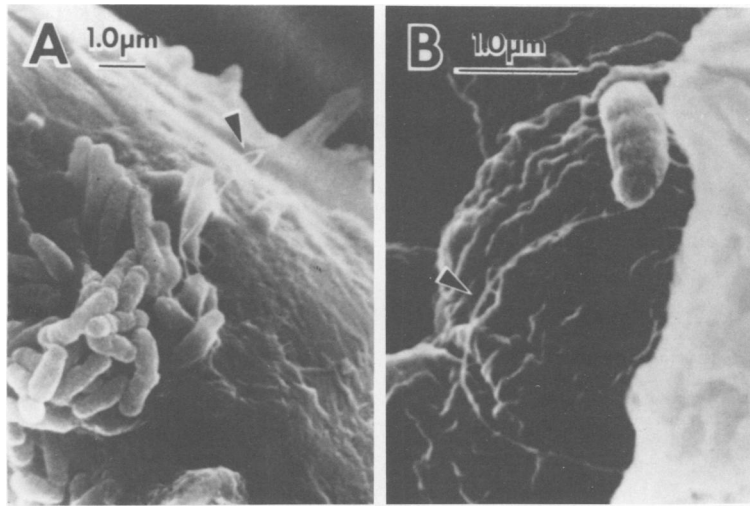

FIG. 5. *In a preparation of A. tumefaciens K14 and carrot cells similar to that shown in Fig. 4, single long flagellae (arrowheads) project from the end of some of the bacteria.*

TABLE 4. *Effect of LPS on the attachment of A. tumefaciens A6 to tobacco tissue culture cells*[a]

Source of LPS	Concn (μg of KDO/ml)	% Attachment of control
None	0	100% (control)
A. tumefaciens A6	0.1	100 ± 8[b]
	0.2	55 ± 20
	2.5	0 ± 15
A. tumefaciens C58	0.1	100 ± 10
	0.7	45 ± 15
	2.5	0 ± 10
A. tumefaciens NT1	0.3	70 ± 15
	1.1	53 ± 20
	2.5	0 ± 15
A. radiobacter 6467	2.5	100 ± 20

[a] LPS was added to the tobacco cells 15 min before the addition of the bacteria.

[b] Standard error of the mean of a minimum of three separate experiments. Measurements were made at 0, 15, 30, 60, 120, and 180 min.

formation by virulent strains in wound sites on bean leaves (16). However, neither of these strains show the same degree of attachment to tobacco tissue culture cells seen with virulent strains. The reason for this discrepancy between the results with wound sites on bean leaves and tobacco tissue culture cells is not known. (Washing of the bacteria with 0.1 M phosphate buffer (pH 7) as used by Lippincott and Lippincott (8) did not increase the ability of the bacteria to attach to the tobacco cells.) LPS from the avirulent strain NT1 inhibited tumor formation in whole plants (16) and attachment to tissue culture cells (Table 4). This result combined with the fact that NT1 does not show the degree of attachment to tissue culture cells shown by virulent strains suggests that LPS may not be the only bacterial substance involved in the attachment of virulent strains to tissue culture cells. The genes for the production of the proper LPS for inhibition of bacterial attachment appear to be chromosomal, although at least one of the genes for the additional requirements for the higher degree of attachment to tissue culture cells shown by virulent strains appears to be on the plasmid.

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